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MICROBIOLOGY AND MICROBIAL TOXICOLOGY:

NUTRITION AND CHEMICAL TOXICOLOGY:

SENSORY EVALUATION AND CONSUMER ACCEPTANCE:

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Certain Physiological and Biochemical Changes in Greenhouse-Grown Tomatoes (Lycopersicon Esculentum Mill.)

SUMMARY

Tomatoes of V. R. Moscow and Fireball varieties grown in a greenhouse varied markedly from previously reported data on field-grown lots of the same varieties with regards to total titratable acidity, color development, free reducing sugars, pectins, volatile reducing substances (VRS), organic acids, and ascorbic acid. Concentrations of all the nonvolatile attributes except total titratable acidity (%) were low as compared with field-ripened tomatoes. Lower concentrations of VRS, organic acids (mg/100 g), and sugars (%) considerably affected flavor, aroma, and taste in greenhouse tomatoes. Color (beta-carotene and lycopene) development was defective. In general, tomatoes grown in the greenhouse were "flat" and tasteless.

INTRODUCTION

A previous paper (Dalal *et al.*, 1965) dealt with the various physiological and biochemical changes occurring in the field-grown tomatoes. This article deals mainly with the studies pertaining to various changes such as acidity, color development, sugars, volatile reducing substances, organic acids, and ascorbic acid of the greenhouse-grown tomatoes.

EXPERIMENTAL

Tomatoes (variety V. R. Moscow and Fireball) were grown in the glass greenhouse in the winter of 1964. Sunshine during the winter months (December through March) averaged 5½ hr per day. No supplemental lighting was provided during the growing months (January to March 1964). Day and night temperatures were kept at 70 and 65°F during the growing period. Tomatoes were harvested at different stages of maturation. The fruits were sorted and classified into nine categories according to size, weight, and color (Table 1). Alcohol slurries were prepared by grinding 250 g (4–40 fruits, depending on maturity stages) of fruit in 250 ml of ethanol. The slurries were preserved at 40°F and analyzed for the free reducing sugars, pectins, organic acids, and ascorbic acid. Fresh fruits were used for the determinations of the total titratable acidity, color measurement, and volatile reducing substances. The following analytical procedures were followed.

Total titratable acidity. Ten grams of fresh fruit from each category were homogenized with 100 ml distilled water and then titrated to pH 8.1 with 0.1*N* sodium hydroxide. Each sample was tested on a Beckman pH meter.

Color measurement. Fresh samples of tomatoes were obtained for determination of total chlorophylls and carotenoids.

Total chlorophylls and chlorophyll *a* and *b* were determined according to AOAC (1960) procedures. Total carotenoids and beta-carotene were determined according to procedures outlined by the Assoc. of Vitamin Chemists (1951).

Free reducing sugars. Free reducing sugars were determined by the Shaffer-Somogyi micro-method (AOAC, 1960).

Total pectic materials and soluble pectins. Total pectic materials and soluble pectins were respectively determined according to procedures outlined by McCready and McComb (1952) and Ruck (1956).

Volatile reducing substances (VRS). Fresh samples of tomatoes were obtained for VRS determinations. Fruit (250 g; number of fruits varied from 4–40, depending upon maturity stages) was blended with a minimum quantity of distilled water and analyzed for VRS content according to the procedure outlined by Luh (1961).

Organic acids. Organic acids were separated by ion-exchange column chromatography and were identified by the method outlined by Marvel and Rands (1950).

Ascorbic acids (vitamin C). Ascorbic acid was

Table 1. Classification of tomatoes (variety V. R. Moscow) according to size, color, and average weight of individual fruit.

Degree maturation	Size (diam, in inches) and/or color	Av. wt. of fruit (g)
1	½ or below, greenish	2.1
2	¾ to 1, greenish	6.2
3	1 to 1¼, greenish	9.0
4	1¼ to 1¾, greenish	33.3
5	2 to 3 or large green	40.1
6	Breakers	45.4
7	Pink	50.0
8	Red	71.4
9	Red-ripe	83.3

determined according to the AOAC (1960) procedure.

RESULTS AND DISCUSSION

Total titratable acidity. Fig. 1 shows the percent total titratable acidity of tomatoes. Total titratable acidity increased consistently up to pink stage, and then declined slightly as the fruit became red. Total titratable acidity was calculated as citric acid. Percent acidity of greenhouse-grown tomato (both varieties) was quite similar to that found in the field-grown tomatoes. Total titratable acidity of tomatoes represents the total amount of free acids present; however, it provides no information about their presence in other forms. Free acids in greenhouse-grown tomatoes were found to increase with fruit maturation.

Color measurement. Total chlorophylls and chlorophyll *a* and *b* continued to increase with the size and maturity of the fruit, but decreased as the fruit started acquiring a pink color (Fig. 2). The decrease of green pigment was considerably less than in field-ripened tomatoes. Greenish-yellow spots were prominent in the pink stage, indicating inadequate color development in fruit during the ripening process. The ratio of chlorophyll *a* to *b* was slightly less than 2:1, as also observed in the field-grown tomatoes. The beta-carotene and lycopene contents of the greenhouse tomatoes are shown in Fig. 3. Beta-carotene content was maximum at the pink stage. Greenhouse tomatoes lacked the intense bright-red color usually found in the field tomatoes. Nonuniform environmental conditions, chiefly light, could be considered as the primary reason for the inadequate color development in the greenhouse tomatoes (McCollum, 1946).

Free reducing sugars. The content of free reducing sugars in greenhouse tomatoes is shown in Fig. 4. Percent free reducing sugars was significantly less than in field-ripened tomatoes. A lower concentration of free reducing sugars in greenhouse tomatoes could be considered as one of the reasons for inferior quality of fruit flavor. Forshey and Alban (1954) indicated that restricted photosynthetic activity due to shading causes the lower sugar concentration in greenhouse tomatoes. Concentrations of free reducing sugars were greater in unshaded tomato fruits than in shaded fruits. The tomato fruit itself may serve as a photosyn-

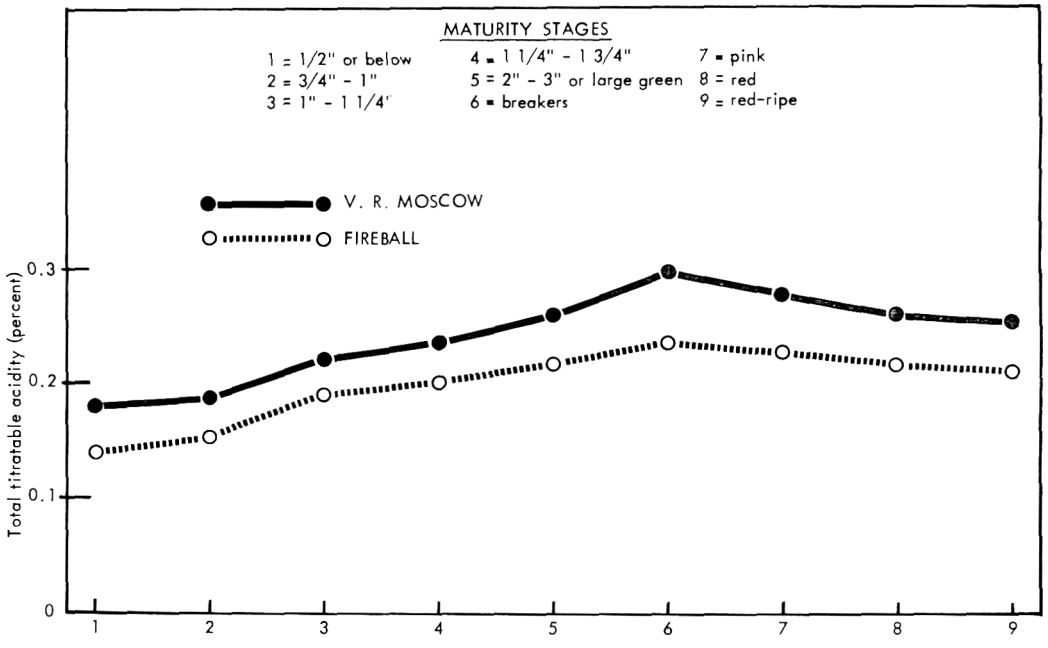

Fig. 1. Total titratable acidity of V. R. Moscow and Fireball tomatoes (greenhouse-grown) at various stages of maturation.

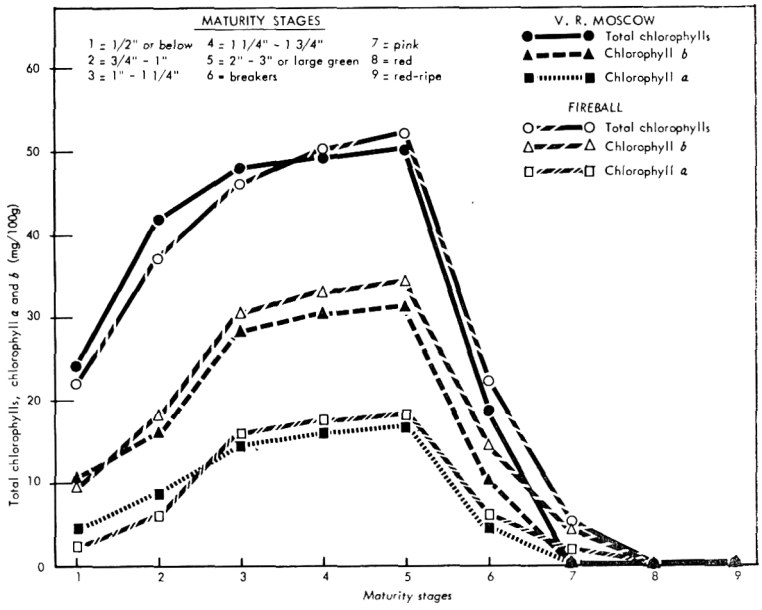

Fig. 2. Chlorophyll content of V. R. Moscow and Fireball tomatoes (greenhouse-grown) at various stages of maturation.

thetic organ even though there are no stomata or lenticels (McCollum, 1946).

Total pectic materials and water-soluble pectins. Total pectic materials increased up to the large green stage, and declined slightly with the ripening of fruit, while water-soluble pectins showed a steady increase (Fig. 5). Quantitatively, both of these attributes were less than in field-grown tomatoes. Lack of sunlight during the growing period, lower concentration of sugars, and soluble solids have been found responsible for the softer fruit (Forshey and Alban, 1954). Moore *et al.* (1958) indicated that the quantity of soluble solids and firmness of tomatoes was reduced by higher fertilization, soil moisture levels, and temperatures. The enzyme protopectinase hydrolyzes the protopectins to water-soluble pectins and pectinic acids, causing softening of fruit with the ripening process.

Volatile reducing substances (VRS). Flavor development is closely associated with the growth of fruit. Environmental

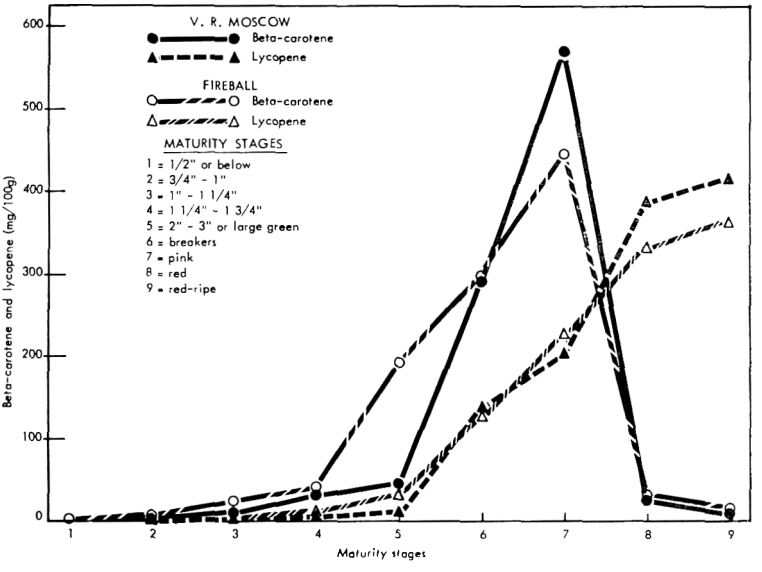

Fig. 3. Beta-carotene and lycopene content of V. R. Moscow and Fireball tomatoes (greenhouse-grown) at various stages of maturation.

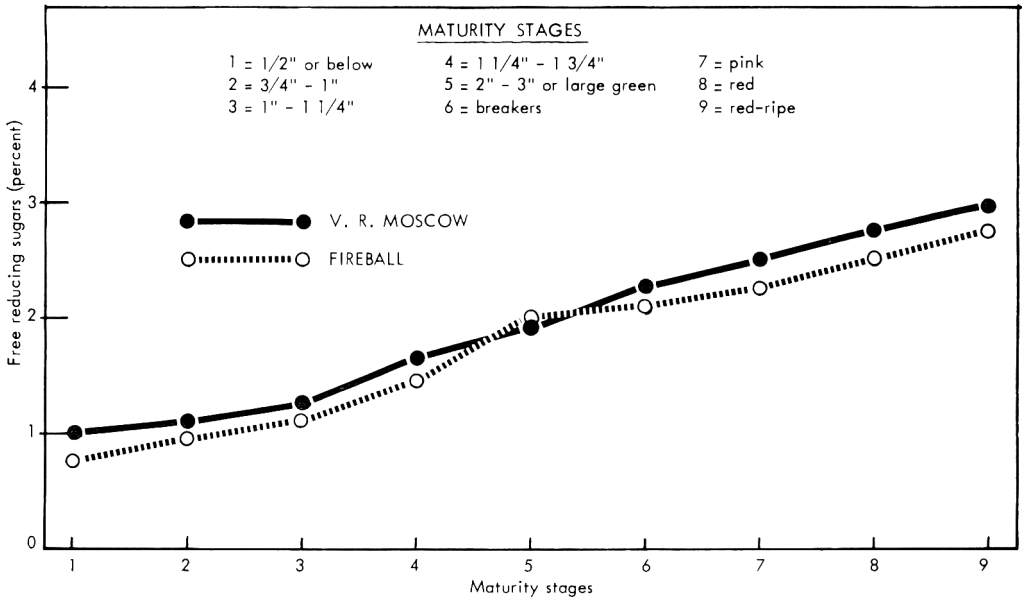

Fig. 4. Free reducing sugars content of V. R. Moscow and Fireball tomatoes (greenhouse-grown) at various stages of maturation.

factors have considerable bearing upon plant growth and the flavor of fruit. VRS increased proportionately with the rate of maturity of the fruit (Fig. 6). The concentration of VRS, partially responsible for typical tomato flavor, was significantly less in greenhouse-grown tomatoes. The distinct aroma and flavor of field-grown tomatoes were lacking. VRS, because of their very sensitive characters, are highly susceptible

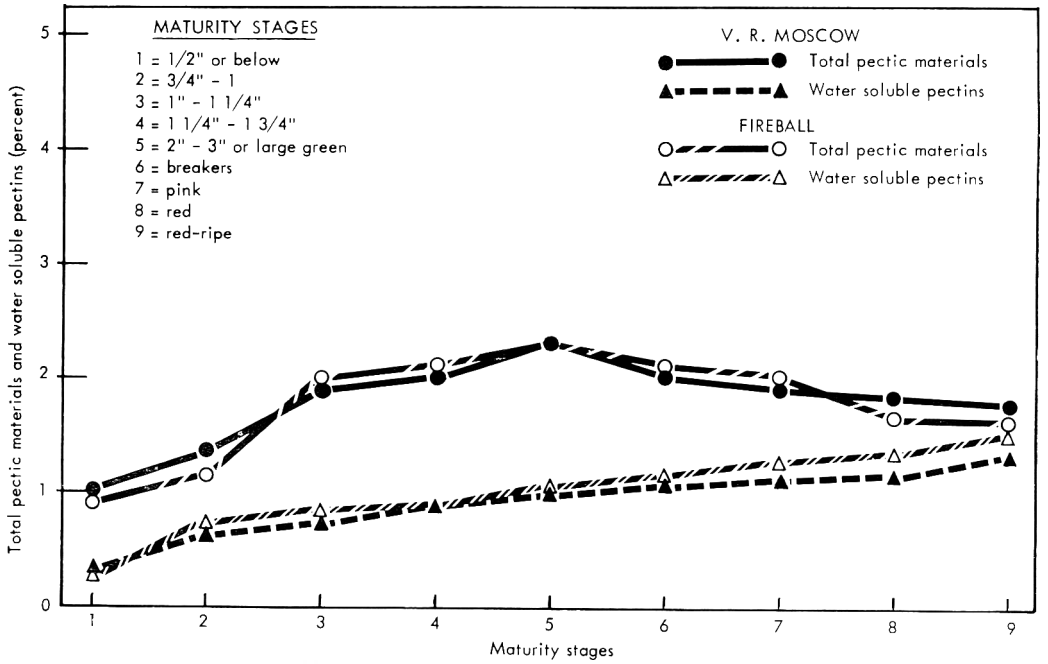

Fig. 5. Pectic values of V. R. Moscow and Fireball tomatoes (greenhouse-grown) at various stages of maturation.

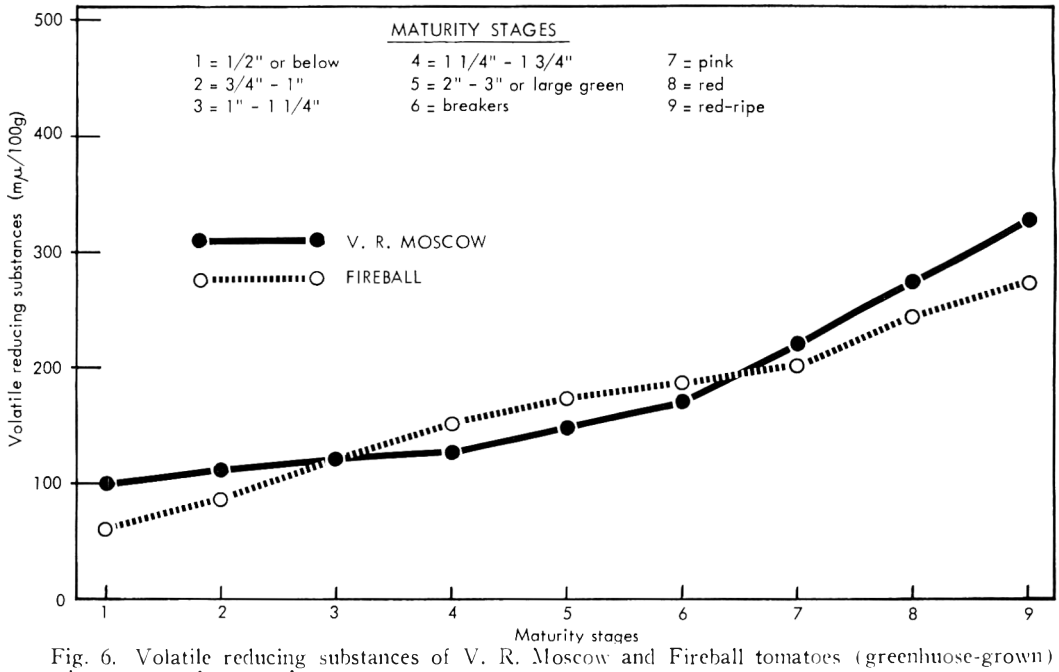

Fig. 6. Volatile reducing substances of V. R. Moscow and Fireball tomatoes (greenhouse-grown) at various stages of maturation.

to variable environmental conditions. Slight alterations in day or night temperatures during the growing period could result in loss of the typical aroma and flavor of tomatoes.

Organic acids. Malic and citric acids were found to be the two major organic acids in the greenhouse tomatoes. The concentrations of malic and citric acids were greater in red-ripe tomatoes than in the other de-

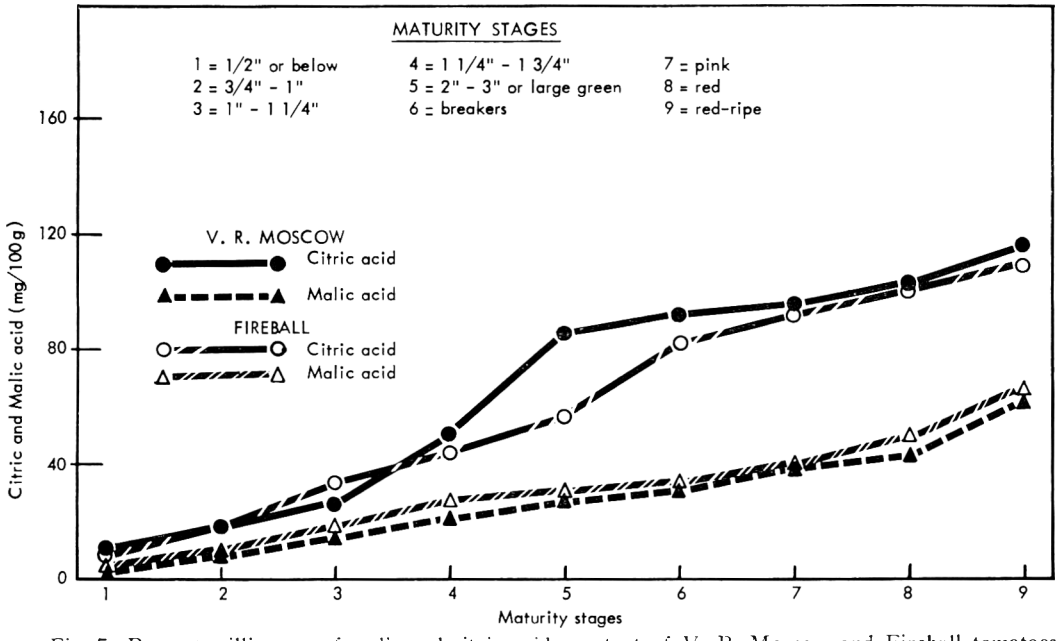

Fig. 7. Percent milligrams of malic and citric acids content of V. R. Moscow and Fireball tomatoes at various stages of maturation.

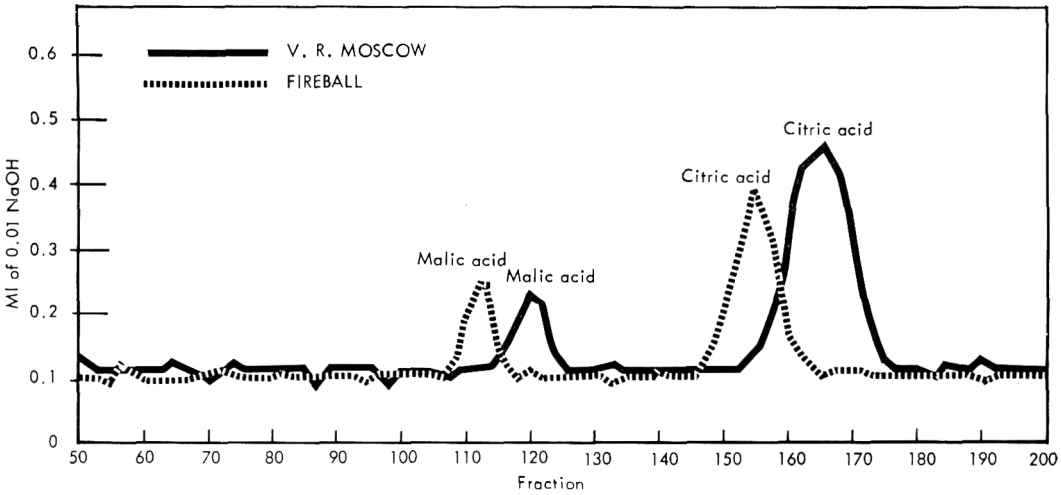

Fig. 8. Schematic representation of chromatographic separation of organic acids from red-ripe V. R. Moscow and Fireball tomatoes (greenhouse-grown).

grees of maturation (Fig. 7). Concentrations of both acids were smaller in these tomatoes than in field-grown tomatoes. Fig. 8 represents a typical chromatogram of red-ripe tomato fruit grown in the greenhouse. The areas under the peaks, representing malic and citric acids, increased progressively with increasing maturity. Quantitatively the total titratable acidity of greenhouse tomatoes was more or less similar to that of field-grown tomatoes, while the concentration of organic acids was considerably lower.

Ascorbic acid (vitamin C). Ascorbic acid increased progressively with ripening (Fig. 9). No decline of vitamin C content was observed in these tomatoes (ascorbic acid content in the field-ripened tomatoes increased up to pink stage and then declined slightly as the fruit attained the red-ripe stage). Ascorbic acid is thought to be consumed in the later stages of the ripening process. No decrease was found in these tomatoes, indicating a different ripening process. Certain experimental results (Schopfer, 1949) indicate that biosynthesis

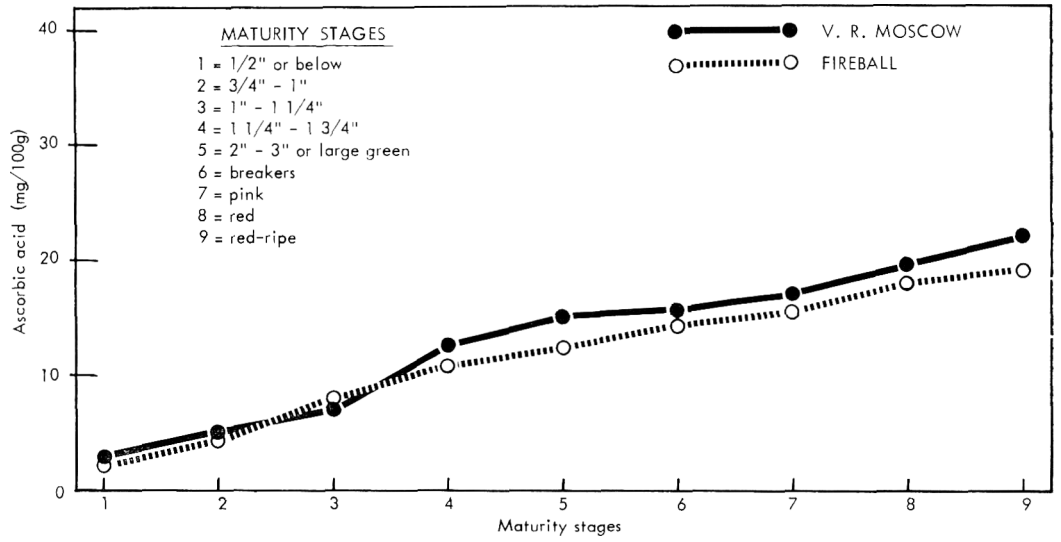

Fig. 9. Percent milligrams of ascorbic acid content of V. R. Moscow and Fireball tomatoes (greenhouse-grown) at various stages of maturation.

of ascorbic acid and photosynthesis are interrelated. However, nothing definite is known as to how enzymatic synthesis of ascorbic acid is connected with the photosynthetic activity. Tombesi *et al.* (1952) reported that the ascorbic acid oxidase activity of the tomato fruit decreases rapidly with ripening and reaches a minimum at the yellowing stage. Light has been recognized as an important environmental factor in determining the ascorbic acid content of fruits and vegetables. Frazier *et al.* (1954), McCollum (1946), and Somers *et al.* (1945) all indicated that light energy impinging directly on the fruits determines their ascorbic acid content. Brown and Moser (1941) reported that greenhouse fruit had only about half the vitamin C concentration of tomatoes grown in the field.

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The Influence of Metallic Ions on the Autoxidation of Oxymyoglobin

SUMMARY

The autoxidation rate of purified oxymyoglobin is extremely variable. Part of the variability has now been traced to contamination of the oxymyoglobin preparations with metals, particularly copper. Copper added in amounts equimolar to the oxymyoglobin concentration resulted in a 25× increase in the autoxidation rate constant. Iron and zinc were much less active than copper in catalyzing the autoxidation. A variety of conditions known to accelerate the autoxidation of oxymyoglobin were tested to see whether the acceleration was due to contamination with metals.

INTRODUCTION

An interest in the autoxidation rate of oxymyoglobin (MbO_2) has been stimulated by the direct connection between this reaction and meat or fish discoloration (Snyder and Ayres, 1961; Brown and Dolev, 1963a,b). (The abbreviations used are Mb for reduced myoglobin, Mb^+ for metmyoglobin, MbO_2 for oxymyoglobin, and similarly for hemoglobin derivatives.) Previous studies have shown the effect of partial pressure of oxygen, pH, and temperature (George and Stratmann, 1952a,b; 1954), the effect of sodium hydrosulfite concentration and temperature (Snyder and Ayres, 1961), and the effect of degree of purification of MbO_2, temperature, buffer strength, and freezing (Brown and Dolev, 1963a,b) on the autoxidation rates of MbO_2.

Despite careful control of the above-named factors, unexplained variations still occur in autoxidation rate constants. A greatly increased rate of autoxidation was observed in this laboratory for an MbO_2 solution which had been Seitz-filtered, and the cause of the increased rate was found to be the metal filter holder. Consequently, a series of experiments were done to evaluate the effect of trace metals on the autoxidation rates of MbO_2 and HbO_2. This paper reports the results.

Weiss *et al.* (1953) reported that the autoxidation of HbO_2 was markedly increased by copper, somewhat increased by iron, and retarded by zinc. Copper and iron were found to be effective catalysts for the reduction of Hb^+ by ascorbic acid (Gibson, 1943; Weiss *et al.*, 1953). An extensive study of the binding of copper and zinc by myoglobin has been completed by Breslow and Gurd (1963) and by Breslow (1964).

METHODS

The myoglobin used was obtained from beef skeletal muscle as reported previously (Snyder and Ayres, 1961) and from sperm whale (Sigma Chemical Co. Type II). Both myoglobin preparations had been crystallized in the oxidized form, and no difference in behavior was noted for the two types of myoglobin.

Hemoglobin was obtained by homogenizing bovine liver with an equal weight of 0.6*M* phosphate buffer at pH 5.7. The homogenate was heated at 55°C for 5 min, cooled, and centrifuged. The hemoglobin solution was diluted with the buffer used for extraction to give an absorbancy of about 0.8 at 574 mμ (1 cm path length). Also, oxyhemoglobin solutions were prepared by precipitating the liver extract with $(NH_4)_2SO_4$ and by dialyzing the rehydrated hemoglobin overnight at room temperature against 0.6*M* phosphate, pH 5.7. As a result of precipitation with $(NH_4)_2SO_4$ and overnight dialysis, the hemoglobin preparation had autoxidized to methemoglobin, and it was necessary to reduce the hemoglobin solution with $Na_2S_2O_4$ prior to measuring the autoxidation rate. The autoxidation rate constant for a crystallized sample of bovine hemoglobin (Sigma Chemical Co. Type I) was also measured.

Autoxidation rate constants were determined for both hemoglobin and myoglobin preparations by using phosphate buffer at a pH of 5.7 and 30°C as previously described (Snyder and Ayres, 1961).

For adsorption of MbO_2, a CM cellulose (Sigma Chemical Co., medium-mesh, 0.62 mequiv/g) column was prepared by equilibrating with 0.005*M* acetate at pH 5.7. MbO_2 was adsorbed on the column, washed with approximately 1 L of distilled water to remove anions, and eluted from the column by using 0.6*M* phosphate at pH 5.7. The adsorption and elution were done at 2°C, but the eluting buffer was at room temperature.

Three 4-g samples of CM-cellulose were wet ashed and analyzed for iron and copper. One sample was used without any pretreatment, one sample was washed with 3 L of distilled water and ashed, and the third sample was washed with 3 L of distilled water followed by 3 L of 0.005*M* acetate at pH 5.7 containing 0.1% EDTA. The wet ashing was done with concentrated HNO_3 at the beginning and was finished with a 5:1 mixture of concentrated HNO_3 and $HClO_3$ (70%). Analysis for iron was according to Diehl and Smith (1960), using bathophenanthroline, and analysis for copper was according to Diehl and Smith (1958), using bathocuproine.

RESULTS

An attempt to remove contaminating microorganisms from an MbO_2 solution by Seitz filtration resulted in a very rapid autoxidation of the MbO_2. The rapid autoxidation was found to be due to the metal filter holder, and a qualitative check of several metal salts (Fe, Zn, Al, Cu) indicated that Cu was extremely active in promoting the autoxidation of MbO_2, while the other metals were less active.

The autoxidation rate of MbO_2 was measured in the presence of varying concentrations of cupric ion, with the results shown in Fig. 1. The MbO_2 concentration was 0.23m*M* for this series of experiments. The copper was added as $CuSO_4$ after reduction and oxygenation of the myoglobin. The rates were found to be first-order with respect to MbO_2 for all concentrations of copper shown. The copper seemed to be acting as a catalyst for the autoxidation rather than as a direct oxidant such as ferricyanide. A qualitative experiment was done to determine if the cupric ion was directly oxidizing the heme iron and then being reoxidized by oxygen, or if the cupric ion was catalyzing the autoxidation. Myoglobin was reduced by adding an excess of sodium hydrosulfite ($Na_2S_2O_4$). A large crystal of $CuSO_4$ was added to the Mb, which was contained in a 13-mm test tube. If the cupric ion were directly oxidizing the heme iron, Mb^+ should have formed first at the bottom of the tube, where the cupric ion concentration was high. The amount of $CuSo_4$ added far exceeded the amount of $Na_2S_2O_4$, so even if the $Na_2S_2O_4$ had to be used up prior to the appearance of Mb^+, there was sufficient $CuSO_4$ present to oxidize all of the $Na_2S_2O_4$ and heme iron. If the cupric ion were acting as a catalyst for the autoxidation,

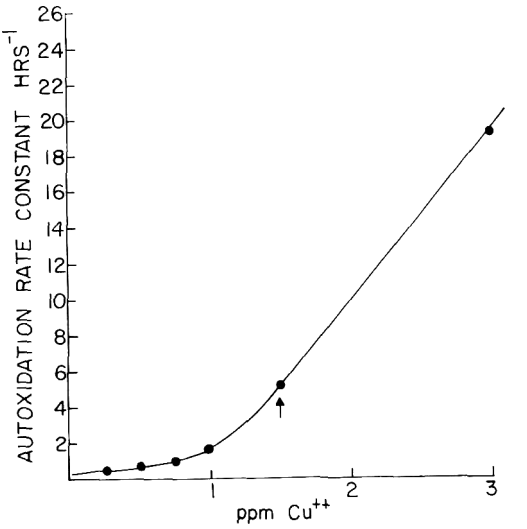

Fig. 1. The effect of Cu^{++} on the autoxidation of MbO_2 at pH 5.7, 0.6*M* phosphate buffer and 30°C. The arrow indicates equimolar concentrations of MbO_2 and Cu^{++}.

then O_2 would still be the oxidizing agent, and Mb^+ would appear first at the surface of the test tube. In the several times this experiment was done, the Mb^+ each time appeared first at the surface exposed to O_2. Therefore, cupric ion acts as a catalyst for the autoxidation rather than as a direct oxidant.

Measurements of autoxidation rate constants for MbO_2 in the presence of small amounts of iron and copper at pH 8.0 are shown in Table 1. Iron had no influence on the autoxidation rate, and the influence of copper was very much less than at pH 5.7. Tris buffer was used to maintain the pH at 8.0. When experiments were done to measure the autoxidation rate constant of MbO_2 with added iron and zinc at pH 5.7, these metals formed insoluble salts with the phosphate buffer. Consequently, 0.5*M* acetate buffer at pH 5.7 was used in place of phosphate buffer, with the result that the autoxidation rate constant of MbO_2 without any added metals was increased severalfold. Sometimes the autoxidation rate in acetate buffer was not first-order. At least some of the increased autoxidation rate with acetate buffer could be controlled by the addition of 0.1% EDTA. Other methods of decreasing the autoxidation rate constant in acetate buffer were by making up the buffer using deionized glass-distilled water or by treating the acetate buffer with dithizone. Rate

Table 1. Autoxidation rate constants for MbO_2 in 0.1*M* Tris, pH 8.0, in the presence of Fe and Cu.

	No metal	5 ppm Fe	10 ppm Fe	1 ppm Cu	2 ppm Cu	4 ppm Cu
Rate constant hr^{-1}	0.09	0.09	0.09	0.28	0.34	0.36

constants resulting from these treatments are shown in Table 2. It appeared that the increased autoxidation rates obtained with acetate buffer could be attributed to contaminating cations. Good reproducibility of autoxidation rates was not obtained when iron and zinc were added to MbO_2 in acetate buffer. However, both iron and zinc were less effective than copper in catalyzing the autoxidation.

The effect of excess $Na_2S_2O_4$ on the autoxidation rate of MbO_2 was described in an earlier paper (Snyder and Ayres, 1961). An obvious method to remove oxidation by-products of $Na_2S_2O_4$ and contaminating metallic cations would be dialysis. Several experiments with dialysis of Mb^+ and MbO_2 were done with the following results.

When a preparation of Mb^+ which had been crystallized from beef skeletal muscle was found to autoxidize with a rate constant higher than normal (0.4–0.5 hr^{-1}, compared with 0.2 hr^{-1}), the autoxidation rate constant could be decreased by extensive dialysis first against 0.01*M* NaCN in 0.6*M* phosphate buffer, pH 5.7, and then against buffer alone until the spectrum of Mb^+ showed no evidence of the cyanide derivative. However, if Mb^+ was first reduced with $Na_2S_2O_4$ and oxygenated, dialysis against buffer to remove oxidation products of $Na_2S_2O_4$ resulted in an autoxidation rate constant higher than with control samples of MbO_2 which had not been dialyzed. Simply incubating MbO_2 with dialysis tubing overnight in the cold was sufficient to increase the autoxidation rate constant. These results are shown in Table 2. The effect could not be decreased by presoaking or washing of the dialysis tubing with deionized water. The effect of dialysis tubing on the autoxidation rate of MbO_2 could be nullified by incubating the MbO_2 and dialysis tubing with 0.1% EDTA.

Another technique which was used to free MbO_2 of oxidation products of $Na_2S_2O_4$ was the adsorption of MbO_2 on a CM cellulose column, washing the adsorbed MbO_2 free of any anionic by-products from $Na_2S_2O_4$, then eluting MbO_2 by using 0.6*M* phosphate buffer and measuring the autoxidation rate. The result was a striking increase in autoxidation rate due to adsorption on the CM cellulose column. The rate constant was 3.72 hr^{-1} for adsorbed MbO_2, compared with 0.21 hr^{-1} for a control sample of MbO_2 which did not receive the column treatment. Washing the CM-cellulose column with buffer containing EDTA was sufficient to decrease the autoxidation rate constant to control values. Further, CM cellulose which had been treated with single-distilled water or with buffer containing EDTA and CM-cellulose which had received no treatment was ashed and analyzed for iron and copper. The results are shown in Table 3. The copper content of the column material was decreased to undetectable amounts by the EDTA treatment. Since copper has a considerable effect on the autoxidation rate of MbO_2 (Fig. 1), it was concluded that copper eluted with MbO_2 was responsible for the striking increase in autoxidation of MbO_2 adsorbed on CM-cellulose.

The MbO_2, which had been adsorbed on CM cellulose, washed with water and then eluted with buffer, containing EDTA, still autoxidized with a rate constant of 0.2 $hr.^{-1}$. Consequently, it was concluded that oxidation by-products of $Na_2S_2O_4$, which would have been washed free of the adsorbed MbO_2 had little effect on the autoxidation of MbO_2.

Table 2. Autoxidation rate constants for MbO_2 using various buffers and buffer treatments to remove metals. General conditions: Phosphate buffer 0.6*M*, pH 5.7, acetate buffer 0.5M, pH 5.7, temperature 30°C.

	Phosphate untreated	Phosphate + 0.1% EDTA	Acetate untreated	Acetate + 0.1% EDTA	Acetate treated with dithizone[a]	Acetate with deionized distilled H_2O[b]
MbO_2	0.29 hr^{-1}	0.25 hr^{-1}	0.50 hr^{-1}	0.39	0.46	0.36
	0.31	0.23	1.10	0.32	0.43	0.36
	0.29		often rapid but	0.35		
	0.35		not first order			
MbO_2						
incubated	0.39	0.29				
with dialysis	0.44	0.23				
tubing[c]	0.40	0.20				
	0.40					
	0.44					

[a] Portions of the buffer were shaken with 0.001% dithizone in chloroform until there was no color change in the dithizone. The buffer was then heated to remove traces of chloroform.

[b] The water used to make up this buffer was distilled, passed over a deionizing column, glass-distilled, and stored in a polyethylene container.

[c] The MbO_2 was incubated for 24 hr in the presence of dialysis tubing at 4°C. Subsequently, the dialysis tubing was removed and the autoxidation rate measured.

Table 3. Analysis for iron and copper in CM-cellulose.

CM-cellulose Treatment	Fe	Cu
Washed with distilled H_2O [a]	40 ppm	18 ppm
Washed with 0.1% EDTA	35	0
No special treatment	51	5

[a] A 4 g sample of CM-cellulose was washed with 3 liters of distilled H_2O or EDTA solution and ashed. The metal concentrations are averages for duplicate samples taken from each acid digest.

The observations on the effect of metallic ions and EDTA on the autoxidation of MbO_2 led to questions concerning other anomalies in the autoxidation of heme pigments. For example, NaCl has been known to increase the autoxidation rate of MbO_2 and HbO_2 (Coleman, 1951). Conceivably, this effect may have been due to trace metals added with the NaCl. Experiments were done to test this hypothesis. The autoxidation of MbO_2 was measured with and without the addition of 10% NaCl and in the presence of 10% NaCl plus 0.1% EDTA. The results are shown in Table 4, and they indicate that part of the NaCl effect on the autoxidation rate is due to cations being complexed by EDTA. However, there is an effect of NaCl not counteracted by the EDTA.

Another possible effect of trace metals which seemed worth exploring was the well-known difference in autoxidation rate between MbO_2 and HbO_2. Usually, HbO_2 autoxidizes at a lower rate than MbO_2. The lower rate could be due to the different methods of preparation of purified hemoglobin and myoglobin. Most studies on the autoxidation of HbO_2 have been done with lysed red blood cells, but for MbO_2 the preparation involves one or more precipitations with $(NH_4)_2SO_4$. Measurements were made on the autoxidation rate of an unpurified HbO_2 preparation extracted from bovine liver and of the same preparation after precipitation with $(NH_4)_2SO_4$ and extensive dialysis. Also, the autoxidation rate was measured for a commercial preparation of bovine HbO_2 which had been crystallized and lyophilized. The results (Table 5) indicate that crude tissue extracts of HbO_2 autoxidize very slowly. After a purification procedure similar to the procedures used for MbO_2, HbO_2 autoxidizes at a rate quite comparable to that for MbO_2. Also, HbO_2 shows a response to added EDTA similar to that of MbO_2. Added EDTA will decrease the rate of autoxidation, but only to a point, and never is the autoxidation rate constant decreased to the extent which can be achieved in crude tissue extracts.

Table 4. Autoxidation rate constants for MbO_2.

Experiment	No added salt	10% NaCl	10% NaCl + 0.1% EDTA
1	0.25 hr^{-1}	1.47 hr^{-1}	0.71 hr^{-1}
2	0.18	0.86	0.62

Table 5. Autoxidation rate constant for different HbO_2 preparations at pH 5.7 and 30°C.

1. Crude beef liver extract	No measurable rate after 3 hr
2. Same as 1 after $(NH_4)_2SO_4$ precipitation	0.16 hr^{-1} 0.18 0.14
3. Crystallized bovine HbO_2	0.41 0.41 0.41
4. Crystallized bovine HbO_2 + EDTA	0.12 0.22 0.28

The increase in autoxidation rate constant with purification of heme pigments might be attributed to the removal of some component which inhibits the autoxidation or the addition of something (possibly trace amounts of copper) which catalyzes the autoxidation. The latter possibility was tested experimentally by purifying myoglobin from beef skeletal muscle in the presence of EDTA. The purification procedure was the same as previously reported (Snyder and Ayres, 1961) except that the muscle extract was kept continually in contact with solutions containing 50 ppm EDTA. After crystals were obtained, the autoxidation rate constant was 0.25 hr^{-1}. Consequently, the presence of EDTA during purification of myoglobin had no effect on the autoxidation rate constant.

DISCUSSION

The effect of cupric ion on the autoxidation of purified MbO_2 could be attributed to a direct oxidation of MbO_2 by Cu^{++} and subsequent autoxidation of Cu^{+} to Cu^{++}. This does not seem to be the mechanism, for two reasons: 1) if MbO_2 were oxidized directly by Cu^{++}, the rate of the reaction for equimolar mixtures of the myoglobin and copper would be expected to be much greater than that shown in Fig. 1; and 2) Mb oxidized only in the presence of excess $CuSO_4$ if O_2 were also present.

The removal of oxidation products of minimal amounts of $Na_2S_2O_4$ had no effect

on the autoxidation rate constant of MbO_2. It has been shown that products of $Na_2S_2O_4$ autoxidation can take up considerable O_2 (Matsuura *et al.*, 1963). Consequently, for best reproducibility, autoxidation rates should be measured with sufficient exposure to air so that the partial pressure of O_2 in solution is not lowered during the measurement.

Numerous experiments (Table 2) showed that the incorporation of EDTA in an autoxidizing solution of MbO_2 is sufficient to lower abnormally high autoxidation rate constants to approximately 0.2 hr^{-1}. However, all attempts failed to decrease rate constants even further by careful exclusion or removal of metal ions. Yet, for unpurified HbO_2 and MbO_2 (Brown and Dolev, 1963a), rate constants are frequently lower than those for crystallized HbO_2 or MbO_2. The reason for the increased susceptibility to autoxidation upon purification of a heme pigment is not known. An attempt to purify MetMb in the presence of EDTA and thereby to minimize contamination with trace metals did not result in a decreased rate constant for autoxidation. Consequently, the presence of protective factors in crude extracts seems the preferable explanation for the slow oxidation found in crude extracts. Perhaps an enzyme system such as that recently described by Stewart *et al.* (1965) may be responsible for the slow autoxidation.

With regard to the effect of salt on the autoxidation of MbO_2, the results shown here indicate that some of the influence can be attributed to metals which can be chelated by EDTA. Yet there remains an effect due to salt which cannot be attributed to metallic cations. Wang (1961) has attributed the stability of HbO_2 to the apolar environment immediately surrounding the heme group. Added salt could be expected to modify the environment of the heme, either directly or indirectly, which may account for the effect of salt on the autoxidation.

The discoveries that MbO_2 autoxidizes rapidly after adsorption and elution from a CM-cellulose column, and that the increased autoxidation rate is probably due to contamination of the preparation with copper, indicate the ease with which MbO_2 solutions can pick up small amounts of metals. This information may be helpful in explaining the multiple myoglobins as revealed by CM- and DEAE-cellulose chromatography (Rumen, 1959; Perkoff *et al.*, 1962; Quinn *et al.*, 1964; Atassi, 1964; Akeson and Theorell, 1960).

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Lipid Hydrolysis in Unblanched Frozen Peas (Pisum sativum)

SUMMARY

Data on hydrolytic changes in the lipids of unblanched (enzymatically active) peas in the range −5 to −20°C are presented. The Q_{10} value for the formation of free fatty acids between these temperatures is about 2.5. The corresponding value for development of off-flavor is about 3.0. Both values are considerably lower than those typical for deteriorative non-enzymatic reactions in blanched vegetables.

Gas chromatographic analysis of the free fatty acid fraction demonstrates further that there is an apparent preference for hydrolysis of polyunsaturated acids. This tendency is especially evident in the lower part of the temperature range studied. A corresponding increase of the proportion of saturated acids in the unhydrolyzed fat can be shown. No net change of any single acid in the combined lipid fractions is observed, except for linoleic and linolenic acids, which decrease somewhat after long storage at the higher temperatures. This indicates a substantial breakdown into smaller molecules.

INTRODUCTION

It has been known for many years that lipids of unblanched peas undergo great changes during storage at freezing temperatures (Lee *et al.*, 1955, 1956). In only about 2–3 weeks at −18°C the formation of free fatty acids and peroxides can be shown. At the same time, characteristic off-flavor can be detected. These changes are catalyzed by enzymes and do not occur in blanched peas at comparable rates. During further storage the hydrolytic and oxidative changes continue, leading eventually to extensive hydrolysis of the lipids and marked decrease of the amount of polyunsaturated fatty acids. A simultaneous formation of characteristic lipid breakdown products can be shown by ordinary chemical methods or indicated by gas chromatographic headspace analysis (Bengtsson and Bosund, 1964).

Data from Lee and Mattick (1961) on fractionated pea lipids indicate further, however, that the decrease of polyunsaturated fatty acids is balanced by a corresponding increase of palmitic acid in the combined pea lipids. In pea material stored for long periods more than a doubling of the original amount of palmitic acid was observed. This is astonishing since one should not expect a direct transformation of unsaturated C_{18} acids into a saturated C_{16} acid. Such changes might occur under dramatic chemical conditions but have never been observed in metabolizing or frozen tissues of any type. In this work the studies of Lee and Mattick were repeated using somewhat different methods for extraction and fractionation of the pea lipids. The hydrolytic changes as well as the off-flavor development were followed at different storage temperatures in the range −5°C to −26°C in order to determine the temperature effect on the rate of hydrolysis and on the composition of the free fatty acid fraction.

The studies will be continued on raw materials such as fish and meat and will also include the effect of freezing rate. The latter is of special interest considering the tendency toward such freezing techniques as flow-freezing and freezing in liquid nitrogen, both involving considerably higher freezing rates than conventional methods.

EXPERIMENTAL

Pea material. Peas (*Pisum sativum* sensu amp. (L.) Govorov var. pachylobom, the wrinkled type) of normal harvesting maturity (tenderometer readings 90–100) were used throughout except in experiments comparing different degrees of maturity. In order to secure a uniform material, ordinary factory peas were sampled from the processing line after mechanical vining and size grading immediately before the blanching step. Part of the sampled material was blanched to a negative peroxidase reaction and used as reference. The material was frozen in a blast freezer to −40°C and immediately stored at the temperatures in question.

Organoleptic evaluation. Five experienced judges graded the cooked samples according to a scale from 1 to 7, where 7 denoted very high quality and 5 just acceptable. The ranks were treated statistically by analysis of variance.

Extraction and fractionation of the lipids. The crude lipids were extracted with chloroform-methanol by three successive 1-min treatments in

a Waring blender. For the first treatment the peas were placed in the organic solvent without previous thawing. The first extraction was made with a more polar mixture than the second and third (chloroform-methanol 1:1 compared with 2:1) in order to break the bonds between phospholipids and protein. After each extraction the solvent phase was separated from the water phase and the pea solids by centrifugation for 15 min. The pooled solvent phases were evaporated to dryness under reduced pressure at temperatures not exceeding 40°C. The whole procedure of extraction and subsequent fractionation is shown in Fig. 1. The resulting three fractions of reasonably pure neutral fat, phospholipids, and free fatty acids were analyzed with regard to dry weight, phosphorus content, and fatty acid composition.

In a few experiments the phospholipids were further fractionated into crude fractions of lecithin, cephalin, and inositol phosphatides according to the scheme in Fig. 1.

Extracted lipids. The amount of extracted lipids was determined by drying in an oven at 80°C for 20 hr.

Phosphorus. Phosphorus was determined according to the method of Allen (*1940*).

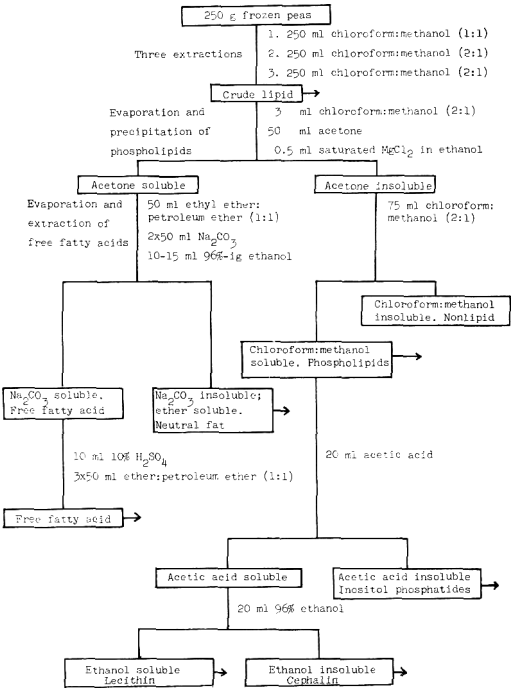

Fig. 1. Extraction and fractionation of crude pea lipids. Arrows (⟶) indicate that determination of dry weight, phosphorus, and amount and composition of fatty acids were made on aliquots of the fractions.

Gas chromatographic analysis of fatty acids. The fatty acid composition of the various fractions was determined at 200°C in a Perkin-Elmer vapor fractometer, model 116E, with a hot-wire detector. The column was 6-ft packed with 20% butanediol succinate polyester on 60–80-mesh chromosorb W. For identification the retention times of the various peaks were compared with those of pure commercial samples. The detector response was calibrated with known mixtures. Esterification of the fatty acids was achieved either by refluxing in 0.5*N* HCl in methanol during 2 hr in an atmosphere of nitrogen after hydrolysis by refluxing in 1*N* KOH in methanol during 3 hr in an atmosphere of nitrogen, or directly by refluxing in 0.4*N* sodium methylate during 30 min in an atmosphere of nitrogen. The two methods gave identical results when tested on the same material. Direct esterification was preferred in the latter part of the work, because of its simplicity. In the case of free fatty acids the first-mentioned method was used throughout, omitting the hydrolysis.

RESULTS AND DISCUSSION

Extraction and fractionation. Fig. 2 shows the amounts of crude lipid, phosphorus, and fatty acids in the material from 6 successive extractions. Of the total amount of lipid present in the peas, 90–95% was recovered in the first 3 extractions. It is evident, however, that a large portion of the crude lipid consists of non-lipid material (higher for each extraction). Most of this is separated from the phospholipids later in the procedure, as chloroform-methanol in-

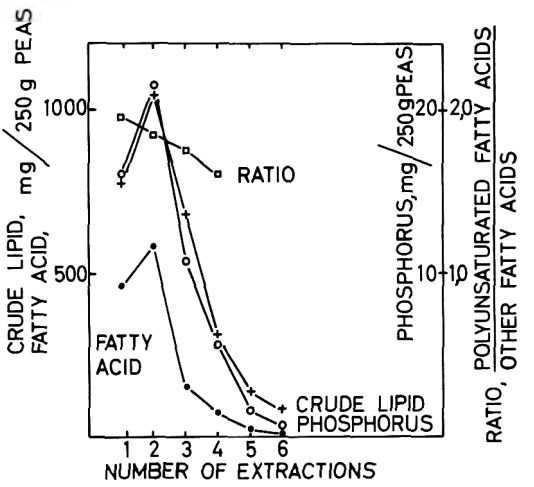

Fig. 2. Composition of crude lipids in repeated extractions.

soluble material, according to Fig. 1. In any case some contamination is tolerable since it does not affect the validity of the results obtained on the composition of the lipids in terms of individual fatty acids.

Fig. 2 also shows that the ratio of polyunsaturated fatty acids to other fatty acids decreases slightly with the number of extractions.

Fractionation of the crude lipids into neutral fat and phospholipids by precipitation of the phospholipids by addition of acetone in the presence of $MgCl_2$ resulted in a neutral fat fraction nearly completely free from phosphorus. The phosphorus content in the phospholipids was close to 4% when calculated on the amount of fatty acids as determined by gas chromatography (assuming that $\frac{2}{3}$ of the phospholipid weight consists of fatty acids).

Fractionation of the crude phospholipids as described in Fig. 1 resulted in inositol phosphatides and cephalin phosphatides essentially free from other material (at least when judged from the phosphorus content), but a very crude lecithin fraction with sugar as the main contaminant. No further purification was attempted, since the main reason for the fractionation was only to give a rough idea of the extent to which each phospholipid group was affected by hydrolytic enzymes.

Fatty acid composition of lipids of fresh peas. A large number of determinations were made throughout this work of the fatty acid composition in fresh peas of varying maturity and in various parts of the pea (cotyledons and skin).

The average fatty acid composition in peas of ordinary harvesting maturity is shown in Table 1. The proportion of, especially, palmitic acid was higher in phospholipids than in neutral fat, while the contrary was true for oleic and linolenic acids. The same picture was obtained by Lee and Mattick (1961). It is interesting to note that the 1961 peas contained twice as much oleic acid as linolenic acid, whereas no significant difference was observed in the 1962 material.

Fig. 3 shows the variation of palmitic, linoleic, and linolenic acids with maturity (here measured as the diameter of the pea) in two experiments. The remaining fatty acid of quantitative importance, oleic acid, did not vary significantly with maturity. Linoleic acid apparently increases with maturity, while palmitic acid and linolenic acid decrease. The total amount of fatty acids, calculated on a dry-weight basis, was about 30% higher in more mature peas (compare Table 2.) The changes of fatty acid composition with varying maturity are not very great, but it should be pointed out that the peas used in this work were of a narrow range of maturities from a physiological point of view.

A comparison of the fatty acid composition of lipids from cotyledons and skins revealed great differences, especially with regard to oleic and linolenic acids. In the cotyledons these acids occurred in about equal amounts (8.5 and 9.7% of the total amount of fatty acid, respectively) while in the skins the amount of linolenic acid was about 9 times as great (2.5 and 22.5%, respectively).

Influence of temperature, oxygen, and pea maturity on rate of off-flavor development. Before the analytical work was started some studies were made on the effect of storage conditions (as temperature and oxygen

Table 1. Fatty acid composition in peas of ordinary harvesting maturity.

Fatty acid	Proportion (%)			
	1961 harvest		1962 harvest	
	Neutral fat	Phospholipids	Neutral fat	Phospholipids
Lower fatty acids [a]	2	1	2	1
Palmitic acid	21	27	20	25
Stearic acid	2	2	1	1
Oleic acid	16	13	10	7
Linoleic acid	51	51	55	58
Linolenic acid	8	6	12	8

[a] Mainly myristic acid.

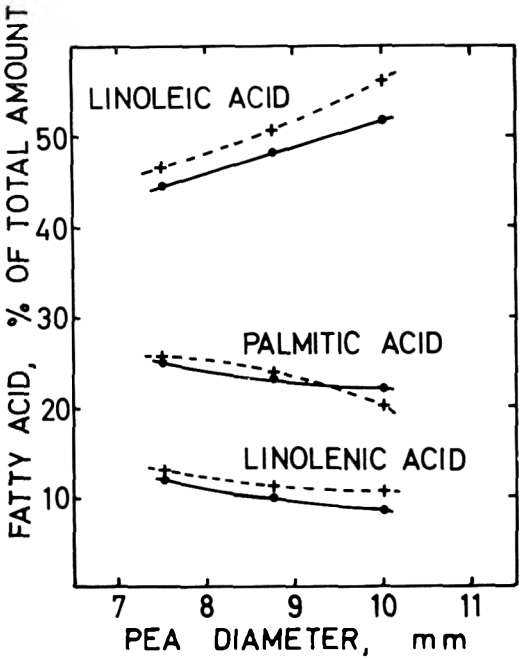

Fig. 3. Content of palmitic, linoleic, and linolenic acids as a function of pea diameter.
+ - - - - + Experiment I
●———● Experiment II

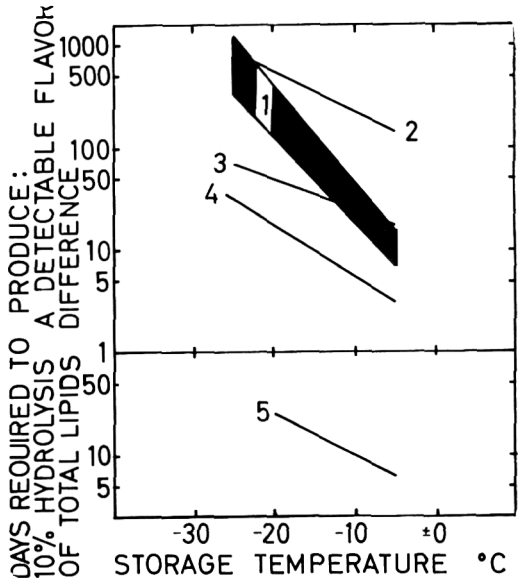

Fig. 4. Time-temperature relationship for storage changes in frozen foods.

1. Q_{10} 10–20 Strawberries; raspberries; beans; peas; spinach; cauliflower. Data: Guadagni *et al.* (1957a); Guadagni *et al.* (1957b); Dietrich *et al.* (1959); Boggs *et al.* (1960); Dietrich *et al.* (1960); Dietrich *et al.* (1962).

2. Q_{10} 3–3.5 Raw chicken. Data: Jul (1963); Klose *et al.* (1959).

3. Q_{10} 2.5 Fatty fish. Data: Jul (1963).

4. Q_{10} 3.0 Unblanched peas. Data: From this work.

5. Q_{10} 2.5 Unblanched peas. Data: Fig. 5 in this work.

availability) and of pea maturity on the rate of off-flavor development. In these studies the off-flavor was evaluated by a trained taste panel. Pea samples stored at −8, −18, −20, and −26°C showed a detectable off-flavor after 4.5, 15, 17, and 35 days. It is evident from Fig. 4 (line 4) that these values correspond to a temperature coefficient (Q_{10}) of about 3.0. This figure for enzymatically active material is much lower than that obtained for blanched peas by Boggs *et al.* (1960), who reported a Q_{10} value of about 13 for non-enzymatic changes in the same temperature range. The considerably lower value for our material is consistent with the fact that enzymatically catalyzed reactions proceed with lower energies of activation than the same reactions in the absence of enzymes. Additional illustration of this is given in Fig. 4, which summarizes data for off-flavor development in various frozen foods. An examination reveals that high Q_{10} values are typical for blanched vegetables. Low Q_{10} values seem to be typical for enzymatically active material such as unblanched peas, raw chicken, fish, etc. Berries treated with sugar fall within the group of blanched vegetables and present very different conditions, which makes a comparison with the other materials difficult.

According to theories of Lee *et al.* (1955), the compounds that contribute off-flavor are formed via peroxidation of unsaturated fatty acids. The formation of these peroxides is, for acids with methylene-interrupted double bonds, catalyzed by lipoxidase. Anyhow, oxygen should be necessary for at least one step in the reaction chain involved. The dependence on oxygen could be easily tested with a biological material completely free from oxygen. Efforts to carry out experiments with such material have been made recently by Fuleki and David (1963) with snap-beans. They showed that the accumulation of certain compounds as acetaldehyde and ethanol could be affected by removing most of the oxygen, but they did not succeed in proving that the rate of off-flavor

development was also affected. An additional difficulty in these experiments is that a complete exchange of air with, for instance, nitrogen might very well lead to the formation of new compounds and off-flavors not easily distinguished from those obtained in the presence of oxygen.

Some attempts were also made along these lines in our studies. The material was frozen in ordinary cans, and air was removed before freezing as completely as possible by repeated evacuation followed by replacement with nitrogen carefully freed from all traces of oxygen. Only when the evacuation procedure was repeated 4 times or more, a significantly (at the 5% level) slower rate of off-flavor development could be demonstrated in the treated material. It was not possible to establish whether the type of off-flavor was different from that developed in the untreated cans.

It has been claimed in the literature that less mature peas deteriorate more rapidly than more mature ones at room temperatures. In our experiments at temperatures below 0°C such a relationship was not observed by the taste panel. During the work it was, however, repeatedly borne out that the hydrolysis is more rapid in less mature peas than in more mature peas (Table 2).

The studies summarized above indicate that many factors might affect the chemical changes of pea lipids at low temperatures. The following report on hydrolytic changes in pea lipids at freezing temperatures deals only with experiments on peas of ordinary harvesting maturity (tenderometer readings 90–100) frozen in normal atmosphere.

Influence of temperature on rate of hydrolysis. The taste thresholds in water phase for short-chain fatty acids are comparatively high, about 5 ppm. It is therefore improbable that free fatty acids should contribute to the off-flavor, especially since hydrolysis of pea lipids at all temperatures studied here was barely detectable when off-flavor was first noticed. The rate of hydrolysis might nevertheless be of prime importance, since lipoxidase is generally considered to attack only free fatty acids.

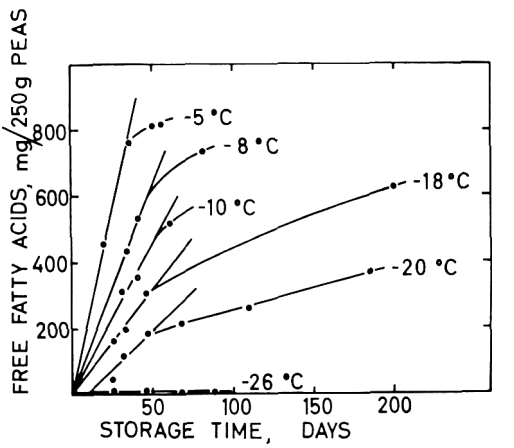

Fig. 5. Rate of free fatty acid information at various temperatures.

The influence of temperature on the rate of free fatty acid formation is illustrated in Fig. 5. Calculation of Q_{10} values, based on the linear part of the curves, is uncertain because of the rather few and scattered points. The value obtained is, however, in the neighborhood of 2.5 (compare lower part of Fig. 4). Repeated experiments have given similar results, and it is clear that the value for this enzyme-catalyzed process is of the same magnitude as for off-flavor development and considerably lower than for deterioration of blanched peas. It is further obvious that the rate of hydrolysis rapidly decreases after a certain time, approximately the same at all temperatures. This means that the rapid phase proceeds to nearly complete hydrolysis of the fat at high temperature, whereas at low temperatures the final degree of hydrolysis is small. The reason for this is further discussed below. Similar results have been reported by other investigators, as

Table 2. Rate of hydrolysis in small, medium, and large-sized peas kept for 7 months at −15°C.

Pea diameter (mm)	Crude lipids (mg/250 g peas)	Fatty acids		% hydrolysis
		mg/250 g peas	mg/g dry weight basis	
7.0–8.0	2,940	960	22.2	32
8.0–9.5	3,680	1,500	29.5	23
More than 9.5	3,520	1,840	30.4	16

for instance for fish by Olley and Lovern (1960) and for cereals by Acker and Beutler (1965).

It could be added that a possible influence of pH changes in the peas is ruled out by the fact that even at extensive hydrolysis the pH does not drop more than 0.2–0.3 unit from the original value of 6.8 in fresh peas.

Influence of temperature on composition of the free fatty acid fraction. Of great interest is the data obtained on the composition of the free fatty acid fraction in terms of individual acids. Fig. 6 shows the ratio of polyunsaturated fatty acids to other fatty acids in free fatty acids formed at various times at different temperatures. It is quite evident that at all temperatures investigated the free fatty acids have a higher degree of unsaturation than the average value for fat in unchanged pea material (1.9; compare Fig. 6). The only exception is the value obtained after about 50 days at −5°C. This is, however, a very long storage time at such a high temperature, and the low ratio was due to break-down of considerable amounts of especially linoleic acid (compare Table 4).

Anyhow, it is of interest to note that the ratios were highest at the lowest temperatures. This might to some extent be due to the fact that the amounts of lipids hydrolyzed were smaller as the temperature was lower (figures at the beginning and the end of each curve give the total amount of free fatty acids in mg fatty acid/250 g of peas at the times indicated). The data show, however, that there is an increasing specificity for hydrolysis of polyunsaturated fatty acids with decreasing temperature, even at comparable levels of free fatty acid formation. One possible explanation could be that unsaturated fat has a low crystallization point and stays longer than saturated fat as a liquid phase when the temperature is lowered. This would facilitate enzymatic reactions by allowing the substrate to diffuse to the enzyme. Such an explanation has been suggested by Acker and Beutler (1965) for all material with a low content of free water on the basis of work with cereals and model mixtures at various levels of relative humidity. Obviously the same explanation could apply to the results in Fig. 5, assuming that the formation of free fatty acids stops when the liquid fat at each temperature has been hydrolyzed.

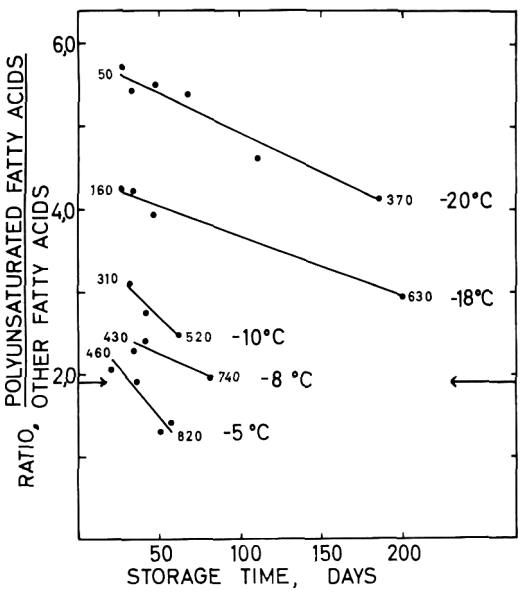

Fig. 6. Ratio of polyunsaturated fatty acids to other fatty acids in the free fatty acids as a function of temperature and degree of hydrolysis. Figures at beginning and end of each curve give the total amount of free fatty acid formed in mg per 250 g of peas.

The high percentage of polyunsaturated fatty acids in the free fatty acids recovered in the above experiment should necessarily result in a percentage below average in the remaining unhydrolyzed fat (the neutral fat and the phospholipid fractions). Such an effect has repeatedly been observed in pea lipids stored at low temperatures.

Rate of hydrolysis with regard to type of lipid. No detailed analysis was made of the influence of factors other than temperature on the hydrolytic rate. Nevertheless it might be worthwhile to point out some observations made during the work.

A comparison between the rate of hydrolysis of neutral fat and phospholipids reveals no significant difference at low storage temperatures but indicates a somewhat more rapid attack on phospholipids than on neutral fat at −5°C and −8°C (compare Table 4). It is doubtful whether this observation has a real significance or is in some way connected with the extraction and fractionation procedure.

A more clear-cut difference is shown in

Table 3. Hydrolysis of different types of phospholipids. The peas were kept 9 months at −20 and −40°C before analyzing.

Fraction	Storage temperature (°C)	Lipids (mg/250 g peas)	Phosphorus	
			mg/250 g peas	% of lipids
Inositol phosphatides	−20	273	11.5	4.2
	−40	295	11.8	4.0
Lecithin [a]	−20	355	4.4	1.2
	−40	535	9.2	1.7
Cephalin	−20	154	6.5	4.2
	−40	446	14.5	3.3

[a] A large part of this fraction consists of sugar.

Table 3 between the three main phospholipid fractions. As is clearly indicated, inositol phosphatides are very resistant to hydrolysis compared with the lecithin and cephalin phosphatides. In the case of lecithin phosphatides it must be remembered that this fraction was very contaminated (note the low phosphorus content) and, consequently, the apparent decrease from 535 to 355 mg. must have been considerably proportionally greater. The resistance of inositol phosphatides to hydrolysis is consistent with the fact that at least phospholipase-A is unable to attack this molecule (Dawson, 1962). A corresponding resistance of inositol phosphatides in cod has been reported by Bligh (1961) and Lovern and Olley (1962).

Net changes of palmitic and linoleic acids. One objective of this work was to reinvestigate the puzzling observation by Lee and Mattick (1961) that a very large decrease of linoleic acid is balanced by a corresponding net increase of palmitic acid. Table 4 summarizes the results obtained for the total amount of these acids present in reference material (at −70°C) and in material stored under various conditions. The degree of hydrolysis in these materials in

Table 4. Content of palmitic and linoleic acids in deteriorated pea lipids.

Storage temp. (°C)	Storage time (days)	% of fat hydrolyzed	Fraction	Total amount of fatty acid (mg/250 g peas)	Amount of palmitic acid (mg/250 g peas)	Amount of linoleic acid (mg/250 g peas)
−70	60	0	Neutral fat	538	121	297
			Phospholipids	709	195	423
			Free fatty acids	0	.....	.....
			Total	1247	316	720
−20	781	41	Neutral fat	316	94	153
			Phospholipids	426	137	210
			Free fatty acids	520	49	353
			Total	1262	280	716
−18	788	64	Neutral fat	206	66	91
			Phospholipids	238	87	100
			Free fatty acids	782	124	471
			Total	1226	277	662
− 8	81	59	Neutral fat	270	84	133
			Phospholipids	252	76	132
			Free fatty acids	736	162	426
			Total	1258	322	691
− 5	56	68	Neutral fat	201	63	75
			Phospholipids	189	74	92
			Free fatty acids	819	204	402
			Total	1209	341	569

terms of free fatty acids as percent of total amount was within the range 41–68%. Although the extent of the hydrolysis is well comparable with the one reported by Lee and Mattick (62%) no substantial decrease of linoleic acid was demonstrated, and consequently no increase of palmitic acid. Only in the material stored at −5°C is there a tendency in this direction. It is doubtful whether the small increase of the palmitic acid peak should be regarded as real or within the experimental error; in any case it could very well be due to contamination with breakdown products from linoleic acid or other transformations. Considering the long storage time and the extensive hydrolysis involved, it was not regarded as worthwhile to investigate this possibility further.

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Acrylamide Gel Disc Electrophoretic Patterns and Extractability of Chicken Breast Muscle Proteins During Post-Mortem Aging

SUMMARY

Acrylamide gel disc electrophoretic patterns of water-soluble extracts consistently showed the presence of 2–4 fast-moving, faint components which increased in relative intensity with post-mortem aging. The electrophoretic patterns of salt-soluble and urea-soluble extracts showed no corresponding pattern changes. The extractability of unbuffered water-soluble proteins was less at 24 hr post-mortem than at 30 min post-mortem in breast muscle obtained from seven 7–10-week-old chickens. The percentage of dialyzable peptides in the water-soluble extracts and the percentages of salt-soluble and urea-soluble proteins in those extracts remained fairly constant during a 24-hr post-mortem aging period.

INTRODUCTION

Changes have been reported in the chemical and physical properties of muscle and muscle proteins during the post-mortem aging period of poultry (de Fremery and Pool, 1960; May *et al.*, 1962; Scharpf and Marion, 1964). Studies using various fractionation procedures have been made on the extractability of chicken muscle proteins at various times after slaughter (Weinberg and Rose, 1960; Fischer, 1963; Khan and van den Berg, 1964), as well as in the aged muscle (Khan, 1962). Electrophoretic patterns of chicken muscle proteins determined with the free-boundary (Weinberg and Rose, 1960) and starch-gel (Neelin, 1964; Neelin and Rose, 1964) techniques have also been reported.

This research was initiated to study chicken breast muscle proteins during post-mortem aging. Acrylamide gel disc electrophoresis, a high-resolution method of zone electrophoresis introduced recently (Ornstein, 1964; Davis, 1964), was utilized in this research. An extraction method was selected which utilized unbuffered solvents and followed a sequential scheme in order to minimize interactions among protein components of the various classes. Muscle samples were extracted successively with water, 1*M* potassium chloride, and 8*M* urea. Protein concentrations of the various extracts were determined by the Folin phenol method (Lowry *et al.*, 1951), and total protein was based on micro-Kjeldahl nitrogen.

EXPERIMENTAL METHODS

Peterson × Arbor Acres day-old chicks were obtained and raised in wire cages to 7–10 weeks old. Commercial starter and grower rations and water were made available *ad libitum*. The birds were slaughtered by severing the neck veins, and, following bleeding, were scalded for 1 min at 50°C. The feathers were removed by hand, and the birds were eviscerated. A muscle sample was removed from the posterior end of one of the pectoralis major muscles 30 min post-mortem. The carcasses were then aged in ice water for 24 hr, and, following removal of the second muscle sample, the breasts, with bone structure intact, were cooked in boiling water for ½ hr. The chilled breasts were placed in rapidly boiling water, and the ½-hr timed cooking period was begun when the water recommenced to boil. Tenderness evaluations were made on the pectoralis major by three members of the laboratory on a 10-point scale, with 7 and above being considered acceptable.

The extraction procedure is outlined in Fig. 1. The muscle sample was weighed and minced with scissors into a VirTis homogenizing flask. Four volumes of iced deionized water were added to the flask, and the mixture was homogenized at high speed for 1 min and at low speed for 5 min. All procedures were carried out at approximately 2°C to minimize protein denaturation. The homogenate was then centrifuged for 20 min at approximately 35,000 × G in a refrigerated centrifuge. The supernatant, consisting of water-soluble substances, was decanted and retained for further study, while the precipitate was returned to the homogenizing flask for washing to remove as completely as possible any water-soluble material remaining. This procedure consisted of homogenizing the precipitate with an additional four volumes of iced deionized water, followed by centrifugation. The supernatant wash was discarded, and the washed precipitate was returned to the homogenizing flask, homogenized with four volumes of 1*M* potassium chloride, and centrifuged. The supernatant, consisting of the salt-soluble proteins, was retained for further experiments. The precipitate was then washed with 1*M* potassium chloride to remove the residual salt-soluble material and was returned to the homog-

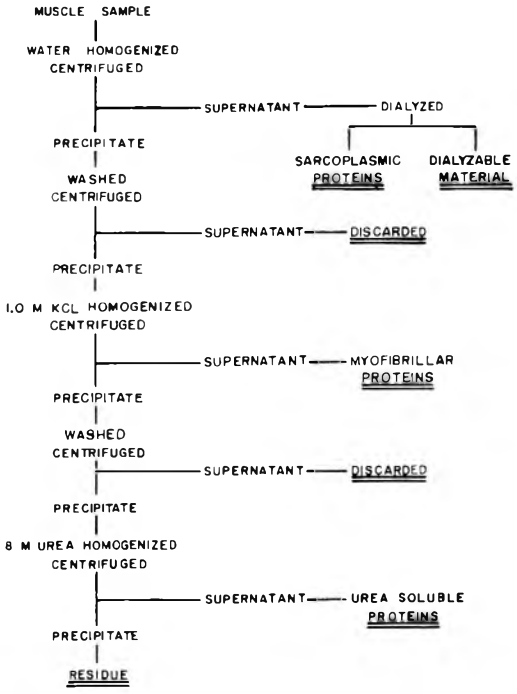

Fig. 1. Chicken breast muscle protein extraction procedure.

enizing flask and homogenized with four volumes of 8*M* urea. Following centrifugation, the supernatant was decanted and retained as the urea-soluble fraction, and the precipitate (consisting of substances not soluble in urea) was discarded. This extraction procedure permits rapid separation of the major solubility classes of muscle proteins with a minimum of interaction between classes. Portions of the water-soluble fractions were dialyzed for three days against deionized water in the cold, with the water replaced at the end of each day.

Protein determinations were made on the various fractions, both before and after dialysis, utilizing the Folin phenol reagent. Protein values for the water-soluble dialyzable material were calculated as the undialyzed value minus the nondialyzable value. The optical-density measurements were made in a Beckman spectrophotometer, model DU, at 750 mμ. Total nitrogen was determined in all samples of intact muscle by the micro-Kjeldahl method.

Acrylamide gel disc electrophoresis was performed on the water-soluble, 1*M* potassium-chloride-soluble, and 8*M* urea-soluble-fractions. The method used was essentially that of Davis (1964) with a few minor modifications. The large-pore gel containing the sample material was omitted, and the samples were applied by pipette directly to the surface of the spacer gel underneath the buffer (Barka, 1961). The water- and urea-soluble extracts were applied directly onto the top of the gel, while the salt-soluble extracts were applied in a diluent of polyethylene glycol. Gel tubes of 8 mm ID were used instead of the standard tubes of 5 mm ID since the larger diameter resulted in the migration of a flatter protein disc, with less tendency to trail off along the sides of the tube. Used in addition to the 7½% acrylamide standard gels were 4% and 15% acrylamide gel systems, respectively, resulting in larger and smaller average pore sizes. All extracts were subjected to electrophoresis on the day prepared so as to minimize pattern variations resulting from secondary and tertiary denaturation. The destaining procedure consisted of diffusion of the unbound stain from the gels by successive changes of 7% acetic acid rather than an electrophoretic migration, since it was found that some of the fast-moving bands in the water-soluble extracts were decolorized somewhat if the electrophoretic destaining was allowed to proceed too long.

RESULTS AND DISCUSSION

Protein determinations on the various chicken breast muscle extracts are summarized in Table 1 and Fig. 2. Table 1 shows

Table 1. Protein content of chicken breast muscle extracts as determined by the Folin method (averages of 7 birds).

Sample	g protein/100 g tissue	% of total protein
Total protein	21.6[a]	100
Water-soluble total		
30 min post-mortem	6.59	31
24 hr post-mortem	5.23	24
Water-soluble protein—nondialyzable		
30 min post-mortem	5.59	26
24 hr post-mortem	4.19	19
Water-soluble dialyzable[b]		
30 min post-mortem	1.00	5
24 hr post-mortem	1.04	5
Salt-soluble protein		
30 min post-mortem	9.25	43
24 hr post-mortem	10.1	47
Urea-soluble protein		
30 min post-mortem	3.01[c]	14
24 hr post-mortem	3.37[c]	16
Insoluble protein		
30 min post-mortem	2.75[c]	12
24 hr post-mortem	2.90[c]	13

[a] Based on Kjeldahl nitrogen.
[b] Primarily small peptides.
[c] Based on 6 birds.

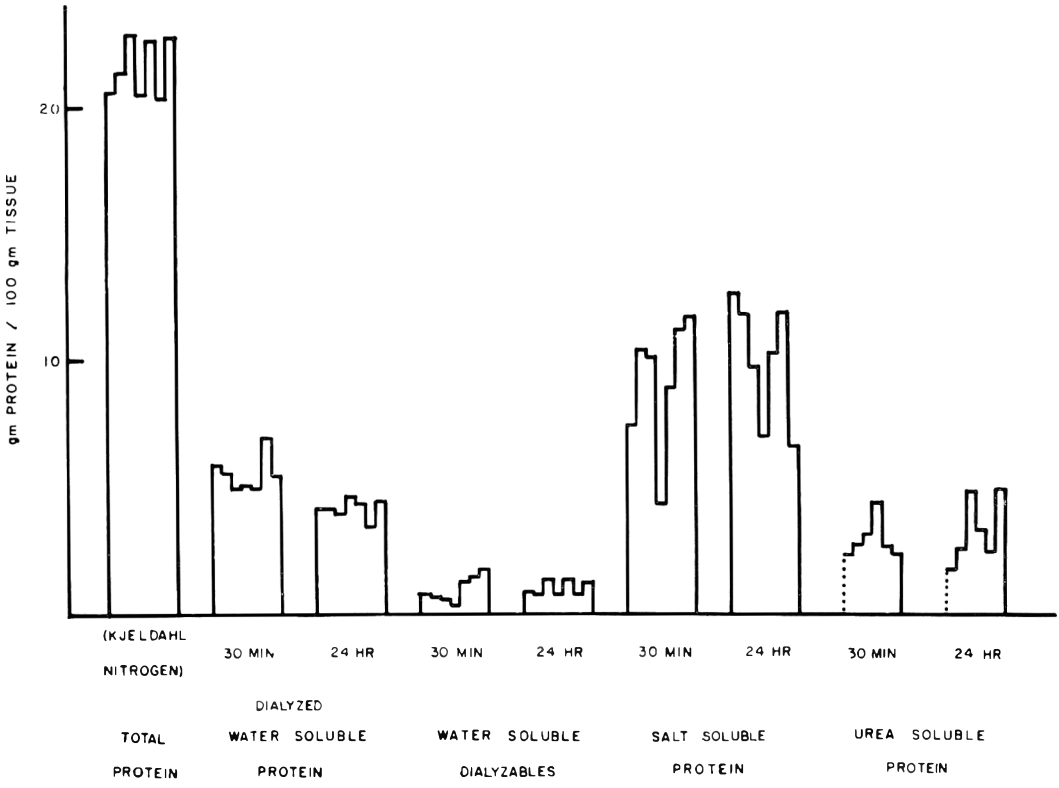

Fig. 2. Variation of Folin values among birds. Data for urea-soluble protein missing for bird 1.

the average values determined by the Folin method for the seven birds. Fig. 2 shows the variation in values among all the birds, each composite column representing the data from birds 1 through 7, left to right. It can be seen that extractable water-soluble material decreases with post-mortem aging. Under the same conditions the extractable salt-soluble and urea-soluble material is seen to remain more nearly constant. The decrease in the water-soluble material may be the result of the decrease in pH between the 30-min and 24-hr extracts, since the extractions are carried out in an unbuffered system. The relatively small change in the salt-soluble protein concentration may be due to the lack of water-solubles (including ATP), since they are extracted first. Therefore, the 30-min salt-soluble extract may contain a large proportion of actomyosin, since little ATP remains to produce the dissociation. This may allow much of the actin, combined with myosin as actomyosin, to be extracted at this time. At 24-hr post-mortem, actomyosin also would predominate. The average values obtained for the protein content of the various 24-hr extracts are in good agreement with values reported by Khan for a buffered system, allowing for variation among birds. The protein values of the dialyzable water-soluble fractions as determined by the Folin method most likely represent small peptides. The decrease in the extractability of the water-soluble proteins with post-mortem aging reported here for chicken may correspond to the decreases reported for pork (Wasilewski, 1961), beef (Fujimaki and Deatherage, 1964; Goll *et al.*, 1964), and rabbit (Sharp, 1963).

Evaluations of the cooked pectoralis major muscles by three members of the laboratory showed that all seven samples were within the range of acceptable tenderness, with no sample being exceptionally tender or tough.

Acrylamide gel disc electrophoresis showed that characteristic band configurations were obtained for each type of extract. There were no major variations observed either

among samples obtained from various chickens or for samples obtained from the same chicken at the same time post-mortem. The electrophoretic patterns obtained with the water-soluble extracts utilizing gels containing 7½% acrylamide (standard gels) consisted of four heavy bands, *A* through *D*, and additional light bands (see Figs. 3A, 3B). These heavy bands showed some slight changes in relative intensities with post-mortem extraction times, but no consistent pattern change developed. The material which accumulated at the pH front (band *F*, Figs. 3A, 3B), representing those components migrating with maximum mobility by reason of molecular size and charge, was observed to increase in relative intensity in the extracts prepared 24 hr post-mortem as compared to the extracts prepared 30 min post-mortem. In a group of 29 birds processed and extracted under similar conditions over several months, an increase in this material was observed in 27 cases, the remaining two appearing to show no change. In order to separate the components of this band, since, as the front, it was expected to consist of more than one component, electrophoresis was performed in gels containing 15% acrylamide, resulting in a smaller average pore size and thus providing more resistance to the migrating molecules. The patterns obtained with this technique were similar to those obtained with the standard gels, except that the relative mobilities of all the bands were proportionately reduced (Figs. 3C, 3D). A number of faint bands located between the fastest heavy band (band *D*, Figs. 3A, B, C, D) and the new front (band *F*, Figs. 3C, 3D) were demonstrated by increasing the total quantity of protein applied to the gels. The slow-moving bands were obscured by this technique. These faint bands represented those components which had not been resolved by the use of standard gels, and which had accumulated at the front. A certain amount of material still remained unresolved at the new front, even with the 15% acrylamide gel. It was observed that a number of faint, fast-moving bands resolved by the 15% acrylamide gel system increased in relative intensity with post-mortem aging. In Figs. 3C and 3D, Bands 1, 2, and 3 are seen to increase with aging, being below the level of visibility in the 30-min post-mortem extract. In these figures it may be seen that the material migrating at the front is also increasing in relative intensity with post-mortem aging, indicating that additional components increasing in relative intensity with aging are present but unresolved in the 15% acrylamide system. Bands 1, 2, and 3 and the front *F* (Fig. 3D), which increase in relative intensity with post-mortem aging of the muscle, may represent peptide fragments of larger molecules, either sarcoplasmic or myofibrillar, which have undergone enzymatic hydrolysis during the post-mortem aging process, as postulated by Whitaker (1964). The non-dialyzable nature of these components indicates that their various molecular weights range above 5,000, allowing the possibility that they may be H-meromyo-

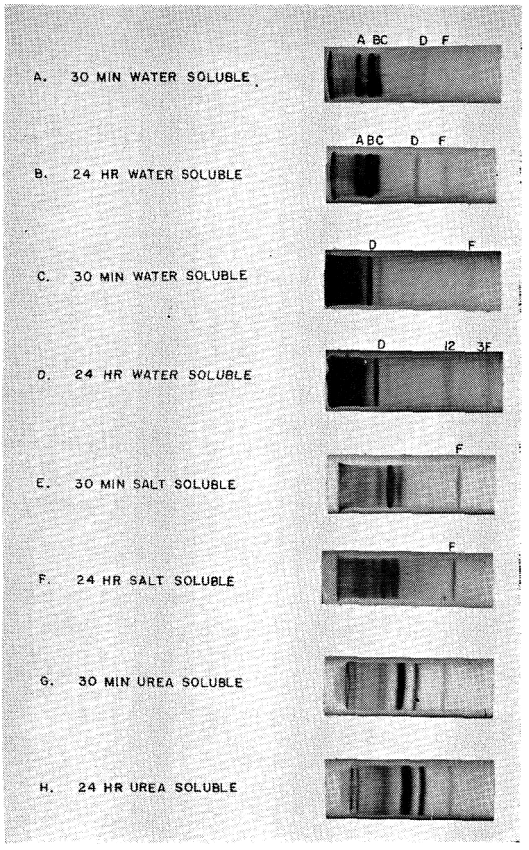

Fig. 3. Acrylamide gel disc electrophoretic patterns of chicken breast muscle protein extracts. *A* and *B*, 7½%; *C* and *D*, 15%; *E* and *F*, 4%; *G* and *H*, 7½% acrylamide gels.

sin contractile polypeptides (Davies, 1963) which have been released from the actomyosin by post-mortem hydrolysis. A similar increase in fast-moving electrophoretic components, as determined by the moving-boundary method, has been reported (Zender *et al.*, 1958) for myofibrillar extracts of rabbit and lamb aged aseptically at elevated temperatures.

It was observed that the electrophoretic patterns obtained with the salt-soluble extracts in a 7½% acrylamide system showed low relative mobilities, and that much of the material did not move beyond the immediate vicinity of the origin. The use of a 4% acrylamide system with the corresponding increase in average pore size allowed the various components to migrate further into the gel. No consistent pattern changes with post-mortem extraction times were observed under the conditions of these experiments. Typical 30-min and 24-hr extract patterns are shown in Figs. 3E and 3F.

The 7½% acrylamide gel system provided a well-distributed band pattern for the separation of the protein present in the urea-soluble extracts. It was observed that with the regular buffer and gel formulations, however, the bands were not migrating as flat discs but were moving faster in the centers than at the edges, resulting in irregular miniscus-like shapes. Since it was possible that the various protein components were becoming less soluble as they moved from the urea extract into the gels, both the primary and spacer gels were prepared with solid urea such that the polymers in the tubes were 8*M* with respect to urea. This resulted in sharp, flat bands (Figs. 3G, 3H). No significant pattern changes between the 30-min and 24-hr samples have been noted thus far. Further studies are now in progress on the salt- and urea-soluble fractions under varied acrylamide gel disc electrophoretic conditions.

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Changes in Color and other Characteristics of Green Beans Stored in Controlled Refrigerated Atmospheres

SUMMARY

The value of controlled atmosphere for holding of green beans prior to marketing or processing was studied. Respiration rates and color changes were studied in bottles under atmospheres ranging from 0 to 10% CO_2 and 2 to 20% O_2. The bottles were flushed periodically. Other studies involved storing green beans at 45°F up to 15 days in a 10% CO_2 and 3% O_2 atmosphere continuously produced by a generator and in air. The tests used to evaluate product included visual color and chlorophyll determinations; organoleptic evaluation; plate counts; pH measurements and nitrogen analyses.

Oxygen concentration had to be reduced to 2% to decrease respiration to 60% of that in air, while carbon dioxide had little or no effect on respiration. The greatest advantage of controlled-atmosphere storage for beans lies in improving the color of stored product by retarding chlorophyll breakdown.

Flavor and texture scores and microbiological studies showed no differences between controlled-atmosphere and air storage.

INTRODUCTION

It has been known for a long time that controlled atmospheres consisting of relatively high carbon dioxide concentrations and low oxygen levels retard undesirable post-harvest changes in apples and certain other fruits in refrigerated storage. In the past forty years, many experiments have been carried out on controlled-atmosphere (CA) storage of apples, pears, and other fruits. This work has resulted in successful CA storage practices for several fruit products. However, only a very limited amount of work has been conducted in the area of CA storage of green beans. In light of the increasing importance of commercial CA storage and the development of generators used in the production of artificial atmospheres, an investigation was undertaken of quality changes in green beans stored in CA.

Post-harvest storage results in a general reduction in the quality reflected by a loss of water (transpiration), biological oxidation (respiration), various chemical changes, and physiological breakdown. Microorganisms are also important in that they cause the deterioration of plant material in storage.

A storage temperature of approximately 45°F in air was found to be optimum for green beans (Lewis, 1958; Platenius *et al.*, 1934), while the optimum relative humidity was 85–90%. Guyer and Kramer (1950) found a significant loss in the green color of green beans stored 10 days in air at 50–70°F. The loss in color was not significant at 34°F. Kramer *et al.* (1949) showed a loss of chlorophyll in asparagus at an air storage temperature above 50°F after four days. Lieberman and Hardenburg (1954), working with broccoli at 75°F, found that the presence of some oxygen could cause yellowing and that CO_2 retarded yellowing. Lyons and Rappaport (1962) found no change in the color of Brussels sprouts over a 16-day storage in air at 32°F. At 41 and 50°F, an increase in carbon dioxide at 21% oxygen delayed the loss of chlorophyll. A lowering of the oxygen content below 10%, in the absence of carbon dioxide, was also effective in retention of the green color. The combination of increased carbon dioxide and reduced oxygen concentration was slightly more beneficial in maintaining quality than the same concentrations applied independently.

James (1953) stated that yellowing is caused by a breakdown of chlorophyll. He suggested that breakdown of the protein which is attached to the chlorophyll molecule within the chloroplasts removes the natural protection it affords the chlorophyll. The chlorophyll is then labile. Michael (1935) and Wood *et al.* (1943) showed a similar protein-chlorophyll relationship in regard to the yellowing of excised grass and leaves.

Respiration, as measured by carbon dioxide output, does not give a clear indication as to what specific chemical and quality changes are occurring within the tissue. In Brussels sprouts, Lyons and Rappaport (1959) found that the total carbon dioxide respired was an indication of quality. However, no general rule exists as to the relation-

ship between respiration and quality; and each product should be considered individually.

Few investigators have studied respiration as a function of carbon dioxide tension because of the difficulties involved in measuring small changes in relatively high concentrations of carbon dioxide. Platenius (1943) studied the effect of oxygen on respiration of various vegetables and found that reducing the oxygen to 4.4% at 20°C had little effect on snap beans. No sign of anaerobic respiration appeared even when the oxygen concentration was as low as 0.8%. In the air storage of green beans, Parker and Stuart (1935) showed that over a four day storage period only a very slight change in nitrogen distribution occurred. Platenius (1943) found that protein breakdown in asparagus was less as the level of oxygen in the storage atmosphere was lowered.

Thornton (1933) determined the pH of the saps of various living tissues exposed to various concentrations of carbon dioxide. Contrary to expectation, when carbon dioxide was increased in the presence of normal oxygen concentrations, the alkalinity of the sap increased. Fife and Frampton (1935) concluded that the effect of carbon dioxide in raising the pH was due to a release of ammonia from organic nitrogen compounds.

This paper covers the effects of air and CA as well as other factors on the storage characteristics of whole green beans. Visual color, chlorophyll determinations, organoleptic evaluations and plate counts were used in this study to determine the effect of controlled atmosphere on the quality of green beans. Respiration studies, nitrogen measurements, and pH determinations were carried out in an attempt to gain some insight on the mechanisms of the actual changes which were occurring.

MATERIALS AND METHODS

The majority of green beans used in this project were Harvester variety grown in the Pompano area of Florida and shipped to Urbana by air express. They always arrived within 36 hr of being picked. Lot 4, Harvester beans, and lot 5, Tendergreen beans, were grown on the University of Illinois farm. The beans were sorted immediately after receipt, and all beans larger than No. 5 and smaller than No. 3 were removed. Also, any discolored and infected beans were discarded. After this operation the initial sample was taken and the beans were placed in the various storage conditions.

In the studies designed to determine the effect of various carbon dioxide and oxygen levels, 250 g of beans were placed on a suspended perforated stainless-steel disk in brown 4-L glass bottles fitted with rubber stoppers. When 0% carbon dioxide was desired, KOH solution was held below the stainless disk.

The gas mixtures were obtained with commercial cylinders of high-purity nitrogen, carbon dioxide, and oxygen. Each tank was connected in series to two pressure-reducing valves and a flow manometer. Flow rates could be held relatively constant over a long period, and it was possible to duplicate the gas mixture to 0.1% as measured on the Orsat (Haldane Cabinet Model VC, Burrell Corp., Pittsburgh, Penn.) apparatus.

Gas samples were taken from the product sample bottles using 50-ml-displacement sample tubes. All lines were thoroughly flushed before the sample was taken. The Orsat apparatus was used to determine oxygen and carbon dioxide contents. The confining liquid in the sampling tubes and the Orsat apparatus was mercury. The product sample bottles were flushed every 12–18 hr to maintain the desired gas concentration. A maximum of 1.5% increase in carbon dioxide and decrease in oxygen was thus obtained.

For comparison of air and CA storage, the whole green beans were weighed into small wire baskets. The baskets, containing about 6 lb each, were then placed in air and CA chambers at 45±1°F with the relative humidity at 90–95%. The 10% CO_2–3% O_2 (10–3) atmosphere in the CA chamber was continuously maintained by a small commercial-type CA generator (Tectrol Division, Whirlpool Corp., Benton Harbor, Michigan).

The beans to be processed as frozen product were snipped and cross-cut into one-inch lengths. This was followed by a wash in cold water, steam-blanching for 2 min at 212°F, and cooling immediately to 70°F in cold water. The beans were then drained, packaged, frozen, and stored at −40°F. Unblanched beans needed for various chemical analyses were snipped, packaged in aluminum-foil containers, frozen, and stored at −40°F.

Prior to extraction for chlorophyll analysis, both blanched and unblanched beans were freeze-dried to a moisture content of about 4%. Four 80%-acetone extractions were made starting with an accurately weighed 5-g sample of dried bean powder. After each addition of acetone the mixture was centrifuged for 5 min at 30,000 rpm, and the liquid was decanted and filtered through fritted glass. The

final volume of the extract was adjusted to 250 ml. A 1:1 80% acetone dilution of the extract was read at 663 and 645 mμ on a Beckman DU spectrophotometer. The chlorophyll values were then calculated with the equation of Mackinney (1941).

The method of Sweeney and Martin (1961) was used to calculate the amount of pheophytin present. Three hours after adding the oxalic acid the samples were read at 642.5, 558, 560, and 535 mμ. The results of this method were checked with those of Tan and Francis (1962), and agreement between the methods was good.

Total and soluble nitrogen were determined by Nesslerization (Umbreit *et al.*, 1957). The beans were blended with 200 ml of water for 3 min in a Waring blender. One aliquot of the resulting suspension was diluted and used to determine total nitrogen. Another aliquot was added to an equal portion of 20% trichloroacetic acid (TCA) and placed in an ice bath for 15 min. The TCA solution was then centrifuged, and the TCA-soluble or non-protein nitrogen content of the supernatant was determined.

The pH of the beans was determined with a Beckman glass electrode pH meter, model H-2 (National Technical Laboratories, South Pasadena, California). About 100 g of beans were ground twice in a food chopper. Juice was obtained with a hand press and used to determine pH.

A panel (10 members from the Department of Food Science) organoleptically evaluated blanched and frozen samples. The samples were defrosted and examined for color. They were then cooked in boiling 2% salt water for exactly 10 min, and graded for flavor, texture, and color. Samples were scored on a basis of 1 to 9, with 9 excellent, 5 just acceptable, and 1 completely unacceptable.

Plate counts for bacteria and molds were made by standard methods for analysis recommended by the Am. Public Health Assoc. (1948).

RESULTS AND DISCUSSION

Cold damage. Previous investigations (Lewis, 1958) on green beans stored in air showed that this product is subject to low-temperature damage. When green beans were stored at temperatures below 45°F and then transferred to room temperature (70°F), russeting generally developed on the beans within one day. The effect of temperature on the quality of beans stored in 10% CO_2 and 3% O_2 and in air was studied. No russeting developed in beans stored in either air or controlled atmosphere at 45°F and subsequently transferred to 70°F for one day. However, below 45°F the beans developed russeting after removal from both air and CA storage to room temperature. Thus, since CA storage did not eliminate cold damage, all further studies were made at 45°F.

Studies in bottles. *Respiration.* The respiration rate is a good measure of the metabolic activity of the product. Since metabolic rate during storage generally parallels loss of quality, it was of interest to measure the respiration of green beans stored in bottles under various concentrations of carbon dioxide and oxygen. The rate was expressed as output of carbon dioxide per unit of time per unit product weight. Table 1 gives the average rates in the various atmospheres for an 8-day storage of two lots. To allow for acclimation of the product to the atmosphere, all data through the first day were excluded. It is obvious from Table 1 that lot 7 respired at a higher rate than lot 6. However, good agreement between lots is shown by the figures in parentheses. These are relative rates based on the rate in 20% oxygen and 0% carbon dioxide taken as 100.

Table 1 clearly demonstrates that reduction of the oxygen concentration from 20 to 10% did not reduce respiration rate appreciably. At 5% oxygen the rate was reduced

Table 1. Effect of oxygen and carbon dioxide concentrations in the atmosphere on respiration rate of Harvester variety green beans from Florida averaged over an eight-day period. (Numbers in parentheses are relative rates based on "air" as 100.)

	Carbon dioxide (%)	Oxygen (%)			
		20	10	5	2
		Respiration rate, mg CO_2/hr/kilo			
Lot 6	0	40.2(100)	39.9(98)	33.2(83)	25.6(64)
	5	34.7(86)	33.9(84)	30.4(76)	23.3(58)
	10	40.6(101)	39.6(99)	30.5(76)	25.2(63)
Lot 7	0	55.0(100)		46.3(84)	34.5(63)
	5	48.1(87)		38.4(70)	33.6(61)
	10	52.8(96)		44.6(81)	32.7(58)

to about 80%, and at 2% oxygen to about 60% that at the 20–0 gas composition. These results are in agreement with those of Platenius (1943).

The effect of carbon dioxide on respiration (Table 1) was more complicated. The respiration rates at 5% CO_2 were generally lower than those for 0 and 10% CO_2 at 20% and 10% O_2. The reason why the 5% CO_2 depressed the rate of respiration more than the 10% level of CO_2 is not obvious. A special study would be needed to explore this further. At low O_2 levels, 5% and 2%, this trend of the depressing effect of 5% CO_2 is not consistent. Probably the lower oxygen level is more important in suppressing respiration rate than a higher carbon dioxide level. Therefore, it is concluded that, within the range studied, carbon dioxide exerted little effect on the respiration rate of the green beans.

Color. One of the most important changes affecting the quality of green beans during storage is that of color. Two methods for the measurement of color were used: chlorophyll determination and visual evaluation. Storage time was 14 days.

Data from the chlorophyll determinations are shown in Fig. 1. The sample stored in air retained 68% of the original, or 0-day, chlorophyll content. The sample stored at 20% oxygen and 0% carbon dioxide, a composition close to that of air, showed a similar retention. All other samples showed much higher chlorophyll contents, with retentions of 84–96%. The reduction of oxygen to 5 and 2% improved retention at 0% carbon dioxide. Both the 5 and 10% carbon dioxide levels were more effective in preserving chlorophyll than the 0% carbon dioxide level. It was concluded that the presence of carbon dioxide in the storage atmosphere is the important factor in preventing chlorophyll degradation.

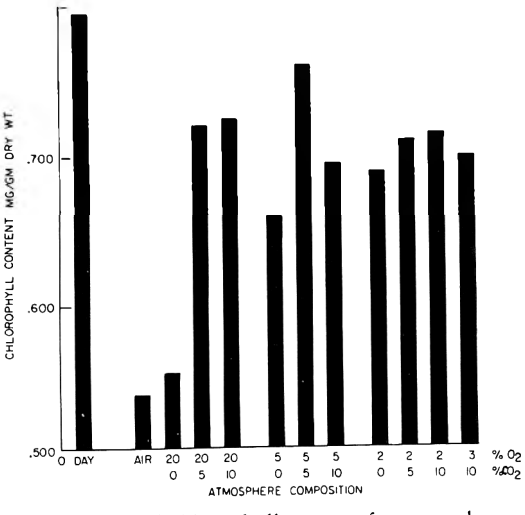

Fig. 1. Total chlorophyll content for green beans stored under various atmospheres.

Duplicate sets of blanched bean samples were evaluated visually for color. Each set of samples was presented to each panel member in three booths. For the first set of samples, each booth represented a single level of carbon dioxide with oxygen concentration as the variable, and for the second set, each booth held the samples stored under the same oxygen level but with different carbon dioxide concentrations.

Before considering the visual color data (Fig. 2) it should be noted that the only valid comparisons are among samples rated in the same booth. For example, when scores for individual samples in Booth 1 of Fig. 2 are compared, it will be noted that the 2% O_2 and 0% CO_2 sample rated nearly 9, compared to about 5.5 for the air and 20% O_2 and 0% CO_2 samples. It is believed that the high value given the sample held in 2% O_2 and 0% CO_2 was due to the fact that the other samples in this booth were substantially inferior by comparison. With samples in Booth 6, (Fig. 2), the 2% and the 0% CO_2 sample rated about 7.5, which is less than that of the other samples held under low oxygen and 5 or 10% CO_2, which is about 1.5 organoleptic units less than the value given this same sample in Booth 1. Fig. 2 also shows that air and atmospheres containing 20% O_2 and 0% CO_2 are generally inferior to other modified atmospheres. The effect of oxygen and carbon dioxide in the atmosphere is not shown as clearly by visual color data as by chlorophyll values. However, the favorable effect of carbon dioxide at 5 or 10% concentration is evident when in combination with 5% or less oxygen content.

CA storage of green beans. Results on respiration and color reported above were obtained by storage of beans in bottles with

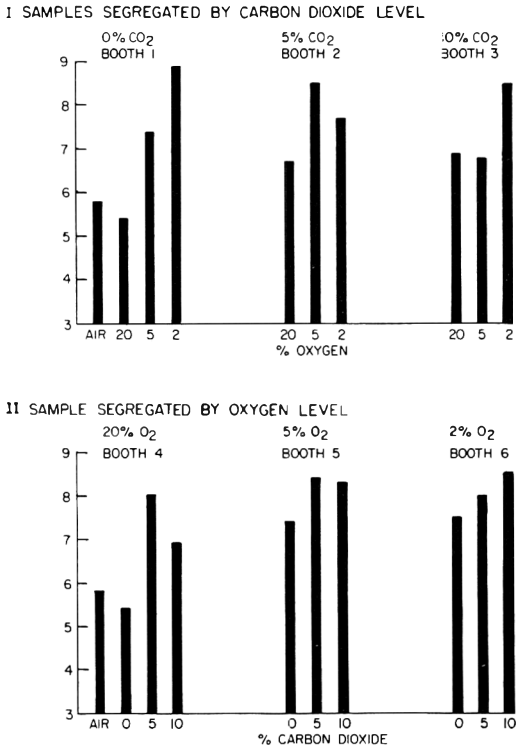

Fig. 2. Organoleptic color scores for green beans stored under various atmospheres for 14 days (Harvester variety, Florida).

intermittent flushing to restore the desired atmosphere. It was desired to expand this work to include continuous control of the atmosphere with a CA gas generator. The gas composition could be regulated over a substantial range. An atmosphere of 10% carbon dioxide and 3% oxygen was chosen on the basis of the results reported above. Control samples were stored in air at the same temperature.

Color. Four lots of beans were evaluated for color. All samples had been blanched and frozen after storage for periods to 15 days. The samples were examined after thawing, and again after cooking in preparation for serving. In most of the individual samples there was considerable variation in the green color. In the 15-day air-stored samples, which generally appeared quite yellowish, about 5% of the beans still possessed a good green color.

Table 2 indicates a marked loss in green in air-stored beans over a 15-day period; at least a slight change was always noticeable after 5 days. The CA-stored beans showed a definite loss of color in lot 2, a slight loss in lot 4, and no loss in lots 3 and 5. However, in every lot, CA storage was superior to air storage, usually by a wide margin. The data for the cooked beans (Table 2) were generally similar to those for the blanched beans. Since blanched beans were not presented to the panel in the same evaluation booth with the cooked beans, the data for each are not directly comparable.

Chlorophyll. Four lots of beans were analyzed for total chlorophyll, and the data for a typical lot are presented in Fig. 3. These data indicate a much greater loss of chlorophyll in air-stored samples than in CA samples, confirming the visual data. The amounts of chlorophyll degradation in the CA samples varied with the different lots of beans. It is believed that growing conditions and ma-

Table 2. Visual color scores for green beans stored in air and controlled atmosphere for periods to two weeks.

Storage time (days)	Lot 2 Harvester, Fla.		Lot 3 Harvester, Fla.		Lot 4 Harvester, Ill.		Lot 5 Tendergreen, Ill.	
	Air	CA	Air	CA	Air	CA	Air	CA
Blanched beans								
0	8.4	8.4	7.3	7.3	8.7	8.7	8.7	8.7
5	6.6	8.6	7.1	8.0	8.0	8.6	7.4	8.5
10	6.9	7.0	5.7	8.8	6.4	8.5	5.2	8.7
15	4.9	6.4	3.9	7.9	5.7	7.5	5.1	8.2
Cooked beans								
0	7.6	7.6	5.9	5.9	8.4	8.4	8.6	8.6
5	6.5	8.2	5.4	8.2	7.3	8.4	7.4	8.6
10	5.2	7.6	5.4	7.7	6.4	8.5	5.1	8.3
15	4.7	6.1	3.2	7.9	5.8	8.0	5.3	8.3

turity account for this difference. In some of the air samples an off-green color, possibly caused by pheophytin formation, was noted after blanching.

Chlorophyll degradation. The method of Sweeney and Martin (1961) was used to determine the conversion of chlorophyll to pheophytin. The results in Table 3, expressed as percent chlorophyll converted to pheophytin, indicated that a small percent of pheophytin was formed in the fresh beans. However, this may be present as an artifact. The amount of pheophytin present appears to depend on the lot of beans, on the storage time, and the atmosphere. Conversion of chlorophyll to pheophytin was greater in the samples stored 10 and 15 days in air and then blanched.

Although no conclusive data were obtained linking nitrogen metabolism with chlorophyll degradation, it was shown that there was a definite increase in the non-protein nitrogen content in the air-stored beans while a much smaller increase occurred in the CA-stored beans. Also, green plant material, when blended with 0.5*M* sucrose solution, forms a suspension of nuclei and chloroplasts which is less stable as chloroplast breakdown increases (Rabinowitch, 1945). Such suspensions from the 10- and 15-day air-stored samples were very unstable, thus indicating chlorophyll degradation, while those from all the CA-stored samples were quite stable.

It was also of interest to examine the data to see if the rate of respiration was related to chlorophyll breakdown. The respiration rate of the sample in CA storage was about 35% lower than that in the air samples. It is also shown that the chlorophyll retention was much greater in the CA samples (Fig. 3). A lowering of the oxygen concentration also reduced both the rates of respiration and chlorophyll degradation. The respiration rate at 20% O_2 and 10% CO_2 was nearly identical to that at 20% O_2 and 0% CO_2; however, the color data indicated a greater chlorophyll degradation in the latter sample.

Flavor and off-flavor. Flavor and off-flavor scores indicated that the CA samples were generally superior to the air samples. However, these scores seemed to follow the visual color scores which were obtained simultaneously. A separate test for flavor and off-flavor was then conducted under a very dim light. These results (Table 4) showed that the panel did not prefer CA- over air-stored samples in regard to flavor and off-flavor. Texture scores are not presented, because they also showed no differences between CA and air storage.

pH changes. pH determinations of lots 1, 2 and 6, tabulated in Table 5, showed that the pH of the CA samples increased by about 0.5 unit in two weeks, while that of the air samples remained fairly constant. These results are in agreement with those reported by Thornton (1933) and Fife and Frampton (1935).

Table 3. Pheophytin formation in green beans stored under air and CA atmospheres for various times as measured both before and after blanching.

	Storage time (days)	Atmosphere	Pheophytin formation (%)	
			fresh beans	blanched beans
Lot 4 Harvester, Ill.	0		6	13
	5	CA	3	16
		Air	7	16
	10	CA	5	16
		Air	10	26
Lot 6 Harvester, Fla.	0		2	12
	5	CA	1	10
		Air	1	13
	10	CA	1	10
		Air	3	18
	15	CA	1	10
		Air	3	20

Table 4. Flavor and off-flavor scores for green beans stored in air and CA for various times. Evaluation conducted under dim light to eliminate color prejudice.

Storage time (days)	Lot 2 Harvester, Fla.		Lot 4 Harvester, Ill.	
	Air	CA	Air	CA
Flavor scores				
0	7.4	7.4	7.0	7.0
5	6.3	7.4	7.1	7.3
10	6.8	7.1	7.2	6.3
15	6.6	6.8	7.1	7.0
Off-flavor scores				
0	7.3	7.3	6.8	6.8
5	6.4	7.1	7.4	7.3
10	6.7	7.0	6.9	6.0
15	6.6	6.6	6.8	7.1

Changes in soluble nitrogen. It has been reported that a relationship exists between chlorophyll breakdown and protein content (Wood *et al.*, 1943). Fig. 4 indicates the percent change in the fraction soluble in trichloroacetic acid (TCA) from that of the initial or zero-day sample with storage time and atmosphere. Each point represents the average of three samples analyzed in duplicate. The standard deviation was $\pm 1.7\%$. Thus, even though the variation is fairly large, the data showed more soluble nitrogen in the air samples than in the CA samples after 10 and 15 days of storage. The CA data also indicated the possibility of a decrease in TCA-soluble nitrogen between 5 and 10 days of storage.

Other chemical and microbiological changes in CA- and air-stored beans. Carbohydrate analyses were performed for reducing sugar, sucrose, dextrins, and acid-hydrolyzable fraction. The results showed the variation within a given sample to exceed the difference between the air and CA treatments.

The titratable acid and total acid anion content increased during storage in air and

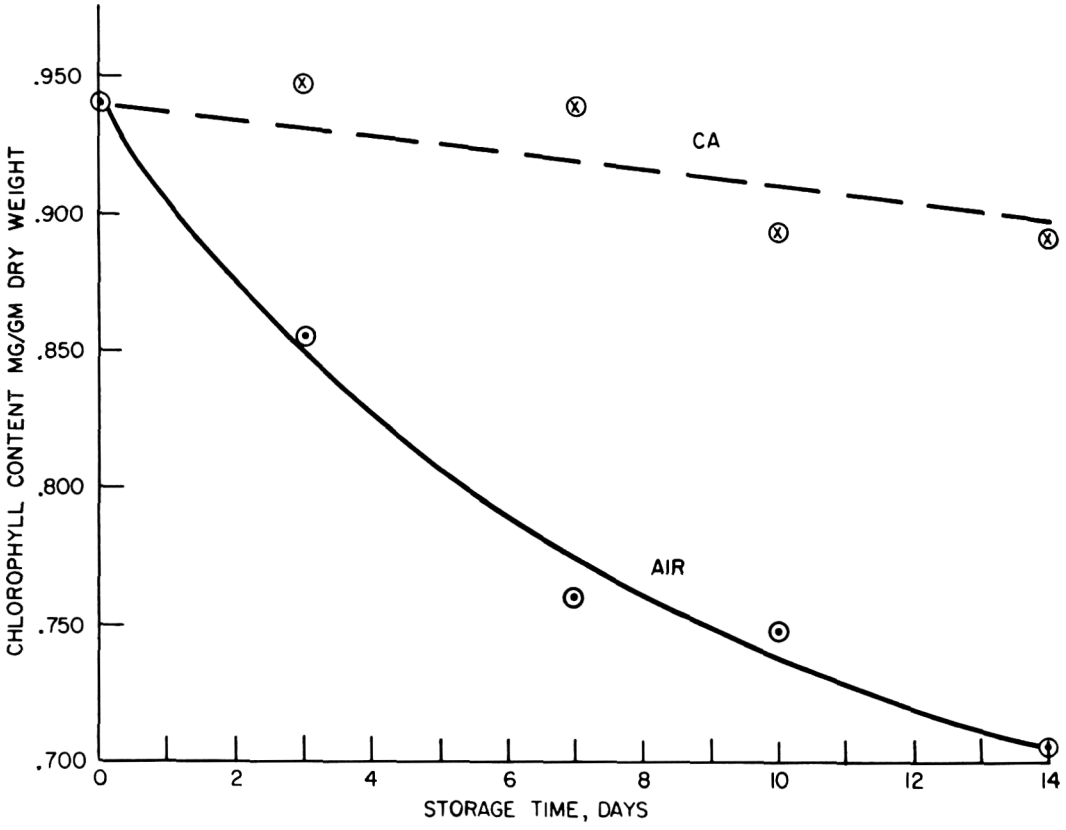

Fig. 3. Effect of storage for 14 days in air and in CA on chlorophyll content of green beans (Harvester variety, Florida).

Table 5. Changes in the pH units of Harvester variety green beans stored for various times in air and CA.

Storage time (days)	Lot 1		Lot 2		Lot 6	
	Air	CA	Air	CA	Air	CA
0	6.1	6.1	6.0	6.0	6.2	6.2
3	6.2	6.2	6.2	6.4	....	....
5	....	....	....	....	6.2	6.5
7	6.2	6.4	6.4	6.4	....	....
10	6.3	6.6	6.2	6.3	6.2	6.6
14	6.2	6.7	6.2	6.7	....	....
15	....	....	....	....	6.2	6.7

decreased slightly in CA. A general increase of acid content rather than a specific increase in any single acid was indicated by the results of a study in which the organic acids were separately determined.

The results of the microbiological studies indicated no real differences between the CA and air treatments in total plate count or potato agar (mold) plate count over a one-week period. Supplementary visual data on mold growth in lots 2 and 3 showed that some mold growth was noticeable under both storage conditions. However, slightly less growth was found in the CA samples than in the air-stored samples.

Usefulness of CA storage for green beans. The primary advantage of CA storage for beans is prevention of the fading of the green color. This is a real advantage for both fresh-produce handlers and freezing companies. At 45°F, visual differences were apparent after approximately 4–5 days. The

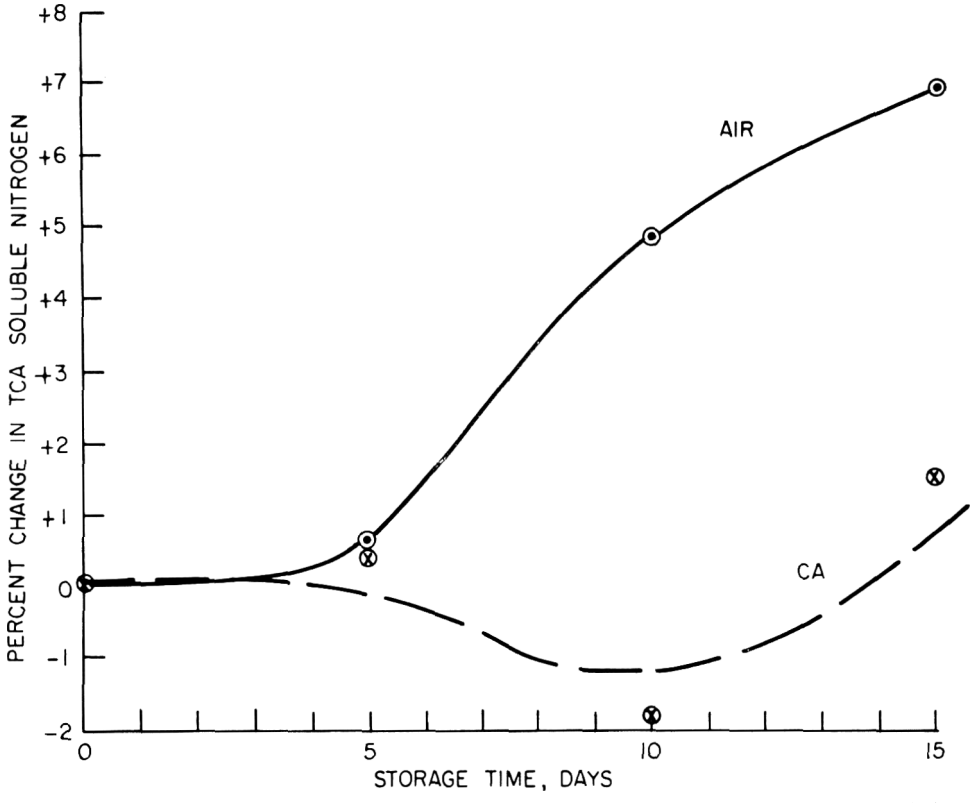

Fig. 4. Change in acid-soluble nitrogen content of green beans during storage for 15 days in air and in CA.

differences became greater with time until the maximum storage period of approximately two weeks was reached. The flavor and texture remained nearly constant for the entire storage period.

The optimum atmosphere for storage of green beans would be low in oxygen content, 2–3%, with 5–10% in carbon dioxide. It seems unlikely that higher concentrations of carbon dioxide would improve the product at low oxygen levels and that carbon dioxide injury could result.

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The Effect of Pre-Slaughter Environmental Temperature and Post-Mortem Treatment Upon Some Characteristics of Ovine Muscle. I. Shortening and pH

SUMMARY

A study was made of the effect of various postmortem incubation temperatures upon some physical and chemical characteristics of ovine muscle obtained from animals subjected, before slaughter, to various environmental temperatures. Unfrozen and pre-rigor frozen muscles were studied.

Pre-slaughter treatment did not significantly affect muscle shortening or pH after 24 hr of incubation of unfrozen or pre-rigor frozen muscle. Incubation temperatures significantly influenced the rate of change of pH and shortening as well as the final values obtained after 24 hr. These values were also affected by pre-rigor freezing and subsequent thawing. Minimum shortening in unfrozen muscle occurred at incubation temperatures between 5 and 20°C. For pre-rigor frozen muscle, shortening values increased with increasing thawing temperatures. Unfrozen and pre-rigor frozen muscle incubated at temperatures between 0 and 40°C showed similar pH patterns after 24 hr, with minimum values for temperatures between 5 and 20°C.

INTRODUCTION

Two major factors cited as affecting the post-mortem biochemical and biophysical properties of muscle, and ultimate meat quality, are variable pre-slaughter temperatures and post-mortem storage temperature. Hall *et al.* (1944) observed that calves adapted to mild environmental temperatures often showed dark muscles after being exposed to extreme temperatures immediately prior to slaughter. Ludwigsen (1954), Judge *et al.* (1959), and Wismer-Pedersen (1959) have shown a significant relationship between high ante-mortem environmental temperatures and the occurrence of soft watery pork muscle. More recently, Forrest *et al.* (1963) have shown a similar effect due to extreme diurnal temperature fluctuation.

The rate of post-mortem biochemical changes in muscle, which in the pig can be altered by subjecting the live animal to elevated pre-slaughter temperatures or the carcass to variations in storage temperature, can have a profound effect on the chemical (Bate-Smith, 1948; Bendall, 1951; Marsh, 1954; Bate-Smith and Bendall, 1956; Sayre *et al.* 1963a,b, 1964; Sayre and Briskey, 1963) and biophysical or structural properties of muscle (Bendall, 1951; Marsh, 1954; Locker and Hagyard, 1963; Cassens *et al.*, 1964; Cook and Wright, 1966).

Most of the intensive work on the effect of pre- and post-slaughter temperatures upon post-mortem changes in muscle have been done on rabbits and pigs, with little done on sheep. The experiment described in this paper was designed to investigate the effects of a range of pre- and post-slaughter temperature treatments upon some chemical and physical characteristics of ovine muscle.

EXPERIMENTAL

Pre-slaughter treatments. Twelve Dorset Horn × Border Leicester-Merino lambs were allocated at random to 3 pre-slaughter treatment groups, each of 4 lambs. Each group was held for 48 hr prior to slaughter at one of the following temperatures (±2°C): 0, 21, and 38°C.

Post-slaughter treatments. The following post-slaughter measurements were made: 1) Muscle fiber shortening and pH of muscle mince of unfrozen semitendinosus incubated at 0, 5, 10, 15, 20, 30, and 40°C for 0, 1, 2, 4, 8, 12, and 24 hr post-mortem. 2) Muscle fiber shortening and pH of muscle mince of pre-rigor frozen biceps femoris after incubation for 24 hr at 0, 5, 10, 15, 20, 30, and 40°C.

Methods. Within 10 min of slaughter, both semitendinosus and biceps femoris muscles were removed and cut into strips, parallel to the long axis of the fibers, as outlined by Locker and Hagyard (1963). Seven strips of biceps femoris which had previously been attached to a thin rod at either end in order to maintain rest length were placed in sealed plastic bags and frozen in a dry ice-ethanol mixture for 40 min. The strips of frozen muscle,

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together with 7 strips of unfrozen semitendinosus muscle, were incubated for 24 hr at 0, 5, 10, 15, 20, 30, and 40°C under an atmosphere of moist nitrogen. The unfrozen samples were placed in the incubation chambers within 20 min of slaughter.

Percent muscle shortening was calculated by measuring the rest lengths of the muscle strips prior to incubation (20 min post-mortem) and after the various time-temperature treatments.

Muscle pH values were determined on ground samples of unfrozen and pre-rigor frozen muscle by the method of Locker and Hagyard (1963) with a Radiometer 25 expanded-scale pH meter. The initial pH values of unfrozen muscle were evaluated within 15 min of slaughter, whereas the pH values of the pre-rigor frozen mince were determined by placing a 1-g sample of frozen muscle in 10 ml of 0.005*M* sodium iodoacetate (pH 7.0) and recording the pH of the sample after it had thawed.

Prior to incubation and grinding, all samples were dipped in a 10.0-ppm solution of chlortetracycline to inhibit bacterial growth.

The data were statistically analyzed with a mixed nested design according to Steel and Torrie (1960). Multiple-range analyses were done by the method of Duncan (1955).

RESULTS AND DISCUSSION

Tables 1 and 3 present the mean percent shortening values. For simplicity, data either for post-slaughter or pre-slaughter treatments are pooled. Tables 2 and 4 present data for ultimate (24 hr) pH, similarly pooled. Tables 5 and 7 are analyses of variance of the full data for shortening and pH.

Pre-slaughter treatment. The percent shortening values and ultimate pH decreased with increase in pre-slaughter temperatures (Tables 1 and 2), but these differences failed to attain statistical significance. The initial pH values were indistinguishable for all pre-slaughter treatments (Table 2). These results differ from those obtained in the pig by Sayre *et al.* (1963a,b) and Briskey (1963). It appears that treatments such as those imposed in this experiment do not depress muscle glycogen sufficiently to affect ultimate pH, as can be achieved in cattle and sheep by the pre-slaughter administration of chemical agents (Hedrick *et al.*, 1959, 1961; Forrest *et al.*, 1964).

Table 1. Mean percent shortening values of unfrozen and pre-rigor frozen muscle at 24 hr post-mortem, grouped according to pre-slaughter treatment.[a]

Treatment and muscle	Pre-slaughter temperature		
	0°	21°	38°
Unfrozen	30.07	29.51	27.71
Pre-rigor frozen	65.17	61.60	60.00

[a] Data pooled for post-slaughter incubation temperature treatment within each pre-slaughter treatment.

Post-slaughter treatment. *Percent muscle shortening after 24 hr.* Post-mortem incubation temperature had a highly significant effect ($P<0.001$) on 24 hr shortening values of both unfrozen and pre-rigor frozen muscle (Tables 3, 5; Fig. 1).

The percentage shortening of frozen muscle increased rapidly from 0° to 5° to 10°C ($P<0.05$), more slowly to 15°C ($P<0.05$), and thereafter did not alter significantly, although a slight increase is evident from 69.5% shortening, at 15°C, to a maximum of 72.2%, at 40°C. There was a significant interaction ($P<0.01$) between incubation temperature and pre-slaughter treatment (Table 5). Examination of the mean effects of post-mortem incubation temperature and pre-slaughter treatment showed that the degree of fiber shortening increased with decreasing pre-slaughter temperatures (Table 6).

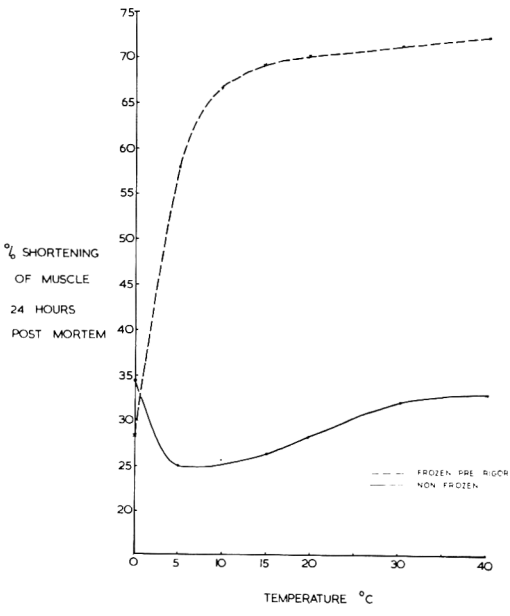

Fig. 1. The percentage of shortening of pre-rigor frozen and unfrozen muscle strips incubated for 24 hr post-mortem at different temperatures.

Table 2. Mean pH values of unfrozen and pre-rigor frozen muscle minces, at slaughter and 24 hr post-mortem, grouped within each pre-slaughter treatment.[a]

Treatment and muscle	Pre-slaughter temperature								
	0°			21°			38°		
	Initial 24 hr		Total decrease[b]	Initial 24 hr		Total decrease[b]	Initial 24 hr		Total decrease[b]
Unfrozen semitendinosus	6.72	6.24	0.48	6.71	6.12	0.59	6.71	6.11	0.60
Pre-rigor frozen biceps femoris	7.07	6.20	0.87	6.99	6.08	0.91	6.88	6.03	0.85

[a] Means of four animals pooled within each pre-slaughter treatment.
[b] Initial pH minus final pH.

Table 3. Mean percent shortening values of unfrozen and pre-rigor frozen muscle at 24 hr post-mortem, grouped within each incubation temperature treatment.[a]

Treatment and muscle	Incubation temperature[b]						
	0°	5°	10°	15°	20°	30°	40°
Unfrozen semitendinosus	33.6[a]	25.4[b]	26.0[b]	26.1[b]	28.2[b]	32.2[a]	32.6[a]
Pre-rigor frozen biceps femoris	28.2[a]	54.5[b]	67.2[c]	69.5[c, d]	70.3[c, d]	71.7[d]	72.2[d]

[a] Means of four animals pooled within each pre-slaughter treatment.
[b] All means within a treatment followed by the same postscript do not differ significantly. All others differ significantly ($P<0.05$).

Table 4. Mean pH values of unfrozen and pre-rigor frozen muscle minces at 24 hr post-mortem, grouped within each incubation temperature treatment.[a]

Muscle	Initial pH	Treatment: 24-hr pH incubation temperature[b]						
		0°	5°	10°	15°	20°	30°	40°
Unfrozen semitendinosus	6.72[a]	6.18[b]	6.15[b]	6.07[b]	6.09[b]	6.07[b]	6.45	6.89[a]
Pre-rigor frozen biceps femoris	6.97[a]	6.21[c]	6.01[d]	5.91[d]	5.93[d]	5.96[d]	6.18[c]	6.49[b]

[a] Means of four animals pooled within each pre-slaughter treatment.
[b] All means within a treatment followed by the same letter do not differ significantly. All others differ significantly ($P<0.05$).

Table 5. Analysis of variance of data for percent shortening at 24 hr post-mortem of strips of unfrozen semitendinosus and pre-rigor frozen biceps femoris muscles.

Source of variation	Degrees of freedom	Mean squares	
		Unfrozen semitendinosus	Pre-rigor frozen biceps femoris
Pre-slaughter treatment	2	51.18	235.87
Animals within pre-slaughter treatment	9	206.08***	194.81***
Incubation temperature	6	158.95***	3100.21***
Incubation temperature × pre-slaughter treatment	12	24.51	68.11**
Incubation temperature × animals within pre-slaughter treatment	54	16.75	24.74
Total	83		

** $P<0.01$.
*** $P<0.001$.

Table 6. Effect of pre-slaughter treatment and post-mortem incubation temperature upon the percent shortening of pre-rigor frozen muscle.

Pre-slaughter treatment (°C)	Post-mortem incubation temperature (°C)						
	0°	5°	10°	15°	20°	30°	40°
38°C	24.00	56.00	64.00	66.00	68.00	70.00	72.00
21°C	30.40	52.00	67.00	68.00	73.00	72.40	68.40
0°C	30.00	66.00	69.40	73.40	69.00	72.00	76.40

The pattern for the unfrozen muscle was quite different. There was a sharp decrease in shortening, from a value of 33.6% at 0°C to 25.4% at 5°C ($P<0.5$), followed by a slight but nonsignificant rise to 28.2% at 20°C, followed in turn, by a significant rise to a maximum of 32.6% at 40°C ($P<0.05$). No significant difference existed among the 24 hr shortening values for muscle incubated at 0, 30, and 40°C.

Observations on unfrozen muscle are in agreement with those of Locker and Hagyard (1963), who observed minimum shortening of unfrozen beef muscle after incubation at 19°C, whereas maximum shortening occurred at 0 and 37°C.

Rate of muscle shortening over a 24 hr period. Fig. 2 shows the rate at which unfrozen semitendinosus muscle strips shortened over the 24 hr post-mortem period when held at various temperatures. The slopes of the curves for 5, 10, 15, and 20°C are indistinguishable. There was very little delay before contraction commenced, at a rate of about 3.5% per hr for 4 hr, reducing to about 1.5% per hr to 8 hr, and continuing thereafter at a reduced rate, stabilizing from 12 hr onward.

The patterns for 0, 30, and 40°C were similar from 8 hr onward, by which time maximum shortening had occurred. Beforehand, however, they differed greatly. At 0°C there was little, if any, delay in the onset of contraction, 16 and 24% being reached in 1 and 2 hr. By 8 hr, maximum shortening of 33.6% had been reached. At 30 and 40°C, a slight delay period was observed before significant shortening was evident. Shortening was most rapid between 1 and 4 hr post-mortem, and ultimate values of 32% were attained by 8 and 4 hr.

The shortening curves illustrated for ovine muscle differ slightly from those of Locker and Hagyard (1963) for beef muscle, possibly because of species differences.

pH values at 24 hr post-mortem. Post-mortem incubation temperature had a highly significant effect ($P<0.001$) on 24 hr pH values of both unfrozen and pre-rigor frozen muscle minces (Tables 4, 7).

Fig. 3 illustrates the mean 24 hr pH values attained at the various incubation tempera-

Table 7. Analysis of variance of data for pH values at 24 hr post-mortem for unfrozen semitendinosus and pre-rigor frozen biceps femoris muscles.

Source of variation	Degrees of freedom	Mean squares	
		Unfrozen semitendinosus	Pre-rigor frozen biceps femoris
Pre-slaughter treatment	2	0.52	0.24
Animals within pre-slaughter treatment	9	0.47***	0.17***
Incubation temperature[a]	7	1.29***	1.60***
Incubation temperature × pre-slaughter treatment	14	0.04	0.01
Incubation temperature × animals within pre-slaughter treatment	63	0.03	0.03
Total	95		

[a] Includes initial pH values.
*** $P<0.001$.

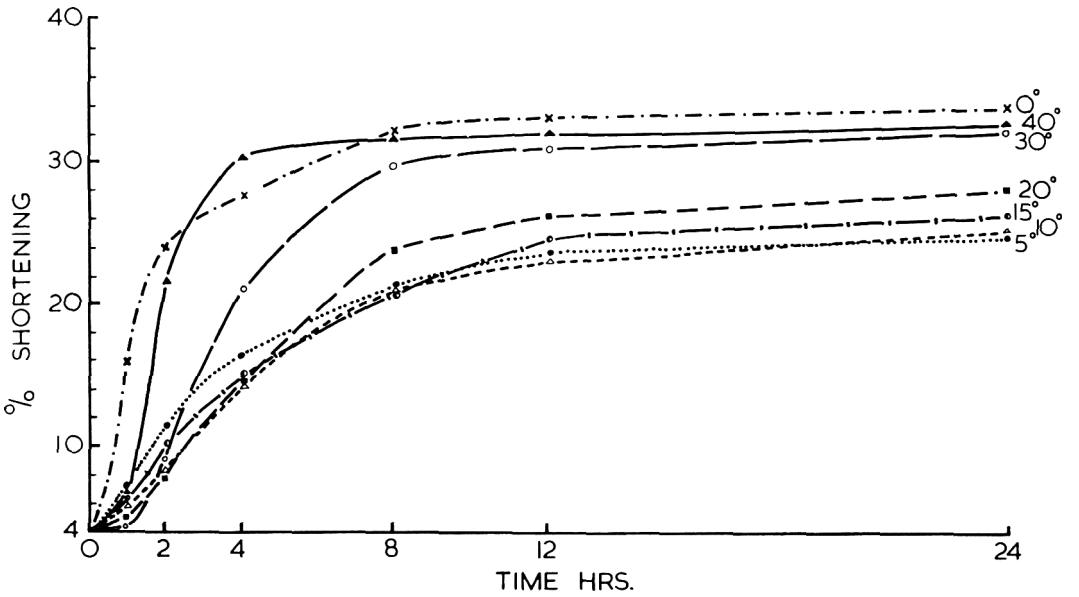

Fig. 2. The rate of shortening of unfrozen semitendinosus muscle during incubation for 24 hr post-mortem at various temperatures.

tures. The slope of the curves for the unfrozen and pre-rigor frozen muscles are similar. The absolute pH values at each incubation temperature for unfrozen and pre-rigor frozen muscle do not differ significantly. The excessively high values noted at 40°C, despite the precautions taken, may have been caused by partial decomposition of the muscle tissue by bacterial action (Ingram, 1965).

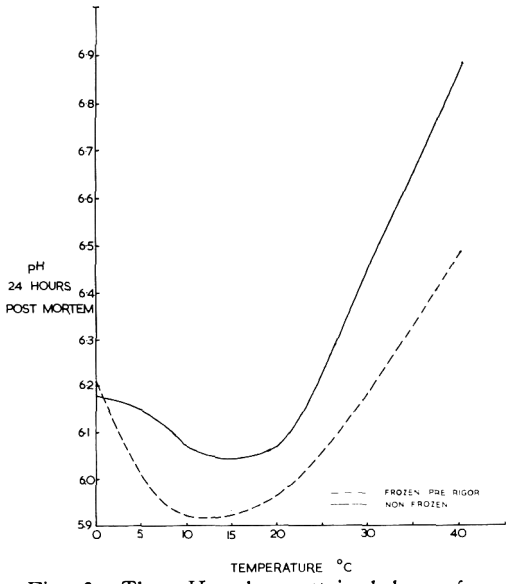

Fig. 3. The pH values attained by unfrozen (semitendinosus) and pre-rigor frozen (biceps femoris) muscles after incubation for 24 hr post-mortem at various temperatures.

Rate of change of muscle pH over a 24-hr period. Fig. 4 shows the rate at which the pH of unfrozen semitendinosus muscle mince changed over a 24-hr post-mortem period, when held at various incubation temperatures. The rate of fall of pH at all temperatures during the first hr post-mortem shows a positive temperature coefficient. Between 1 and 2 hr post-mortem, the slopes of the curves for 0, 5, 10, and 15°C show a marked reduction in the rate of fall. After 2 hr the rate increases to a maximum at 4 hr, continuing thereafter at a reduced rate until the final 24 hr values are reached. The pattern for 20°C was similar to those for 0, 5, 10, and 15°C, except that no reduction in the rate of fall of pH was noted between 1 and 2 hr post-mortem.

The slopes of the curves for 30 and 40°C differed significantly from the others. Minimum pH values at 30 and 40°C were attained 4 and 8 hr post-mortem, after which the values increased steadily at the rate of 0.02 and 0.04 pH unit per hr. A similar phenomenon was reported by Briskey and Wismer-Pedersen (1961) in isolated samples of pork muscle that had been subjected to elevated temperatures during the onset of rigor. Although it is generally accepted that

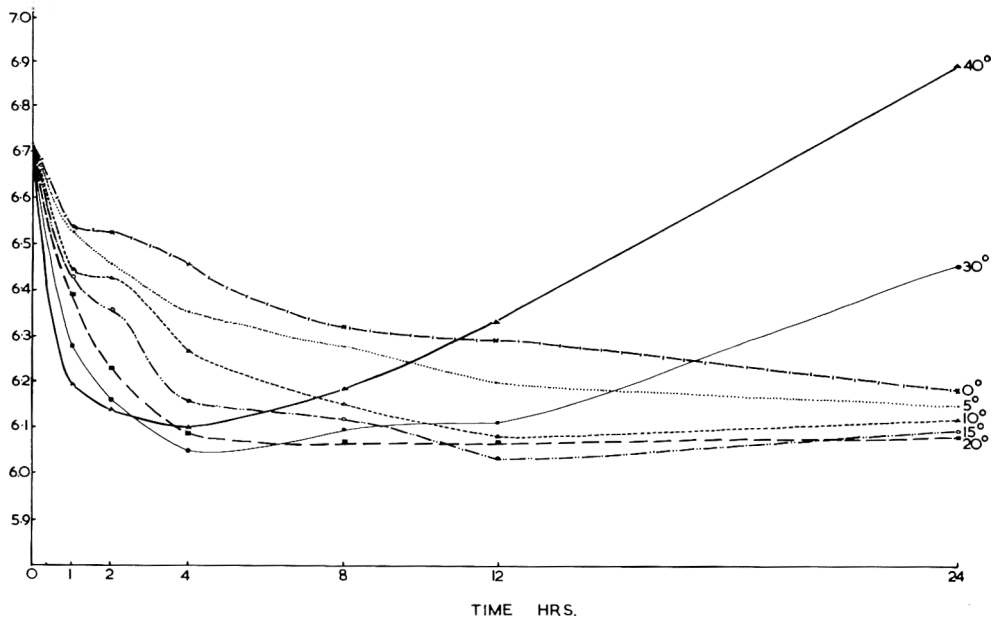

Fig. 4. The rate of change of pH of unfrozen semitendinosus muscle during incubation for 24 hr post-mortem at various temperatures.

the ultimate pH value of muscle is associated with glycogen and lactic acid reserves at the time of slaughter, Bendall (1963) postulated that the increase in ultimate pH observed in muscle subjected to elevated temperatures during the onset of rigor may be due to a decrease in the pK value of histidine.

The curves in Fig. 4 show that the rate of fall of pH, at all temperatures, during the first 2 hr post-mortem is more rapid than expected. These observations were made on muscle minces, rather than on intact muscles. Newbold (1965) showed that the initial rate of glycolysis in muscle minces is approximately 2–3 times as rapid as in intact muscle. Although the initial rate of fall in pH differs significantly between intact muscle and mince, the ultimate values attained are not significantly different.

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The Effect of Pre-Slaughter Environmental Temperature and Post-Mortem Treatment Upon Some Characteristics of Ovine Muscle. II. Meat Quality

SUMMARY

Samples of unfrozen and pre-rigor frozen ovine longissimus dorsi muscle, obtained from animals subjected to various pre-slaughter temperatures, were incubated at temperatures ranging from 0 to 40°C for 24 hr post-mortem.

Pre-slaughter treatment had no effect upon shear value, exudate loss during storage or cooking loss.

Post-mortem treatments—incubation temperature and pre-rigor freezing—each had a significant effect upon all traits examined. The shear values of unfrozen muscle decreased with increasing incubation temperatures from 0 to 10°C, remained constant from 15 to 30°C, and fell for 40°C. By contrast, shear values for pre-rigor frozen muscle increased for temperatures from 0 to 20°C and fell sharply between 20 and 40°C. Exudate and cooking losses increased with increasing incubation temperatures for both unfrozen and pre-rigor frozen muscle. Pre-rigor freezing brought about an increase in exudate and cooking loss.

INTRODUCTION

The rate and extent of post-mortem biochemical and biophysical changes in muscle have been cited as affecting ultimate meat quality. The rate of fall of pH in porcine musculature has been shown to influence its color, texture, flavor, and water-binding capacity (Wismer-Pedersen, 1959; Briskey and Wismer-Pedersen, 1961; Briskey, 1963; Sayre and Briskey, 1963; Sayre *et al.*, 1963a,b, 1964). Locker (1960) and Herring *et al.* (1965) have postulated that beef tenderness is associated with the contracture band patterns of the fibers and the extent of shortening, while Marsh (1963) presented evidence that variations in tenderness of beef muscle are associated with the phase of rigor mortis.

The experiment described in this paper was designed to investigate the effect of variations in pre-slaughter environmental temperature and post-mortem incubation temperature upon shear value, exudate, and cooking loss of unfrozen and pre-rigor frozen muscle.

EXPERIMENTAL

Pre-slaughter treatment. The animals described in Part I of this investigation were used (Cook and Langsworth, 1966).

Post-slaughter treatments. Shear value, exudate loss during storage, and cooking loss were measured on samples of unfrozen and pre-rigor frozen longissimus dorsi which had been incubated for 24 hr post-mortem at one of the following temperatures: 0, 5, 10, 15, 20, 30, and 40°C.

Methods. The complete longissimus dorsi muscles were removed from each animal within 10 min of slaughter. Eight samples, approximately 8 cm long and 4 cm^2 in cross-section, were prepared immediately from each muscle.

The eight samples from one muscle from each lamb were placed in separate plastic bags, sealed, and frozen for 90 min in a dry-ice-ethanol mixture (pre-rigor frozen samples). Seven of these samples, together with 7 samples from the other muscle from each lamb (unfrozen) were then placed in incubation chambers at the various temperatures for 24 hr. Incubation of the unfrozen samples began 15 min after slaughter.

Prior to incubation, all samples were dipped in a 10.0-ppm solution of chlortetracycline to inhibit bacterial growth.

At the conclusion of the incubation period the samples were cooked in boiling water until an internal temperature of 82°C was reached. The samples were then cooled for 6 hr at 10°C, and their shear values were determined, in triplicate, on ½-inch cores of muscle with a Warner-Bratzler shear.

The remaining two samples of unincubated muscle (unfrozen and pre-rigor frozen) were cooked in a similar manner and their shear values determined. The unfrozen sample was cooked 15 min post-mortem, whereas the pre-rigor frozen samples was cooked upon removal from the freezing mixture.

Exudate losses and cooking losses were calculated from the loss of weight incurred during incubation and during cooking and subsequent cooling.

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The data were handled as described earlier (Cook and Langsworth, 1966).

RESULTS AND DISCUSSION

Pre-slaughter treatment. Pre-slaughter treatments had no significant effects on shear value, exudate loss, or cooking loss. The data are therefore pooled for presentation.

Post-slaughter treatment. Tables 1 and 4 present data for mean shear values and exudate and cooking losses, with data for pre-slaughter treatment pooled. Tables 2 and 5 present analyses of variance for the total data.

Shear value. Post-mortem incubation temperature and freezing pre-rigor each had a highly significant ($P<0.001$) effect upon shear values (Tables 1, 2), and there was a significant interaction between them ($P<0.001$, Table 2, Fig. 1). The shear values for the unfrozen samples fell from a value of 19.10 at 0°C to a minimum of 8.26 at 40°C with an intermediate plateau between 10 and 30°C. Thus at 5°C values were significantly lower than those at 0°C ($P<0.05$); there were no significant differences between the values at 10, 15, 20, and 30°C, while the 40°C value was significantly lower. By contrast, the shear values for the pre-rigor frozen samples rose significantly from 0°C to a maximum at 20°C ($P<0.05$) and then fell to a minimum at 40°C ($P<0.05$).

The initial shear values for the unfrozen and pre-rigor frozen samples were not significantly different from each other, nor was there a difference between these values and that for the unfrozen sample incubated at 40°C.

Numerous inherent properties of muscle, both chemical and physical in nature, have been cited as influencing meat tenderness. Marsh (1963) recently presented evidence that the tenderness of bovine musculature varies concomitantly with the phases of rigor

Table 1. The mean shear values of unfrozen and pre-rigor frozen muscle at 24 hr post-mortem, grouped within each incubation treatment.[a]

Treatment and muscle	Initial shear	Incubation temperature[b]						
		0°	5°	10°	15°	20°	30°	40°
Unfrozen	8.97[a]	19.09	14.95[b]	12.11[c]	13.05[b,c]	11.67[c]	11.70[c]	8.26[a]
Pre-rigor frozen	8.24[a]	8.09[a]	9.83[a]	12.31[b]	12.75[b]	12.61[b]	8.45[a]	4.31

[a] Means of 12 animals.
[b] All means within a treatment followed by the same postscript do not differ significantly. All others differ significantly.

Table 2. Analysis of variance of data for shear values 24 hr post-mortem.

Source of variation	Degrees of freedom	Mean square
Pre-slaughter treatment	2	23.50
Animals within pre-slaughter treatment	9	247.31***
Freezing treatment	1	2129.40***
Freezing × pre-slaughter treatment	2	113.75
Animals × freezing	9	52.72
Incubation temperature	7	760.31***
Temperature × pre-slaughter treatment	14	37.21
Animals × temperature	63	38.49
Temperature × freezing	7	450.57***
Pre-slaughter treatment × freezing × temperature	14	52.97
Animals × freezing × temperature	63	31.87
Total	191	

*** $P<0.001$.

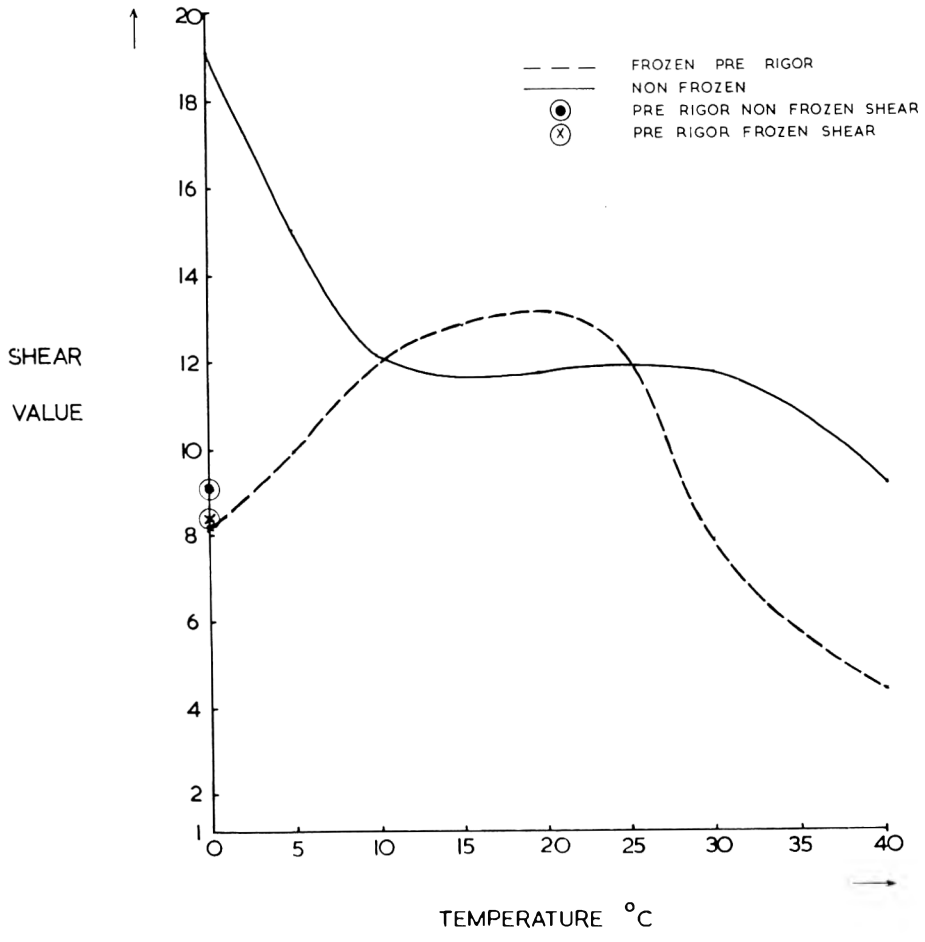

Fig. 1. Shear values of unfrozen and pre-rigor frozen longissimus dorsi after incubation for 24 hr post-mortem at various temperatures.

development. Maximum shear values were observed while the muscle was in full rigor, while the lower shears were reported for muscle in the pre- and post-rigor phases. Data presented in Part I of this investigation (Cook and Langsworth, 1966) and unpublished results of Cook and Wright (1966) suggest that variations in post-mortem incubation temperature bring about differences in the extent of muscle shortening and in the nature of the contracture band patterns of the fibers.

Simple correlation coefficients between the percent shortening values presented in Part I and shear values show no statistically significant relationship at the 5% level of probability between these two variables when calculated either within a temperature treatment or pooled over all incubation temperatures (Table 3).

It thus appears that the variation in shear values observed among samples incubated at the various temperatures may be partially due to the different phases of rigor development (Marsh, 1963), or variations in the contracture band pattern of the fibers (Cook and Wright, 1966) which affect the ordered structure of the myofibrilar proteins (Mandelkern *et al.*, 1965), or subsequent post-rigor changes of the proteins (Wierbicki *et al.*, 1954).

Exudate loss and cooking loss. Post-

Table 3. Simple correlation coefficients between the percent shortening of unfrozen semitendinosus and the shear value of the longissimus dorsi after 24 hr of post-mortem incubation.

	Incubation temperature °C							
	0°	5°	10°	15°	20°	30°	40°	
	Shear values							Pooled
% shortening of muscle	−0.16	−0.36	−0.45	−0.28	−0.49	−0.15	−0.51	−0.11

Value greater than 0.55 significant at $P<0.05$.

mortem incubation temperature and freezing pre-rigor each had highly significant effects on exudate and cooking losses ($P<0.001$, Tables 4, 5) and there were highly significant interactions between them ($P<0.01$ and <0.001). In both the unfrozen and pre-rigor frozen muscle there was a significant increase in the percent exudate lost with increasing incubation temperatures ($P<0.001$). There was no significant difference between them at the lower temperatures, 0 and 5°C, but at 10°C and above the percent exudate was significantly greater in the pre-rigor frozen than in the unfrozen samples (Table 4, Fig. 2).

These data are consistent with the observa-

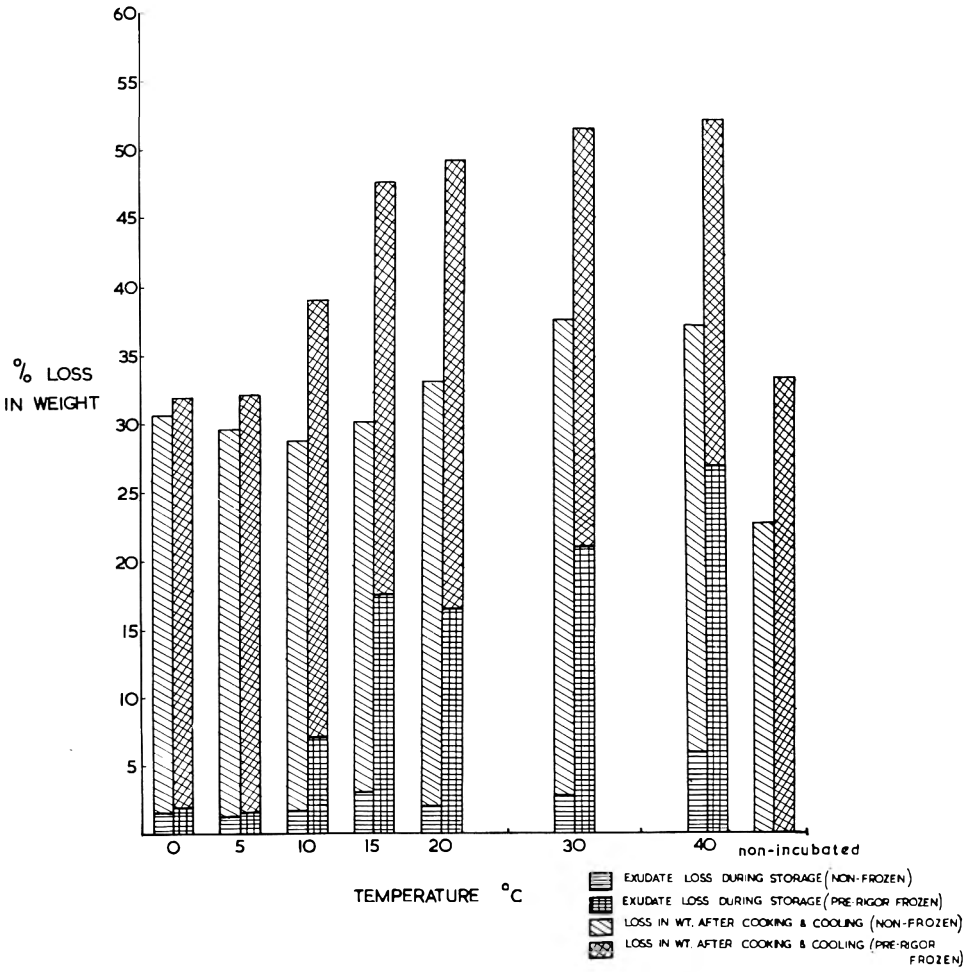

Fig. 2. Exudate loss during incubation and cooking loss after cooling of unfrozen and pre-rigor frozen longissimus dorsi incubated for 24 hr post-mortem at various temperatures.

Table 4. The mean exudate loss during storage and cooking loss at 24 hr post-mortem, grouped within each incubation treatment.[a]

	Incubation temperature[b]						
	0°	5°	10°	15°	20°	30°	40°
% exudate loss							
Unfrozen	1.5[a]	1.3[a]	1.6[a]	3.1[b]	2.1[a]	2.9[b]	6.1
Pre-rigor frozen	1.8[a]	1.5[a]	5.9	17.8[b]	16.5[b]	24.3[c]	28.2[c]
% cooking loss							
Unfrozen	30.0[a,b]	29.4[a,b]	28.5[a]	29.3[a,b]	32.1[b]	37.1[c]	36.4[c]
Pre-rigor frozen	32.1[a]	30.7[a]	37.5	44.4[b]	44.0[b]	47.2[b]	46.8[b]

[a] Means of 12 animals.
[b] All means, within a treatment, followed by same postscript do not differ significantly. All others do differ significantly ($P<0.05$).

tions of Marsh and Thompson (1958), who reported that pre-rigor freezing caused the formation of larger quantities of exudate during thawing.

Similarly, the percent cooking loss increased with increasing incubation temperatures for both the pre-rigor frozen and unfrozen samples ($P<0.001$, Table 4). The percent cooking losses were higher for the pre-rigor frozen samples than for the unfrozen samples at incubation temperatures above 10°C (Fig. 2).

Numerous investigators (Hamm, 1960; Briskey, 1963; Howard, 1963; Jay, 1964) have reported that high ultimate muscle pH in muscle, not subjected to elevated temperatures post-mortem, is associated with increased water-binding capacity. The data presented in this study substantiate these observations for pre-rigor samples incubated at 10°C or below; however, for samples incubated at temperatures above 10°C a positive relationship exists between total moisture loss and pH. Hence, samples of unfrozen muscle incubated at 30 and 40°C had respective 24 hr pH values of 6.45 and 6.89, released the highest quantities of exudate during incubation, and also lost the greatest percentage weight during cooking. The differences reported in this study and those of the literature may have been due to slight bacterial decomposition in samples incubated at high temperatures, resulting in a decrease in water-binding capacity of the muscle as postulated by Jay and Kontou (1964) and Jay (1965), or because of severe protein denaturation due to the combined effect of elevated post-mortem temperatures and high pH (Cook, 1966).

Table 5. Analysis of variance of data for percentage exudate and cooking loss.

Source of variation	Degrees of freedom	Mean squares	
		% exudate loss[a]	% cooking loss
Pre-slaughter treatment	2	0.07	28.17
Animals within pre-slaughter treatment	9	0.56**	45.70***
Freezing treatment	1	119.16***	4069.53***
Pre-slaughter × freezing	2	0.04	0.95
Animals × freezing	9	0.52	26.63*
Temperature of incubation	6	21.11***	705.33***
Pre-slaughter × temperature	12	0.06	23.20
Animals × temperature	54	0.20	11.85
Freezing × temperature	6	9.2**	152.52***
Pre-slaughter × freezing × temperature	12	0.29	118.13***
Animals × freezing × temperature	54	0.15	10.46
Total	167		

[a] Analysis of variance conducted on $\sqrt{\times + 1}$ transformed data.
* $P<0.05$.
*** $P<0.001$.

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Effects of Modified Storage Atmospheres on Ascorbic Acid and Other Quality Characteristics of Spinach

SUMMARY

It has been shown that gas composition and temperature of the atmosphere can affect respiration and the quality of vegetables. This study was undertaken to determine the effect of these factors on the storage quality of spinach and New Zealand spinach. Both showed typical respiratory drift curves. Increasing CO_2 around New Zealand spinach reduced respiration rate, and product in 13% CO_2 was not acceptable after one week. A gas containing 9.5% CO_2 and 3.3% O_2 was compared with air at 34 and 45°F. The leaves tended to increase in oxalate but this increase was not related to ascorbate reduction. Samples in controlled atmosphere had fewer microorganisms than air samples at the same temperature.

The ascorbic acid content of the leaves was affected by atmosphere, temperature, and time. Oxygen depletion of the atmosphere resulted in a marked decrease of ascorbic acid. When oxygen content was maintained at 3.3% by intermittent flushing, the leaves retained their ascorbic acid. This phenomenon was intensively investigated with addition of analysis for dehydroascorbic acid. Since the total of dehydroascorbic and ascorbic acids did not vary with atmosphere composition, it was concluded that modified storage atmospheres neither improve nor impair antiscorbutic value.

INTRODUCTION

Retention of the original quality of fresh fruits and vegetables is a problem of primary concern. Most of these products are grown on a seasonal basis, so that only one crop is harvested per year and made available to the public as "fresh" produce. Various preservation methods are used to assure a continuous supply to consumers. However, these practices modify the raw materials, and the preserved products are often considered to be less desirable than the fresh. Modified atmospheres of controlled concentrations of CO_2 and O_2 have been used as an aid in maintaining the quality of fresh produce.

McKenzie (1931), in studying the effect of temperature on the respiration rate of lettuce, noted that as the CO_2 increased there was a decrease in rate of CO_2 evolution. Thornton (1931) studied the effect of storage in CO_2 on several vegetables. The response of the vegetable was found to vary with both temperature and CO_2 in the storage atmosphere. At 4°C, beans showed no injury at 18% CO_2 but did show injury at 30% CO_2. The ability of beans to withstand CO_2 injury was reduced when the temperature was lowered; beans stored in 18% CO_2 at 0°C developed injury. Cauliflower in 25% CO_2 showed no injury at 0° and 10°C, but was damaged when the temperature was increased to 15°C. Celery stored in 50–80% CO_2 showed greater injury at 0° than at 4°C. Lettuce was injured by any concentration of CO_2 above 7%. Spinach tissues became soft and developed off odors when concentrations greater than 20% CO_2 were used at temperatures above 0°C.

Platenius (1943) found that lowering the O_2 concentration to 1.2% could reduce the respiration rate of asparagus by 55%. Decreasing the O_2 below the normal atmospheric level was also found effective for other vegetables such as snapbeans and peas. Claypool and Allen (1948) found that when oxygen levels were reduced below that of normal air the respiration rates of apricots, grapes, peaches, pears, and plums were reduced. Lieberman and Hardenburg (1954) stored broccoli at 75°F and at various oxygen concentrations. Their results indicated a reduction in respiration rate as oxygen concentration was lowered from 20.9 to 0%.

This study was initiated to determine the extent to which gas composition, storage temperature, and storage time affect the chemical, physiological, microbiological, and organoleptic quality of New Zealand Spinach *(Tetragonia expansa)* and true spinach *(Spinacia oleracea)*.

MATERIALS AND METHODS

New Zealand spinach was grown in the greenhouse and harvested periodically throughout the late fall, winter, and spring months. Only leaves with a surface area of greater than 2 in.^{2} were

used in this study. Regular spinach was grown out of doors; American variety was used for the spring crop, and Early Hybrid No. 7 for the fall crop.

The leaves were harvested and immediately sorted to remove yellow, injured, or diseased leaves. This was followed by a thorough washing in cold water. After draining, the leaves were placed in refrigerated storage (34°F) for at least 2 hr, but no longer than 6 hr, in order to equilibrate the temperature.

Initial product respiration rates were determined by using the method and equipment described by Platenius (1942). Carbon dioxide and oxygen analyzers manufactured by Thermco Instrument Corporation, La Porte, Indiana, were used to measure concentrations of gases in the storage chambers. The instruments were calibrated periodically by the Orsat method, using the Burrell-Haldane Gas Analyzer. The storage chambers used in these studies were 4-L bottles. One-hundred-gram samples were placed in each test chamber, in which the RH was maintained at 100%. A screen stand was placed in each chamber to prevent product from coming in direct contact with the water that usually accumulated on the bottom of the chambers. In the initial studies the desired atmospheres were obtained by mixing CO_2, O_2, and N_2 cylinder gases. In later studies, cylinders of specific gas mixtures were used.

Spinach leaves were analyzed for dextrose, sucrose, and starch. All three determinations were made on the same extract of leaf material by the Official Methods of AOAC (1955). Total oxalic acid was measured by a method of Kiharo (1952). The ascorbic acid procedure used in this study consisted of the extraction procedure of Loeffler and Ponting (1942), modified by using an N_2 atmosphere (Assoc. Vitamin Chemists, 1951), and titration with 2,6-dichlorophenol-indophenol (McKenzie, 1931). Dehydroascorbic acid was determined on the filtrate from the ascorbic acid analysis. The dehydroascorbic acid in the extract was reduced to ascorbic acid using H_2S according to the procedure of Rubin *et al.* (1945). Moisture of shredded leaf material was determined by drying in a vacuum oven for 16 hr at 70°C. The dried material was saved for the oxalic acid analysis. Chlorophyll analysis followed the method of Jacobson and Oestli (1956). Chlorophyll concentrations were calculated by using the optical densities observed and the specific adsorption coefficients for chlorophyll *a* and *b* published by MacKinney (1941).

The standard method of analysis recommended by the Am. Public Health Assoc. (1948) was followed for bacteriological total plate counts. Twenty grams of leaves were aseptically transferred into a presterilized, chilled Waring blender jar with 180 ml of 1.0% sterile peptone and the material blended for 3 min. Decimal dilutions were made in 0.1% peptone solution. Triplicate plates were poured for each dilution using tryptone glucose extract agar. The plates were incubated 3 days at 20°C before counting.

Samples were prepared for organoleptic evaluation by simmering 50 g of leaves in one cup of water for 10 min. The cooked materials were arranged in random order and presented on a blindfold basis to ten experienced panel members from the Department of Food Science.

Respiratory drift curves were determined for New Zealand and regular spinach leaves at 34°F under conditions which allowed continual adsorption of carbon dioxide and continual replacement of utilized oxygen.

New Zealand spinach was held in atmospheres containing various CO_2 and O_2 concentrations at 34°F to determine the effect of initial gas composition on respiration rate and organoleptic qualities. Initial atmospheres of 25, 13, 7.5, 4.0, and 0.03% CO_2, all in conjunction with 20% O_2, were used to determine the effect of CO_2. To determine the effect of oxygen, initial atmospheres of 4, 10, 14, and 18% O_2 were used in conjunction with 4% CO_2. Normal air was used as the control.

RESULTS AND DISCUSSION

Both types of spinach demonstrated typical respiratory drifts (James, 1953) with the New Zealand type generally showing a slightly lower rate.

Increasing carbon dioxide in the atmosphere around the product reduced rates of respiration. Twenty-five percent CO_2 caused rapid physiological breakdown of the product, and product in 13% CO_2 was not acceptable after one week. As the oxygen level was decreased to 4%, respiration was slightly reduced but the effect was less than that with increased CO_2. However, reduction of respiration rate did not result in improved flavor, color, or texture of the spinach. The organoleptic evaluation showed that samples stored in air were generally as good as the best products stored in modified atmospheres.

Several experiments were carried out to determine the effect of various storage atmospheres and temperatures on certain chemical constituents. In one of the experiments, 100 g of New Zealand spinach leaves were placed in 4-L bottles at 34 and 45°F. Half of the bottles at each temperature were sealed

in air and labeled 34-A and 45-A, while the other bottles were purged with a gas mixture of 9.5% CO_2 and 3.3% O_2 and labeled 34-CA and 45-CA. A large sample of leaves on a perforated stainless screen was exposed to normal air at 34°F and labeled 34-Control.

Sucrose, which was zero at the beginning, showed no increase. Chemical analyses for this experiment are presented in Table 1. Starch content of all samples varied with storage time but showed no definite trends. The reducing sugar analyses showed considerable variation, without any consistent patterns related to storage time, atmosphere, or temperature. This variation may be caused by the formation of other reducing substances. Since some apparent discrepancies had appeared in the reducing sugar results from earlier experiments, a check on the precision of reducing sugar measurements was made during this study. After extraction of the sugars, the solutions were divided into two aliquots and a standard glucose solution was added to one of the aliquots. The glucose recovery was generally very good, averaging 104% over all the experimental samples. Therefore, the tendency for samples to increase in reducing sugar cannot be attributed to faulty measurements in the latter two storage periods.

Dry matter (Table 1) decreased with storage time in all samples except the 34-Control. Since the latter sample was opened to the storage atmosphere, it is conceivable that desiccation of the leaves resulted in an increase in percentage of dry matter.

Total chlorophyll data (Table 1) were erratic, and no trends were evident which could be related to storage atmosphere, time, and temperature. Chlorophyll *a* and *b* data varied in a similar manner, and are not presented in Table 1.

Oxalate, initially lower in these samples than in earlier experiments, was maintained or slightly increased in concentration during the experiment (Table 1). The data seemed to suggest that oxalic acid is not readily oxidized during the storage of New Zealand spinach leaves. This characteristic of the stored leaves to retain or show increases in oxalate seems to contradict the theory that oxalic acid is used by the leaves as respirable substrate. Oxalic acid may be formed as a result of the oxidation of ascorbic acid. Thus, ascorbic acid is reversibly oxidized to dehydroascorbic acid, which is unstable in an environment above pH 5 and breaks down to form oxalic and threonic acids. The New Zealand spinach leaves have a pH of about 6.5, so the formation of oxalate from ascorbate is a possibility. Table 1 discloses that the changes of oxalic acid are apparently not closely related to the loss of ascorbic acid. Therefore, the accumulation of oxalate at this time can only be attributed to leaf anabolism. Bonner (1950) suggested that oxalic acid formation takes place through glycolic and glyoxylic acids.

The ascorbic acid content of the leaves was found to be affected by storage time, temperature, and atmosphere (Table 1). All samples showed a loss of ascorbic acid with time. The ascorbic acid content was reduced more quickly at the higher storage temperature. The 34-Control and the 34-A samples showed similar ascorbic acid changes. The more rapid reduction of ascorbic acid in *CA* samples than in *A* samples presented an unexpected change. The lower initial oxygen concentration in the *CA* bottles was expected to give a longer retention of the ascorbic acid in storage. The opposite was shown to be true; when the oxygen was depleted to levels near zero, the ascorbic acid appeared to be more easily oxidized.

Microbial plate counts made on all 34-Control, 34-A, 34-CA, 45-A, and 45-CA samples covering the 14-day storage period are shown in Fig. 1. These are the same samples as described in Table 1. Examination of these data reveals that both temperature and atmosphere affected microbial plate counts during storage. The 45-A samples showed the most rapid rate of microbial increase, and the *CA* samples always had fewer microorganisms than the similar *A* samples. The 45-CA condition, although at a higher temperature, gave less microbial growth than the 34-Control. This suggests that part of the effectiveness of "controlled atmosphere" in prolonging the quality of some products in storage may be in the reduction in growth of microorganisms.

Table 1. Gas and chemical analyses based on fresh weight of New Zealand spinach leaves stored for periods to two weeks at two temperatures and initially controlled atmospheres.

Storage temp. (°F) and atmosphere	Storage time (days)	CO_2 (%)	O_2 (%)	Dry matter (%)	Ascorbic acid (mg/100 g)	Oxalate (%)	Reducing sugar (%)	Starch (%)	Total chlorophyll (mg/g)
34-Control [a]	0	....	....	7.0	19.1	1.41	.024	.32	.77
34-A	0	0.03	20.8	7.0	19.1	1.41	.024	.32	.77
34-CA	0	9.5	3.3	7.0	19.1	1.41	.024	.32	.77
45-A	0	0.03	20.8	7.0	19.1	1.41	.024	.32	.77
45-CA	0	9.4	3.3	7.0	19.1	1.41	.024	.32	.77
34-Control	5	....	....	6.6	15.1	1.54	.063	.41	.89
34-A	5	2.0	18.8	5.4	13.6	1.20	.030	.33	.55
34-CA	5	11.7	2.2	6.1	12.5	1.64	.020	.33	.58
45-A	5	2.8	17.1	5.0	9.9	1.10	.017	.26	.56
45-CA	5	12.4	1.7	5.1	7.7	1.38	.016	.33	.70
34-Control	7	....	....	6.0	10.3	1.83	.009	.20	.59
34-A	7	2.6	16.5	5.7	9.5	1.28	.009	.28	.73
34-CA	7	12.2	0.3	5.7	9.5	1.59	.023	.37	.67
45-A	7	3.3	15.5	5.5	6.3	1.67	.006	.31	.78
45-CA	7	13.1	0.0	4.9	5.0	....	.008	.28	.52
34-Control	11	....	....	7.6	7.9	1.41	.016	.28	.63
34-A	11	3.3	17.0	5.4	7.1	2.12	.012	.33	.74
34-CA	11	13.1	1.2	5.4	4.9	1.89	.037	.36	.67
45-A	11	6.0	13.5	5.0	4.6	2.58	.015	.39	.59
45-CA	11	13.7	0.7	5.4	0.0	1.79	.069	.36	.73
34-Control	14	....	....	8.9	7.8	0.59	.025	.34	.69
34-A	14	4.0	14.7	4.3	5.7	1.96	.026	.34	.57
34-CA	14	12.9	0.0	5.5	4.0	1.29	.027	.38	.70
45-A	14	7.6	9.9	5.3	4.0	2.09	.033	.46	.62
45-CA	14	13.3	0.0	5.3	0.0	1.69	.052	.36	.53

[a] Control means samples exposed to normal air atmosphere.
A Means samples sealed in air.
CA Means samples sealed in a mixture of 9.5% CO_2 and 3.3 O_2.

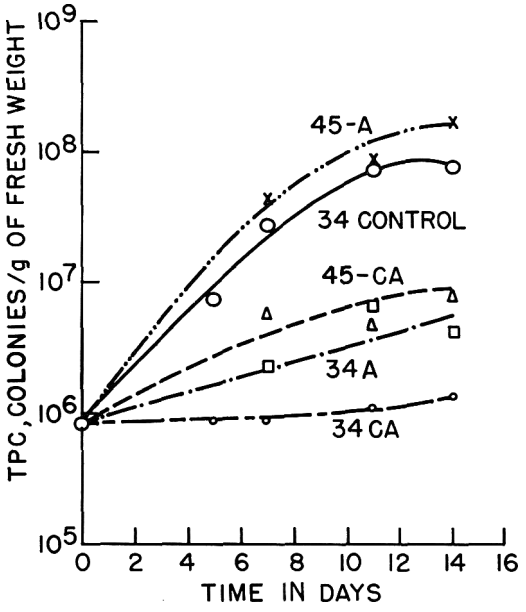

Fig. 1. Effect of temperature and atmosphere on the increase in total plate count during storage of New Zealand spinach leaves.

The previous experimental conditions were duplicated in a second study except that the bottles were flushed at various intervals to renew the initial atmospheres. The primary objective was to prevent anaerobic conditions from developing in the storage chambers. This procedure more nearly approaches the state of "controlled atmosphere." During the first 36 hr of storage, the atmosphere was renewed after each 12-hr period. Thereafter, the respiration rate slowed and longer periods elapsed between atmosphere renewals. Carbon dioxide and oxygen concentrations were determined before each renewal of the atmosphere in each bottle. The samples were labeled as before, but no 34-Control sample was used in this study.

Chemical analyses (Table 2) revealed that the changes in dry matter, reducing sugar, sucrose, chlorophyll, and oxalate were similar to changes in the previous section.

The starch content of the stored leaves was much higher here than in the other studies. In contrast to earlier observations, it showed a steady decline as storage progressed. This approaches agreement with results from other laboratories (James, 1953). The previous study had indicated that when oxygen was depleted to a very low level, such as in the *CA* samples after longer storage times, the remaining ascorbic acid was lost more rapidly from the product; in addition, ascorbic acid was lost more readily at the higher storage temperature. In the second experiment (Table 2), similar effects of temperature and time were obtained; however, the *CA* samples retained ascorbic acid at about the same level as the *A* samples. Since in this experiment a given oxygen level was intermittently maintained in the *CA* samples, the conclusion follows that when the atmospheric conditions approach or

Table 2. Changes in chemical constituents based on fresh weight of New Zealand spinach as affected by storage time, temperature, and periodically controlled atmosphere.

Storage temp. (°F) and atmosphere	Storage time (days)	Dry matter (%)	Ascorbic acid (mg/100 g)	Oxalate (%)	Reducing sugar (%)	Starch (%)	Total chlorophyll (mg/g)
34-A	0	7.3	22.3	1.98	.064	.69	.63
34-CA	0	7.3	22.3	1.98	.064	.69	.63
45-A	0	7.3	22.3	1.98	.064	.69	.63
45-CA	0	7.3	22.3	1.98	.064	.69	.63
34-A	7	6.1	17.6	2.54	.011	.33	.57
34-CA	7	6.2	15.0	1.81	.016	.26	.60
45-A	7	6.2	13.4	2.44	.019	.43	.55
45-CA	7	6.4	11.0	2.36	.033	.42	.56
34-A	14	5.9	8.1	2.50	.025	.25	.52
34-CA	14	6.1	8.9	2.48	.047	.27	.55
45-A	14	5.8	4.0	3.02	.031	.35	.48
45-CA	14	6.1	5.2	2.62	.035	.29	.43

become anaerobic the ascorbic acid is lost more rapidly.

Another study was made using 100 g of regular spinach sealed in 4-L bottles. This experiment was conducted to determine whether the ascorbic acid in regular spinach reacted to low oxygen concentrations in the same manner as the New Zealand spinach. The atmospheres were not renewed during this study, and samples were examined after 8 and 15 days of storage. Storage temperatures of 34 and 45°F and atmospheres of normal air and a standard gas mixture of 9.5% CO_2 and 3.3% O_2 were used.

The results are shown in Table 3. The 34-CA, 45-A and 45-CA samples lost the oxygen from their atmosphere during the first 8 days. The two samples which initially had low oxygen concentrations, 34-CA and 45-CA, contained no ascorbic acid at the end of the 8-day storage period. The only one containing ascorbic acid at 15 days of storage was the 34-A sample, which still retained over half the original ascorbic acid at a final oxygen content of 3.4%.

The phenomenon of an increase in the rate of loss of ascorbic acid from leaves stored in low oxygen was investigated. Regular spinach was stored under atmospheres of O_2, CO_2, N_2, and normal air, while New Zealand spinach was stored only under normal air. The spinach samples were sealed in 4-L brown jars. At 24-hr intervals, carbon dioxide, oxygen, and nitrogen from commercial gas cylinders were flushed through the jars for 10 min at a rate in excess of 2 L/min. The spinach and New Zealand spinach samples stored in normal air were placed on screens with water below the screens. All samples were stored at 34°F, and samples were analyzed for ascorbic and dehydroascorbic acid.

Table 3. Ascorbic acid content of regular spinach during storage at two temperatures and initially controlled atmospheres.

Storage temp. (°F) and atmosphere	Storage time (days)	CO_2 (%)	O_2 (%)	Ascorbic acid (mg/100 g fresh wt.)
34-A	0	0.03	20.8	71.3
34-CA	0	9.5	3.5	71.3
45-A	0	0.03	20.8	71.3
45-CA	0	9.5	3.5	71.3
34-A	8	8.8	8.0	53.1
34-CA	8	14.8	0.2	0.0
45-A	8	15.4	0.0	31.2
45-CA	8	14.6	0.0	0.0
34-A	15	11.8	3.4	37.3
34-CA	15	13.4	0.2	0.0
45-A	15	16.6	0.0	0.0
45-CA	15	14.0	0.0	0.0

Table 4 shows the results of storage through seven days. The high-oxygen atmosphere appeared to have caused a more rapid loss of ascorbic acid after 7 days than did the normal-air atmosphere. However, there was no tendency for the dehydroascorbic acid to increase in the oxygen atmosphere. The samples stored in carbon dioxide contained less ascorbic acid after seven days than either the oxygen or air samples. However, dehydroascorbic acid in the product stored in carbon dioxide was found to have increased substantially. The nitrogen samples showed a very rapid decrease in ascorbic acid, with conversion of most of the lost ascorbic acid to dehydroascorbic acid. By measuring both ascorbic and dehydroascorbic acid, regular spinach leaves stored in air, nitrogen, oxygen, and carbon dioxide for 7 days were found to have total "ascorbic" acid contents between 47 and 68 mg/100 g (Table 4).

These findings are in agreement with those in the previous studies, where the ascorbic acid was found to disappear more rapidly when spinach samples were stored in oxygen-depleted atmospheres. Ascorbic acid is considered to be reversibly oxidized to dehydroascorbic acid in plant tissues. The analysis for dehydroascorbic acid demonstrated that, under such conditions, the loss of ascorbic acid is generally accompanied by proportionate increases in dehydroascorbic acid. Since the original leaf samples contained a predominance of ascorbic acid, we may assume that the normal equilibrium in the leaf is toward a high concentration of ascorbic acid and low concentration of dehydroascorbic acid. Table 4 shows that this equilibrium was upset toward the dehydroascorbic acid when only minute quantities of oxygen were present in the atmosphere.

Table 4. Ascorbic acid content on fresh-weight basis of spinach and New Zealand spinach stored seven days in several initially controlled atmospheres at 34°F.

Sample	Storage atmosphere	Storage time (days)	Ascorbic acid (mg/100 g)	Dehydro-ascorbic acid (mg/100 g)	Total "ascorbic" acid (mg/100 g)	Dehydro-ascorbic acid (percent of total)
Spinach	.....	0	77.7	7.0	84.6	8.2
New Zealand spinach	.....	0	28.5	2.2	30.7	7.2
Spinach	O_2	3	69.3	6.6	75.9	8.7
Spinach	CO_2	3	73.2	12.7	85.9	14.8
Spinach	N_2	3	1.5	74.0	75.6	98.0
Spinach	Normal air	3	65.4	9.6	75.0	12.7
New Zealand spinach	Normal air	3	29.6	3.8	33.4	11.3
Spinach	O_2	7	40.6	6.3	46.9	13.5
Spinach	CO_2	7	27.0	29.6	56.7	52.3
Spinach	N_2	7	1.8	62.5	64.3	97.2
Spinach	Normal air	7	58.7	9.3	68.0	13.7
New Zealand spinach	Normal air	7	15.9	5.2	21.1	24.9

This suggests that oxidation by transfer of hydrogen from ascorbic acid is proceeding normally, while the reductase system required to reduce dehydroascorbic acid to ascorbic acid is not functioning properly and abnormally low oxygen concentrations appear to be causing this change in metabolism.

As shown in Table 4, storage time exerted a similar effect on the ascorbic and dehydroascorbic acid contents of both regular and New Zealand spinach stored in air.

Dehydroascorbic acid content as a percent of the total "ascorbic" acid (last column in Table 4), showed that nitrogen storage gave nearly complete conversion to the dehydro form after 7 days of storage, while oxygen storage showed a very low percent increase during storage. Upon checking back to initial dehydroascorbic contents, it is found that oxygen storage gave essentially no conversion to the dehydro form over the 3-day storage and only a moderate increase over the 7-day storage. The important consideration, however, is that there is relatively little difference among all samples at 3 days of storage in total "ascorbic" or antiscorbutic content. Modified storage atmospheres do not appear to give added protection to total "ascorbic" acid, but neither do they seriously reduce the vitamin content.

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The Relation of Structure to Antioxidant Activity of Quercetin and Some of Its Derivatives
I. Primary Activity

SUMMARY

The primary antioxidant activity of quercetin and some of its derivatives was studied in the 36–70°C range in dry systems, using two fatty-ester substrates, respectively with linoleate and linolenate, as the main constituent undergoing oxidation. Metal contamination was avoided as far as possible, and any residual traces of metals in the thoroughly purified esters were chelated with citric acid.

Methylation of the 3, or 5, or 3 and 7, or 5 and 7 hydroxyls of the quercetin molecule led to considerable reduction of the antioxidant activity, while reduction due to methylation of the 7 hydroxyl was slight. Methylation of the 3′, or 4′, or 3′ and 4′ hydroxyls, or of any single hydroxyl of the B ring, and of an additional hydroxyl or hydroxyls of the A ring, led to a drastic reduction (to 11% or less); the 3,7,3′,4′ tetramethoxy derivative was found to be completely inactive. Hydrogenation of the 2,3 double bond resulted in an antioxidant (dihydroquercetin) with only about half the activity of quercetin.

The primary antioxidant activity of quercetin seems to be a function of the molecule as a whole and cannot be regarded as an additive property of active hydroxyls. The effect produced by methylation of a particular hydroxyl may, however, be related to the probability of formation of a stabilized free radical by the hydroxyl in question.

The type of the substituted alkyl radical had little or no effect on the activity of the derivative, but replacement of a hydroxyl with hydrogen failed to produce the same effect as methylation of the same hydroxyl.

No indication was found of pro-oxidant activity of the meta-hydroxyl grouping in the A ring.

INTRODUCTION

Among naturally occurring substances of potential antioxidant activity, flavonoids are of particular interest. They are widely distributed in the plant kingdom (Bate-Smith, 1954) and some of them combine in their molecules both chain-breaking and metal-deactivating functions (Lea, 1956, 1958).

Relation between the structure of flavonoids and their antioxidant activity has been investigated by several workers. Heimann and Reiff (1953) studied the antioxidant effect of a number of flavonoids in ethyl linoleate at 25°C and deduced that the α,β-unsaturated ketone structure of the pyrone ring, the free 3-hydroxyl group, and the 3′,4′-o-diphenolic grouping in the B ring were the main contributors to antioxidant capacity in flavonoids, while the meta-diphenolic grouping in the A ring impaired that capacity. Simpson and Uri (1956) used aqueous emulsions of methyl linoleate for evaluation of flavonoids as antioxidants. They also claimed that meta-diphenolic groupings either had a pro-oxidant effect or were of no importance. These conclusions were not supported by Mehta and Seshardi (1958), who compared the antioxidant activities of 27 flavonoids with lard as substrate. Their results showed that gossypetin (3,5,7,8,3′,4′-hexahydroxyflavone) was a particularly powerful antioxidant, confirming findings of Lea and Swohoda (1956). The arrangements of hydroxyl groups mainly responsible for the activity seemed to be a 7,8- or 5,7,8-grouping in the A ring, and a 3′,4′- or 3′,4′,5′-grouping in the B ring.

Crawford *et al.* (1961) investigated the overall (chain-breaking and metal-deactivating) effect of quercetin and some of its derivatives as antioxidants in lard at 97.8°C. They found that methylation of one or more hydroxyl groups (and of the 4′- and/or 3′-hydroxyls in particular), or hydrogenation of the 2,3 double bond, resulted in decrease of the antioxidant activity, while benzylation of the 7-hydroxyl yielded an antioxidant superior to quercetin. Rhamnetin (the 7-*O*-methylated derivative of quercetin) was found by Heimann and Reiff (1953), Simpson and Uri (1956), and Uri (1958) to be also slightly superior to quercetin.

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The experiments described here are a further contribution to the study of the relationship between the molecular structure of polyhydroxyflavones and their primary (chain-breaking) antioxidant activtiy. The effects of the secondary (metal-complexing) antioxidant activity of the investigated compounds were eliminated as far as possible by preliminary chelation (with citric acid) of trace amounts of metals possibly present in the substrate.

Quercetin (3,5,7,3',4'-pentahydroxyflavone) was chosen for this study as one of the commonest and apparently nontoxic plant phenols (Bate-Smith, 1954). Its antioxidant properties were compared with those of a number of *O*-alkylated and dehydroxylated derivatives in a dry ester system. The importance of the 2,3 double bond of the α,β-unsaturated ketone structure was studied by including in the series dihydroquercetin (3,5,7,3',4'-pentahydroxyflavanone), a naturally-occurring dihydro-derivative of quercetin.

Both quercetin and dihydroquercetin qualify as quite good antioxidants, the former being considered the more powerful of the two (Kurth and Can, 1951; Heimann *et al.*, 1953; Heimann and Reiff, 1953; Crawford *et al.*, 1961).

EXPERIMENTAL

Preparation of fatty-acid methyl esters. Five g of sodium were dissolved in 1,150 ml of absolute methanol, and the solution was refluxed for 1.5 hr with 1,000 g of sunflower or linseed oil. The methanol was then driven off *in vacuo* below 50°C, and the residual esters were washed with warm water until free from alkali, dried over anyhydrous Na_2SO_4, and passed through a colum (8 × 1-inch) of anhydrous alumina. The purified esters were distilled and redistilled at a few mm pressure (with CO_2 "bleed") at a temperature not exceeding 200°C. A short fractionation column charged with glass beads was used, and small initial and final portions of the distillate were rejected. A single distillation removed 97% of the reducing substances (estimated as alpha-tocopherol by the Emmerie and Engel method, 1938), while two distillations removed them completely.

The distilled esters were immediately ampouled under vacuum (with minimum exposure to air and light) and stored at −20°C until required.

The composition of the esters was determined by gas chromatography. Those prepared from sunflower oil contained 10.0% saturated, 21.5% monounsaturated, and 68.5% diunsaturated constituents; those prepared from linseed oil contained 8.5% saturated, 16.5% monounsaturated, 15.0% diunsaturated, and 60.0% triunsaturated constituents.

Stock solutions of antioxidants. Stock solutions of the following antioxidants in purified ethanol (13.4 μM/ml at 15°C) were prepared and stored in darkness at −20°C: quercetin and its various methoxy-, benzoxy-, and allyloxy-derivatives, luteolin and fisetin (dehydroxylated derivatives), and dihydroquercetin.

Pretreatment of the esters. The ampoules with the esters were heated 15 min at 210°C to destroy any peroxide present, cooled to room temperature, and opened. A calculated volume of a solution of citric acid in ethanol (2600 μg/ml) was added to the esters to yield a concentration of 130 μg (0.67 μM) anhydrous citric acid per gram of autoxidizing esters. The ethanol was subsequently driven off under vacuum at 50°C in a rotary evaporator. An aliquot of the same esters, previously oxidized in the presence of the same proportion of citric acid to a peroxide value of about 25 μM 0_2/g, was added to the heat-treated esters in an amount sufficient to yield after mixing a peroxide value of about 1 μM 0_2/g. As a result of the procedures described above, a fatty substrate was obtained, free from catalytically active metal and with a standardized concentration of a pre-formed fat hydroperoxide as starter.

The prepared esters were then divided into 5-ml portions, to which were added 0.25-ml portions of the ethanolic stock solutions of the antioxidants. One sample, serving as control, received pure ethanol. The solvent was driven off in the manner already described. The antioxidant concentration was 0.67 μM/g ester.

Ten to twelve 0.2-ml portions of each of the stabilized esters and of the control samples were carefully delivered (without touching) into 12-mm-diameter flat-bottomed glass cups with a calibrated pipette, and stored in thermostatically-controlled ovens at 36, 50, 60, or 70°C.

Precautions. Accurate temperature control during incubation of the samples was found essential. Only the center of an accurately-controlled oven was used. Samples were removed soon after the end of the induction period, to prevent accelerated oxidation of other samples by volatile reaction products.

The glassware was cleaned with utmost care. Hot concentrated sulfuric acid yielded the best results, after preliminary cleaning with hot detergent solution.

As an example of the error sources involved, it was found that results were erratic with glass cups

rinsed with glass-distilled water and oven-dried. Ethanol was used for final rinsing, and it is believed that alkali, leached from the glass and trapped in some of the cups, was responsible.

Determination of oxidation level. Individual samples were withdrawn from the oven at suitable intervals and subjected to iodometric determination of hydroperoxides by the Lea (1952) method (which calls for deaeration of the dissolved sample), or, alternatively, by a less accurate rapid method adequate after the induction period. In the latter procedure the reaction time is reduced (3 min) and no deaeration is required. According to the author's experience the error involved was comparatively small, presumably due to offsetting of the slightly incomplete reaction by the oxygen error.

RESULTS

Calculation of protection factors and quercetin indices. The activity of the various antioxidants was compared by means of the protection factor (PF) and the quercetin index (QI) (Tables 1, 2). The former is defined as $PF = (t_a - t_c)/t_c$, and the latter as $QI = (t_a - t_c)/(t_q - t_c)$, where:

t_a = time to reach an arbitrarily chosen peroxide level, with antioxidant.

t_q = ditto, with quercetin.

t_c = ditto, without antioxidant (control).

Evaluation of antioxidants. A "good" (G) rating was assigned to substances with QI above 0.67; "fairly good" (F) to those with QI = 0.26–0.67; "poor" (P) to those with QI = 0.11–0.25; "very poor" (VP) to those with QI = 0.02–0.10; "inactive" (I) to those with QI below 0.02.

Relative activities of the antioxidants. Figs. 1 and 2 show, for illustration only, a very small fraction of the actual results (autoxidation curves obtained by plotting peroxide value vs. time). The calculated results of the experiments (averaged protection factors and quercetin indices) are summarized in Tables 1 and 2.

Table 3 shows the antioxidants in order of decreasing efficiency, those of similar activity (averaged QI of all experiments) being grouped together.

DISCUSSION

It can be seen (Table 3) that alkylation of the 7-hydroxyl in the quercetin molecule

Table 1. Relative antioxidant activities of quercetin and its derivatives in sunflower oil esters.[a]

	36°C		50°C		60°C		60°C	
Antioxidant	PF	QI	PF	QI	PF	QI	PF	QI
3-OMeQ	7.4	0.64	16.4	0.59	6.0	0.53	....	....
5-OMeQ	4.8	0.45	14.0	0.42	7.0	0.48	18.9	0.53
7-OMeQ	8.4[b]	1.02	31.4	0.94	13.3	1.03	26.7	0.76
3′-OMeQ	1.7	0.16	4.8	0.14	2.5	0.19	....	....
4′-OMeQ	0.6	0.05	1.3	0.04	0.9	0.07	....	....
3,5-diOMeQ	0.2	0.01	1.9	0.06	1.2	0.09	....	....
3,7-diOMeQ	5.7[b]	0.66	17.3	0.52	7.5	0.59	....	....
3,3′-diOMeQ	0.3	0.02	0.4	0.01	0.8	0.06	....	....
5,7-diOMeQ	1.4	0.12	7.7	0.23	3.0	0.22	4.7	0.13
3′,4′-diOMeQ	0.3	0.01	2.0	0.06	0.8	0.06	....	....
3,5,7-triOMeQ	0.3	0.03	2.5	0.08	....	....	3.4	0.10
3,5,3′-triOMeQ	0.1	0.01	0.3	0.01	0.3	0.02	....	....
3,7,4′-triOMeQ	0.0	0.00	0.2	0.01	0.4	0.02	....	....
3,3′,4′-triOMeQ	0.5	0.04	1.5	0.05	1.0	0.07	....	....
7,3′,4′-triOMeQ	0.2	0.01	0.7	0.02	0.8	0.08	....	....
3,7,3′,4′-tetraOMeQ	−0.1	−0.01	0.1	0.00	0.4	0.02	....	....
7-OBeQ	8.4[b]	1.02	26.3	0.78	12.2	0.95	30.8	0.86
7,4′-diOBeQ	0.4	0.02	1.8	0.05	0.8	0.06	....	....
7,4′-diOAllQ	1.5	0.12	3.6	0.11	1.2	0.09	....	....
2,3-dihydroquercetin (taxifolin)	7.0	0.67	22.3	0.66	....	....	....	....
5-desoxyquercetin (fisetin)	6.3[b]	0.76	25.2	0.75	....	....	....	....
Quercetin (Q)	10.8	1.00	33.6	1.00	12.9	1.00	36.0	1.00

Luteolin (3-desoxyquercetin) was compared with quercetin at 70°C.
The PF of luteolin was 8.8, and QI 0.26; the PF of quercetin was 33.8.

[a] The PF and QI are averaged results at peroxide values 20, 50, and 100, except for the results in the first experiment at 60°C; there, the results were averaged at peroxide values 20 and 50 only.

[b] Results at peroxide value 20; the PF of quercetin at that peroxide value was 8.2.

Table 2. Relative antioxidant activities of quercetin and its derivatives in linseed oil esters.[a]

Antioxidant	36°C		36°C		60°C	
	PF	QI	PF	QI	PF	QI
3-OMeQ	6.0[b]	0.34	5.3	0.33	....	....
5-OMeQ	4.6	0.26	11.6	0.70	....	....
7-OMeQ	16.4	0.85	15.4	0.93	....	....
3'-OMeQ	1.9	0.10	1.4	0.08	....	....
4'-OMeQ	0.6	0.03	0.5	0.03	....	....
3,5-diOMeQ	0.8	0.04	1.2	0.07	....	....
3,7-diOMeQ	5.4[b]	0.30	5.6	0.33	....	....
3,3'-diOMeQ	0.5	0.02	0.1	0.01	....	....
5,7-diOMeQ	2.4	0.12	3.2	0.15	7.1	0.16
3',4'-diOMeQ	0.4	0.02	0.3	0.02	1.0	0.02
3,5,7-triOMeQ	0.6	0.03	1.0	0.06	3.3	0.07
3,5,3'-triOMeQ	0.2	0.01	0.1	0.00	....	....
3,7,4'-triOMeQ	0.4	0.02	0.1	0.00	....	....
3,3',4'-triOMeQ	0.5	0.03	0.4	0.02	....	....
7,3',4'-triOMeQ	0.4	0.02	0.3	0.02	....	....
3,7,3',4'-tetraOMeQ	0.3	0.02	−0.2	−0.01	....	....
7-OBeQ	12.3[b]	0.68	16.0	0.96	....	....
7,4'-diOBeQ	0.6	0.03	0.2	0.01	....	....
7,4'-diOAllQ	1.6	0.08	0.9	0.05	....	....
2,3-dihydroquercetin (taxifolin)	7.3	0.38	6.1	0.36	....	....
5-desoxyquercetin (fisetin)	18.2	0.95	13.2	0.80		
Quercetin (Q)	19.1	1.00	16.6	1.00	41.0	1.00

Luteolin (3-desoxyquercetin) was compared with quercetin at 50°C. The PF of luteolin was 3.6, QI 0.23; the PF of quercetin was 15.6.

[a] The PF and QI are averaged results at peroxide values 20, 50, and 100.

[b] Averaged results at peroxide values 20 and 50; the average PF of quercetin for these peroxide values was 18.1.

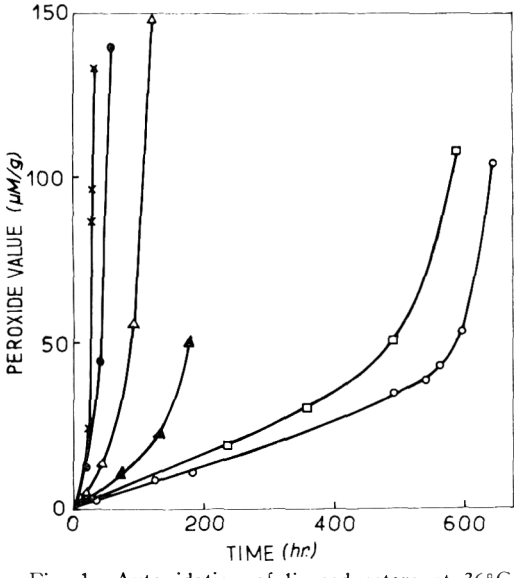

Fig. 1. Autoxidation of linseed esters at 36°C. Antioxidants added at 0.67 $\mu M/g$: Q, quercetin (○); 7-OMeQ, rhamnetin (□); 3-OMeQ (▲); 5,7-diOMeQ (●); none (×).

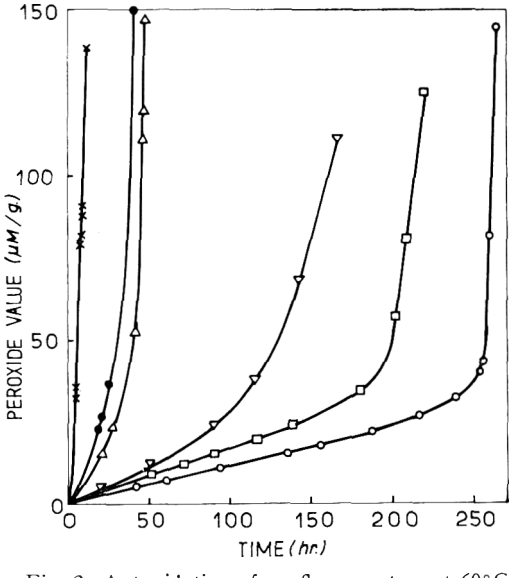

Fig. 2. Autoxidation of sunflower esters at 60°C. Antioxidants added at 0.67 $\mu M/g$: Q, quercetin (○); 7-OMeQ, rhamnetin (□); 5-OMeQ (▽); 5,7-diOMeQ (△); 3,5,7-triOMeQ (●); none (×).

Table 3. Relative activities of quercetin and its derivatives as primary antioxidants in dry ester system.

Class and range of QI	Compound	QI (av.)
G (good), (above 0.67)	Q (quercetin)	1.00
	7-OMeQ (rhamnetin)	0.93
	7-OBeQ	0.88
	5-HQ (fisetin)	0.81
F (fair), (0.26–0.67)	2,3-dihydroQ (taxifolin)	0.52
	3-OMeQ	0.48
	5-OMeQ	0.47
	3,7-diOMeQ	0.47
P (poor), (0.11–0.25)	3-HQ (luteolin)	0.24
	5,7-diOMeQ	0.16
	3′-OMeQ	0.11
VP (very poor), (0.02–0.10)	7,4′-diOAllQ	0.09
	3,5,7-triOMeQ	0.06
	3,5-diOMeQ	0.05
	4′-OMeQ	0.05
	7,4′-diOBeQ	0.04
	3,3′-diOMeQ	0.03
	3′,4′-diOMeQ	0.03
	3,3′,4′-triOMeQ	0.03
	7,3′,4′-triOMeQ	0.03
I (inactive), (below 0.02)	3,5,3′-triOMeQ	0.01
	3,7,4′-triOMeQ	0.01
	3,7,3′,4′-tetraOMeQ	0.00

had very little effect on the antioxidant activity, rhamnetin (*7-O*MeQ) and *7-O*BeQ being only slightly (about 10%) inferior to quercetin. The type of the alkyl group, methyl or benzyl, also had little or no effect. Rhamnetin has been reported by several workers to be superior to quercetin (Heimann and Reiff, 1953; Simpson and Uri, 1956), whereas Crawford *et al.* (1961), using lard as substrate, found its activity to be only 70% of that of quercetin. On the other hand, the last-named found the *7-O* benzyl derivative to be slightly (about 7%) superior to quercetin.

Since the 7-hydroxyl contributes little to the antioxidant activity of quercetin, it seems that a long-chain *7-O* alkyl derivative might be its equivalent in antioxidant properties while conveniently more soluble in oil.

Methylation of the 3, or 5, or 3 and 7 hydroxyls has nearly the same effect, reducing activity to about 50% of that of quercetin. Again, the indication is that the 7-hydroxyl contributes compartively little to activity, while both the 3- and 5-hydroxyls are obviously of importance. Surprisingly, however, methylation of both the 5- and the 7-hydroxyls reduces activity still further (to below 20%).

The 3- and 5-hydroxyls are supported by stronger conjugation systems than the 7-hydroxyl, and their hydrogens are therefore given up more easily. It seems that methylation of any hydroxyl reduces the antioxidant activity of the derivative, and that this effect is related to the probability of formation of stabilized free radical from the hydroxyl in question.

Hydrogenation of the 2,3 double bond (leading to destruction of the α,β-unsaturated ketone structure, and to breaking up of the conjugated chain between the B ring and the 4-keto group) reduces the activity to about 50% (in good agreement with Crawford's figure, 66%). This indicates that the structure of the molecule as a whole is of primary importance and not only the number and position of the hydroxyls (identical in quer-

cetin and dihydroquercetin). It is of interest that replacement of a hydroxyl with hydrogen did not necessarily have the same effect as methylation of the same hydroxyl group. Fisetin (5-HQ), for example, proved to be a considerably better antioxidant than 5-OMeQ (81 and 47%, respectively), whereas luteolin (3-HQ) was markedly inferior to 3-OMeQ (23 and 48%, respectively).

Our results show no indication that the meta-hydroxyl grouping of the A ring has any pro-oxidant effect, as suggested by Heimann and Reiff (1953).

Methylation of the three hydroxyls (3, 5, and 7) of the A ring reduces the activity to 6%, despite the fact that the important 3',4' orthodiphenolic grouping in the B ring is still intact. On the other hand, methylation of the 3', or 4', or 3' and 4' groups also drastically reduces activity (to 3–11%), indicating the expected and very marked contribution of the o-dihydroxyl grouping in the B ring. Obviously, the functions of the two parts of the molecule cannot be considered as additive in any way.

These observations are incompatible with those of Heimann and Reiff (1953), who found that 3,5,7-triOMeQ showed about two-thirds, and 3',4'-diOMeQ about one third, of the activity of quercetin.

Methylation of the single 4'-hydroxyl reduces activity quite considerably (to 5%), while, when the 3' hydroxyl alone is methylated, some activity (11%) is retained. The 4'-hydroxyl is more important, being supported by a stronger conjugated system than 3', and thus tends to form stabilized free radicals more easily. Blocking of both the 3'- and 3-hydroxyls reduces activity to 3%. This result was also obtained when both the 3'- and 4'-hydroxyls were methylated.

While it was observed occasionally that the relative activity of a series of antioxidants depends in some degree on the fatty-acid composition of the oxidizing substrate, no considerable differences were observed in the present work for the quercetin derivatives in the linoleate (sunflower) and linolenate (linseed) systems. The only striking exception was dihydroquercetin, which was 66% as active as quercetin in the linoleate system, and only 37% as active in the linolenate system.

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Anthocyanins of the Garden Huckleberry, Solanum guineese

SUMMARY

The anthocyanin pigments of the garden huckleberry *(Solanum guineese)* were isolated and identified from their chromatographic, spectral and chemical properties. The major pigment was petanin (petunidin-3-(p-coumaroyl rutinoside)-5-glucoside) (93%). The minor pigments were petunidin-3-rutinoside-5-glucoside (3.5%), negretein (malvidin-3 (p-coumaroyl rutinoside)-5-glucoside (2.0%), and a new compound, guineesin, petunidin-3-rutinoside-5-glucoside acylated with two molecules of p-coumaric acid (1.5%).

INTRODUCTION

The attractive black fruits of the garden huckleberry (*Solanum guineese*) have been used for preserves and pies, and as a colorant for apple sauce and fruit juices. They are particularly appropriate as colorants because the pigment is present in high concentration, and the plants are vigorous and easy to grow, and yield berries in great abundance. This paper is concerned with the identity of the anthocyanin pigments.

MATERIALS AND METHODS

The berries were grown at Amherst, Mass. One kilogram was macerated with 1% conc HCl in methanol and the filtered extract was concentrated under vacuum for chromatography.

Chromatographic methods. Whatman No. 3 paper was used for pigment purification, and No. 1 for determining R_f data by descending method in all cases. The following solvents were used for chromatography.

1. BAW	n-butanol, glacial acetic acid, water (4:1:5). Upper phase. Made up fresh for R_f data.
2. BAcW	n-butanol, glacial acetic acid, water (6:1:2). Upper phase.
3. BEW	n-butanol, 95% ethanol, water (4:2:2.2).
4. BuHCl	n-butanol, 2*N* hydrochloric acid (1:1). Upper phase. Paper equilibrated 24 hr after spotting and before running, in tank containing aqueous phase of BuHCl mixture.
5. BuN	n-butanol, 2% ammonium hydroxide (1:1). The papers were dipped in a saturated solution of boric acid in water and dried prior to use.
6. BBPW	n-butanol, benzene, pyridine, water (5:1:3:3).
7. Forestal	glacial acetic acid, conc. hydrochloric acid, water (30:3:10).
8. Formic	formic acid, conc. hydrochloric acid, water (5:2:3).
9. HAc-HCl	water, glacial acetic acid, 12*N* hydrochloric acid (82:15:3).
10. Phenol	phenol, water (4:1).
11. 15% HAc	glacial acetic acid, water (15:85).
12. 50% HAc	glacial acetic acid, water (50:50).
13. PrN	n-propanol, conc. ammonium hydroxide (7:3).
14. TAW	toluene, glacial acetic acid, water (4:1:5).
15. 1% HCl	conc. hydrochloric acid, water (3:97).

Aglycones. The aglycones were obtained by heating approximately 1 mg of purified pigment with 2*N* HCl in a water bath for 30 min. The aglycones were extracted with *n*-amyl alcohol, evaporated to dryness, and spotted on No. 1 paper together with authentic markers. The papers were run in Forestal, formic, and BAW solutions.

Sugars. The solutions remaining after extraction of the aglycones were treated with di-n-octylmethylamine (Harborne, 1958a) to remove the mineral acid, evaporated to dryness at room temperature, and spotted on No. 1 paper together with authentic markers. The papers were run in BAW, BEW, BBPW, and phenol. After development for 24 hr the papers were dried, dipped in aniline hydrogen phthalate (Partridge, 1959), and heated in an oven at 105°C for 2 min. The spots were clearly visible when examined under ultraviolet light.

Acyl groups. Approximately 1 mg of purified pigment was dissolved in aqueous 2*N* NaOH solution and allowed to stand for 2 hr at room temperature. Oxygen was removed from the reagents and headspace by flushing with nitrogen since petunidin is easily decomposed by oxygen in alkaline media.

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The mixture was acidified with 2*N* HCl and extracted 3 times with ethyl ether. The ether extracts were combined, concentrated, spotted on No. 1 paper, and run in BAW, TAW, PrN, and BuN. Authentic markers, caffeic, coumaric, and ferulic acids had been synthesized for previous work (Harborne, 1958b).

Peroxide hydrolysis. Hydrolyses for removal of the sugars at the 3 position were performed by the method of Chandler and Harper (1961). Approx. 1 mg of purified pigment was dissolved in methanol, treated with hydrogen peroxide and then ammonia, concentrated, and spotted directly on No. 1 paper with authentic sugar markers. Authentic rutinose was obtained by carrying rutin through the same hydrolysis procedure. Chromatographic solvents were the same as listed for sugars above.

Controlled hydrolyses. Acid hydrolyses for determination of intermediate pigments were performed by dissolving approx. 5 mg of purified pigment in 2*N* HCl and heating in a water bath. Portions were withdrawn at intervals of 1, 2, 4, 8 min, etc., up to 1 hr, and spotted directly on No. 1 paper. The papers were run in BAW, BuHCl, 1% HCl, formic, and Forestal.

Authentic pigments. Negretein, petanin, petunidin-3-rutinoside-5-glucoside (abbreviated here as Pt3RG5G), and malvidin-3-rutinoside-5-glucoside (Mv3RG5G) were available from previous work, on the potato variety "Congo" (Harborne, 1959).

Spectral data. All spectral data were determined with a Unicam SP500 spectrophotometer. The $AlSl_3$ shifts were determined by dissolving the purified pigment in 0.1% HCl in methanol and adding one drop of 5% $AlCl_3$ in ethanol to 3 ml. Determination of spectral maxima and visible color before and after addition of $AlCl_3$ showed the presence or absence of the shift.

RESULTS AND DISCUSSION

Chromatographic separation. The crude pigment mixture in 1% HCl in methanol was streaked on No. 3 paper and run in BAW for 16 hr. Three bands appeared, a major one at R_f 0.3 (2), and two minor bands at 0.2 (1) and 0.4 (3). The minor band 1 was cut out, eluted with methanol, glacial acetic acid, and water (90:5:5, MAW), and rerun in 1% HCl. The band was eluted again with MAW, and rerun in BAW. After a fourth purification, in 15% HAc, the pigment was used in identification. The major band 2 was eluted with MAW and purified by successive development and elution through BAW, 1% HCl, BAW, and 15% HAC. During the third purification in BAW, the pigment would often separate into two widely separated bands which upon subsequent purification would recombine. This was probably due to the acid-front phenomenon described by Albach *et al.* (1965). Band 2 contained petunidin and malvidin derivatives which could not be separated by chromatography without excessive labor because even a fifth purification in BuHCl failed to separate the two. Band 2 after four purifications was hydrolyzed with NaOH under nitrogen and chromatographed in BAW for 60 hr. The alkaline hydrolysis removed the acyl group, and the petunidin and malvidin derivatives could be separated with prolonged chromatography. The pure petunidin derivative (Pt3RG5G) crystallized very easily when its methanol concentrate was allowed to stand in the refrigerator for three days. The minor band 3 was purified by successive development and elution through BAW, 1%

Table 1. Chromatographic data (R_f) for anthocyanins.

Band	Solvent system			
	BAW	BuHCl	Forestal	1% HCl
1	0.22	0.06	0.79	0.37
1 (alk hyd.)	0.22	0.06	0.80	0.36
2.1	0.32	0.32	0.85	0.54
2.1 (alk hyd.)	0.23	0.06	0.81	0.38
2.2 (alk hyd.)	0.29	0.05	0.83	0.39
3	0.40	0.67	0.88	0.30
3 (alk hyd.)	0.20	0.06	0.80	0.37
Pt3RG5G	0.22	0.06	0.80	0.37
Mv3RG5G	0.29	0.05	0.84	0.40
Negretein	0.34	0.32	0.86	0.20
Petanin	0.30	0.29	0.82	0.19

Table 2. Chromatographic data (R_f) for anthocyanidins.

Band	Solvent system		
	BAW[a]	Forestal	Formic
1	0.50	0.45	0.21
2.1	0.50	0.45	0.21
2.2	0.55	0.58	0.28
3	0.51	0.46	0.20
Petunidin	0.52	0.46	0 20
Malvidin	0.56	0.59	0.28
Delphinidin	0.42	0.30	0.13
Peonidin	0.70	0.63	0.29

[a] Paper washed with dilute HCl and dried prior to use.

Table 3. Chromatographic data (R_g; glucose = 1) for sugars.

Band	Solvent system			
	BAW	BEW	BBPW	Phenol
Acid hydrolysis				
1	1.02, 2.20	1.00, 2.00	1.00, 2.10	1.01, 1.72
2.1	1.02, 2.19	1.00, 2.10	1.00, 2.10	1.02, 1.73
2.2	1.01, 2.18	0.99, 2.10	0.98, 2.15	1.00, 1.74
3	1.01, 2.18	0.98, 2.05	0.98, 2.15	1.02, 1.72
Peroxide hydrolysis				
2.1	0.74	0.71	0.50	0.56
Rutin	0.74	0.70	0.50	0.55
Markers				
Galactose	0.85	0.90	0.81	1.14
Glucose	1.00	1.00	1.00	1.00
Arabinose	1.29	1.23	1.23	1.49
Xylose	1.48	1.40	1.50	1.28
Rhamnose	2.14	2.00	2.10	1.74

Table 4. Chromatographic data (R_f) for cinnamic acids.

Band	Solvent system			
	BAW	TAW	PrN	BuN
1	.....	.....	.....	.....
2.1	0.91	0.36	0.68	0.49
2.2	0.91	0.36	0.68	0.49
3	0.91	0.36	0.68	0.49
p-Coumaric acid	0.91	0.36	0.68	0.49
Caffeic acid	0.82	0.02	0.50	0.30
Ferulic acid	0.89	0.98	0.60, 0.66[a]	0.38

[a] Cis and trans ferulic acid.

HCl, BAW, 15% HAC, BuHCl, and 50% HAC.

Pigment identification. The R_f data in Table 1 for band 1 are similar to those for Pt3RG5G (Harborne, 1960). Alkaline hydrolysis did not change the R_f data, indicating the absence of an acyl group. The R_f data for band 2 are similar to those for petanin, whereas those for band 3 are suggestive of the same pigment with two acyl groups. Alkaline hydrolysis of both bands 2 and 3 produced a pigment identical with band 1.

Acid hydrolysis of bands 1, 2.1, 2.2, and 3 provided evidence (Table 2) that the aglycones were respectively petunidin, petunidin, malvidin, and petunidin. A positive $AlCl_3$ shift for bands 1, 2.1, and 3 and a negative result for 2.2 supported the above conclusion. The purple color of the aglycones from 1, 2.1, and 3 and the bright red of 2.2, when viewed under ultraviolet light, provided further evidence. The sugars in all four bands were identical, with the spots approximately twice as dense for glucose as for rhamnose as judged by visual examination. Glucose and galactose were clearly distinguished because of the reversal of the R_f values in BBPW and phenol.

The peroxide analysis provided evidence that the sugar at position 3 was rutinose (rhamnosyl α→6 glucose), since this procedure specifically removes the sugar at the 3 position. Since acid hydrolysis gave twice as much glucose as rhamnose, the sugar at the 5 postion must be glucose.

Table 5. Spectral data for anthocyanidins in methanolic 0.01% HCl solution.

Band	λ_{max} (nm)	E_{440}/E_{max} (%)	E_{328}/E_{max} (%)	Pigment
1	538	10	10	Pt3RG5G
2	540	10	70	Petanin
3	540	12	130	Guineesin

Table 6. Products of controlled hydrolysis of band 2.1 in order of appearance (R_f).

Pigment	Solvent				
	BAW	BuHCl	1% HCl	Formic	Forestal
Petanin	0.32	0.32	0.19	0.91	0.82
Pt3RG5G	0.22	0.08	0.36		
Pt3RG	0.30	0.18	0.13	0.84	0.70
Pt3G5G	0.20	0.04	0.09	0.86	
Pt3G	0.31	0.14	0.04	0.66	0.61
Pt5G	0.34		0.03	0.62	
Pt	0.50	0.42	0.01	0.22	0.44

The chromatographic data for cinnamic acids (Table 4) indicate that the acyl group in pigments 2.1, 2.2, and 3 is p-coumaric acid. This acid is easily recognized on a chromatogram because of its deep-blue fluorescence under ultraviolet light in the presence of ammonia. The R_f data provided in Table 5 differ somewhat from those of Albach *et al.* (1965), possibly because descending chromatography was used.

The spectral data (Table 5) are in agreement with the pigments suggested by the R_f data. The E_{440}/E_{max} values indicate that the 5 position is substituted (Harborne, 1958b). Since rutinose is on the 3 position, glucose must be on the 5 position. The E_{308}/E_{max} ratio for pigment 1 indicates the absence of an acyl group, whereas that for pigment 2 indicates one acyl group. The data for pigment 3 indicate two acyl groups (Harborne, 1958b). The acylated petunidin derivatives were very difficult to purify since cinnamic acids were present in the berries in large quantities and fluorescent impurities were evident during the purification steps. Pigment 2 was probably contaminated with a small amount of negretein, but it could not be separated by the usual chromatography.

The products of controlled hydrolysis (Table 6) of pigment 2.1 are in agreement with the proposed structure of the pigment. All the theoretical intermediates were isolated in at least one of the five solvent systems and identified by their R_f values (Harborne, 1960). The data for pigment 1 were identical with those in Table 6 except that the acylated compound was not present. Pigment 3 is a new compound, for which the name "guineesin" is proposed. It could not be obtained in sufficient quantity for further analysis, so that the positions of the two acyl groups in the molecule still remain to be determined.

The approximate concentrations of each pigment are listed in Table 7. The total pigment was estimated by measuring the absorbance of a berry extract in an 0.1*N* HCl and 95% ethanol (15:85) solution, and assuming an E (1%, 1cm, 540 nm) value of 900. The Pt3RG5G concentration was determined by measuring the ratio of absorbances of pigment 1 to that of 2 and 3 after elution from the original BAW chromatogram. The concentration of negretein was estimated from the absorbance of pigments 2.1 and 2.2 after alkaline hydrolysis and

Table 7. Approximate concentration of anthocyanins in fresh berries.

Number	Pigment trivial name	Structure	% of total pigment
1	Pt3RG5G	Petunidin-3-rutinoside-5-glucoside	3.5
2.1	Petanin	Petunidin-3-(p-coumaroyl rutinoside)-5-glucoside	93
2.2	Negretein	Malvidin-3-(p-coumaroyl rutinoside)-5-glucoside	2.0
3	Guineesin	Di-p-coumaroyl petunidin-3-rutinoside)-5-glucoside	1.5

chromatography. The concentration of guineesin was estimated from the ratios of absorbance of pigment 3 to those of 1 and 2 from the original BAW chromatogram. The total pigment was approximately 0.1% of the fresh weight.

Acylated anthocyanins are common among members of the Solanaceae family. Harborne (1960) identified the 3-(p-coumaroyl rutinoside)-5-glucosides of pelargonidin, cyanidin, peonidin, malvidin, petunidin, and delphinidin in the potato, *Solanum tuberosum*. Sakamura (1963) reported the presence of delphinidin-3-(p-coumaroyl rutinoside)-5-glucoside in eggplant, *Solanum melongena* L., and Harborne (1965) discussed the occurrence of this type of compound in many other members of the genus, and also in other genera of the Solanaceae. All the pigments now found in the garden huckleberry are thus characteristic of this family.

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Deterpenation of Citrus Essential Oils by Solvent Partition with Dimethyl Sulfoxide

SUMMARY

A comparison of deterpenation of cold-pressed lemon and orange oils by silicic acid, steam distillation, and dimethyl sulfoxide (DMSO) partition is presented. The recoveries by each method for lemon and orange oils and the ultraviolet absorbance of the orange oil fractions are given. Deterpenation by DMSO compared favorably with the steam-distillation and silicic acid methods. The recoveries of 34 naturally occurring citrus constituents from aqueous DMSO after solvent partition with pentane are presented to indicate the separation capabilities of DMSO for flavor and aroma studies in citrus and citrus products and possible application to residue analyses.

INTRODUCTION

Essential oils are present in a number of fruits and vegetables upon which the residue analyst is required to work. Essential oils are composed mainly of terpene hydrocarbons and their oxygenated derivatives—the terpene hydrocarbons generally predominating. In citrus, terpene hydrocarbons make up 80–90% of the total essential oils. Essential oils are readily extracted from plant tissue by a variety of aliphatic, aromatic, or chlorinated solvents. The terpene hydrocarbons can be separated from oxygenated components by steam distillation (Langenau, 1948; Burchfield and Storrs, 1962; Rotondaro, 1964) or silicic acid chromatography (Kirchner and Miller, 1952).

Dimethyl sulfoxide (DMSO) is a polar solvent in which most aromatic and unsaturated hydrocarbons are soluble but in which paraffinic hydrocarbons are only slightly soluble. It is used as an extraction solvent for highly unsaturated condensed aromatic hydrocarbons (Haenni *et al.*, 1962a,b; Crown Zellerbach, 1963a; Howard and Haenni, 1963, 1965) and for many organic pesticides (Crown Zellerbach, 1963b; Beroza and Bowman, 1965a,b). In connection with cleanup studies on citrus extracts, the oxygenated terpenes and other oxygenated or aromatic components were found to be preferentially soluble in DMSO when citrus constituents were partitioned with pentane. This solvent partition permitted the selective removal of the major terpene fraction. This paper compares deterpenation by steam distillation, silicic acid, and solvent partition between DMSO and pentane and presents data on the recovery of several natural citrus constituents (Kirchner *et al.*, 1951; Stanley *et al.*, 1961; Miller and Kirchner, 1963; Peyron, 1963; Stanley, 1963) from aqueous DMSO after solvent partition with pentane. Information of this type indicates the separation capabilities of DMSO for citrus constituents and is useful both in flavor and in aroma studies and in developing cleanup procedures for residue analyses in citrus and citrus products.

METHOD

Apparatus

a) Rotary flash evaporator. Buchler Instruments Model PTDE-1.

b) Spectrophotometer. Bausch and Lomb Model 505.

c) Gas chromatograph. Loenco Model 150–15A equipped with dual hydrogen flame ionization detectors, temperature programming, and a Cary Model 31 vibrating reed electrometer and a Minneapolis Honeywell Model 143X 10-mv recorder.

d) Gas Chromatographic columns. Dual columns, 12-ft × ⅛-inch ID, stainless steel, containing 10% SE-30 silicone rubber on 50–60-mesh Anakrom ABS.

e) Gas chromatographic gas flows. Nitrogen carrier gas, 50 ml/min; hydrogen, 20 ml/min; and air, 300 ml/min.

f) Separatory funnels. 125-ml capacity equipped with tetrafluoroethylene polymer stopcocks.

g) Steam distillation apparatus. Standard apparatus equipped with a modified Clevenger trap as described by Stanley *et al.* (1957).

Reagents

a) n-Pentane. Pure grade (Phillips Petroleum Co.), redistill and pass through activated silica gel before use. Pre-equilibrate with DMSO for partitions.

b) DMSO. Spectrophotometric grade (Crown Zellerbach Co.). Pre-equilibrate with n-pentane.

c) Silica gel. Grade 08, mesh 12–28 (Davison Chemical Co.). Activate for 2 hr at 550°C immediately before use.

d) Silicic acid. 100-mesh. Mallinckrodt No. 2847. Activate the silicic acid by heating 3 hr at 105°C and cool in a desiccator.

e) Sodium sulfate. Anhydrous, granular.

f) Citrus constituents. Dodge & Olcott, Glidden Co., Eastman, Matheson Coleman & Bell, and Aldrich Chemical Co. Cold-pressed lemon and orange oils and crude lemon wax were obtained from the Sunkist Research Department, Ontario, California.

Procedure. *Deterpenation by silicic acid.* Ten ml of essential oil was weighed and added to 75 ml of pentane and 15 g silicic acid. The mixture was allowed to stand at room temperature with occasional shaking for 2 hr. The pentane fraction was filtered through a sintered-glass funnel under reduced pressure, and evaporated to dryness on a flash evaporator. The oxygenated fraction retained on the silicic acid was eluted with absolute ethanol and the ethanol evaporated to dryness on a flash evaporator. The residue from each fraction was weighed.

Deterpenation by steam distillation. Ten ml cold-pressed oil was placed in a 250-ml distilling flask with 50 ml water and 0.1 ml 1*N* HCl. The flask was connected to an all-glass distillation assembly with a modified, graduated Clevenger trap. The mixture was distilled until the volatile fraction was completely separated. The distillate in the trap was allowed to cool and the volume of oil read.

Deterpenation by solvent partition. Ten ml cold-pressed oil was weighed and quantitatively transferred to a 125-ml separatory funnel. n-Pentane pre-equilibrated with DMSO was added to make a total volume of 50 ml. The mixture was shaken vigorously with 3 successive 10-ml portions of DMSO pre-equilibrated with n-pentane (Haenni *et al.*, 1962a,b; Howard and Haenni, 1963; Beroza *et al.*, 1965a,b). After each lower DMSO phase had separated it was removed and these fractions were combined in a clean separatory funnel. The combined DMSO fractions were diluted with 2 volumes of cold 10% NaCl. The aqueous DMSO was extracted with 3 successive 10-ml portions of n-pentane. The combined pentane extracts were washed with 3 successive 10-ml portions of distilled water. The pentane extracts and the original oil-pentane mixture were filtered through anhydrous sodium sulfate into tared 50-ml round-bottom flasks. The pentane was evaporated off on a flash evaporator and the residue weighed.

Partition of citrus constituents. Known concentrations of each citrus constituent were prepared in n-pentane; 1 mg in 100 ml was usually sufficient. An appropriate aliquot was transferred to a 125-ml separatory funnel. The mixture was extracted as described under deterpenation by solvent partition. The residue was dissolved in 10.0 ml n-pentane for spectrophotometric measurements. A few of the citrus constituents lacked sufficient spectral detail for quantitative spectrophotometric measurements. Recoveries of these compounds were determined by weighing the residues or by gas chromatographic measurements.

For gas chromatographic measurements, the residues were dissolved in 2.0 ml of carbon disulfide containing 20 mg/ml of n-butyl benzene (Ikeda *et al.*, 1962) as an internal standard. Column temperatures for separation of butyl benzene from some citrus constituents were 75°C for beta-pinene, p-cymene, and linalool; 95° for alpha-terpineol; 140° for geranyl acetate; and 75° programmed to 150° for alpha-citral.

The percent recovered from aqueous DMSO

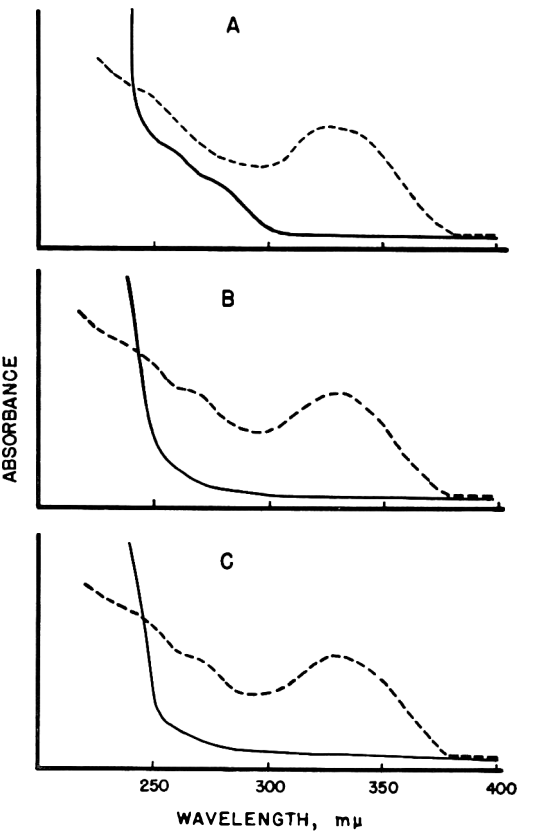

Fig. 1. Ultraviolet absorbance of orange oil fractions in purified n-pentane after: A) silicic acid; B) steam distillation; and C) dimethyl sulfoxide deterpenation. Terpene fraction: solid line. Oxygenated fraction: dotted line.

Table 1. Deterpenation of crude orange and lemon oils by three procedures.

Procedure	Fraction	% recovery [a] Orange oil	Lemon oil
Silicic acid adsorption	Pentane eluate [b]	90.0±1.2	85.5±2.4
	Ethanol eluate [c]	5.3±0.4	7.0±0.4
Steam distillation	Volatile [b]	93.1±1.8	95.5±0.7
	Non-volatile [c]	6.9±1.8	4.5±0.7
Solvent partition	Pentane [b]	96.8±1.9	94.1±1.9
	Dimethyl sulfoxide [c]	3.2±1.9	5.9±1.9

[a] Mean of 3 determinations, expressed as percentage ± standard deviation.
[b] Terpene fraction.
[c] Oxygenated fraction.

was calculated from the absorbance, weight, or gas chromatographic peak area of the sample before and after extraction.

RESULTS

Table 1 gives the recovery of cold-pressed orange and lemon oils deterpenated by silicic acid, steam distillation, and DMSO partition. Steam distillation and DMSO partition gave a higher percent recovery of the terpene fraction than silicic acid adsorption. Fig. 1 presents the ultraviolet absorbance of cold-pressed orange oil deterpenated by the 3 methods. Dimethyl sulfoxide and steam distillation gave the cleanest separation of the oxygenated fraction, whereas the terpene fraction separated by silicic acid had a higher absorption between 250 and 290 mμ, indicating the presence of a small amount of oxygenated compounds in the terpene fraction.

Table 2 summarizes the recovery of several

Table 2. Partition of terpenes and other citrus constituents between dimethyl sulfoxide (DMSO) and pentane.[a]

Citrus constituents	Total recovery from DMSO (%)	Citrus constituents	Total recovery from DMSO (%)
Crude orange oil [b]	3.2	Esters	
Crude lemon oil [b]	5.9	Ethyl anthranilate	80.2
Crude orange terpenes [b]	9.6	Linalyl proprionate	73.5
Crude lemon wax [b]	7.0	Geranyl formate	71.9
Terpenes		Methyl anthranilate	71.4
gamma-Terpinene	20.3	Citronellyl	70.9
p-Cymene [c]	5.2	Methyl-N-methyl anthranilate	70.7
Myracene	5.0	Octyl formate	47.9
Camphene	4.8	Geranyl acetate	23.1
Limonene [b]	4.6	Geranyl butyrate	40.8
beta-Pinene [c]	0.7	Methyl isoferulate	18.8
Aldehydes		Flavones	
Citral [c]	89.7	Pentamethoxyflavone	29.3
Cinnamaldehyde	65.9	Psoralens	
Citronellal	44.7	Imperatorin	103.5
Lauric	0	Bergapten	97.2
Ketones		Xanthotoxin	80.7
L-carvone	63.3	Pigments	
Alcohols		beta-Carotene	0
Linalool [c]	63.4	Chlorophyll	100.3
alpha-Terpineol [c]	60.9	Xanthophyll	98.9

[a] All measurements are spectrophotometric unless otherwise stated.
[b] Measured by weight.
[c] Measured by gas chromatography.

natural citrus constituents from aqueous DMSO after solvent partition with pentane. All measurements are spectrophotometric unless otherwise stated. The terpenes were only slightly soluble in DMSO. The recovery of the aldehydes, esters, ketones, and alcohols varied from approximately 20 to 90 % in 3 DMSO extractions. Beta-carotene remained in the pentane fraction, whereas DMSO extracted 100% of the chlorophylls and 99% of the xanthophylls. Only 7% of crude lemon wax was recovered by DMSO.

DISCUSSION

Preliminary separation and concentration of the oxygenated fraction are essential steps in flavor and aroma studies. Solvent partition between DMSO and pentane is comparable to steam distillation for separating the oxygenated fractions from the terpene hydrocarbons. This separation facilitates the analysis of essential oils. Solvent partition has the advantage over steam distillation in that it is faster and eliminates possible decomposition or structural rearrangement of heat-labile compounds. Terpene hydrocarbons may also isomerize when passed through silica gel columns (Hunter and Brogden, 1963). The recovery values of the natural citrus constituents from aqueous DMSO after solvent partition with pentane indicate the separation capabilities of DMSO for studies of essential oils (Table 2).

The separation capabilities of DMSO suggest potential application in the development of residue analyses of citrus. The number of DMSO extractions was set arbitrarily at three. The optimum number of extractions would be dependent upon the solubility of the particular residue under study in relation to the solubility of the naturally occurring citrus constituents.

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Pressure Losses and Rheological Properties of Flowing Butter

SUMMARY

The pressure losses and rheological properties of butter flowing through stainless-steel tubing were investigated. Since viscosity is a measure of internal resistance to flow, an extrusion viscometer was constructed to measure apparent viscosity. The apparent viscosity was calculated from the extrusion viscometer data using the Hagen-Poiseuille equation for flow through tubing. Flow profiles were obtained by alternately forcing butter of different colors through the various lengths of tubing at different temperatures. The velocity gradient was small within the butter except near the wall. A linear relationship was found between the logarithm of apparent viscosity and the logarithm of bulk velocity for a range of 0.001 to 1 ft/sec. The average slope of the regression line was −0.846. As the length of the tubing increased, the average apparent viscosities decreased, though at a decreasing rate. Very small differences were found between the apparent viscosities obtained with 10.5-in. and 14.0-in. lengths of tubing. The influence of temperature on the logarithm of the apparent viscosity was found to be linear, having a slope of −0.059 for the range of 55–75°F. A general empirical equation was determined relating the influence of bulk velocity (v, ft/sec) and temperature (T, °F) to the decrease in apparent viscosity (η, lb/ft sec):

$$\log \eta = 4.762 - 0.846 \log v - 0.59T$$

which is considered valid for temperatures between 55 and 75°F.

INTRODUCTION

Butter has been transferred from the churn to the printer or bulk container primarily by manual methods in the United States. Attempts have been made to develop methods of emptying the churn mechanically to eliminate the labor involved and to reduce the possibility of contamination. The two main methods are the butter truck and the gear-type pump. Both of these methods are utilized with churns incorporating specific designs.

To develop better methods of handling butter mechanically, a knowledge of the physical characteristics of flowing butter would be very important. Butter is usually subjected to empirical tests which evaluate characteristics of immediate interest to the consumer, and, except in isolated cases, little effort has been made to consider the fundamental flow properties.

The rheological properties of butter (those associated with flow) will be influenced by the volume of both the dispersed phase (fat globules and aqueous solution) and the continuous phase (fat crystals), by the flow properties of the continuous phase, by the deformability of the dispersed phase, and by the proportion, form, and arrangement of the dispersed particles.

King (1964) described the structural elements of the butter as a complicated type of emulsion. The fat globules and crystals, moisture droplets, and cured particles are about 0.5 to 20 μ in size. He stated that under the microscope in polarized light the crystalline fraction of free fat in conventional butter can be perceived as minute crystals resulting from an eventual partial crystallization taking place in the free fat.

Several authors have stressed the formation of a three-dimensional network from the needle-like fat crystals. The high structural viscosity of butter was explained by Dolby (1941) as a crystalline network. Mulder (1953) stated that, because milk fat can easily be supercooled and liquid fat often occurs in freshly churned butter in the supercooled state, the increase in hardness is due to the formation of a "skeleton-like structure" as a result of the growing together of the fat crystal. As pointed out by deMan and Wood (1958), the minute fat crystals are responsible for the thixotropic changes in butter because of their anisometric shape (needles) and size (below 1 μ). Upon moving, working, or stirring of butter, the links between the interlaced crystals, presumably van der Waals' forces, are broken, and the system becomes softer. On standing, the crystals in the butter rearrange into a continuous pattern and the system becomes harder. This process is known as "setting." Sone *et al.* (1962) related the recovery of

viscosity with the recrystallization of the fat and the formation of a crystalline network.

X-ray studies by Knoop and Samhammer (1962) indicated less than 20% of the fat in butter is in the crystallized form. They suggested this amount does not significantly influence hardness of butter. Haighton (1965) reported that the fat crystals in butter do not move in respect to each other. They are fixed in a tridimensional network. This network causes about 60–80% of the hardness. It can be measured by kneading the sample isothermally. Prentice's studies (1953) showed that changes in spreadability were similar to changes of butter firmness. He reported that sweet cream butter 1 wk old with a 1% deformation at 9.5°C had to increase to 14.0°C to remain at 1% deformation when held 19 wk.

PROCEDURE

Three manufacturers supplied 60 lb of butter processed commercially on two days. The boxes of bulk butter were stored at 34°F until all were received. Each sample was cut into 2- to 3-lb blocks to facilitate tempering. The blocks were wrapped in parchment paper. Portions were stored at 50, 55, 60, and 70°F for at least 48 hr. The composition was determined by the Kohman method (Milk Industry Foundation, 1949). The iodine value was determined by the Hanus method (AOAC, 1960). The density of the butter was determined by extruding a sample from the tubing in a weighed container of known volume.

The equipment for extrusion viscometer (Fig. 1) consisted of four interchangeable stainless-steel tubings of equal diameter (0.313 in.) and lengths of 3.5, 7.0, 10.5, and 14.0 in. (Fig. 1). A disc containing a hole in its center was made to fit the tubing. The disc was attached to a sample container by a 1.5-in. stainless-steel female fitting. The container was made from a 1.5-in.-diameter stainless-steel tubing. Compressed nitrogen was used as a source of pressure.

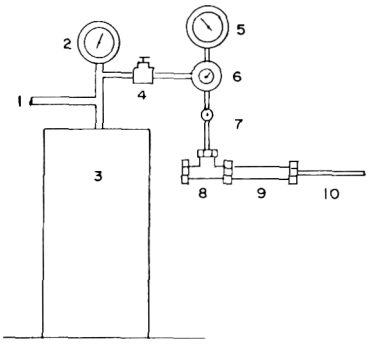
The shape of the velocity profile was obtained by forcing dark-blue and regular butter through various lengths of tubing at several temperatures. The resulting cylindrical samples were conditioned at a low temperature and cut in half to show the flow profiles.

The apparent viscosity of flowing butter was determined from the extrusion viscometer data using the Hagen-Poiseuille equation:

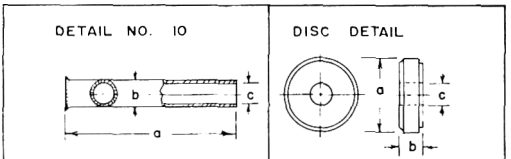
$$\eta = \frac{\pi \Delta P R^4}{8QL}$$ where η is the viscosity; ΔP is the pressure loss; R is the radius of the tubing; Q is the volumetric flow rate; and L is the length of the tubing. The equipment and supplies were tempered and the data were obtained from tests conducted in rooms with controlled temperatures.

A test was conducted by placing the butter in the sample container and attaching it to the pressure equipment. The tubing was connected to the other end. Pressure was applied directly to the surface of the butter by increments of 1 or 2 lb/sq in. at 70°F and increments of 5 lb/sq in. at 65, 60, and 55°F. Data were gathered for each length of tubing at 55–70°F. But, some studies were limited to three temperatures.

The pressure loss for the sample container was determined by forcing the butter through a disc similar to the disc holding the tubing except that the diameter of the hole in its center was equal to the diameter of the inside of the tubing (Fig. 1). The pressure loss was determined by slowly applying pressure to the system until the butter started to flow out of the hole in the disc. After several replicates, the gauge pressure noted just before the



1. Pressure source connector
2. Pressure gauge
3. Air cylinder
4. Shutoff valve
5. Pressure gauge
6. Pressure regulator
7. Two-way valve
8. Stainless steel tee
9. Sample container
10. Stainless steel tubing



TUBING NO.	DIMENSIONS (IN.) a	b	c
A	3.5	0.375	0.313
B	7.0	0.375	0.313
C	10.5	0.375	0.313
D	14.0	0.375	0.313

DISC NO.	DIMENSIONS (IN.) a	b	c
A	1.875	0.313	0.313
B	1.875	0.313	0.375

Fig. 1. Extrusion viscometer and tubing equipment arrangement.

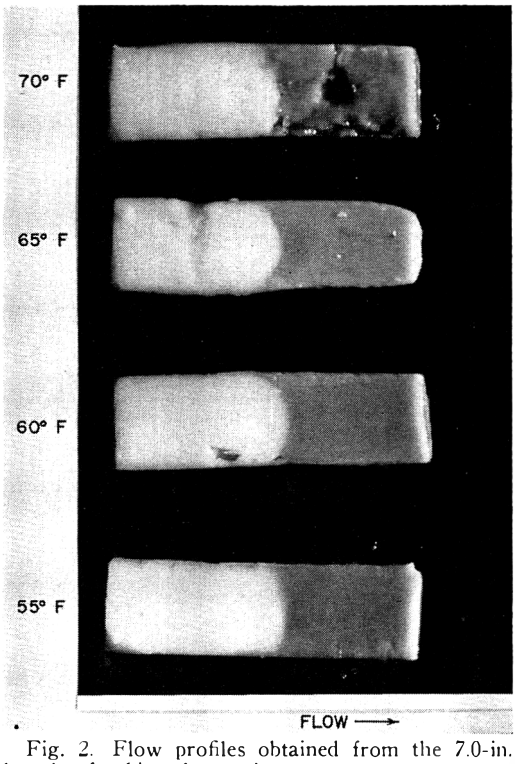


Fig. 2. Flow profiles obtained from the 7.0-in. length of tubing for various temperatures.

observed movement was recorded to the nearest lb/sq. in. The sample container loss was subtracted from the measured pressure loss obtained for each tubing length to obtain the pressure loss along the tubing.

The extruded butter was cut at the exit of the tubing with a spatula. At the end of 15 sec the edge of the weighed container was used to cut the butter at the exit of the tubing. Three samples were taken for each pressure. The temperature of the butter was determined by inserting the thermometer into the butter within the tubing after each test.

The regression coefficients and the standard error of estimate were used in the equation: $Y = a + bX \pm SE$ where $Y = \log \eta$ and $X = \log v$.

The continuous loss of pressure resulting when a fluid flows in a tube must be determined. Many investigators have established experimentally that, for adiabatic flow, the pressure loss (h, ft) due to friction is a function of the length (L, ft) and diameter (D, ft) of the tube and of the velocity (v, ft/sec) of the fluid (King, 1954). Thus, $h = f\frac{L}{D}\frac{v^2}{2g_c}$. In flow calculations, L, D, and v are usually known; therefore, if the friction factor (f) can be determined, the pressure loss or loss of head can be calculated.

For laminar flow the friction factor is:

$$f = \frac{64}{\mathrm{Re}} \text{ and } \mathrm{Re} = \frac{Dvl^2}{\eta} \text{ (Reynolds number).}$$

The apparent viscosity and bulk velocity data from the extrusion viscometer were used to calculate the Reynolds number and friction factor. An equation for calculating the apparent viscosity at various temperatures and bulk velocities was also determined. The above relationships were used to calculate the loss of head and power requirements of butter at 55, 60, and 65°F when forced through tubing with 1.5- and 3.0-in. diameter.

RESULTS AND DISCUSSION

The flow profiles (Figs. 2, 3) revealed that a variation in velocity occurs in the butter across the diameter of the tubing. The velocity gradient is small in the butter except near the wall. The plug flow profile becomes flatter as the temperature is lowered from 70 to 55°F, because of the increase in the viscous properties of the continuous phase. As the distance of butter-flow increased, the profile became more cone-like in shape (Fig. 3), indicating that the velocity gradient, even

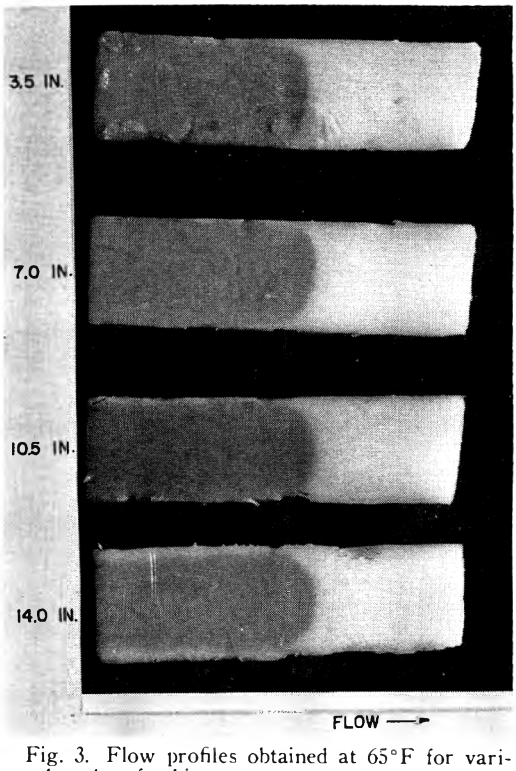


Fig. 3. Flow profiles obtained at 65°F for various lengths of tubing.

though small, is still present. Mulder *et al.* (1956), using colored butter in their moisture dispersion studies, concluded that the gradient of velocity was large in the butter near the walls of a perforated disc through which it was forced, and small for the remainder.

The relationship between the logarithm of apparent viscosity and the logarithm of velocity was found to be linear. The average slope of the regression lines was −0.846. This linear relationship is in agreement with results of Sone *et al.* (1962) with a parallel-plate plastometer at lower rates of shear and 68 and 77°F. The results of the correlation and regression analysis on the apparent viscosity versus velocity are presented in Table 1. The correlation coefficients ranged from 0.9738 to 0.9975. The regression equations have negative slopes ranging from −0.764 to −0.924. The decrease in apparent viscosity with an increase in bulk velocity can be illustrated by comparing the results from the 10.5-in. length of tubing at 62.2°F for various velocities. The average apparent viscosity was 4,380 lb/ft sec at 0.001 ft/sec, while at 0.01 ft/sec it had decreased to 582 lb/ft sec for the length of tubing. Likewise, for 0.1 ft/sec the apparent viscosity is only 77 lb/ft sec.

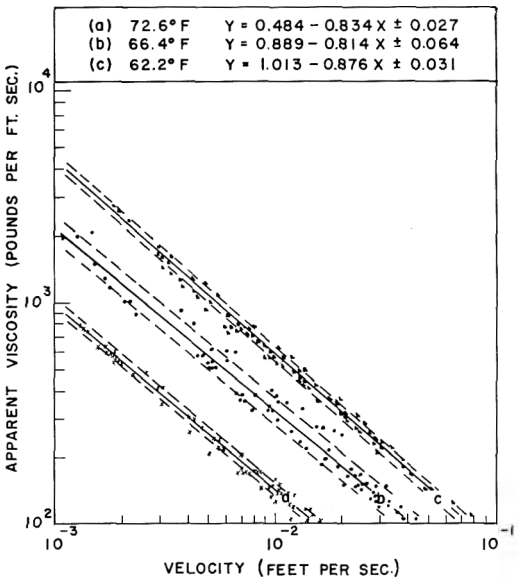


Fig. 4. Effect of temperature on the apparent viscosity of flowing butter with the dotted lines showing the standard error of estimate (10.5-in. tube length).

The influence of the length of tubing on the apparent viscosity for butter flowing at different temperatures is also shown in Table 1. At a constant velocity of 0.1 ft/sec and at 62°F the apparent viscosity is 142 lb/ft sec for a distance of 3.5 in. For a distance of 7.0 in. the apparent viscosity is 89 lb/ft sec,

Table 1. Results of the correlation and regression analysis for the apparent viscosity versus velocity.

Tube length (in.)	Temperature (°F)	Regression coefficients		Standard error of estimate *SE*	Correlation coefficient[a]	Degrees of freedom *DF*	Apparent viscosity (lb/ft sec)	
		a	*b*				Bulk velocity 0.01 ft/sec	Bulk velocity 0.1 ft/sec
3.5	72.9	0.732	−0.861	0.034	0.998	88	254	31
3.5	66.4	1.120	−0.819	0.055	0.995	70	573	87
3.5	62.3	1.246	−0.906	0.081	0.990	82	1140	142
3.5	56.6	1.194	−0.924	0.131	0.976	67	1100	131
7.0	72.9	0.482	−0.862	0.044	0.992	88	160	22
7.0	66.4	1.040	−0.764	0.064	0.984	85	369	64
7.0	62.4	1.089	−0.864	0.080	0.976	88	655	89
7.0	57.2	1.096	−0.881	0.094	0.974	82	719	95
10.5	72.6	0.484	−0.834	0.027	0.997	88	142	21
10.5	66.4	0.889	−0.815	0.064	0.984	85	328	50
10.5	62.2	1.013	−0.876	0.031	0.996	85	582	77
14.0	72.9	0.439	−0.829	0.035	0.993	88	125	18
14.0	66.4	0.850	−0.825	0.036	0.994	88	317	47
14.0	62.1	0.951	−0.899	0.037	0.994	85	560	71

[a] *DF* corrected.

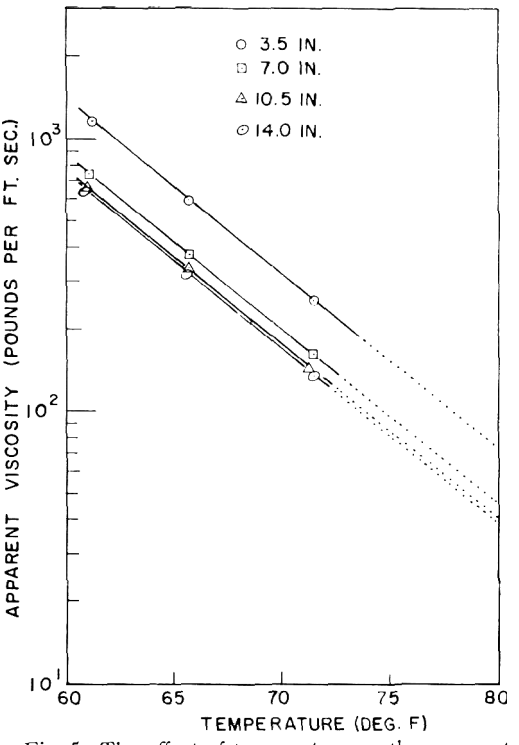


Fig. 5. The effect of temperature on the apparent viscosity of butter flowing at 0.01 ft per sec for different lengths of tubing.

which is a decrease of 53 lb/ft sec as the distance increased 3.5 in. Less decrease (39 lb/ft sec) is found between the 7.0- and 10.5-in. lengths of tubing, and only 3 lb/ft sec between the 10.5- and 14.0-in. lengths of tubing. Fig. 4 shows the influence of temperature on the apparent viscosity of flowing butter at various bulk velocities. Fig. 4 illustrates the location of the standard error of estimate for the regression lines for the data obtained with the 10.5-in. tube.

The apparent viscosity versus temperature is shown in Fig. 5 for a constant rate of shear of 0.01 ft/sec. The slope of the lines is −0.059. Thus, apparent viscosity decreases rapidly as the temperature increases. The apparent viscosity with the 10.5-in. length of tubing at 55°F is 1,150 lb/ft sec. It decreases to 773 lb/ft sec at 60° F, and to 396 lb/ft sec at 65°F. This relationship is in agreement with the equation given by Weltmann (1960) for some non-Newtonian materials.

The results on the apparent viscosity from the different samples of butter showed a variation, but no definite pattern (Table 2). The variation was small within a given sample of butter, as indicated by the high correlation coefficients (ranged from 0.994 to 0.999). However, the variations among the samples of butter were greater (correlation coefficients ranged from 0.974 to 0.992). The variations among the results from different samples of butter also became greater at 55 and 60°F. The variations within the same sample are probably due to temperature fluctuations and small differences in the amount of softening which occurred just prior to flowing through the tubing. The variations between the samples are probably due to variation of triglycerides, which have different melting points, and to the effects of the processing conditions upon them.

The general relationship for apparent viscosity versus bulk velocity and temperature for flowing butter is:

$$\log \eta = 4.762 - 0.847 \log v - 0.059T$$

which is considered valid for temperatures between 55 and 75°F.

Pressure loss versus logarithm of velocity was a linear relationship. The results of the correlation and regression analysis on the pressure loss versus velocity for different lengths of tubing and temperatures are presented in Table 3. The correlation coefficients ranged between 0.351 to 0.938. The low correlation coefficients were from data obtained with the shorter tubing (3.5 and 7.0 in.) and the lower temperatures (55 and 60°F). The

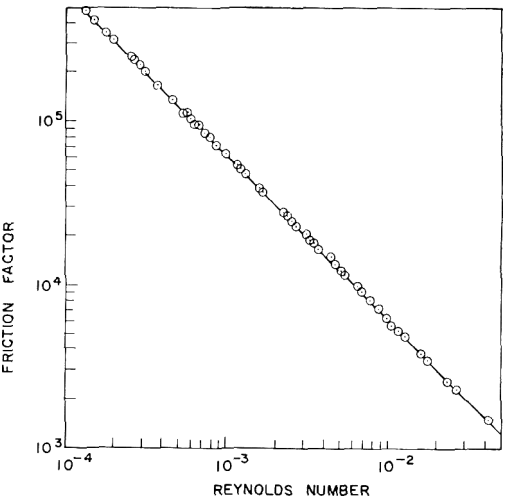


Fig. 6. Friction factor versus Reynolds number flowing through stainless-steel tubing for butter.

Table 2. Results of the correlation and regression analysis on the apparent viscosity versus velocity of butter.

Butter sample	Temperature (°F)	Regression coefficients		Standard error of estimate	Correlation coefficient[a]	Degrees of freedom	Apparent viscosity (lb/ft sec)	
		a	*b*	*SE*		*DF*	Bulk velocity 0.01 ft/sec	Bulk velocity 0.1 ft/sec
A	72.8	0.473	−0.879	0.022	0.998	13	170	23
B	72.3	0.469	−0.900	0.016	0.999	13	160	22
C	73.3	0.492	−0.868	0.014	0.999	13	170	23
D	72.6	0.530	−0.808	0.027	0.994	13	140	22
E	73.3	0.512	−0.815	0.022	0.997	13	139	21
F	72.8	0.697	−0.730	0.018	0.994	13	144	26
A	65.4	1.067	−0.816	0.028	0.997	13	500	76
B	66.6	1.139	−0.691	0.024	0.996	13	333	68
C	67.5	0.990	−0.733	0.013	0.999	10	286	53
D	65.2	0.985	−0.776	0.026	0.998	13	345	58
E	66.4	1.026	−0.775	0.015	0.999	13	372	63
F	66.0	1.049	−0.769	0.019	0.998	13	388	66
A	62.6	1.237	−0.753	0.020	0.998	13	554	98
B	63.7	1.022	−0.839	0.021	0.999	13	502	73
C	61.7	1.150	−0.828	0.026	0.997	13	641	95
D	61.0	1.110	−0.860	0.021	0.998	13	854	117
E	60.8	1.110	−0.821	0.028	0.994	13	712	108
F	62.4	1.115	−0.797	0.016	0.999	13	513	82
A	56.9	1.262	−0.854	0.015	0.999	13	934	131
B	57.2	1.221	−0.835	0.009	0.999	13	780	114
C	56.6	1.237	−0.852	0.016	0.999	13	874	123
D	57.3	1.027	−0.865	0.020	0.999	10	772	78
E	56.9	1.106	−0.835	0.018	0.999	13	597	85
F	57.1	0.998	−0.868	0.021	0.999	10	542	73

[a] *DF* corrected.

Table 3. Results of the correlation and regression analysis on pressure loss versus velocity.

Tube length (in.)	Temperature (°F)	Regression coefficients		Standard error of estimate	Correlation coefficient[a]	Degrees of freedom	Pressure loss (lb/sq in.)	
		a	*b*	*SE*		*DF*	Bulk velocity 0.01 ft/sec	Bulk velocity 0.1 ft/sec
3.5	72.9	11.641	2.397	0.583	0.918	88	6.9	9.2
3.5	66.4	33.950	8.110	2.382	0.916	70	17.7	25.8
3.5	62.3	50.368	7.803	6.817	0.582	82	34.8	42.6
3.5	56.6	47.390	6.548	10.497	0.351	67	34.3	40.8
7.0	72.9	16.431	3.424	1.050	0.782	88	9.6	13.0
7.0	66.4	52.363	14.950	4.511	0.838	85	22.5	37.4
7.0	62.4	67.569	13.879	8.359	0.559	88	39.8	53.7
7.0	57.2	67.614	11.633	9.804	0.470	82	44.4	56.0
10.5	72.6	24.591	5.954	0.988	0.938	88	12.7	18.6
10.5	66.4	58.668	14.326	5.160	0.771	85	30.0	44.3
10.5	62.2	86.098	16.957	4.000	0.852	85	52.2	69.1
14.0	72.9	29.073	7.053	1.203	0.906	88	15.0	22.0
14.0	66.4	72.766	17.111	2.999	0.916	88	38.5	55.7
14.0	62.1	102.006	17.486	6.170	0.728	85	67.0	84.5

[a] *DF* corrected.

Table 4. Loss of head and power requirements for various quantities of butter at different temperatures flowing through tubing of various lengths.

Inside diameter (in.)	Temperature (°F)	Weight[a] (lb/min)	Length (ft)	Bulk velocity (ft/sec)	Apparent viscosity (lb/ft sec)	Reynolds number	Friction factor	Loss of head (ft)	Horsepower (hp)
1.402	65	10	10	0.262	30	0.0613	1,045	10	0.05
		10	20	0.262	30	0.0613	1,045	19	0.09
		10	30	0.262	30	0.0613	1,045	29	0.14
		50	20	1.310	8	0.1130	567	1,974	46.80
		100	20	2.620	1	0.4030	159	2,900	137.00
1.402	60	10	10	0.262	58	0.0315	2,030	191	0.91
		10	20	0.262	58	0.0315	2,030	382	1.81
		10	30	0.262	58	0.0315	2,030	764	3.62
		50	20	1.310	16	0.0580	1,100	5,020	119.00
		100	20	2.620	9	0.2080	308	5,620	226.00
1.402	55	10	10	0.262	112	0.0162	3,950	358	1.70
		10	20	0.262	112	0.0162	3,950	716	3.39
		10	30	0.262	112	0.0162	3,950	1,074	5.08
		50	20	1.310	30	0.0298	2,150	9,800	232.00
		100	20	2.620	17	0.1060	603	11,000	521.00
2.870	65	10	10	0.0626	105	0.0084	7,600	19	0.09
		10	20	0.0626	105	0.0084	7,600	39	0.18
		10	30	0.0626	105	0.0084	7,600	60	0.27
		50	20	0.3130	27	0.1640	390	50	1.17
		100	20	0.6260	15	0.5900	108	55	2.60
2.870	60	10	10	0.0626	205	0.0043	14,800	38	0.18
		10	20	0.0626	205	0.0043	14,800	75	0.35
		10	30	0.0626	205	0.0043	14,800	113	0.53
		50	20	0.3130	53	0.0843	760	98	2.31
		100	20	0.6260	29	0.3030	211	107	5.07
2.870	55	10	10	0.0626	400	0.0022	28,800	73	0.35
		10	20	0.0626	400	0.0022	28,800	146	0.69
		10	30	0.0626	400	0.0022	28,800	220	1.04
		50	20	0.3130	102	0.0435	1,470	187	4.43
		100	20	0.6260	57	0.1560	411	209	9.90

[a] Density of butter: 59.3 lb/cu ft.

pressure losses increased as the velocity increased. For the 7.0-in. length of tubing at 66.4°F the increase was from 22.5 to 37.4 lb/sq in. as the velocity increased from 0.01 to 0.1 ft/sec. The pressure increased as the length of tubing increased. The increase was from 42.6 to 84.5 lb/sq in. for lengths of tubing of 3.5 to 14.0 in. at a bulk velocity of 0.1 ft/sec and approximately 62°F. The pressure increased from 18.6 to 69.1 lb/sq in. as the temperature decreased from 72.6 to 62.2°F at a bulk velocity of 0.1 ft/sec.

Large variations in pressure losses were found among the various samples of butter (correlation coefficients ranged from 0.351 to 0.938). However, no pattern could be found for composition or processing influences to explain the variations. The variation within the same sample was small as indicated by the correlation coefficients, which ranged from 0.866 to 0.994.

The calculated friction factor versus the calculated Reynolds number is illustrated in Fig. 6. Since the data were determined for laminar flow, a logarithm scale was used for the friction factor to show a wider range of values.

The power requirements and loss of head are presented in Table 4 for butter flowing under various conditions. The calculated power requirements revealed that a 5°F decrease in temperature about doubles the

horsepower (0.18, 0.35, and 0.69 hp at 65, 60, and 55°F) required to move the butter. Increasing the diameter from 1.5 to 3.0 in. reduced the power required from 1.81 to 0.35 hp for moving butter at 60°F through 20 ft of tubing at 10 lb/min. This reduction is even greater as the quantity of flow of butter increases.

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Seasonal Variation of Naringin and Certain Other Flavanone Glycosides in Juice Sacs of Texas Ruby Red Grapefruit

SUMMARY

A recently developed chromatographic-fluorometric method was used for individual quantitative determination of naringin, neohesperidin, poncirin, naringenin-7-β-rutinoside, hesperidin, and isosakuranetin-7-β-rutinoside in juice sacs of Texas Ruby Red grapefruit harvested in late July and late September, 1964, and monthly thereafter through late April, 1965. The concentrations of the six glycosides in these juice sacs were found to decrease rapidly and approximately porportionally during the period from late July through late November. In December–April, the concentrations of all six glycosides tended to decrease, although the monthly changes were slight. These results are compared with data obtained from the same samples by the Davis method for "naringin" determination and with taste-panel evaluations of grapefruit juice bitterness.

INTRODUCTION

Naringin, the 7-β-neohesperidoside of naringenin (4′,5,7-trihydroxyflavanone), has long been known to be a major bitter constituent of grapefruit, and until recently was the only flavanone glycoside known to be present in this fruit. Recent studies, however, have shown grapefruit to contain significant quantities of naringenin-7-β-rutinoside, which is a tasteless isomer of naringin, as well as lesser quantities of several other flavonoid compounds (Mizelle *et al.*, 1965; Horowitz and Gentili, 1961; Dunlap and Wender, 1962). These include two other bitter flavanone glycosides, namely poncirin (7-β-neohesperidoside of isosakuranetin [5,7-dihydroxy-4′-methoxyflavanone]) and neohesperidin (7-β-neohesperioside of hesperetin [3′,5,7-trihydroxy-4′-methoxyflavanone]), and their tasteless isomers, isosakuranetin-7-β-rutinoside and hesperidin (7-β-rutinoside of hesperetin). The discovery of these compounds in grapefruit has indicated a need for information concerning the concentrations of naringin and these other bitter and tasteless glycosides in grapefruit of different stages of maturity. Such information is necessary for clarification of the effect of maturity on the flavanone glycoside content of grapefruit, and for elucidation of the effect of the individual glycosides on grapefruit bitterness, including their role in the debittering of grapefruit which occurs with maturity. Several previous investigations of the seasonal variation of flavanones, measured as naringin, in grapefruit have been reported (Harvey and Rygg, 1936; Maurer *et al.*, 1950; Kesterson and Hendrickson, 1953; Lime *et al.*, 1954), but the quantitative methods employed in these studies were incapable of determining concentrations of the individual flavanone glycosides. Therefore, a study has been conducted in our laboratory to determine the individual quantities of the neohesperidosides and rutinosides of naringenin, hesperetin, and isosakuranetin in the juice sacs of Texas Ruby Red grapefruit harvested in late July and late September, 1964, and monthly thereafter through late April, 1965. A recently developed chromatographic-fluorometric procedure (Hagen *et al.*, 1965) has been employed for quantitative determination of the individual glycosides. "Naringin" values have also been obtained for these same samples by means of a slight modification of the much used colorimetric method of Davis (1947) in order that the effectiveness of the Davis method for quantitative determination of naringin and other flavanone glycosides in grapefruit might be evaluated. This paper presents the results of these studies.

EXPERIMENTAL

Sample preparation. The fruit employed in this study was obtained from Ruby Red grapefruit trees in an orchard at the Texas Agricultural Experiment Sub-Station at Weslaco, Texas. Fruit samples representative of the orchard were harvested on July 28, 1964, and on about the 28th of each month from September, 1964, through April, 1965. Samples consisted of 28 fruit each, except for the July sample (16 fruit) and April sample (20 fruit).

Grapefruit samples were shipped immediately after harvest to the University of Oklahoma for processing and analysis. When a shipment was received, the fruit were scrubbed in water and dried, and weights and diameters for individual

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whole fruit were recorded. The peel (albedo and flavedo) and core were removed by hand from the segments, which consisted of the juice sacs, segment walls, and seeds. Juice-sac samples for flavanone glycoside analysis were prepared by careful removal of segment walls and seeds from the juice sacs of four segments from each fruit in a shipment. The combined juice sacs comprising a sample were placed in tared polyethylene bags, weighed, quick-frozen in powdered dry ice, and stored at −10°C until needed for analysis. The remaining whole segments were also weighed, quick-frozen, and stored.

Analysis of juice sacs for flavanone glycosides. A chromatographic-fluorometric procedure which has recently been described in detail (Hagen *et al.*, 1965) was employed, with slight modification, for quantitative determination of the individual rutinosides and neohesperidosides of naringenin, hesperetin, and isosakuranetin in the grapefruit juice-sac samples. The essential steps of this procedure were: preparation of a stock solution from each sample by macerating the juice sacs with 2-propanol and thoroughly extracting the solid residue with hot 2-propanol and methanol; chromatography of stock-solution aliquots on columns packed with Polyclar AT polyvinylpyrrolidone (General Aniline and Film Corp., Grasselli, N. J.), with water, and with 25% methanol–75% water as solvents, to achieve preliminary quantitative isolation of the desired flavanone glycosides; further separation and purification of these flavanone glycosides by chromatography on thin-layers of Polyamide Woelm (Alupharm Chemicals, New Orleans, La.), with nitromethane-methanol (5:2) as solvent; removal of the adsorbent zones containing the individual flavanone glycosides from the thin-layer plates and elution with methanol of the glycosides from the polyamide; and quantitative determination of each individual compound by measurement with a Turner model 110 fluorometer of the fluorescence of the complex formed by addition of $AlCl_3$ to the flavanone solution. In this work, the Turner fluorometer was equipped with an ultraviolet phosphor lamp (Westinghouse FS4T5), with a 325-mμ interference filter (Baird Atomic, Inc., 33 University Road, Cambridge, Mass.) as primary (activation) filter, and with a Wratten 2A-12 filter, which passes all wavelengths greater than 510 mμ, as secondary (emission) filter. With this lamp and filter system, the high sensitivity conversion kit for the Turner Fluorometer which was used in the original procedure was not required.

The stock solutions prepared from the grapefruit juice-sac samples were also analyzed for flavanone content, as naringin, by a slight modification of the Davis method (Davis, 1947) in order to compare values obtained by this method with those produced by the chromatographic-fluorometric method. Aliquots of stock solution (25 or 50 μl) were mixed with 0.2 ml of 4*N* NaOH and sufficient 90% diethylene glycol to give a final volume of 10 ml, and the solutions were allowed to stand for 10 min before optical density was measured. The absorbances of these solutions at 420 mμ, as determined with a Beckman DU spectrophotometer using blanks consisting of stock solutions and diethylene glycol but no added alkali, were compared with absorbances obtained for similar alkaline diethylene glycol solutions containing known quantities of pure naringin.

Other analyses. Solutions for acid and Brix determination and organoleptic evaluation were prepared as needed from frozen whole segments not used for the preparation of juice-sac stock solutions. Segment walls and seeds were carefully removed from a representative sample (at least one segment from each fruit in a monthly shipment), and the partially frozen juice sacs thus obtained were thoroughly macerated in a Waring blender to obtain a purée.

For acid determination, 10-ml samples of strained juice from the juice-sac purée were titrated to pH 8.2 with 0.1479*N* sodium hydroxide. Acidity was calculated as percent citric acid.

Brix was determined with an Abbé refractometer; appropriate corrections were made for temperature and acid content of the juice sac preparations.

A taste panel of 12 members conducted organoleptic evaluations of the juice sac samples. Panel members were selected from a group of 17 candidates on the basis of their ability to distinguish between dilute citric acid solutions, distilled water, and solutions of naringin in distilled water ranging in concentration from 1 part/6,250 to 1 part/50,000. The grapefruit juice-sac purées were filtered through fluted filter paper prior to tasting in order to remove excess solids. Samples were evaluated according to the following scoring system:

10—extremely high bitterness
9, 8—high bitterness
7, 6—medium-high bitterness
5—standard medium bitterness
4, 3—medium-low bitterness
2, 1—trace of bitterness
0—no detectable bitterness

Table 1. Monthly variation in concentrations of six flavanone glycosides in juice sacs of Texas Ruby Red grapefruit.

Harvest date	Concentration in juice sacs (μg/g wet weight)								
	Naringin 1	Naringenin rutinoside 2	Neohesperidin 3	Hesperidin 4	Poncirin 5	Isosakuranetin rutinoside 6	Sum of 1 and 2	Sum of 1 thru 6	"Naringin" by Davis method
7/28/64	1630	836	79.5	45.5	59.9	25.1	2466	2676	3370
9/28/64	1010	501	45.4	27.9	42.7	17.5	1511	1645	2100
10/28/64	786	370	35.5	21.0	31.7	12.9	1156	1257	1770
11/28/64	615	288	27.6	15.7	22.4	9.30	903	978	1270
12/28/64	642	264	31.4	15.9	23.9	8.57	906	986	1290
1/28/65	494	249	24.2	14.4	18.8	8.80	743	809	1090
2/28/65	499	207	29.4	12.5	21.3	7.20	706	776	995
3/28/65	507	230	21.4	13.1	20.0	8.74	737	800	1030
4/28/65	484	195	26.2	13.3	25.7	5.37	679	750	879

The November sample was arbitrarily assigned a score of 5 and presented to the panel as a comparison standard. It was also included as one of the coded samples to be evaluated by the panel members. No organoleptic evaluation was made of the April sample.

RESULTS AND DISCUSSION

Table 1 gives the concentrations of the rutinosides and neohesperidosides of naringenin, isosakuranetin, and hesperetin determined by the chromatographic-fluorometric procedure in the various grapefruit juice-sac samples, together with the concentrations of flavanones, as naringin, indicated by the modified Davis method. Table 2 presents acid, brix, and taste-panel data for the juice-sac samples. Fig. 1 shows the concentration of naringin, the sum of the concentrations of the two naringenin glycosides, the sum of the concentrations of all six glycosides measured, and Davis "naringin" values.

Table 2. Acid, Brix, and taste-panel data for Texas Ruby Red grapefruit juice sacs analyzed for flavanone glycosides.

Harvest date	Acid as citric (%)	° Brix	Brix-acid ratio	Av. taste panel scores[a]
7/28/64	1.96	11.2	5.7	9.3
9/28/64	1.15	11.2	9.7	7.0
10/28/64	1.18	11.2	9.5	6.2
11/28/64	0.79	9.2	11.6	4.2
12/28/64	0.88	10.3	11.7	3.6
1/28/65	0.93	10.7	11.5	3.4
2/28/65	1.06	11.6	10.9	3.9
3/28/65	1.02	12.0	11.8	3.1
4/28/65	0.74	11.0	14.9	—

[a] Scoring system: 10, extremely high bitterness; 8,9, high bitterness; 6,7, medium high bitterness; 5, standard, medium bitterness; 3,4, medium low bitterness; 1,2, trace of bitterness; 0, no detectable bitterness.

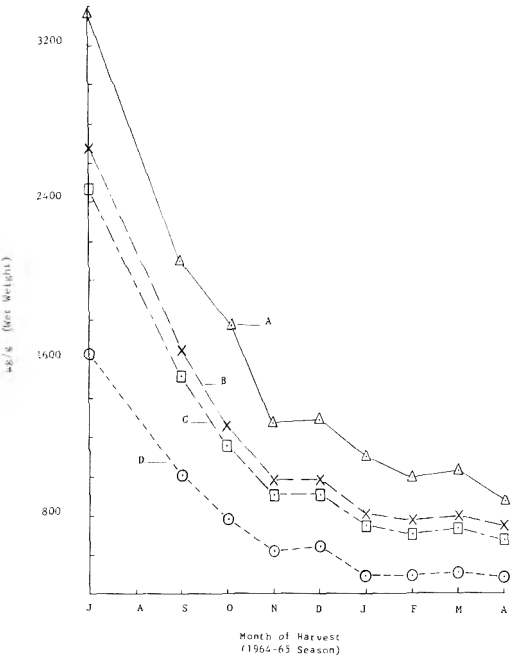

Fig. 1. Concentrations of flavanone glycosides determined to be present in juice sacs of Texas Ruby Red grapefruit by the chromatographic-fluorometric method and the Davis method: A, "naringin" by Davis method; B, sum of concentrations of rutinosides and neohesperidosides of naringenin, hesperetin, and isosakuranetin (6 glycosides); C, naringin + naringenin-7-β-rutinoside; D, naringin.

These data show that the change in concentration of flavanone glycosides in the grapefruit juice sacs was most significant

during the period from late July through late November. During this period the concentrations of the glycosides decreased rapidly and approximately proportionally until, at the end of November, the concentrations of all six glycosides were in the range of 34–38% of their July concentrations. In December–April all six glycosides tended to decrease in concentrations in the juice sacs, but the magnitudes of the decreases were generally much less than those occurring earlier in the season, and concentration changes from month to month, particularly those of the neohesperidosides, were more erratic than earlier.

Although the concentrations of the flavanone glycosides in juice sacs of the grapefruit harvested at different stages of maturity were often found to be quite different, the average quantities of flavanone glycosides in the juice sacs of a single grapefruit appeared not to be subject to much seasonal variation, as shown in Table 3. This suggests that the

Table 3. Average quantities of flavanone glycosides in the juice sacs of a single grapefruit.

	Weight of glycosides (mg)			
Harvest date	Naringin	Naringenin-7-β-rutinoside	Total naringenin glycosides	Total six flavanone glycosides
7/28/64	158	81	239	260
9/28/64	177	88	265	288
10/28/64	168	79	247	269
11/28/64	169	79	248	268
12/28/64	192	79	271	295
1/28/65	150	75	225	245
2/28/65	160	66	226	249
3/28/65	156	71	227	246
4/28/65	169	68	237	261
Av.	167	76	243	265

decrease in flavanone glycoside concentration of the juice sacs which occurred with maturity may have been principally a matter of dilution resulting from increased size of the fruit, as postulated by Kesterson and Hendrickson (1953). However, the average quantities of glycosides in the juice sacs per single fruit recorded in Table 3 show enough variation at different intervals within the growing season that the possibility cannot be eliminated that other factors in addition to dilution are involved in the changes in flavanone glycoside concentrations.

The ratios of the concentrations of the six flavanone glycosides in the grapefruit juice sacs did not, in most cases, appear to be significantly different for fruit harvested at different stages of maturity. This suggests that the relative concentrations of the six glycosides were probably established early in the development of the grapefruit, and remained relatively constant throughout the period studied. Analyses of small samples of juice sacs from fruit harvested in late December, 1963, and early April, 1964, from the orchard providing fruit for this study, indicated that the neohesperidoside–rutinoside ratios were somewhat higher in the 1963–64 fruit than in the 1964–65 fruit, which may indicate that these ratios were controlled at least partially by exterior factors other than heredity, such as weather and husbandry techniques. There is no evidence in these data of significant interconversions between either rutinosides and neohesperidosides or glycosides of different aglycones, but no detailed experiments have been undertaken specifically designed to explore this possibility in depth. Horowitz (1961, 1964) postulated that the decrease in bitterness of grapefruit with maturity might be the result of a trans-glycosylation reaction which changes the bitter flavone neohesperidosides to tasteless rutinosides, but this did not appear to be the case in the grapefruit utilized in this study. If anything, the ratio of the bitter naringin to the tasteless naringenin-7-β-rutinoside appeared to increase very slightly with maturity of the fruit.

The minor neohesperidosides, poncirin and neohesperidin, usually comprised only about 8% of the total measured neohesperidosides in the grapefruit juice sacs studied in this investigation. Since poncirin has been reported to be about equal in bitterness to naringin, and neohesperidin to be only one-tenth as bitter as the latter compound (Horowitz and Gentili, 1963), it appears likely that the contribution of these minor neohesperidosides to the bitterness of the grapefruit studied was small compared to the contribution of naringin.

The concentrations of flavanones, as naringin, indicated by the slightly modified Davis method to he present in the grapefruit

juice-sac samples were proportional to, but considerably higher than, the concentrations of naringin and other flavanone glycosides determined to be present in these samples by the chromatographic-fluorometric procedure. "Naringin" concentrations obtained by the Davis method were about 2.1 times the true naringin concentrations, about 1.4 times the combined concentrations of naringin and naringenin-7-β-rutinoside, and about 1.3 times the combined concentrations of the neohesperidosides and rutinosides of naringenin, hesperetin, and isosakuranetin. Although the Davis method gave absolute values which were high, it did accurately reflect the decrease, with maturity, of flavanone glycoside concentrations in the juice sacs studied here, as shown in Fig. 1. Obviously, substances in the juice-sac samples in addition to the neohesperidosides and rutinosides of naringenin, hesperetin, and isosakuranetin contributed to the values obtained by the Davis method. The ratios of the concentrations of these substances in the grapefruit juice sacs to those of the six flavanone glycosides apparently remained approximately constant throughout the study. Qualitative studies of the grapefruit juice sacs samples employed in this work have shown that they probably contained several other flavanones, but the quantities of these compounds appeared so small that they were likely to account for, at most, only a very small fraction of the difference between flavanone concentrations obtained for the samples by the Davis method and the total concentrations of the neohesperidosides and rutinosides of naringenin, hesperetin, and isosakuranetin in these samples. The juice-sac samples also contained several flavone and flavonol-like compounds which appeared to be present in relatively small quantities. The extent to which these compounds contributed to "naringin" values obtained by the Davis method is not known.

Taste-panel evaluations of the bitterness of the juice-sac samples analyzed in this study showed good correlation with "naringin" content determined by the Davis method, as well as with true naringin content. However, by clearly showing the lack of specificity of the Davis method for bitter flavanone neohesperidosides, this study does suggest a possible explanation for the rather frequent inability of taste panels to correlate bitterness of grapefruit with Davis "naringin" values (Hendrickson and Kesterson, 1958; Griffiths and Lime, 1963). It is evident that a change in concentration of any of the non-bitter components of grapefruit measured by the Davis method which was not accompanied by a directly proportional change in flavanone neohesperidoside concentration, would result in poor correlation of Davis values with flavanone neohesperidoside content, and hence, probably with bitterness. No such non-proportional concentration changes occurred in the grapefruit juice sacs studied in this investigation, but fruit grown in a different year, or obtained from other trees grown in a different location might not be the same in this respect. Another possible explanation for poor Davis "naringin" value–bitterness correlations is suggested by the recent report that grapefruit juice contains a significant quantity of limonin (Maier and Dreyer, 1965), a bitter limonoid which would probably not be measured by the Davis method. The extent to which limonin contributed to the bitterness of the grapefruit studied in this investigation is not known.

The data obtained provide a relatively good picture of the seasonal variation of naringin, naringenin-7-β-rutinoside, and four minor flavanone glycosides in the particular grapefruit juice sacs studied, but there is no way of knowing at present if the variation pattern noted here is typical of all grapefruit. A need is clearly indicated for similar seasonal studies of other grapefruit, grown in other locations and in other seasons.

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Effect of Wet and Dry Heat on Structure of Cellulose

SUMMARY

Heating of cellulose (purified cotton and cuprammonium-regenerated cellulose) under nitrogen in the presence of liquid water at 100° and at 150°C brought about a small amount of hydrolysis and some dehydration, the extent of which was directly proportional to the temperature. Heating in the absence of water caused a much greater amount of hydrolysis and dehydration, which was particularly marked at the higher temperature. Heating affects the amorphous regions of cellulose more than the crystalline regions; hence changes are more evident in the less crystalline regenerated cellulose than in purified cotton cellulose.

INTRODUCTION

It has been shown that heat degrades cellulose (Burney, 1954; Fletcher and Houston, 1940; Higgins, 1958). It has also been claimed (Wiegerink, 1940) that moisture accelerates this thermal breakdown, particularly in the presence of air (Waller *et al.*, 1948). On the other hand, high-temperature treatment decreases the swelling power of cellulose, reportedly by the formation of new junction points in the network of the gel (Hermans, 1949). In seeming substantiation of this interpretation, the tensile strength of viscose was increased by heating at 120°C even though the tensile strength of other cellulosic materials was lowered by the same treatment (Rogovin *et al.*, 1943).

In baking, frying, roasting, and similar processes, cellulose may be heated to temperatures in excess of the boiling point of water. The changes that occur in the physical structure of cellulose during these processes are largely unknown. It seemed useful to explore the effects of heating in model systems. Thus it could be determined whether any different evidence exists for the formation of new associations in the gel network of cellulose. In these experiments, native and regenerated celluloses were heated in the absence of oxygen, both with and without moisture. The methods of analysis involved determination of molecular weight, measurement of crystallinity, infrared spectrometry, and chromatography of the hydrolytic products.

MATERIALS AND METHODS

Absorbent cotton was extracted with ethanol, 2% HCl, and 2% NaOH (with intervening washings in water), dried in air, and ground to pass a 60-mesh sieve (Shimazu and Sterling, 1961). A cuprammonium-regenerated cellulose was prepared from the extracted cotton by dispersal in the Döring cuprammonium reagent (Jayme and Lang, 1963), dilution, adjustment to pH 7, and precipitation in an ice-water bath (Sharples, 1958). The regenerated cellulose was washed and centrifuged 10×, lyophilized, and reground to pass a 60-mesh sieve.

Two g of each sample were placed in glass tubes, under a positive pressure of dry N_2, and the tubes were sealed. Other 2-g samples were placed in tubes with 2 ml H_2O, also under N_2 gas, and those tubes were likewise sealed. Two tubes of each sample remained at room temperature, two were heated 5 hr at 100°C, and two were heated 5 hr at 150°C. The samples were removed from the tubes, dried in air if necessary, and held at room temperature and humidity for 5 days.

Intrinsic viscosity was determined in cupriethylene diamine solutions (Swenson, 1963) at 25.0± 0.01°C, with concentrations of 0.5, 0.3, 0.2, and 0.1% air-dry cellulose. The concentrations were later corrected for the moisture content of the air-dry material (determined at 70°C, under a vacuum of 100 mm Hg). Intrinsic viscosity was converted to degree of polymerization (D.P.) on the basis: D.P. = 150 $[\eta]$ (Gloor and Klug, 1954). Similar determinations were made as a check with solutions of the control celluloses in cuprammonium solvent (Jayme and Lang, 1963), the D.P./$[\eta]$ relational constant being 200 (Gloor and Klug, 1954). There was close agreement of the values from cuprammonium solutions of the control specimens with those from cupriethylene diamine solutions.

Crystallinity was estimated by 3 different methods. *Sorption ratio.* Water vapor sorption was measured dynamically, with oil-pumped N_2 being bubbled first through 3 solutions of sulfuric acid and water, of appropriate concentration to give the desired water vapor content in the N_2 atmosphere, and then through the sample. The sample and the 2 immediately preceding sulfuric acid–water solutions were held in a water bath at 25.0±0.01°C. The sample was weighed after equili-

bration at 20, 40, 60, and 80% RH. The values of the sorption ratio (based on sorption of purified cotton) were averaged and converted to percent crystallinity on the basis that the relative amount of sorption is proportional to the relative amount of amorphous material.

X-ray diffraction. X-ray determinations of crystallinity were made at 40% RH by the method of Hermans and Weidinger (1948, 1949) with samples in a stream of N_2. The moisture content of the N_2 stream was achieved through the same system that was used in water vapor sorption measurements.

Deuteration. Infrared spectrograms (single beam) were obtained for cellulose deuterated by immersion in liquid D_2O for 24 hr (1 sample: 5 D_2O by gross weight) and then dried at 10^{-4} mm Hg vacuum (room temperature) for 48 hr. N_2 gas was introduced, and the sample was suspended in hexachloro-1,3-butadiene (HCBD) under positive nitrogen pressure. HCBD ($CCl_2 = CClCCl = CCl_2$) can supply no protons and has minimal absorption in the range 2000–4500 cm^{-1}. Crystallinity was given by the relative areas under the peaks at the stretching frequencies of OH (at *ca.* 3350 cm^{-1}, interpreted as inaccessible or crystalline region) and OD (at *ca.* 2550 cm^{-1}, interpreted as representing the accessible or amorphous region): percent crystallinity = OH/(2OD + OH). [Theoretically the OH intensity is greater than the OD intensity by a factor of 2 (Wilson *et al.*, 1955).] Peak areas were calculated on the basis of optical density and wave-number.

Infrared spectrograms were also made of cellulose in the air-dry, protonated state. The samples were mixed with KCl and compressed into pellets, for examination in a double-beam infrared spectrometer.

For paper chromatography, each sample was dissolved in 72% H_2SO_4, which was diluted after 1 hr, and then hydrolyzed by heating at 100°C for 4 hr. The mixture was neutralized, diluted, and centrifuged (Saeman *et al.*, 1963). The supernatant was concentrated and refrigerated. Thirty μg were spotted on Whatman No. 1 paper. The chromatographic solvent was ethyl acetate–acetic acid–water (3:1:3 v/v) epiphase. Development of the spots was made with $AgNO_3$-saturated acetone, 5% NaOH in ethanol, and photographic fixing solution, in succession. Sensitivity of this method, by experimentation, is 0.8 γ of reducing sugar (glucose). Standard substances used for comparison were glucose, galactose, mannose, xylose, arabinose, and cellobiose.

RESULTS

Color changes in the treated specimens are given in Table 1. In all cases, dry heating produced a darker coloration than heating in the presence of water.

Table 1. Color appearance of treated celluloses.

Treatment	Purified cotton	Regenerated cellulose
Air dry	white	white
100°C, wet	white	white
100°C, dry	white	light yellow-brown
150°C, wet	white	light brown
150° C, dry	light yellow	brown

Values for the intrinsic viscosities of the cellulose samples and the derived D.P. values are presented in Table 2. To a small extent, the process of regeneration reduced the intrinsic viscosity. Heating at 100°C scarcely affected the moist cellulose, but in the dry state a slight decrease was noted for both purified cotton and regenerated cellulose. Heating at 150°C caused a greater drop in the intrinsic viscosity of regenerated cellulose than in that of purified cotton. The effects of moisture were opposite in the 2 different celluloses at 150°C.

The results of the crystallinity determinations are summarized in Table 3. By virtually all methods of measurement, treatment at 100°C caused an increase in cellulose crystallinity, and to be sure, the dry treatment gave a somewhat greater increase than the wet. At 150°C there was virtually no further change in X-ray crystallinity of wet or dry samples. However, in the determinations by sorption ratio and deuteration, dry heating at 150°C produced a marked increase in crystallinity over wet heating.

The infrared spectrum of deuterated samples may be evaluated further. If the areas under the OD- and OH-stretching peaks be compared with the height of the CH-stretching peak, an estimate may be made of the relative change in total OH groups [(2OD + OH)/CH treated]/[(2OD + OH)/CH control] as well as the relative change in the amount of OH groups in both the crystalline and amorphous regions, considered separately [(OH/CH treated)/(OH/CH control) and 2OD/CH treated)/(2OD/CH control), respectively].

Table 2. Intrinsic viscosity and D.P. of treated celluloses in cupriethylene diamine.

Treatment	Purified cotton		Regenerated cellulose	
	$[\eta]$	D.P.	$[\eta]$	D.P.
Air dry	1.07	161	0.96	144
100°C, wet	1.07	161	0.94	141
100°C, dry	1.05	158	0.89	134
150°C, wet	1.03	155	0.67	100
150°C, dry	1.02	153	0.75	112

Table 3. Estimates of percent of crystallinity for treated celluloses.

Treatment	Purified cotton			Regenerated cellulose		
	Sorption	X-ray	Deuteration	Sorption	X-ray	Deuteration
Air dry	70	68	67	40	40	35
100°C, wet	71	69	67	42	43	37
100°C, dry	73	74	69	44	45	38
150°C, wet	72	73	68	43	45	37
150°C, dry	78	74	72	58	45	46

The results are shown in Table 4. It is apparent that the total OH decreases with treatment at 150°C and particularly in the dry state. Also, the apparent increase in crystallinity with dry heating at 150°C is shown to be the result primarily of the loss of amorphous OH. Thus, X-ray determinations showed no change, while those based on accessible OH groups indicated a relative increase in crystallinity in dry heating at 150°C.

The infrared spectra of the undeuterated samples were virtually identical with one another, regardless of the experimental treatment. There were slight differences, however, between the spectra of samples of purified cotton and those of regenerated cellulose. In all, there was no evidence for a real change in any of the chemical groups or for the appearance of any new group.

The chromatograms of all the hydrolyzed celluloses were also identical, and no new sugars were found after any of the treatments. Only 3 spots appeared upon hydrolysis and chromatography: one of these was identified as glucose, and the second as cellobiose, and the third appeared to be cellotriose, but no standard was available for comparison. All 3 spots appeared in the hydrolyzed starting materials (Table 5).

DISCUSSION

The results show that a major breakdown occurs in samples of cellulose exposed to 150°C, that the breakdown is more pronounced in the dry than in the wet state, and that it takes place predominantly in the amorphous regions, particularly in regenerated cellulose. Results cited above (Wiegerink, 1940; Waller *et al.*, 1948) indicate that thermal breakdown is accelerated by the presence of moisture. One difference, however, between this experiment and the previous ones is the immersion of the samples here in liquid water. It is perhaps possible to speculate that if the heating process produces free radicals in cellulose (Pakhomov, 1958), these are rapidly quenched when water is abundantly available.

The nature of the breakdown is indicated in the analyses. Some of the changes (decline in intrinsic viscosity, unchanged infrared spectrum, and occurrence of glucose and its polymers as the sole hydrolytic products) point to a simple hydrolysis of the 1–4 glucosidic linkage. Other changes (decrease in relative area of the OD-stretching peak in infrared spectra of deuterated specimens, marked drop in water-vapor sorption capacity, and darker color of the dry-heated specimens) suggest a further chemical reaction. This reaction seemingly involves the splitting off of hydroxyl groups.

According to the investigations of Golova *et al.* (1958, 1960), the main product of the thermal decomposition of cellulose *in vacuo* is 1,6-anhydro-1,5-glucopyranose (levoglucosan), involving therefore a simultaneous dehydration, rearrangement of the glucopyranose moiety, and rupture of the 1–4 glucosidic bond. These reactions are indicated by the data of this experiment. Levoglucosan is also the breakdown product upon pyrolysis

Table 4. Estimates of total, crystalline, and amorphous OH (as fraction of air dry control) for treated celluloses.

Treatment	Purified cotton			Regenerated cellulose		
	Total OH	Crystalline OH	Amorphous OH	Total OH	Crystalline OH	Amorphous OH
Air dry	1.00	1.00	1.00	1.00	1.00	1.00
100°C, wet	Lost	Lost	Lost	1.02	1.03	0.99
100°C, dry	1.00	1.02	0.96	0.95	1.03	0.91
150°C, wet	0.99	1.02	0.96	0.98	1.03	0.93
150°C, dry	0.89	1.03	0.84	0.86	1.06	0.72

Table 5. R_f and R_g* values of identified spots in paper chromatograms of hydrolyzed cotton cellulose.

Sugar	Reported value	Measured value	
		Purified cotton	Regenerated cellulose
Glucose	0.17	0.17	0.17
Galactose	0.15		
Mannose	0.21		
Xylose	0.32		
Arabinose	0.27		
Cellobiose	0.38*	0.38*	0.38*
Cellotriose (?)		0.24*	0.24*

of starch (Horton, 1965). Further confirmation is given by the work of Murphy (1962). Levoglucosan was not detected in paper chromatography, possibly because not enough was present, possibly because its R_f value was not sufficiently different from that of glucose under the conditions of this experiment.

No evidence was found for increased absolute crystallinity nor for appreciable cross-linking in cellulose as a result of heating. To be sure, any cross-linking that involved small changes in di- or trisaccharide structure might be undetected in paper chromatography here. However, there was no indication by viscosity measurements of an increase in molecule size that would be a consequence of cross-linking, nor was there any marked change of the infrared spectrum that would show a new type of bonding or grouping.

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Thiobarbituric-Acid-Reactive Substances from Autoxidized or Ultraviolet-Irradiated Unsaturated Fatty Esters and Squalene

SUMMARY

The water-soluble 2-thiobarbituric-acid-reactive substances (TBRS) from oxidized and UV-irradiated unsaturated fatty acid esters and squalene were fractionated on Sephadex G-10 columns, and the fractions were characterized by elution volume, UV-absorption spectra, and behavior on TLC plates. By these criteria the main component in all cases was unequivocally identified as malonaldehyde (MA). Other TBRS accompanied MA but were clearly distinguishable. They were not identified, however. With squalene, the total TBRS per double bond were only one-sixth of those of the fatty acid esters, and MA accounted for about half.

INTRODUCTION

The pink chromogen (max. 532 mμ) obtained from the reaction of 2-thiobarbituric acid (TBA) with oxidized lipids is used widely as a measure of extent of lipid oxidation. During the early stages of oxidation, the amounts of TBA-reactive substances (TBRS) in oxidized unsaturated fatty acids are closely correlated with peroxide value, oxygen uptake, diene conjugation, etc. (Kenaston *et al.*, 1955; Tarladgis and Watts, 1960; Dahle *et al.*, 1962). It has been assumed that malonaldehyde (MA) was the TBRS. Recently, however, Saslaw *et al.* (1963) and Saslaw and Waravdekar (1965) reported that several different TBRS were produced when unsaturated fatty acids were irradiated, and that none of these were MA.

The experiments to be described were designed to investigate the nature of the TBRS produced during autoxidation and UV-irradiation of polyunsaturated fatty esters and squalene, and to clarify the relationship between TBRS and MA. We have already reported that MA is the principal TBRS in oxidized methyl linolenate (Kwon and Olcott, 1966). Some limitations of the TBA reaction as an index of lipid oxidation are discussed.

EXPERIMENTAL

Materials and analytical methods. Methyl linolenate (Mann Research Lab., IV 260), methyl arachidonate (Cal. Biochem., IV 322), and squalene (Eastman), purified by silicic acid column chromatography (Olcott and Van der Veen, 1963), were used. MA was prepared by acid hydrolysis of the distilled acetal (Kay-Fries) (Kwon and Watts, 1963) or by distillation of the bisulfite addition product (Saslaw and Waravdekar, 1957). TBRS were determined by heating equal volumes of sample and TBA reagent (0.02*M* TBA in 90% acetic acid) for 35 min at 100° or, for the eluants from the Sephadex G-10 columns with citrate-phosphate buffer, by allowing the reaction mixtures to stand at room temperature for 22 hr. When Tris-HCl or KCl-HCl buffer was used as eluant, the color was developed with 0.02*M* aqueous TBA solution. Glass-distilled water was used throughout. Thin-layer chromatography (TLC) was conducted with silica-gel G (E. Merck AG, Darmstadt, Germany). Solvents used were n-butanol-dichloromethane-water (20:4:1 v/v) and n-butanol-dichloromethane (1:10 v/v). After development, plates were allowed to dry, sprayed with TBA reagent, and heated at 100° for several minutes.

Autoxidation of fatty acids and squalene in air. Several 100-mg portions of the polyunsaturated substrates in covered 10-ml Pyrex beakers were allowed to oxidize in air at room temperature. At intervals, samples were weighed to determine the amount of oxygen absorbed (Olcott and Einset, 1958) and were extracted five times with 2 ml of water. The combined extracts were filtered through wet Whatman No. 42 filter paper and made up to 10 ml. Saslaw and Waravdekar (1965) reported that more than 95% of the TBRS were extracted by a similar procedure. The extracts were subjected to the TBA reaction and UV examination. All measurements were made with a Cary recording spectrophotometer, model 15.

Fractionation of the water-soluble TBRS. Five g of methyl linolenate was allowed to oxidize at 40° in a 10-ml Pyrex beaker with continuous mechanical stirring. At intervals, 2 ml of water was added to the rancid sample and the mixture was stirred for 5 min. The water extract was separated by centrifugation. The residue was returned to the beaker and allowed to oxidize further. Squalene was treated in the same manner. Methyl arachidonate (0.17 g) was oxidized at room temperature without stirring for 20 days, and then extracted with water. Water extracts were subjected to fractionation in a column (2 × 75 cm) contain-

ing Sephadex G-10 (Pharmacia) by elution with 0.05*M* Tris-HCl buffer containing 0.1*M* NaCl, pH 7.4, as described by Kwon and Olcott (1966), or in a longer column (2 × 105 cm) of the same material by elution with 0.05*M* KCl-HCl buffer containing 0.1*M* NaCl, pH 2.3.

In a separate experiment, 2-g quantities of methyl linolenate and squalene were respectively irradiated with UV light for 18 and 40 hr, as described by Saslaw *et al.* (1963). The irradiated samples were extracted with 2 ml of water and the extracts were separated by centrifugation as described above. They were then separately eluted from the longer Sephadex column with 0.0064*M* citrate-0.086*M* phosphate buffer containing 0.1*M* NaCl, pH 7.

RESULTS AND DISCUSSION

Production of TBRS. The production of TBRS by the autoxidation of arachidonate, linolenate, and squalene increased linearly, with apparent respective zero-order rate constants of 9.6×10^{-9}, 4.4×10^{-9} and 1.1×10^{-9} *M*/min during the early stages of autoxidation (Fig. 1). Later, the rate of production decreased, as has been shown previously with irradiated fatty acids (Saslaw and Waravdekar, 1965) and with autoxidizing lipids (Tarladgis and Watts, 1960). The amounts of TBRS at the peaks were 9.6, 6.3, and 3 μmoles MA equivalents per g substrate for arachidonate, linolenate, and squalene, respectively. The increment of TBRS paralleled that of weight gain, indicating a close correlation between oxygen uptake and TBRS production. TBRS production reached a peak at about the same time that the rate of oxygen uptake started to decline (Fig. 1 and 2); Tarladgis and Watts (1960) observed similar phenomena.

Properties of the water extracts of oxidized fatty acids and squalene. The water extracts from the autoxidized or UV-irradiated methyl linolenate, arachidonate, and squalene all gave the typical pink color after TBA reaction, identical to that seen with MA (max. 532 mμ). However, there was an additional absorption band at 455 mμ, particularly evident in the water extracts from oxidized squalene, and the ratio $A_{455m\mu}/A_{532m\mu}$ gradually increased during oxidation.

The water extract from methyl linolenate which had been oxidized for 1 day at room temperature had a UV-absorption spectrum before the addition of TBA with maxima at 234, 260, 270, and 280 mμ (Fig. 3). With the extract made after 4 days, however, the latter 3 absorption bands were no longer dis-

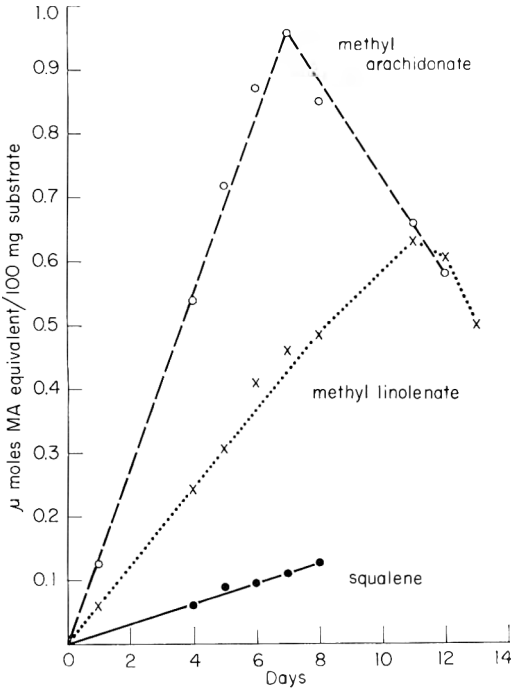

Fig. 1. Production of TBRS during autoxidation of methyl linolenate, methyl arachidonate, and squalene at room temperature.

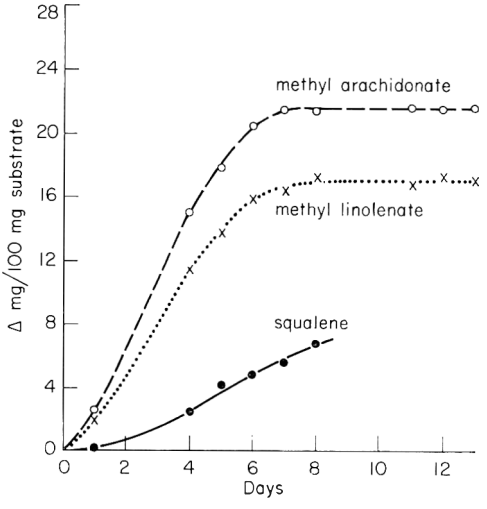

Fig. 2. Increase of weight during autoxidation of methyl linolenate, methyl arachidonate, and squalene at room temperature.

tinct but formed a broad shoulder around 270 mμ, while the band at 234 mμ had shifted to 225 mμ. The latter pattern was seen with a water extract of methyl linolenate that had been UV-irradiated for 18 hr at 40°. Water extracts of autoxidized methyl arachidonate and squalene also had absorption spectra with maxima at 225 mμ and shoulders at 270 mμ (Fig. 3), and the shape of the spectra did not change further with time of oxidation.

In no case was there a correlation between the absorbances at 532 mμ after TBA reaction and the UV absorbances at 225 mμ of the water extracts; this differs from observations of Saslaw and Waravdekar (1965). The compound with the absorption band at 225 mμ was isolated by gel-filtration of the water extracts (see below) and did not give a pink color with TBA.

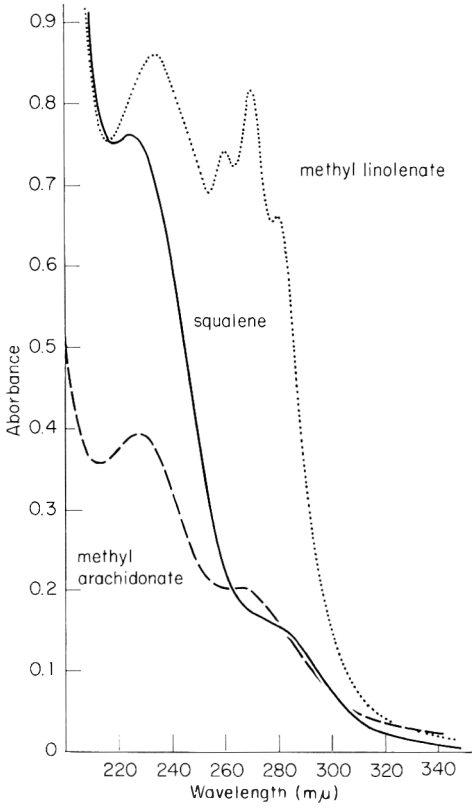

Fig. 3. Ultraviolet absorption spectra of the water extracts of autoxidized methyl linolenate, methyl arachidonate, and squalene. 100 mg of substrates were oxidized at room temperature for 1 day and extracted with 10 ml of water. Squalene was oxidized for 4 days.

Fractionation of water extracts from autoxidized substrates. When water extracts of autoxidized methyl linolenate were fractionated on Sephadex, MA was the principal TBRS of the three obtained (Kwon and Olcott, 1966). Further extended oxidation of linolenate (10 days at 40° with continuous stirring) showed only one TBA-pink peak (max., 532 mμ) with elution volume (Ve), 148 ml, from the short column (Fig. 4). However, this peak was overlapped by a TBA-yellow peak (max. 445 mμ; Ve, 152 ml). With continued oxidation of linolenate, the yellow peak gradually increased while the pink peak decreased. The yellow peak coincided with an intense UV absorption at 225 mμ before the TBA reaction. The absorption band at 267 mμ of the fractions disappeared upon acidification, but the typical absorption band at 245 mμ for acidified MA was not observed, probably because of the overlapping intense absorption at 225 mμ. The

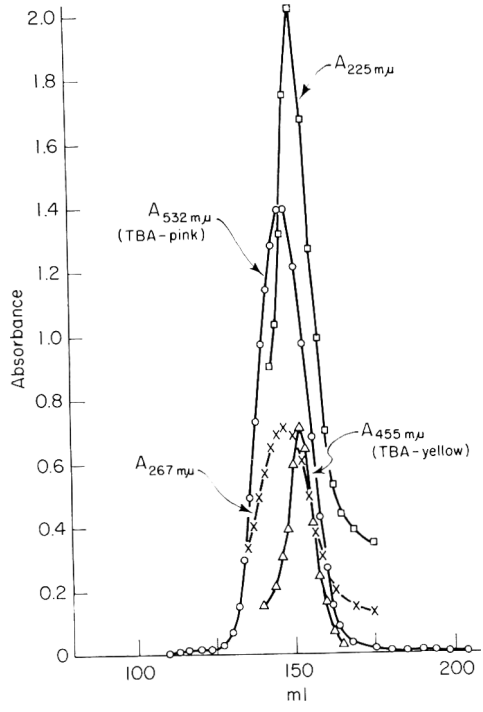

Fig. 4. Fractionation of the water extract obtained from autoxidized methyl linolenate by Sephadex G-10 column (2 × 75 cm) with 0.05*M* Tris-HCl buffer containing 0.1*M* NaCl, pH 7.4. Five g of methyl linolenate was oxidized at 40° for 10 days with continuous stirring, and extracted with 2 ml water.

pink peak is assigned to MA by the following criteria (Kwon and Olcott, 1966): 1) the elution volume at neutrality was the same as that of MA; 2) the ratio of $A_{532m\mu}/A_{267m\mu}$ was about 2; 3) on acidification, the absorption maximum at 267 mμ disappeared; 4) with elution by the KCl-HCl buffer (pH 2.3) from the longer Sephadex column, the pink peak appeared at the same elution volume (Ve, 275 ml) as did authentic MA.

The water extract from the 20 days' autoxidized arachidonate had a chromatographic pattern similar to that of the linolenate which had been oxidized for 16 hr at 40° (Kwon and Olcott, 1966).

The water extract made from squalene, oxidized for 16 hr at 40°, showed two TBA-pink peaks in the chromatogram, eluted from the short column. The elution volume of the first peak (Ve, 148 ml) was the same as that of MA; that of the second was 225 ml. The first peak was overlapped by a TBA-yellow peak (max., 445 mμ). A water extract made from squalene that had been oxidized for 5 days at 40° had a similar pattern, but the absorbance at 455 mμ for the first peak was higher than that at 532 mμ and for the second peak was lower. The UV-absorption spectra without TBA of the first peak had an intense absorption at 225 mμ with a shoulder around 270 mμ. Addition of acid or base to this fraction did not give the characteristic pH-dependent UV-absorption spectra of MA, but this may also have been interfered with by the intense absorption at 225 mμ. A water extract eluted by KCl-HCl buffer from the long column had two TBA-pink peaks with elution volumes at 206 and 275 ml. The first peak was overlapped by a TBA-yellow peak. The second peak had the same elution volume as that of authentic MA. The base difference spectrum had an absorption maximum at 267 mμ, and could thus be assigned to MA. MA can be differentiated from the TBA-yellow peak from water extracts of oxidized linolenate and squalene, because the Ve of MA is pH-dependent (Kwon, 1966) while that of the TBA-yellow peak is pH-independent. Production of MA from autoxidation of squalene has thus been demonstrated without question, but MA accounts for only about half of the total TBRS. The other TBA-pink peak has not yet been identified.

Fractionation of water extracts from UV-irradiated substrates. The water extracts from the irradiated substrates were fractionated by the longer Sephadex column with citrate-phosphate buffer. For the TBA reaction with these fractions, 0.02*M* TBA in 90% acetic acid gave full color development. The Ve of MA was 221 ml. The water extract from the irradiated linolenate showed two TBA-pink peaks with respective Ve of 179 and 221 ml. The second main peak coincided with absorbance at 267 mμ before TBA reaction, and was identified as MA by the criteria described previously. A TBA-yellow peak, coincident with absorbance at 225 mμ, had a Ve of 234 ml. Saslaw *et al.* (1963) isolated two pink and one yellow spots on a TLC plate in the water extracts obtained from irradiated fatty acids, but they reported that neither of the pink spots was MA. The water extract of irradiated squalene gave a pattern similar to that of autoxidized squalene.

Polymerization of MA. MA prepared from acid hydrolysis of its acetal or from distillation of its bisulfite addition compound, and MA acetal could be separated on TLC plates. When n-butanol-dichloromethane-water was used to develop the samples, MA prepared from acid hydrolysis of its acetal gave two spots (R_f 0.77 and 0.16), and MA prepared from distillation of the bisulfite addition product had only one spot (R_f 0.16) with tailing, as Saslaw *et al.* (1963) reported. The acetal gave one spot with R_f 0.82. Although the R_f values of the first spot of MA obtained by acid hydrolysis of its acetal and that of the acetal itself were very close, a mixture of the two could be clearly separated with n-butanol-dichloromethane. Thus, the spot of R_f 0.77, was monomeric MA, and that of R_f 0.16, appeared to be polymeric MA (Kwon and Olcott, 1966), as was the distillate of the bisulfite addition product.

When 15 ml of 0.3*M* aqueous MA-bisulfite solution was distilled to collect 10 ml of the distillate, MA concentration in the distillate was $1.83 \times 10^{-3}M$ and the UV absorption maximum was 245 mμ, as Saslaw and Waravdekar (1957) reported. In basic solution

the maximum was at 267 mμ. However, the ratio $A_{267m\mu}/A_{245m\mu}$ was 13.4, while monomeric MA has a ratio of 2.3 (Kwon and Watts, 1963). In a separate experiment in which the solution had been subjected to somewhat more heat, the distillate had its absorption maximum at 275 mμ and upon acidification the absorbance was increased. When the acidified distillate was heated for 15 min at 100°, the maximum was at 245 mμ with increased absorbance and it was shifted to 267 mμ in alkaline solution. The same distillate was eluted from the longer Sephadex column with citrate-phosphate buffers. As shown in Fig. 5 when the fractionation was carried out at pH 2.6, Ve was 131 ml and the UV absorption was negligible. At pH 2.6, monomeric MA was eluted at 275 ml. At pH 7, Ve was 207 ml, close to that of monomeric MA (Ve, 221 ml). These and other findings strongly suggest that the distillate was polymeric MA and that the polymer can be partially depolymerized by either acid and heat, or by alkali treatment.

Fate of TBRS in the oxidizing systems. The kinds of TBRS produced depend on the substrate and the conditions used for oxidation. In several cases, MA was the most important component. The other TBRS are not yet identified, but appear to be unstable

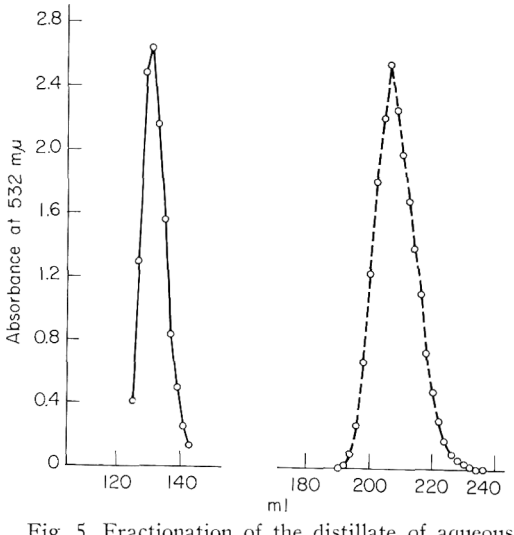

Fig. 5. Fractionation of the distillate of aqueous malonaldehyde-sodium bisulfite addition product by Sephadex G-10 column (2 × 105 cm) with sodium citrate-phosphate buffers containing 0.1 *M* NaCl, pH 2.6(—) and 7.(- - -).

precursors of MA, possibly 2,4-dienals or vinyl ketones. The concentrations of TBRS in the oxidized substrates at the peaks (Fig. 1) were equivalent to from 10^{-3} to 10^{-2} *M* MA and may possibly be polymerizing under the oxidizing condition. Furthermore, MA and the other TBRS may react with other oxidation products during the oxidation. At the latter stages of the oxidation in these experiments both the residual fatty acid esters and squalene formed a layer under water after centrifugation, indicating that the yellowish residues had higher densities than those of the original substrates. Highly polymerized MA is not water-soluble. Thus, the reduction in the amounts of water-soluble TBRS in the latter stages of the oxidation may be explained by the high reactivity of MA and other TBRS in the oxidizing system. These observations support the concept that use of the TBA test as a quantitative measure of lipid oxidation may be meaningful only during the initial stages of oxidation.

On the other hand, the fate of TBRS produced in complicated food systems is somewhat different from that in the pure lipid systems. According to Watts (1962), the amounts of TBRS recovered from meat products during storage never reach a peak, but form a plateau following a rapid initial increase. The differences are probably explainable as follows. MA occurs in both free and bound forms with food constituents (Kwon *et al.*, 1965). Bound MA can be completely recovered through acid and heat treatments, if the foods had been stored at low temperature. Thus, the reactivity of MA with other constituents is responsible for the plateau in the recovery of MA from such food systems. In the later stages the unsaturated fatty materials are exhausted, but the accumulated TBRS have reacted with food constituents and are thus protected from the polymerization or other reactions which account for the loss in total TBRS seen in the pure systems. Despite the above drawbacks the TBA test can be meaningful as a measure of lipid oxidation if the limitations are kept in mind.

The chromatographic pattern obtained with the water-soluble TBRS produced from

UV-irradiated linolenate was slightly different from that of autoxidation, although, again, MA was the main TBRS. The failure of Saslaw *et al.* (1963) and Saslaw and Waravdekar (1965) to identify MA among the TBRS obtained from irradiated fatty acids appears to be due to their use of polymeric MA as a standard in the TLC separations. Their MA had been obtained by distillation of the MA-bisulfite addition compound.

During autoxidation or irradiation of squalene the amount of TBRS produced per double bond of squalene was about 1/6 of those of fatty acids. MA accounted for about half of the total TBRS. The occurrence of MA in oxidized squalene was unexpected since squalene contains only ethylene-interrupted double bonds. Some migration of bonds presumably precedes the oxidative scission.

These combined observations demonstrate that the water-soluble extracts from oxidizing unsaturated esters or squalene can be satisfactorily fractionated on Sephadex columns, that MA is the most important TBRS of these substances and that it is accompanied in oxidizing esters or squalene by other water-soluble compounds, some of which give pink and some yellow colors with TBA. The structures of all of these other than MA have yet to be identified.

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Direct Gas Chromatographic Analysis of Volatiles Produced By Ripening Pears

SUMMARY

Direct sampling of volatiles emanating from pears is shown to be a potentially useful and non-destructive means of assessing fruit ripeness as it is affected by time or treatment such as exposure to ionizing radiation.

INTRODUCTION

Maturation before harvest and ripening thereafter are recognized as important complex physiological processes that directly affect the ultimate quality of fruit (Lott, 1956). The need is manifest for objective measures of fruit maturity and ripeness, and especially for measures that relate well with taste acceptance (Lim and Romani, 1964; Romani *et al.*, 1962). Volatiles offer considerable promise in this regard, on the assumption that they contribute to the aroma and taste of either fresh or processed fruit.

Early attempts at utilizing volatiles as indices to maturity or ripeness were thwarted by the lack of sensitive analytical techniques (Gerhardt, 1954). With the advent of gas chromatography (GC), emphasis was placed on the analysis of extracted pear essence (Jennings *et al.*, 1964). This work has resulted in the identification of many volatile fractions as well as an evaluation of their contribution to aroma (Heinz *et al.*, 1964). Esters of trans:2-cis:2-decadienoate have been found to be especially important contributors to pear aroma (Jennings *et al.*, 1964), and the parent compound has been shown to be readily extractable from pears and potentially useful as a fruit maturity index (Heinz *et al.*, 1965).

However, a maturity index is generally most functional if it is non-destructive and allows for repetitive monitoring of fruit as they ripen or respond to some imposed condition. Direct GC sampling of volatiles meets this requirement. Using direct sampling techniques, Lim and Romani (1964) demonstrated that degree of maturity at time of harvest will greatly influence the subsequent production of volatiles by ripening peaches. In this present study, direct sampling methods have been used to characterize emanations from normal ripening pears as well as from pears that have been previously irradiated.

MATERIALS AND METHODS

Mature pre-climacteric Bartlett pears (*Pyrus communis*, L.) were obtained from local orchards at the time of commercial harvest. These were placed in cold storage (32°F) for several weeks until used, accounting for the immediate onset of the climacteric when placed at 20°C (Fig. 3).

Detailed experimental methods have been described elsewhere (Lim, 1963; Lim and Romani, 1964). Duplicate batches of approximately 1.5 kg of fruit were placed in gallon jars and thereafter kept at 20°C. A measured continuous flow of water-saturated air was passed through the jars. Fruit respiration (CO_2 evolution) was determined by the method of Claypool and Keefer (1942). At daily intervals the jars were sealed for 30 min, and then 5-ml samples of air were removed with a syringe and injected into a Loenco gas chromatograph equipped with a flame ionization detector. Gas chromatographic conditions were as follows:

Column: 8% diethylene glycol succinate (DEGS) on acid-washed chromosorb P (30/60-mesh)
Column size: ¼ inch × 10 feet
Oven temperature: 95 ± 2°C

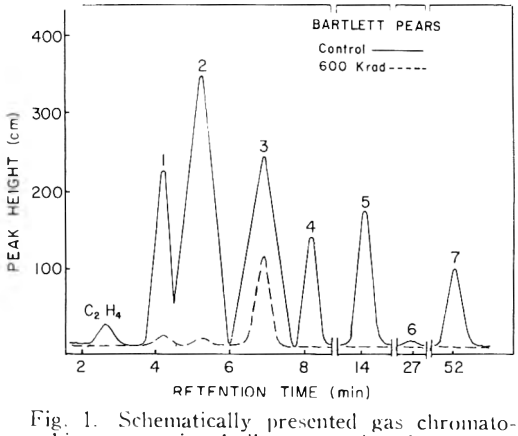

Fig. 1. Schematically presented gas chromatographic traces of volatiles emanating from pears ripened for 10 days at 20° with (– – –) and without (———) prior irradiation. Each fraction is numbered sequentially as eluted through the gas chromatographic column.

Injector temperature: 150°C
Recorder: 1-millivolt full-scale Minneapolis Honeywell
Chart speed: ¼ inch/min
Carrier gas and flow rate: Nitrogen at 12.5 ml/min.
Hydrogen gas flow rate: 20 ml/min
Air flow rate: 30 ml/min

Quantitative estimates of the volatile components were made by a method of approximating triangles (Pecsok, 1959) and expressed in square centimeters of chromatographic peak area per kilogram fruit. Ethylene was identified by absorption in mercuric perchlorate as described by Young *et al.* (1952). Other volatiles were tentatively identified by co-chromatography with known standards.

RESULTS AND DISCUSSION

GC traces of volatiles from normal ripe pears and pears treated with a large dose of gamma radiation are shown in Fig. 1. Following the elution of ethylene each peak is numbered sequentially according to elution time. The known inhibition of pear ripening by ionizing radiation (Maxie and Nelson, 1959) is well reflected in the suppressed production of volatiles. Direct GC analysis of the volatiles suggests itself as a plausible method with which to assess the influence of radiation, or other treatments, on the development of aroma and, indirectly, on fruit quality.

Fig. 2 shows production rates of several low-boiling volatile fractions by ripening Bartlett pears. Table 1 gives tentative identification of each volatile fraction by co-chromatography with known standards. These data have been substantiated in more extensive studies by Jennings and Sevenants (1964). Beginning on the second day, volatile fractions 1, 2, 3, and 4 increase in their

Table 1. Tentative identification of volatiles emanated by ripening Bartlett pears.

Fraction (peak) no.	Retention time		Compound
	sec	min	
C_2H_4	2	43	ethylene
1	4	1	acetaldehyde
2	4	36	methyl acetate
3	5	24	ethyl acetate
4	7	12	propyl acetate
5	10	40	butyl acetate
6	18	2	amyl acetate
7	31	6	hexyl acetate

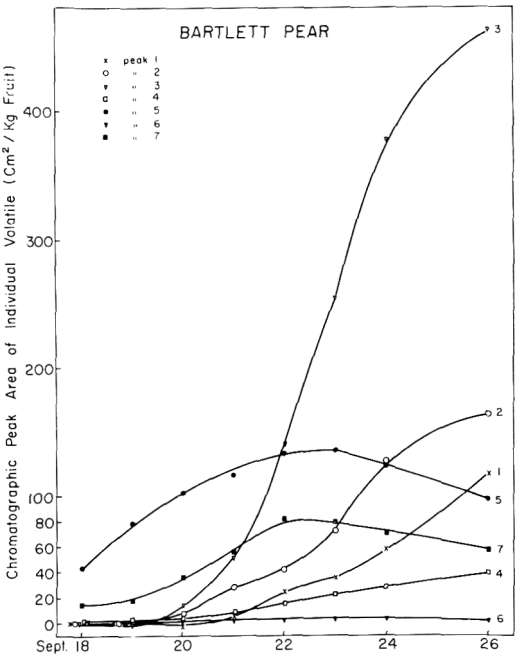

Fig. 2. Emanation of volatile fractions from ripening Bartlett pears. Each fraction is numbered sequentially as eluted through the gas chromatographic column as shown in Fig. 1.

rates of production throughout the ripening period. Fractions 5, 6, and 7, on the other hand, increase for the first 3–4 days but gradually decline thereafter. Divergencies in the pattern of volatile production have also been observed with ripening peaches (Lim and Romani, 1964) and ripening apples (Brown *et al.*, 1966).

Fig. 3 compares the total production of volatiles (less ethylene) with the climacteric respiration sequence and ethylene production by the ripening pears. The maximum production rates of volatile fractions 5, 6, and 7 (Fig. 2) is coincident with the maximum ethylene production in the general region of the climacteric respiratory peak. However, because of the pronounced and sustained increase in the rate of production of fraction 3, total volatiles evolved continue to increase with time and ripening to a point when the fruit is yellow, soft, and overripe.

Total volatile production, as such, may obviously be misleading as a quality index. What is required at this point, beyond the obvious desirability of increased instrumental sensitivity, is a correlation of the volatile

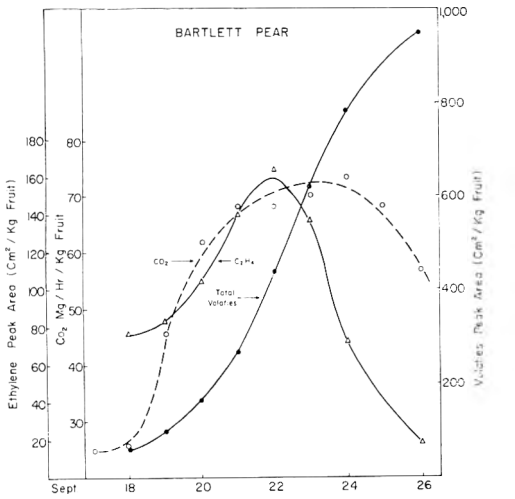

Fig. 3. Total volatiles, carbon dioxide, and ethylene produced by Bartlett pears during their climacteric sequence.

fractions with tangible quality attributes along the lines initiated by Heinz *et al.* (1964). With such correlations established, direct sampling GC could well facilitate the non-destructive monitoring and selection of those stages of ripeness most desirable for the processing or marketing of specific lots or varieties of fruit.

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The Thermal Degradation of Sugars
I. Thermal Polymerization of Glucose

SUMMARY

This paper describes separation and identification of oligosaccharides in caramel made by heating glucose at 150°C without a catalyst. Kojibiose, sophorose, nigerose, laminaribiose, maltose, cellobiose, isomaltose, gentiobiose, and 1,6-anhydroglucose were isolated, and identified as their crystalline β-acetates from the pyrolysate of glucose. Trehalose, isomaltotriose, and panose were tentatively identified by paper chromatography and paper ionophoresis.

INTRODUCTION

One of the oldest problems of food manufacture is the thermal degradation of sugars during processing at elevated temperature. While the caramelization of sugars by heating is well known, previous investigations of caramel have dealt almost exclusively with browning of sugars (Zerban, 1947; Watanabe and Hase, 1956), and little attention has been paid to thermal polymerization of sugars.

This investigation is concerned with the composition of oligosaccharides in caramel made by heating glucose without a catalyst. Pictet and Castan (1920) reported that α-D-glucose readily loses one mole of water at 150°C, yielding α-glucosan (1,2-anhydroglucose). Those workers, and later Cramer and Cox (1922), produced evidence in support of α-1,2-anhydroglucose structure for this material. However, Hurd and Edwards (1949) reported that the thermal dehydration of α- and β-glucose predominantly gave materials of higher molecular weight, and no evidence was found for the formation of an α-glucosan. O'Colla and Lee (1956) and Mora and Wood (1958) reported that α-D-glucose is transformed to high-molecular polymer by heating over 100°C in the presence of a catalyst, cation-exchange resin or phosphorus acid (H_3PO_3). O'Colla *et al.* (1962) recently reported that nigerose, laminaribose, cellobiose, isomaltose, gentiobiose, and 1,5-linked disaccharide were isolated from the polymerization product made by heating glucose with resin-catalyst at 130°C.

EXPERIMENTAL

Pyrolysis of glucose and ether extraction. Dried glucose (100 g) was heated in a retort connected with suction at 150°C until it softened to a brown semi-liquid (2.5 hr). The pyrolysate was dissolved in 200 ml of water and then extracted with ether for 24 hr to remove the ether-soluble substances.

Fractionation of oligosaccharides by carbon column chromatography. The pyrolysate solution, after ether extraction, was adjusted to pH 6.8 by the addition of sodium carbonate solution, and the neutralized solution was then placed on a carbon-Celite column (1:1 w/w; 12.5 × 50 cm) (Whistler and BeMiller, 1962). After standing overnight the column was eluted successively with water and dilute ethanol (2.5–30%) as shown in Table 1. The change of ethanol concentration was made when the effluent reacted negatively to Molisch's reagent (Dische, 1962). The effluent was collected in 5-L fractions which were concentrated to 5 ml under reduced pressure. The sugar components of each fraction were examined by paper chromatography and paper ionophoresis. From the results of chromatography, the effluent portions containing the same sugar component were combined, and classified as fractions I through X (Table 1). The α-linked sugars emerged before the β-linked sugars, and the order within the former group was 1,6- and 1,2-linked sugars before 1,4- and 1,3-, and in the latter group, 1,6-, 1,2-, 1,4-, 1,3- as reported by Aso and Shibasaki (1955).

Paper chromatography and paper ionophoresis. The sugar components of each fraction were tentatively identified by paper chromatography irrigated in a solvent, pyridine–*n*-butanol–water (4:6:3 v/v). The chromatograms were developed with aniline hydrogen phthalate for reducing sugars, and ammoniacal silver nitrate for reducing and non-reducing sugars.

Paper ionophoresis was carried out by the method of Aso and Hamada (1955). Borate buffer solution consisted of 0.05*N* borax and 0.1*N* sodium hydroxide adjusted to pH 9.8. This ionophoresis was operated with constant voltage (600 VDC) and currents (15–25 mAmp), requiring about 3 hr. The acidic aniline hydrogen phthalate was used chiefly as spraying reagent, and the ionophoretogram was developed by heating for 10 min at 120–130°C. The

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Table. 1. Fractionation of the sugars by carbon column chromatography.

Fraction	Number of eluate fraction	Volume of eluate (liters)	Solvent	Sugar component	Yield (g)
I	1	5	Water	No sugar	0
	2–4	15	Water	Glu	8.0
	5	5	Water	Glu, Levoglu	2.1
II	6–10	25	Water	Levoglu	
III	19–25	35	2.5% EtOH	Treha, Isomal, Koji	2.9
IV	26–27	10	5% EtOH	Mal, Niger	1.3
V	28–30	15	5% EtOH	Mal, Niger, Gentio	2.9
VI	31–34	20	5% EtOH	Gentio, Sopho, Cello	1.4
VII	35	5	5% EtOH	Cello	0.2
	36–39	20	7.5% EtOH	Cello, Isomaltotriose	1.1
VIII	40–41	10	10% EtOH	Lami, Isomaltotriose	0.5
	42–44	15	10% EtOH	Lami, Isomaltotriose, Oligo	1.3
IX	45–50	30	10% EtOH	Isomaltotriose, Panose, Oligo	2.5
X	51–54	20	15% EtOH	Oligo	32.0
	55–56	30	30% EtOH	Oligo	

Abbreviations: Glu = glucose, Levoglu = levoglucosan, Isomal = isomaltose, Koji = kojibiose, Trcha = trehalose, Mal = maltose, Niger = nigerose, Gentio = gentiobiose, Sopho = sophorose, Cello = cellobiose, Oligo = higher oligosaccharides.

ammoniacal silver nitrate was used in cases of levoglucosan and trehalose. The R_f values of paper chromatography and the M_G values of ionophoresis are shown in Table 2.

Quantitative analysis of disaccharides. The amount of disaccharides in each fraction was determined according to the method of Dimler *et al.* (1952), in conjunction with paper chromatography. The areas corresponding to those on chromatogram were eluted with water, and determined approximately by Anthrone method. The summation of those sugars is shown in Table 3

Acetylation of oligosaccharides and identification. Acetylation was accomplished by heating sugar fraction and anhydrous sodium acetate with acetic anhydride (Wolfrom and Thompson, 1963), and the acetates thus obtained were identified by mixed melting point with authentic compounds. Infrared spectra of the acetates were determined on KBr disc, on a Shimadzu infrared photometer IR-27C.

Further separation by Magnesol-Celite column. The procedure followed that of Thompson *et al.* (1954). For fraction III, the mother liquor from the crystallization of β-isomaltose octaacetate was concentrated to a sirup. This sirup was dissolved

Table 2. R_f and M_G values of the sugars found in glucose caramel, and tabulation of the order of their emergence from carbon column.

Common name	Linkage	R_f	M_G
Glucose		0.75	1.00
Levoglucosan		0.88	−0.10[a]
Trehalose	1,1-	0.56	0.10
Isomaltose	1,6-α	0.48	0.67
Kojibiose	1,2-α	0.56	0.30
Maltose	1,4-α	0.60	0.30
Nigerose	1,3-α	0.65	0.67
Gentiobiose	1,6-β	0.48	0.67
Sophorose	1,2-β	0.60	0.30
Cellobiose	1,4-β	0.56	0.27
Laminaribiose	1,3-β	0.70	0.65
Isomaltotriose	1,6-α-1,6-α	0.27	0.55
Panose	1,6-α-1,4-α	0.40	0.23

R_f values were determined by four times multiple paper chromatography irrigated in a solvent system of pyridine–*n*-butanol–water (4:6:3 v/v).

M_G values were determined as the ratio of the distances traveled by sugar and glucose from 2,3,4,6-tetramethylglucose.

[a] Levoglucosan was traveled to the cathode side in paper ionophoresis.

Table 3. Yields (%) of disaccharides from glucose acid reversion, catalytic polymerization, and thermal polymerization

		1,1-			1,2-		1,3-		1,4-		1,5-	1,6-	
		α	αβ	ββ	α	β	α	β	α	β		α	β
Thompson *et al.* (1954) O'Colla *et al.* (1962)	Reversion			0.04	....	0.09	0.11	....	0.20	0.16	....	2.02	1.70
	Catalytic polymerization [a]	Trace			....	....	2.40	1.45	....	0.48	0.30	7.00	5.00
Present work [b]	Thermal polymerization	Trace			0.25	0.75	1.75	1.12	1.75	0.50	....	2.65	2.00

[a] Classified as catalytic polymerization, expressed more precisely as catalytic thermal polymerization.
[b] The amounts of these disaccharides were determined approximately by anthrone method, in conjunction with paper chromatography.

in 20 ml of benzene, and the mixture was placed on a column (30 × 4 cm) of Magnesol-Celite (5:1 w/w) and chromatographed with 1 L of benzene-*t*-butanol (100:1 v/v) as a developer. The column was extruded from a tube and streaked with indicator (1% potassium permanganate in 10% sodium hydroxide) to locate the zones.

Sugar components of fractions I through X. *Fraction I.* Analysis by paper chromatography indicated the presence of glucose, and 8 g of glucose was recovered.

Fraction II. Paper chromatogram showed the presence of levoglucosan and a trace of glucose. Amorphous white powder (2.1 g) was acetylated by the ordinary method described above. Crystallization from ethanol gave 1.3 g of levoglucosan triacetate, mp 110°C.

Fraction III. Chromatogram analysis indicated the mixture of isomaltose, kojibiose and a trace of trehalose. The presence of trehalose in this fraction was confirmed by paper ionophoresis. Acetylation gave 2.5 g of white amorphous powder; β-isomaltose octaacetate (1.3 g) was crystallized directly from ethanol, mp 144–145°C. After separation of isomaltose acetate, the mother liquid was evaporated to a sirup which was rechromatographed with Magnesol-Celite column. The zone appeared in the upper part of the column, 5–12 cm from the top. This zone was sectioned and eluted with acetone. The acetone eluate was evaporated to a sirup and crystallized from ethanol, and 0.2 g of isomaltose was obtained as crystalline β-acetate. The effluent from the column left 0.2 g of sirup on removal of the solvent. The sirup was crystallized slowly from ethanol at 25°C, and gave 20 mg of β-kojibiose octaacetate, mp 118°C.

Fraction IV. White amorphous powder (1.3 g) containing nigerose and maltose was obtained from fraction IV. After acetylation, the acetate was treated by the same procedure using Magnesol-Celite column as above. The zone appeared in the upper part of the column, 3–11 cm from the top. Elution with acetone and removal of the solvent left 0.9 g of sirup. β-Nigerose octaacetate (60 mg) was obtained upon crystallization from ethanol, mp 149–150°C. The effluent from the column left 0.2 g of sirup on removal of the solvent. The sirup gave 60 mg of β-maltose octaacetate upon crystallization from ethanol. Recrystallized product showed an mp of 158–159°C.

Fraction V. Fraction V, 2.9 g amorphous powder containing gentiobiose, nigerose, and maltose, was acylated and crystallized from ethanol to give 0.9 g of β-gentiobiose octaacetate, mp 193°C. After separation of gentiobiose, 1 g of crystalline substance was recovered as the mixture of their acetates from the mother liquor.

Fraction VI. White amorphous powder (1.4 g) containing sophorose and gentiobiose was obtained from fraction VI. These disaccharides were separated by thick-paper chromatography. The sample was dissolved in 5 ml of 20% ethanol, and 1 ml was spotted on each paper (Toyo filter paper No. 527, 40 × 40 cm, 0.7 mm thick) and irrigated in the same solvent as above. The areas corresponding to those of sophorose and gentiobiose on the chromatogram were eluted with water. An amorphous powder (0.3 g) of sophorose was collected from the eluate, acylated, and crystallized from ethanol, giving 50 mg of β-sophorose octaacetate, mp 189–190°C. Gentiobiose acetate (0.1 g) was obtained from this fraction by the same procedure.

Fraction VII. The fraction of cellobiose, treated by the same procedure, upon thick-paper chromatography gave 0.3 g of β-cellobiose octaacetate on acetylation. Recrystallized product from ethanol showed an mp of 193–194°C.

Fraction VIII. White amorphous powder (0.5 g) containing laminaribiose and isomaltotriose was acylated by the ordinary method, and gave 20 mg of β-laminaribiose octaacetate, mp 160°C. However, the separation of isomaltotriose acetate from the mother liquor could not be achieved.

Fraction IX. This fraction contained isomaltotriose, panose and a trace of higher oligosaccharides. Isomaltotriose and panose were separated by multiple chromatography on thick paper. These trisaccharides were tentatively identified by paper chromatography and paper ionophoresis, although an attempt to obtain crystalline derivatives, acetate and benzoate, was unsuccessful.

Fraction X. This fraction was collected from 15% ethanol and finally 30% ethanol eluate portions, yielding 32 g of sirup. The fraction consisted of the mixture of higher oligosaccharides, and the fractionation is still in progress.

RESULTS AND DISCUSSION

The results are summarized in Table 1. The sugar fractions obtained from glucose caramel are numbered I through X, in the order of the eluate from carbon column. Two monosaccharides, nine of the possible eleven pyranose disaccharides, and two trisaccharides have been found. Most of the sugars described herein were isolated as their crystalline acetates, and identified by melting point, mixed melting point with authentic substance, paper chromatography, paper ionophoresis, and infrared spectra. The acetylation condition employed herein leads to an equilibrium mixture in which the β-acetate predominates; in this work the α-acetate could not be isolated. Three anomers of trehalose, α,α-, α,β-, and β,β-linkages, are known, and it is necessary to obtain them in crystalline form for identification of the structure. In this work, since isolation of trehalose could not be identified.

It has been known for many years that glucose undergoes condensation to form the oligosaccharide when treated with mineral acid or cation-exchange resin; one was designated "reversion" (Thompson *et al.*, 1954; Peat *et al.*, 1958; Anno *et al.*, 1959), the other "catalytic polymerization" (Mora and Wood, 1958; O'Colla *et al.*, 1962). Hence it is of interest to compare the disaccharides of reversion product or catalytic polymerization product with the product obtained in this work (see Table 3). It can be seen that 1,6-linked sugars are formed predominantly in reversion, and 1,3- and 1,6-linked sugars are formed more readily than other linked sugars in catalytic polymerization or thermal polymerization. O'Colla *et al.* (1962) did not find kojibiose (1,2-α) and maltose (1,4-α) in their catalytic polymerization, but did find a 1,5-linked disaccharide. Kojibiose and laminaribiose (1,3-β) were found in reversion product (Peat *et al.*, 1958), the latter in resin-catalyzed reversion (Anno *et al.*, 1959). However, 1,3-linked sugars in reversion product were much less than those in catalytic or thermal polymerization, yield of 1,3-linked sugars 0.1% in reversion, over 2% in this work.

The thermal polymerization mechanism cannot be declared definitely herein, but it is evident that the glucosidic hydroxyl group reacted with the alcoholic hydroxyl group of another glucose, the carbonium ion at the first carbon as in glucose reversion might react with any of hydroxyl group of another glucose molecule. However, the formation of trehalose indicates the probability of dehydration in each glucosidic hydroxyl group. Levoglucosan was isolated, and the formation of this substance is predictable. Wolfrom *et al.* (1959) reported that levoglucosan is capable of polymerization when heated at 235–240°C, and some of the disaccharides were separated from the acid hydrolysate of this polymer.

Table 4. Composition of the sugar fraction in glucose caramel.

Saccharide	Recovery (A)[a] (%)	Separated amount (B) (g)	Calcd. amount (C) $C = B/A \times 100$ (g)
Mono-	100	10.1	10.1
Di-	80	10.7	13.7
Tri-	60	4.3	7.3
Higher oligo-	50	32.0	64.0

[a] Aso *et al.* (1960).

Table 4 shows the amounts of oligosaccharides formed by the pyrolysis of glucose. These amounts were estimated approximately according to the rates of sugar recovery by carbon column chromatography (Aso *et al.*, 1960).

The fractionation of higher oligosaccharides could not be achieved, because of difficulty in chromatographic separation of this fraction. However, it is hoped that the work still in progress will determine at least some of these.

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Identification of Components in the Stale Flavor Fraction of Sterilized Concentrated Milk

SUMMARY

The more volatile components of the stale flavor fraction of sterilized concentrated milk (SCM) were studied by a nitrogen-purge on-column trapping technique. Higher-boiling components were isolated by solvent extraction of the fat from lyophilized SCM, followed by low-pressure reduced-temperature steam distillation of the extracted fat. Components in the resulting flavor extract were identified by gas chromatography and mass spectrometry.

The following compounds were identified in the extract from stale SCM: 2-heptanone, 2-nonanone, 2-tridecanone, benzaldehyde, acetophenone, naphthalene, a dichlorobenzene, δ-decalactone, benzothiazole and *o*-aminoacetophenone. Of these compounds, only the dichlorobenzene and 2-heptanone were identified in the extract from the control SCM.

INTRODUCTION

Development of a stale flavor upon storage is the major obstacle to successful commercial utilization of sterilized concentrated milk (SCM). The stale flavor develops within 2–3 months of storage at 70°F. Patel *et al.* (1962) have referred to the defect as an "old rubber flavor."

The volatile flavor components of SCM have been studied to a limited extent (Patel *et al.*, 1962; Bingam, 1964), but the compounds responsible for the stale flavor defect as it occurs in this product have not been identified. The purpose of this work was to identify these compounds to better understand the staling phenomenon.

LITERATURE REVIEW

Whitney and Tracy (1950) initiated the study of stale flavor with the discovery that the stale flavor components of dry whole milk could be partitioned into the fat phase by solvent extraction of the fat. It was further discovered that the stale flavor components could be steam-distilled from the extracted fat (Whitney *et al.*, 1950). The stale flavor components were not identified, however.

Numerous studies have been reported relating to identification of the flavor volatiles of stored milk products, particularly dried milk products. The various flavor compounds identified in these products are summarized in Table 1.

Kurtz (1965) made an important observation related to the nature of stale flavor components. In attempting to recover the volatile components from foam-dried milk powder by short-path migration at one-micron pressure to a cold finger, he found that powders exhibiting an oxidized flavor could be improved by this treatment, whereas stale powders were not improved. This observation suggests that the compounds responsible for the stale flavor defect are of low volatility, or are in some manner tightly bound by the milk powder.

EXPERIMENTAL METHODS

Commercially processed samples of SCM were obtained. A portion of each sample was stored at 21°C to hasten the development of stale flavor. Control samples were stored at 1°C.

That there was a significant difference between the stale and control samples was determined by subjecting the samples to evaluation by a trained flavor panel. The panel and score card used were similar to those described by Sather *et al.* (1965). The stale and control samples, along with a sample of fresh whole milk, were scored by the panel on three different occasions.

The nitrogen-purge on-column trapping technique described by Morgan and Day (1965) was used to compare the more volatile components of the stale and control samples. The equipment and operating parameters used were identical to those described by those authors. The volatile components from 6 ml samples of both fresh and stale SCM were analyzed by this technique.

The success of Whitney and Tracy (1950) in fractionating the stale flavor components of dry whole milk into the fat phase by extraction of the fat with organic solvents, and of Parks *et al.* (1964) in isolating *o*-aminoacetophenone from nonfat dry milk by solvent extraction, suggested that this was the most suitable method for isolation of the less-volatile compounds. It was determined that stale SCM could be lyophilized prior to solvent extraction without loss of the stale flavor components. Hydration of the lyophilized SCM to the concentration of whole milk yielded a product possessing the same characteristic stale flavor

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Table 1. Volatile flavor compounds of stored milk products as reported in the literature.

Author	Product	Compounds identified
Bassette, 1958	Instant Nonfat Dry Milk	*n*-aldehydes: C_1,C_2,C_6,C_7,C_8,C_{10},C_{12},C_{14} *n*-methyl ketones: C_3,C_4,C_5 furfuraldehyde, 2-methyl heptanal
Parks and Patton, 1961	Dry Whole Milk	*n*-aldehydes: C_1-C_3,C_5-C_7,C_9,C_{10},C_{12} *n*-methyl ketones: C_3,C_4,C_9,C_{11},C_{15} furfuraldehyde, benzaldehyde
Nawar *et al.*, 1963	Dry Whole Milk	*n*-aldehydes: C_1-C_8,C_7 *n*-methyl ketones: C_3,C_5
Muck *et al.*, 1963	Aged Evaporated Milk	*n*-methyl ketones: C_8,C_7,C_9,C_{11},C_{13} *n*-fatty acids: C_6,C_8,C_{10},C_{12},C_{14},C_{16} δ-decalactone, δ-dodecalactone, γ-dodecalactone
Parks *et al.*, 1964	Nonfat Dry Milk	*o*-aminoacetophenone
Patel *et al.*, 1962	SCM	ethanal, dimethyl sulfide, acetone, 2-pentanone, pentyl acetate [a]
Bingam, 1964	SCM	ethanal, propanal, pentanal, acetone, 2-hexanone, 2-heptanone, dimethyl sulfide

[a] Pentyl acetate shown by Bingam to be artifact from antifoam.

as the original stale SCM diluted to the concentration of whole milk.

Various solvent extraction techniques were attempted. The procedure finally adopted was similar to that of Parks *et al.* (1964). One-hundred-gram quantities of the lyophilized SCM were added to 400 ml of hexane (high-purity-grade, Phillips Petroleum Co.; treated for removal of carbonyls by method of Hornstein and Crowe (1962) and subsequently redistilled) in a Waring blender and mixed for 5 min, whereupon 10 ml of distilled water was added, and blending continued for another 5 min. The resulting mixture was then placed in the extraction thimble of a large Soxhlet extractor, and extracted for 4 hr. The extraction thimble was of sufficient capacity to hold 800 g of the lyophilized powder. Three 800-g batches of both stale and control SCM were extracted in this manner. The bulk of the solvent was evaporated in the Soxhlet apparatus after removal of the sample. The three extracts were then pooled, and the remaining hexane evaporated on a rotary evaporator.

The extracted fat was subjected to a reduced-pressure (2–3 mm Hg) reduced-temperature (40°C) steam distillation. The distillate was collected in liquid-nitrogen-cooled Dewar-type traps, and subsequently extracted with ethyl chloride (U.S.P.-grade; Matheson Company). The ethyl chloride extract was dried overnight with sodium sulfate, and the ethyl chloride then evaporated.

The resulting flavor extracts were studied by gas chromatography and mass spectrometry. A Barber-Colman Series 5000 temperature-programming gas chromatograph with a hydrogen flame detector was used for the GLC work. A 12-ft × 1/8-inch-OD stainless-steel GLC column packed with 3.8 g of 20% Apiezon M on 80–100-mesh Celite 545 was used for the initial GLC separation of components. A temperature program of 50°C per minute from 100°C (5-min hold) to 200°C was selected as optimum for chromatographing the flavor extracts. It was found that certain peaks, which appeared to be the result of fatty acids in the flavor extract, were overshadowing other components of the extract. Consequently a 2-ft × 1/8-inch-OD stainless-steel GLC column was packed with 0.6 g of 20% Apiezon M on 80–100-mesh Celite 545 which had been impregnated with 5% sodium hydroxide by weight. When inserted in front of the regular GLC column, this base-treated pre-column successfully removed fatty acids from the flavor mixture, allowing the resolution of components previously hidden by the fatty acid peaks.

Mass spectra were obtained on an Atlas-MAT CH-4 Nier-type mass spectrometer (a 9-inch 60-degree-sector single-focusing instrument). The operating parameters of the mass spectrometer were:

Filament current	60 μ-amps
Electron energy	35 eV
Analyzer pressure	2×10^{-7} mm Hg
Scanning speed	5 seconds to scan m/e 25 to m/e 250
Accelerating voltage	3000 V
Multiplier voltage	1.85 KV

The mass spectrometer was equipped with a Honeywell 1508 Visicorder, enabling the recording of rapid-scan mass spectra of the GLC effluent.

Some of the earlier eluting components of the Apiezon chromatogram were identified from the mass spectral patterns obtained from the Apiezon column effluent. It became apparent that some means of improving separation of the later-eluting components and of increasing their concentration was needed. These requirements were satisfied by the development of a technique for trapping the GLC effluent. Components eluting from the Apiezon column, which appeared to be of significance because of their odor properties, were collected from several successive Apiezon chromatograms in 1½-ft × ⅛-inch-OD stainless-steel GLC columns packed with 20% Apiezon M on 80–100-mesh Celite 545. The collection traps were immersed in methyl cellosolve-dry ice, and were sealed with Swagelok caps between collections. The components in two regions of the Apiezon chromatogram were collected in separate traps from six successive chromatograms.

The components collected in this manner were subsequently rechromatographed by inserting the collection traps in front of a 12-ft × ⅛-inch-OD stainless-steel GLC column packed with 3.8 g of 20% Carbowax 20M on 80–100-mesh Celite 545. The repeated trapping technique was effective in building up the concentration of components in the absence of any solvent, and rechromatographing on the polar GLC column greatly improved separation of the components for mass spectral analysis.

RESULTS AND DISCUSSION

Flavor-panel evaluations are shown in Table 2. Analysis of variance of the data indicated a significant difference between samples at the 1% level of significance, for both total score and stale-flavor criticism. Calculation of the least-significant difference (LSD) indicated that the stale SCM was significantly different from the control SCM at the 1% level for both total score and stale criticism. It was concluded that the stale and control samples were indeed different, and that a comparison of the flavor components of these two samples would give some indication of the compounds contributing to the development of a stale flavor.

Table 2. Average scores for each criticism and average total scores of milk samples evaluated by trained panel.[a]

	Fresh whole milk	Control sterile concentrate	Stale sterile concentrate
Criticism			
Astringent	10.0	9.70	8.87
Caramelized	10.0	9.73	7.90
Chalky	10.0	9.63	9.40
Cooked	9.20	8.33	8.33
Lactone	9.67	9.27	8.67
Scorched	9.80	9.30	7.70
Stale [b]	9.84	6.97	5.36
Total score [c]	8.24	5.86	3.20

[a] Score scale from 1, flavor-criticism intense, to 10, no criticism.
[b] LSD (.01) = 1.30 (stale criticism).
[c] LSD (.01) = 0.786 (total score).

Fig. 1 compares the more volatile components of the stale and control SCM as determined by the nitrogen-purge on-column trapping technique. It can be seen that there is little difference between the composition of the more volatile components of the stale and control SCM. This finding supports the hypothesis that the compounds contributing to the stale flavor defect are relatively nonvolatile.

Some of the more volatile components appearing in the chromatograms in Fig. 1 were tentatively identified on the basis of GLC retention times. These components are listed in Table 3. The largest peak in these chromatograms, peak 8, as well as peak 21, was present in a blank sample consisting of distilled water, sodium sulfate, and antifoam. It was concluded that these peaks came from the antifoam emulsion.

Fig. 2 shows chromatograms of the flavor extracts obtained by the solvent extraction technique from stale and control SCM. Peak 5 of the fresh SCM (Fig. 2A) and peak 7 of the stale SCM (Fig. 2B) were identified as 2-heptanone by GLC and mass spectrometry. These peaks are of comparable size in the flavor extracts from fresh and stale SCM, while a number of the later eluting peaks (peaks 18–34) appearing in the stale SCM are not present in the fresh SCM, or are considerably smaller in the fresh SCM. Since the two flavor extracts were obtained in an identical manner, these components appear to be of importance in the stale flavor defect.

The effluent from the Apiezon chromatogram was collected from two different regions of the chromatogram, as indicated by Trap I and Trap II in Fig. 2B. Fewer than 10 components appear to be present in the region of the Apiezon chromatogram where Trap II was used. When the components

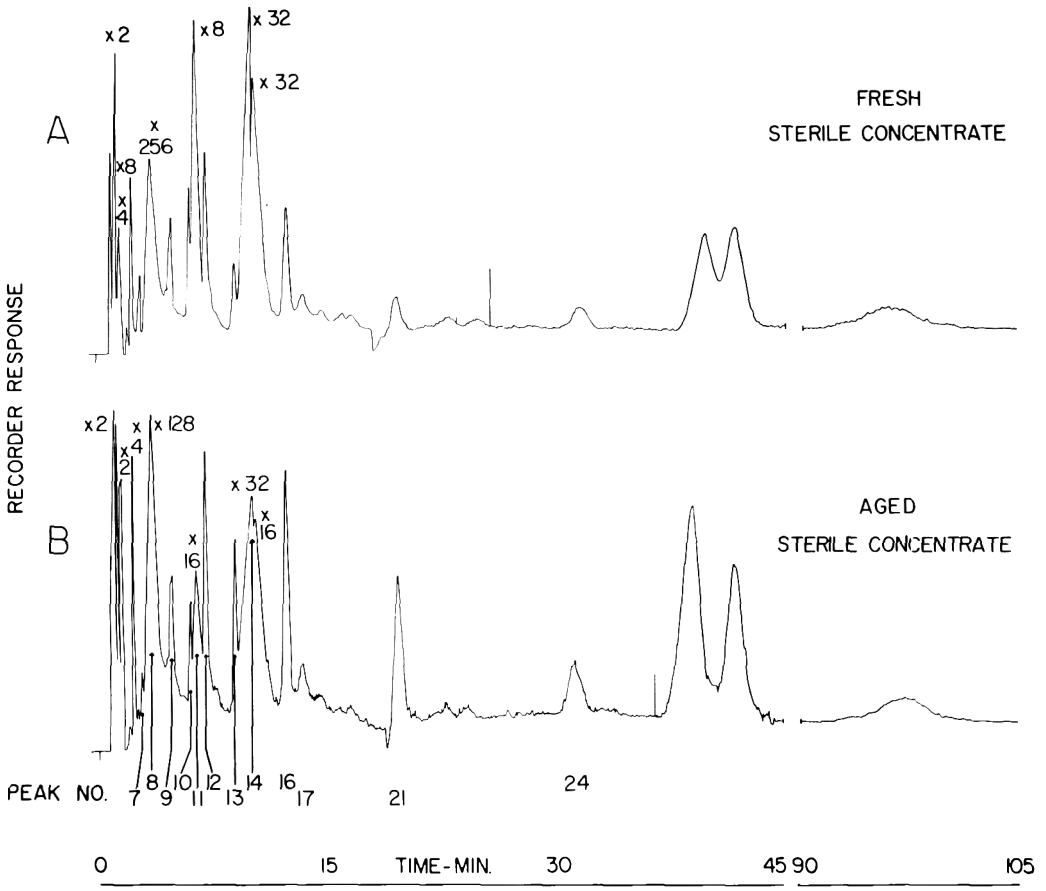

Fig. 1. Chromatograms of volatile headspace components of (A) fresh and (B) aged sterile concentrated milks; 1,2,3-tris-(2-cyanoethoxy) propane column, 80°C.

Table 3. Tentative identity of peaks shown in Fig. 1 (chromatogram of volatile headspace, 80°C, on 1,2,3-tris-(2-cyanoethoxy) propane column).

Peak no.	Relative retention time (to acetone)	Tentative identity	Relative retention time of known	Agreement in 50°C chromatograms
7	0.438	Acetaldehyde	0.443	+
8	0.549	From antifoam	0.552	+
9	0.755	Isobutyraldehyde	0.748	+
		or		
		ethyl formate	0.770	—
10	0.950	Ethyl acetate	0.947	+
11	1.00	Acetone	1.00	+
12	1.10	Ethanol	1.12	?
13	1.41	2-Butanone	1.43	+
14	1.59	Methyl butyrate	1.57	+
16	1.93	2-Pentanone	1.92	+
17	2.10	Dimethyl disulfide	2.10	+
21	3.36	From antifoam	3.43	+
24	4.92	2-Heptanone	4.94	+

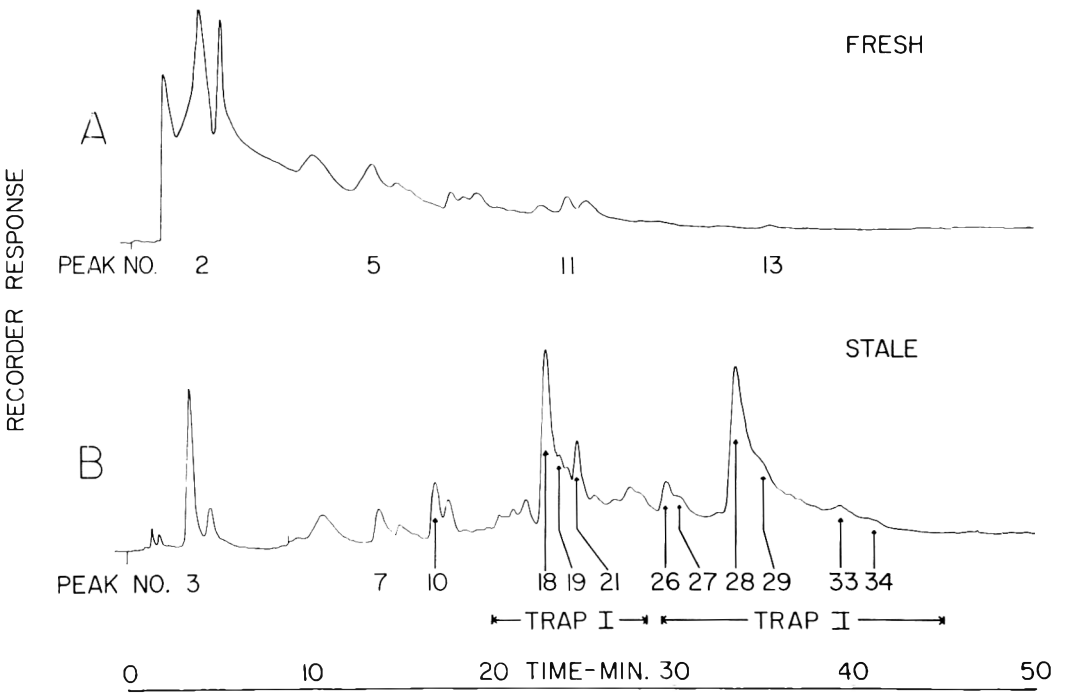

Fig. 2. Chromatograms of flavor extracts from (A) fresh and (B) stale sterile concentrated milks; Apiezon column, temperature-programmed at 5°C/min from 100°C (5-min hold) to 200°C.

collected in Trap II were rechromatographed on the Carbowax 20M column (Fig. 3), 26 components were resolved. This illustrates the utility of the trapping and rechromatographing technique in improving the resolution of components.

The components of Trap II were chromatographed isothermally at 200°C on the Carbowax 20M column in order to elute the slower-moving components, several of which possessed significant odors, as sufficiently sharp peaks to obtain usable mass spectra. Consequently, several of the earlier eluting components of Trap II were not sufficiently separated to obtain good spectra. Temperature programming of the Trap II components should achieve optimum separation of these components.

As an example of the identification of stale flavor components by these techniques, Fig. 4 shows the background mass spectrum and the mass spectrum of peak 22 of the components from Trap II (Fig. 3). The m/e 135 and m/e 108 peaks are increased

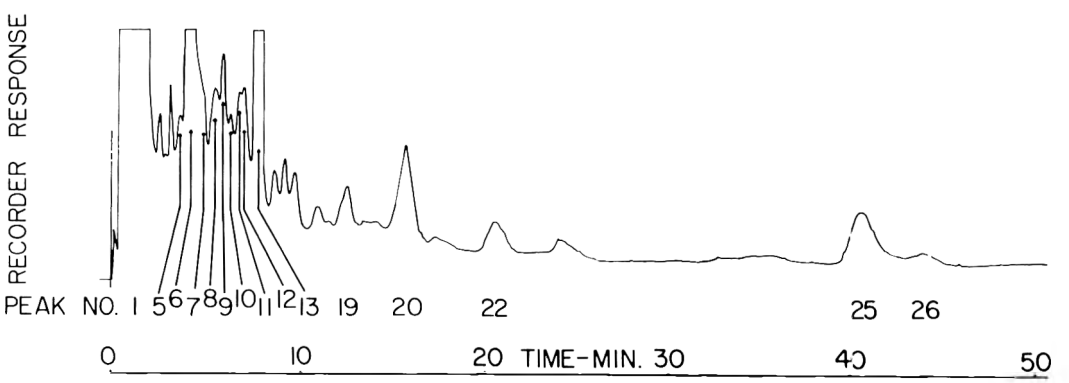

Fig. 3. Chromatogram of components trapped in trap II; Carbowax 20M column, 200°C.

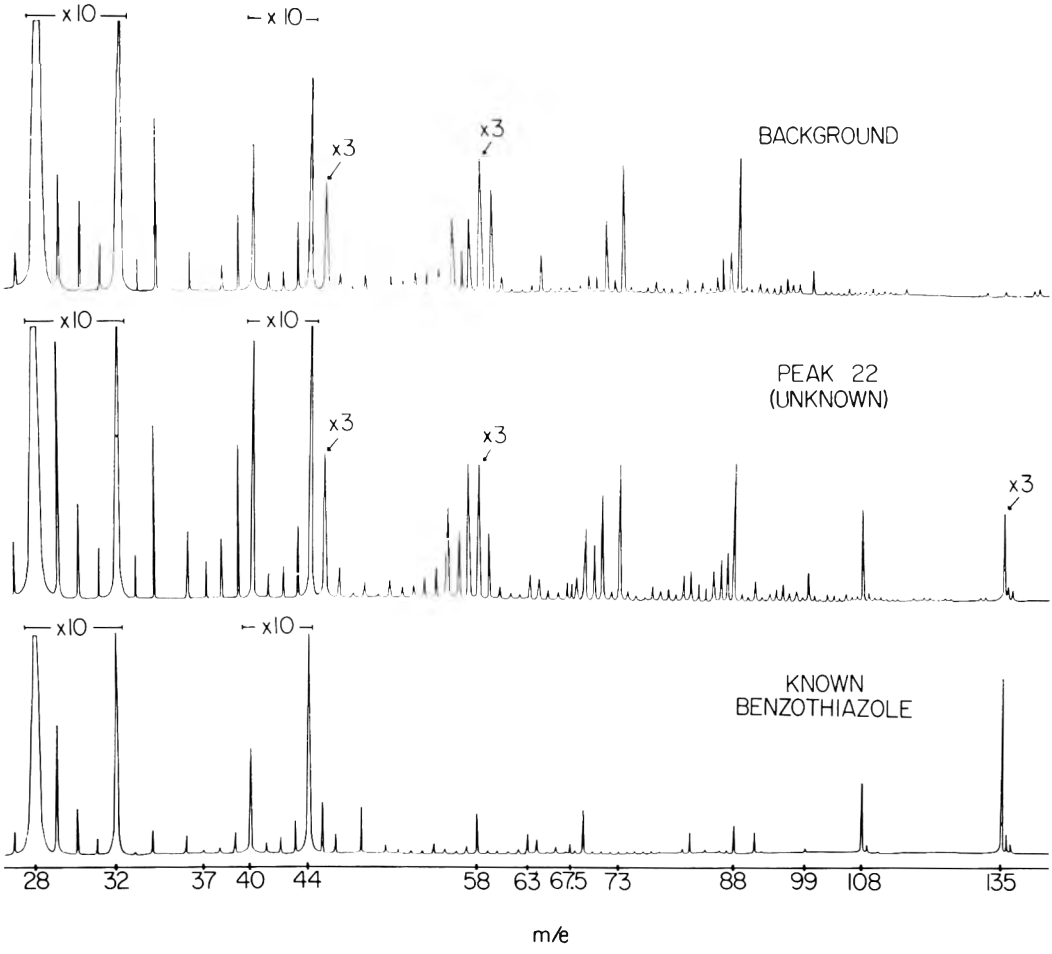

Fig. 4. Background mass spectrum and mass spectrum of peak 22 of the components of Trap II (Fig. 3), and mass spectrum of known benzothiazole.

considerably in the spectrum of the unknown compound. The peaks at m/e 58, 73, and 88, the result of column bleed from the Carbowax column, remain constant in both spectra. Similarly, the peaks at m/e 28, 32, 40, and 44, respectively the results of carbon monoxide and nitrogen, oxygen, argon, and carbon dioxide, remain constant. Peaks which are increased to some extent are those at m/e 37, 38, 39, 45, 63, 69, and 82. The double-charged ion 135^{++}, appearing as m/e 67.5, is new to the spectrum of the unknown. With this information the ASTM Index (Am. Soc. for Testing and Materials, 1963) was consulted. The most probable identity of the unknown compound appeared to be benzothiazole, which has a base and parent peak of m/e 135, with m/e 108 equal to 35% of the base peak. Good agreement was also shown for the other m/e peaks mentioned above. Hence, a sample of benzothiazole was obtained from a chemical supplier, and the retention time was determined under identical GLC conditions. The retention times agreed on both the Apiezon and Carbowax columns. A mass spectrum of the known compound was obtained, again showing agreement with that of the unknown. This mass spectrum is also shown in Fig. 4. The unknown peak was thus presumed identified as benzothiazole.

The compounds identified in this manner in the flavor extract from stale SCM are listed in Table 4. Only the dichlorobenzene and 2-heptanone were identified in the extract from the control SCM.

Table 4. Summary of compounds identified in the flavor extract from stale steril concentrated milk.

Compounds	Retention time data		Mass spectral identification	Agreement of odor
	Apiezon	Carbowax		
2-Heptanone	+		Positive	+
2-Nonanone	+	+	Positive	+
2-Undecanone	+	+	Positive	+
2-Tridecanone	+	+	Positive	?
Benzaldehyde	+		Positive	+
Acetophenone	+	+	Tentative	?
Naphthalene		+	Positive	?
A dichlorobenzene	+	+	Positive	?
δ-Decalactone	+	+	Positive	+
Benzothiazole	+	+	Positive	+
o-Aminoacetophenone	+	+	Positive	+

It is of interest to consider the significance of the compounds identified. The dichlorobenzene is considered to be of no significance in the stale flavor defect, in that it was identified in both the stale and control samples. Its origin is unknown. The longer-chain methyl ketones, δ-deca-lactone, benzaldehyde, and *o*-aminoacetophenone, are likely of significance in the stale flavor defect. These compounds had previously been identified in stored dried milk products. Their identification in stale SCM in this study further implicates them as important compounds in the stale flavor defect.

The significance of *o*-aminoacetophenone in the stale flavor defect was further determined by including a sample of fresh sterile concentrate containing five ppb added *o*-aminoacetophenone in the flavor-panel studies. The average stale-criticism score and average total scores of the samples were as follows:

	Fresh SCM	Stale SCM	Fresh SCM + 5 ppb *o*-aminoacetophenone
Stale criticism	6.97	5.36	5.75
Total score	5.86	3.20	4.63

The scores received by the fresh SCM containing 5 ppb *o*-aminoacetophenone were similar to those of the stale SCM, particularly the stale-criticism score. These results indicate that *o*-aminoacetophenone is indeed an important compound in the stale flavor defect.

The significance of benzothiazole in SCM is currently being studied. This compound has a characteristic "rubber" odor, which might be the cause of the "old rubber flavor" of stored SCM reported by Patel *et al.* (1962).

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The Effect of Environmental Variables on the Processing of Sweetpotatoes into Flakes and on Some Properties of Their Isolated Starches

SUMMARY

Freshly harvested uncured sweetpotatoes were successfully processed into precooked dehydrated sweetpotato flakes by the controlled addition of amylolytic enzymes to the cooked puree prior to drying. The enzyme concentration required decreased as the harvesting season and storage period progressed. Reaction time was not critical when the enzyme concentration was not excessive. The puree was readily liquefied by commercial amylases, but the reducing sugar content of the flakes was not increased.

To determine whether there might be a correlation between starch properties and the decreasing enzyme requirement, the effects of postharvest treatments of the sweetpotato were studied. The amylose content, pasting properties, and susceptibility to amylolysis of the isolated starch were determined. Curing with both optimum and prolonged storage effected changes in the pasting properties of the isolated starches. However, neither the susceptibilities of the starches to amylolysis nor their amylose contents were affected. Time of harvest and short-term storage of the uncured roots did not alter any of the starch properties investigated. The processing characteristics of the sweetpotato could not be correlated directly to the properties of the isolated starches. It was observed, however, that although the starch fraction in the cooked puree was not saccharified by the amylolytic enzymes used in processing, β-amylase was not inhibited in its action on the isolated starches.

INTRODUCTION

At present an acceptable precooked dehydrated sweetpotato flake with excellent rehydration properties can be produced from cured sweetpotatoes stored approximately three weeks or more after curing. Flakes produced from uncured or inadequately cured or stored sweetpotatoes, using the basic process described by Deobald *et al.* (1962), have a thick, pasty consistency and a bland starchy taste when reconstituted. Process modification which would permit the utilization of uncured sweetpotatoes would eliminate the regular curing step, permit processing of sweetpotatoes to begin immediately after harvest, and significantly broaden the range of raw product from which an acceptable flake could be produced.

The present investigation consists of two phases. The first involved a study of the use of amylolytic enzymes during processing as a means of producing a flake with acceptable rehydration properties from uncured sweetpotatoes. During these experiments it was demonstrated that the processing characteristics of the sweetpotato were altered by time of harvest, short-term storage of the uncured roots, curing, and prolonged storage of the cured roots. The second phase of the study consists of an investigation of the effect of these postharvest environmental variables on the nature of the isolated starch fraction. The starch properties investigated were amylose content, pasting properties, and susceptibility to amylolytic attack.

Schermerhorn and Hinz (1952) developed a method of treating sweetpotato puree with a diastatic enzyme in order to produce a commercially acceptable canned mashed product. Hoover (1965) has developed a technique for blending with untreated puree a puree whose starch has been hydrolyzed by an amylolytic enzyme, so as to obtain a puree from which an acceptable precooked dehydrated flake can be produced.

Carbohydrate transformations in the sweetpotato during curing and storage have been studied by a number of investigators (Hasselbring and Hawkins, 1915; Culpepper and Magoon, 1926; Hopkins and Phillips, 1937; Sistrunk *et al.*, 1954). Changes in the nature of the starch itself have also been studied. Barham *et al.* (1944) investigated the effects of time of harvest and curing on granule size, granule size distribution, gela-

tinization temperature, and the viscosity of starches isolated from five varieties of sweetpotatoes grown in Kansas, and concluded that curing lowered both the temperature of transition and peak viscosity. Barham and Wagoner (1946) studied the effect of time of cure over a period of 19 weeks on moisture content, granule diameter, and pasting properties of the starches isolated from sweetpotatoes of the improved Big Stem Jersey variety. They found that the "rate of gelatinization" varied directly with granule diameter and density. Hammett and Barrentine (1961) studied the effect of curing on the amylose contents of starch of the Allgold and Porto Rico varieties grown in Mississippi, and reported that both varieties decreased in amylose during curing. Doremus *et al.* (1951) demonstrated that the amylose contents of the starch from 22 varieties of sweetpotatoes grown in Louisiana fell within the narrow range of 17.5–21.7%, the majority of the varieties falling between 20.0 and 21.7%. Hodge *et al.* (1948) presented evidence that sweetpotato starch might contain molecules intermediate between amylose and the average amylopectin in extent of branching.

The Goldrush variety of sweetpotatoes was selected as the source of raw material since it is the principal variety from which precooked dehydrated sweetpotato flakes are produced. The process modification involved in the first phase of this study, named "enzyme curing," consists essentially of treating the whole puree with a commercial amylolytic enzyme to effect partial starch hydrolysis prior to drying on a double-drum dryer. Four commercial amylase preparations were evaluated. The principal variables investigated were enzyme concentration and reaction time. The properties of the starch fraction isolated by a standardized procedure from sweetpotatoes harvested, cured, and stored for varying periods were amylose content, pasting properties, and susceptibility to amylolytic attack. These investigations were undertaken to provide information upon which to base further variations in processing techniques and to explain observed differences in processing characteristics attributed to curing and storage variables.

EXPERIMENTAL

Enzyme curing. The first phase of the study was conducted from August 28, 1962, through October 22, 1962. The Goldrush sweetpotatoes used were grown in the vicinity of St. Francisville, Louisiana. They were harvested and shipped to the Laboratory at weekly intervals from August 27, 1962, to October 15, 1962. They were stored immediately at 60°F. The basic processing technique used in the production of the flakes is that described by Deobald *et al.* (1962). The enzymes were incorporated as part of the dilution water added to reduce the solids content of the puree to 20%. The reaction temperatures used were those recommended as optimum by the respective suppliers of the commercial enzymes employed. The optimum enzyme concentration and reaction times required to produce an acceptable flake product from uncured sweetpotatoes were determined experimentally in the pilot plant.

Four amylolytic enzyme preparations were evaluated. Mylase P, derived from the fungus *Aspergillus oryzae*, was purchased from Wallerstein Company, Staten Island, New York. It had a diastatic power of 1300° Lintner and optimum activity between 125 and 135°F.

Rhozyme S is derived from the fungus *Aspergillus oryzae*. It has a diastatic power of 340° Lintner and optimum activity between 121 and 132°F. Rhozyme H39 is derived from a bacterial source and is heat-stable. It had a diastatic power of 1940° Lintner and optimum activity at 170°F. Rhozyme 33 had a diastatic power of 600° Lintner and optimum activity between 122 and 131°F. The latter three preparations were purchased from Rohm and Haas Company, Philadelphia, Pennsylvania. The diastatic powers were determined by the procedure described in the AOAC (1960).

The flakes were packaged in 211×300 metal cans in an atmosphere of nitrogen containing less than 2% oxygen. They were evaluated by an experienced panel of 3–5 persons whose ratings on both flavor and consistency were restricted to either "acceptable" or "off."

Starch properties. The Goldrush sweetpotatoes used were grown in the vicinity of St. Francisville, Louisiana. They were harvested and shipped to the laboratory on the same day, arriving at weekly intervals from September 3, 1963, through October 29, 1963. Three of the six 50-lb crates received were immediately stored at 60°F, and three were cured for 14 days at 90°F and 85–90% relative humidity, after which they were also stored at 60°F.

Starch was isolated from each shipment of the uncured sweetpotatoes (stored immediately at 60°F) 1, 3, and 7 days after harvest, and from the

cured sweetpotatoes 90 days after harvest, when the roots had optimum processing characteristics, and again between March 25, 1964, and April 6, 1964, when the sweetpotatoes began to show signs of deterioration. The technique used to isolate the starch is essentially that given by Doremus *et al.* (1951). However, a "tabling" step was introduced between the initial screening out of the pulp and the five washings to remove the residual pigments. The "table" was a cypress trough 10 ft long, 3 inches wide, and 2 inches deep inclined at about 0°8′. A distilled-water slurry of the starch was permitted to flow slowly down the table, allowing the starch granules to settle. This procedure was repeated three times by reslurrying the settled starch. Starch samples prepared in this way were free of color, contained less than 0.01% nitrogen as determined by the Kjeldahl method, and were shown to be 98.78 and 98.94% pure by methods of Balch (1941) and Clendenning (1945), respectively. Approximately 2 kg of peeled sweetpotatoes were utilized for each isolation during and immediately after the harvesting season. As much as 4 kg were required for cured sweetpotatoes which had undergone prolonged storage, since the yield per kg of these sweetpotatoes was significantly lower.

The amylose contents of the starch samples were determined colorimetrically by the method of McCready *et al.* (1950) as modified by Williams *et al.* (1958). However, in order to permit the determination of optical density of the starch-iodine complex on the spectrophotometer, the volume of the iodine reagent was reduced from 2 to 1 ml, and the starch concentration from 5 to 1 mg. The standard curve was prepared by mixing amylose and amylopectin in the following quantities: 0.0, 1.0; 0.2, 0.8; 0.4, 0.6; 0.6, 0.4; 0.8, 0.2; and 1.0, 0.0 mg. The amylose and amylopectin used to prepare the standard curve were obtained from a pooled sample of the isolated starches by the method of McCready and Hassid (1943).

The pasting properties of the starch samples were determined with the Brabender viscograph (C. W. Brabender, South Hackensack, New Jersey). The concentration of starch in the paste was set at 27 g (dry basis) in 450 ml distilled water to accommodate the requirements of the instrument and to facilitate the determination of susceptibility to liquefaction by α-amylase. The viscograph was programmed so that the temperature of the starch slurry was elevated from 25 to 95°C at the rate of 1.5° per minute, held at 95°C for 15 min, and then cooled at the same rate to 35°C and held there for 15 min, after which the gelatinized slurry was used in determination of susceptibility to liquefaction by α-amylase.

The α-amylase used in determination of relative susceptibility to liquefaction was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. Its source was bacterial, and it had an activity of 1800° Lintner and 2500 SKB units per gram. The rate of decrease in viscosity when the gelatinized slurry was treated with 1 ml of a water solution containing 0.135 mg α-amylase (5 ppm with respect to the starch) was followed for 30 min at 35°C. The results are expressed as percent of the original viscosity remaining after the incubation period (Fig. 1).

The susceptibilities to saccharification by β-amylase were also determined. The β-amylase used was purchased from Wallerstein Company,

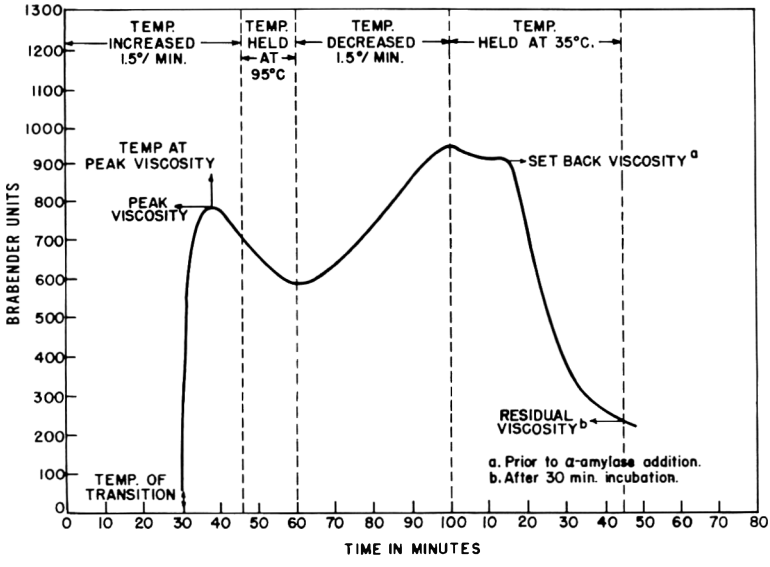

Fig. 1. Typical Brabender curve illustrating origin of the pasting properties and measurement of susceptibility to α-amylase.

Staten Island, New York. It was isolated from barley and had an activity of 2000° Lintner. The starch samples were prepared by the following procedure. Five grams (dry basis) of the starch sample was suspended in approximately 100 ml of a phosphate-citrate buffer pH 6.1, heated with constant stirring to 80°C, cooled, and diluted to 250 ml with the buffer. Five ml of a 1 mg/ml solution of β-amylase in deionized water was added to 50 ml of the 2% starch substrate which had been preheated to 30°C—the temperature at which the incubation was carried out. Hydrolysis was permitted to proceed for 20 min and was stopped by the addition of 10 ml of 0.5*N* NaOH. The reacted sample was diluted to 100 ml with distilled water, and the reducing substances present were determined by the Lane-Eynon general volumetric method (AOAC, 1960). Because of the extremely low concentration of reducing substances in the control samples, where the 10 ml of the 0.5*N* NaOH was added prior to the β-amylase, the following modification was made. A 10-ml portion of the sample was added to 10 ml of the Soxhlet reagent, and the titration was completed with the standard maltose hydrate solution. The difference between the mg maltose hydrate needed to reduce the Soxhlet reagent and that required to complete the titration represents the reducing substances present in the aliquot. In the determination of susceptibility to saccharification, the differences between the mg maltose hydrate in the reacted and control samples, multiplied by the dilution factor 10, represent the total reducing substances formed by the action of β-amylase.

RESULTS AND DISCUSSION

Enzyme curing. The extent to which the starch fraction is hydrolyzed during "enzyme curing" significantly affects both the drying characteristics of the treated puree and the consistency of the reconstituted flake. If the hydrolysis is permitted to proceed until all of the starch has been hydrolyzed to the dextrin stage, the resulting puree is extremely fluid and difficult to dry. It tends to caramelize when subjected to the high temperatures of the drum surface, yielding a wet sticky sheet. The resulting flakes have an unacceptable fluid consistency when reconstituted. If, however, the starch fraction is not sufficiently hydrolyzed, the resulting flakes will be light colored and have a very low bulk density. When reconstituted, they have an unacceptable pasty consistency and a starchy taste. Fortunately, neither the drying characteristics of the puree nor the quality of the flake product are overly sensitive to the extent of starch hydrolysis in the puree, and thus enzyme curing can be readily incorporated into the existing processing technique.

The extent of hydrolysis of the starch fraction of the puree can be controlled by varying the enzyme concentration and/or reaction time when the whole puree is treated. When the amylase concentration was not excessive, however, the length of time the enzyme was permitted to act on the puree was not critical. A series of flake samples with acceptable rehydration properties were produced using reaction times of 5 to 90 minutes where Mylase P was utilized at 50 ppm. These results are included in Table 1. The particular lot of sweetpotatoes used was harvested October 1, 1962, and had been stored 3 days at 60°F. Although all the enzyme-treated flakes in this series were acceptable, those produced from puree which had been incubated with the enzyme less than 15 min had a slightly pasty consistency when reconstituted, whereas those whose incubation period was in excess of 30 min tended to be slightly fluid. The best flakes were obtained when the reaction time was approximately 20 min. Therefore, this holding time was used in evaluation of the other commercial enzyme preparations and in determination of the effect of the environmental variables on the concentration of enzyme required to produce a flake with acceptable rehydration properties.

It was found that the concentration of the amylase with respect to sweetpotato (dry-weight basis) required to produce an acceptable flake from uncured sweetpotatoes depended upon both time of harvest and the length of the storage period at 60°F after harvesting. In general, the range of the concentration had to be decreased steadily as the harvesting season progressed, and, simultaneously, the specific concentration required daily had to be decreased as the length of the storage period increased. The concentrations of Mylase P required throughout the harvesting season are presented in Table 2. The same trends held for all of the enzyme preparations evaluated. The diastatic powers (°Lintner) of the individual preparations

Table 1. The effect of the specific enzyme employed, reaction time, and enzyme concentration on the taste and on the sugar content of precooked dehydrated sweetpotato flakes.

Puree treatment			% sugar [a]		
Enzyme	Enzyme concentration (ppm)[a]	Reaction time (min)	Nonreducing	Reducing	Taste
Control	0	0	14.6	17.3	off
Mylase P	50	5	15.3	17.4	acceptable
Mylase P	50	20	15.5	17.3	acceptable
Mylase P	50	30	15.9	16.8	acceptable
Mylase P	50	50	14.9	17.7	acceptable
Mylase P	50	70	15.8	17.4	acceptable
Mylase P	50	90	15.6	18.0	acceptable
Control	0	0	18.2	14.7	off
Mylase P	50	20	18.0	14.5	acceptable
Mylase P	75	20	18.1	14.5	acceptable
Mylase P	100	20	17.2	14.6	acceptable
Control	0	0	20.4	14.4	off
Mylase P	25	20	20.0	14.9	acceptable
Rhozyme S	100	20	20.5	14.2	acceptable
Rhozyme H39	7	20	20.8	14.7	acceptable
Rhozyme 33	80	20	20.5	14.6	acceptable

[a] Dry-solids basis.

were helpful in determining the equivalent concentrations required only when the enzymes had been obtained from the same organism.

Of the four enzymes evaluated, Mylase P or the equivalent concentration of Rhozyme S, both of which are obtained from the fungus *Aspergillus oryzae*, were the most efficient with respect to ease of handling of the puree during processing. They effect liquefaction of the puree at a relatively rapid but controllable rate. The only apparent disadvantage is that the puree temperature must be lowered to 120–130°F since the enzymes are rapidly inactivated at higher temperatures.

Table 2. Relative concentrations of Mylase P required to produce an acceptable precooked dehydrated sweetpotato flake from uncured sweetpotatoes.

Date harvested (1962)	Days stored at 60°F			
	1	2	3	7
Aug. 27	150[a]	150	125	75
Sept. 4	150	150	125	75
Sept. 10	125	125	100	60
Sept. 17	125	125	100	60
Sept. 24	100	100	75	40
Oct. 1	100	100	75	40
Oct. 8	75	75	50	25
Oct. 15	75	75	50	25

[a] ppm based on the dry-solids content of the puree.

Rhozyme H39 has the advantage of eliminating the necessity for cooling the puree to 130°F. However, liquefaction is so rapid and extensive that the puree is difficult to control during the drying step. This amylase must be used at a much lower concentration than would be indicated by its diastatic power relative to the amylases from *Aspergillus oryzae*.

Although an acceptable flake was produced from puree which had been treated with Rhozyme 33, some difficulty was encountered in the drying step during the early part of the harvest season. It had to be used at a higher concentration than was indicated by its diastatic power relative to the enzymes from *Aspergillus oryzae*.

Organoleptic evaluation was the only reliable criterion for flake quality. Although an acceptable flake was produced using the enzyme curing technique, these flakes did not possess the same degree of sweetness as those made from naturally cured sweetpotatoes and lacked the typical sweetpotato flavor.

Table 1 shows the effects of the specific enzyme used, the relative concentrations of Mylase P, and the length of the reaction time on the reducing and nonreducing sugar content of sweetpotato flakes. Although both

the reducing and nonreducing sugar content of the flakes varied with the particular lot of fresh sweetpotatoes from which they were produced, the reducing sugar content was not increased by "enzyme curing." The purees, however, were rapidly liquefied by these treatments.

In order to attempt to account for both the observed decrease in enzyme requirements as the harvest season and storage period progressed and to explain the contrast between the susceptibility of the sweetpotato puree to saccharification and liquefaction by the commercial enzyme preparations, the starch fraction of the sweetpotato was selected for the initial investigation into the factors influencing the processing of sweetpotatoes into flakes.

Starch properties. *Amylose content.* The quantities of amylose in each of the starch samples are compiled in Table 3. Since amylopectin is determined by difference, the amylose-amylopectin ratio was thus unaltered. No trends were detected which could be related to the environmental variables investigated.

Pasting properties. The following four measurements, taken from the Brabender viscograms as illustrated in Fig. 1, are presented in Table 4 : 1) the temperature at which the first perceptible increase in viscosity occurs [temperature of transition (Cook and Axtmayer, 1937)] ; 2) the peak (maximum) viscosity ; 3) the temperature at which peak viscosity occurred ; and 4) the extent of increase in viscosity on cooling to and holding at 35°C (setback viscosity).

Curing with optimum storage lowered the temperature of transition of the starches. However, neither time of harvest nor storage of the uncured sweetpotatoes for 1–7 days at 60°F altered this property, and prolonged storage of the cured roots did not result in a further decrease in the temperature. Barham *et al.* (1944) demonstrated that curing lowered the transition temperature of starch samples isolated from sweetpotatoes of the Nancy Hall, Improved Big Stem Jersey, Little Stem Jersey, Big Stem Jersey, and Red Bermuda varieties grown in Kansas. Evidently the starch from cured sweetpotatoes swells at a faster rate, and thus the initial increase in viscosity is registered at a lower temperature.

Curing with optimum storage resulted in an elevation of the peak viscosity of the starches, indicating an increase in the extent of granule swelling and solubilization (Kite *et al.*, 1957). Prolonged storage of the cured roots caused an additional increase, but neither time of harvest nor short-term storage of the uncured roots had any noticeable effect. Barham *et al.* (1944), however, reported that curing lowered the peak viscosity of the starches of the five varieties of sweetpotatoes grown in Kansas.

Peak viscosity occurred at a higher temperature in starch samples isolated from cured sweetpotatoes. Prolonged storage of the cured roots did not result in a further increase, and neither time of harvest nor short-term storage of the uncured sweetpotatoes had any effect. Thus, curing with optimum storage can be simultaneously correlated with a lowering of the temperature at which the initial rapid and unrestricted rise in viscosity occurred and an elevation of the

Table 3. Effect of harvesting, curing, and storage variables on the amylose content of the isolated starches.

	Succession of sweetpotato shipments								
	Amylose content (%) at:								
Days after harvest	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk	9th wk
Uncured									
1	17.4	18.6	19.5	17.1	19.8	21.3	17.8	17.4	17.4
3	17.2	18.2	17.6	18.4	19.6	18.6	18.6	17.9	16.1
7	19.7	20.5	18.8	19.6	18.9	19.6	18.5	18.2	18.5
Cured 14 days									
90	17.1	17.0	17.9	17.4	16.7	18.0	17.4	17.0	19.6
150–200	17.4	17.2	18.2	18.1	17.6	18.3	18.1	17.9	18.2

Table 4. Effect of harvesting, curing, and storage variables on the pasting properties of the isolated starches.

Days after harvest	Succession of sweetpotato shipments								
	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk	9th wk
Transition temperatures (°C)									
Uncured									
1	73.8	73.0	73.8	73.0	73.0	72.6	73.0	71.5	72.3
3	73.8	73.8	73.4	73.0	72.3	72.3	73.3	72.3	73.0
7	73.8	73.8	73.0	73.4	71.7	73.0	72.3	72.5	72.3
Cured 14 days									
90	72.3	71.9	71.5	71.5	70.8	72.3	71.5	70.8	71.5
150–200	71.5	71.5	71.5	70.8	70.8	71.5	70.0	70.8	71.5
Peak viscosity temperatures (°C)									
Uncured									
1	79.0	79.0	80.5	79.0	77.5	77.5	79.0	79.0	79.0
3	79.0	79.0	79.0	79.0	77.5	77.5	77.5	80.5	79.0
7	79.0	79.0	79.0	80.5	77.5	77.5	77.5	80.5	80.5
Cured 14 days									
90	82.0	82.0	82.0	80.5	79.0	83.5	80.5	82.0	82.0
150–200	82.0	82.0	82.0	83.5	83.5	85.0	82.0	82.0	82.0
Peak viscosity (Brabender units)									
Uncured									
1	690	660	670	660	675	665	695	730	680
3	690	660	660	675	675	665	630	740	685
7	680	660	610	695	650	640	665	725	695
Cured 14 days									
90	775	688	715	730	675	735	820	795	745
150–200	820	720	775	795	795	780	880	735	695
Setback viscosity (Brabender units)									
Uncured									
1	750	685	740	730	785	735	785	800	800
3	740	740	710	745	710	760	695	780	820
7	785	690	700	775	715	695	755	820	835
Cured 14 days									
90	815	820	800	820	770	880	890	915	920
150–200	880	930	900	960	940	1000	990	910	950

temperature at which maximum swelling takes place.

Setback viscosity is essentially a measure of gel rigidity. Kite *et al.* (1957) and Majurs *et al.* (1957) related the formation of a gel to the association of the ends of the linear molecules to give an interlacing network through the paste. Setback viscosity was increased by curing with optimum storage, and further increased by prolonged storage of the cured roots.

Susceptibility to amylolysis. The relative susceptibilities of the starches to liquefaction by α-amylase and saccharification by β-amylase are presented in Table 5. The viscosity curves were also studied and compared with respect to the average lowering of viscosity per minute, the rate of dropoff during the first 10 min of the incubation, when the major portion of the viscosity is lost, and the time required to reduce the original viscosity by 50%. These criteria gave results similar to those presented in Table 5. It can be concluded that environmental conditions do not alter the susceptibility of sweetpotato starch to amylolytic attack, since neither time of harvest, short-term storage of the uncured sweetpotatoes, curing with optimum storage, nor prolonged storage of the cured roots altered the relative susceptibilities of the starches isolated from them to either liquefaction by α-amylase or saccharification by β-amylase.

Table 5. Effect of harvesting, curing, and storage variables on the susceptibility of the isolated starches to amylolysis.

Days after harvest	Succession of sweetpotato shipments								
	1st wk	2nd wk	3rd wk	4th wk	5th wk	6th wk	7th wk	8th wk	9th wk
Residual viscosity[a] (% of original)									
Uncured									
1	34.3	31.0	35.4	33.9	31.7	34.5	37.7	42.9	64.5
3	33.3	40.3	47.0	52.9	40.0	35.5	35.3	62.5	40.6
7	31.9	34.1	46.9	42.2	40.1	46.0	37.8	41.0	35.2
Cured 14 days									
90	29.1	38.5	33.0	48.9	49.0	35.7	34.5	39.9	32.1
150–200	32.6	36.5	50.3	42.3	56.2	36.5	40.3	41.8	38.7
Maltose[b] (mg)									
Uncured									
1	344	342	343	342	347	350	341	319	332
3	340	322	342	343	326	315	340	339	334
7	338	348	342	337	353	322	318	337	330
Cured 14 days									
90	326	332	329	319	315	332	325	337	312
150–200	328	324	318	314	323	318	332	327	332

[a] After 30 min of incubation with α-amylase.
[b] Formed by β-amylase.

General discussion. The changes effected by curing on the pasting properties of the isolated starches could not be related directly to the observed differences in processing characteristics. Although the relative quantities of amylase required to produce an acceptable flake from uncured sweetpotatoes had to be decreased steadily as the harvest season progressed and as the length of the storage period at 60°F increased, and can be eliminated after natural curing, the isolated starches did not differ in their respective susceptibilities to amylolytic attack. The starch content of the sweetpotato has been shown to decline continually during curing and storage (Sistrunk *et al.*, 1954; Scott and Matthews, 1957; Hammett and Barrentine, 1961). It would therefore be expected that the enzyme concentration would have to be decreased steadily in order to maintain a constant concentration with respect to the quantity of starch present. The extent of this decrease, however, was significantly in excess of what would be anticipated based on a normal pattern of starch decline during storage and curing. Although neither curing nor storage altered the susceptibility of the isolated starch to saccharification by β-amylase, it was demonstrated that these isolated starches could be saccharified, whereas treatment of the puree with commercial enzyme preparation which contained both α- and β-amylase did not increase the reducing sugar content of the resulting flakes. Both the puree and the isolated starches were, however, rapidly liquefied by α-amylase. These observed differences between isolated and sweetpotato starch in a puree with respect to susceptibility to amylolysis cannot, therefore, be accounted for by changes in the properties of the starch itself, and the explanation for the observed difference in processing characteristics must be sought in changes in other constituents of the root.

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Anthocyanins in the Lowbush Blueberry, Vaccinium angustifolium

SUMMARY

Fifteen anthocyanin pigments were isolated from the lowbush blueberry, *Vaccinium angustifolium* Ait., and identified by chromatographic, spectral, and chemical properties. The 3-monoglucosides and 3-monogalactosides of delphinidin, malvidin, petunidin, peonidin, and cyanidin were isolated; also present in small quantities were the 3-monoarabinosides of the same five anthocyanidins. The glucosides were present in much greater quantity than the galactosides or the arabinosides. Several diglycosides were also present, but in quantities too low for identification.

INTRODUCTION

The lowbush blueberry of northeastern North America, *Vaccinium angustifolium* Ait., grows in acid soils on barren lands over extensive areas. It is currently receiving considerable research attention because it represents a cash income in depressed areas. It is sold in the canned, frozen (Hard and Ross, 1959), and fresh fruit markets (Hruschka and Kushman, 1963), and is also suitable for freeze-drying and puff-drying (Eisenhardt *et al.*, 1964). It has been suggested as a source for a sweet wine (Fuleki, 1963, 1965), blended juices (Abdalla *et al.*, 1963; Chandler and Highlands, 1950), and fruit pie fillings (Strachan *et al.*, 1960).

Suomalainen and Keranen (1961) reported the presence of arabinose and glucose derivatives of cyanidin, petunidin, malvidin, and delphinidin in *V. myrtillus* L., a close relative of the lowbush blueberry, but the pigments were not fully characterized. Wood and Barker (1963 a, b) found that the pigments present in one-year-old shoots of *V. angustifolium* showed clonal variation. This paper is concerned with the pigment content of the ripe fruits of *V. angustifolium*.

MATERIALS AND METHODS

The berries for this work were grown in Fredericton, N. B. Five hundred g were extracted with 1% HCl in methanol and concentrated, and the extract was shipped to the John Innes Laboratory.

Chromatographic methods. Whatman No. 3 paper was used for all separation and purification steps, and Whatman No. 1 for r_f data in the descending manner in both cases. The following solvents were used for chromatography:

1) BAW — *n*-butanol, glacial acetic acid, water (4:1:5). Upper phase. Made up fresh for r_f data.

2) BEW — *n*-butanol, 95% ethanol, water (4:2:2.2).

3) BuHCl — *n*-butanol, 2*N* hydrochloric acid (1:1). Upper phase. Paper equilibrated 24 hr after spotting and before running in tank containing aqueous phase of BuHCl mixture.

4) BBPW — *n*-butanol, benzene, pyridine, water (5:1:3:3).

5) Forestal — glacial acetic acid, conc. hydrochloric acid, water (30:3:10).

6) Formic — formic acid, conc. hydrochloric acid, water (5:2:3).

7) HAc-HCl — water, glacial acetic acid, 12*N* hydrochloric acid (82:15:3).

8) 1% HCl — conc. hydrochloric acid, water (3:97).

9) Phenol — phenol, water (4:1).

Aglycones. The aglycones were obtained by heating approx. 1 mg of purified pigment with 2*N* HCl in a water bath for 30 min. The aglycones were extracted with amyl alcohol, evaporated to dryness, and spotted on No. 1 paper together with authentic markers. The papers were run in Forestal, formic, and BAW solutions.

Sugars. The solutions remaining after extraction of the aglycones were treated with di-n-octylmethylamine (Harborne, 1958) to remove the mineral acid, evaporated to dryness at R.T., and spotted on No. 1 paper together with authentic markers. The papers were run in BAW, BEW, BBPW, and phenol. After development the papers were dried, dipped in aniline hydrogen phthalate (Partridge, 1949), and heated in an oven at 105°C for 2 min. The spots were clearly visible when viewed under ultraviolet light.

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Authentic pigments. The 3-glucosides of delphinidin, petunidin, malvidin, peonidin, and cyanidin were available from previous work (Harborne, 1957). The 3-galactosides of cyanidin and peonidin were extracted from cranberries (Zapsalis and Francis, 1965).

Spectral data. All spectral data were determined with an Unicam SP500 spectrophotometer. The $AlCl_3$ shifts were determined by dissolving the purified pigment in 0.01% HCl in methanol and adding 1 drop of 5% $AlCl_3$ in ethanol to 3 ml.

RESULTS AND DISCUSSION

Pigment separation. In preliminary work (Barker, Wood and Harborne, unpublished), a crude acid methanol pigment extract was streaked on No. 3 paper and run overnight in BAW. The bands were eluted with MAW (methanol, glacial acetic acid, water 90:5:5), combined, and rerun in 1% HCl. The bands were eluted with MAW, concentrated, hydrolyzed in 2*N* HCl, and analyzed for aglycones and sugars. The data indicated that five aglycones (delphinidin, petunidin, malvidin, peonidin, cyanidin) and three sugars (glucose, galactose, and arabinose) were present. The E_{440}/E_{max} ratios indicated that the pigments were probably all monoglycosides.

The preliminary observations were confirmed in this work, and it was evident that, with such a complex pigment mixture, extensive paper chromatography was necessary. For adequate isolation and purification, the scheme listed in Fig. 1 was developed.

Thirty-two sheets of No. 3 paper were streaked with the crude pigment extract and developed in BAW overnight. The five bands were eluted with MAW and rerun in BAW, producing 19 bands. The first 16 bands were run in 1% HCl and then in Forestal. Each of the 20 bands from the first Forestal purification were analyzed for aglycones, using the four routine solvents (Forestal, formic, BAW, BuHCl) and for sugars with the four routine solvents (BBPW, phenol, BAW, BEW). It was

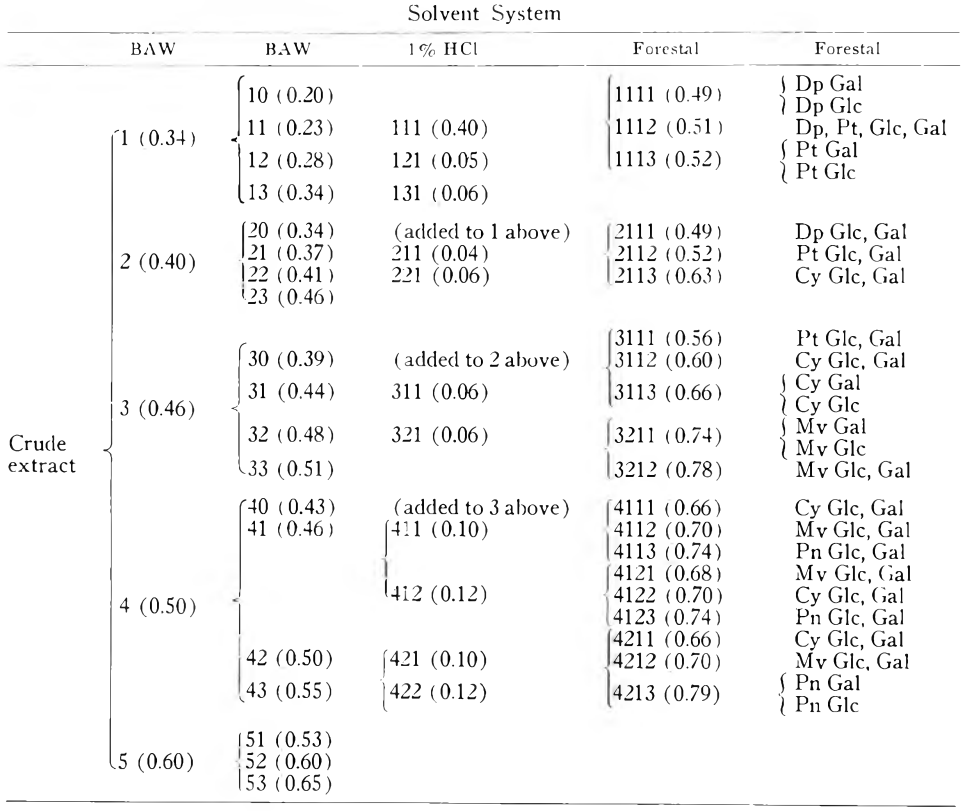

Fig. 1. Separation scheme for blueberry anthocyanins. The figure in parentheses after the band number indicates the r_f value.

apparent at this stage that all ten possible combinations of delphinidin, petunidin, malvidin, peonidin, and cyanidin derivatives of glucose and galactose were present. The band which provided each pigment in the largest quantity was isolated and rerun in Forestal. The products of acid hydrolysis for these bands are presented in Table 1, and r_f data for the pigments in Table 2. Spectral data (Table 3) for the same pigments were obtained on the bands eluted from the first Forestal purification. These solutions were

Table 1. Hydrolysis products of blueberry pigments.

			Sugars			
	Anthocyanidin		Solvent			
Band	Forestal	Formic	BBPW	Phenol	Aglycone	Sugar
	Distance travelled (cm)					
	16 hr	16 hr	16 hr	40 hr		
11111	14.6	11.7	10.0	29.0	Dp	Glc
11112	14.7	11.6	12.5	26.2	Dp	Gal
11131	22.9	19.0	9.9	28.9	Pt	Glc
11132	23.0	19.1	12.4	26.2	Pt	Gal
31131	30.0	27.2	10.0	29.0	Mv	Glc
31132	29.8	27.0	12.5	26.0	Mv	Gal
32111	24.0	20.6	10.1	26.2	Cy	Glc
32112	23.8	20.4	12.5	29.1	Cy	Gal
42131	30.6	26.6	10.0	26.0	Pn	Glc
42132	30.5	26.6	12.4	28.9	Pn	Gal
Markers						
Dp	14.8	11.8				
Pt	22.9	19.0				
Mv	20.0	27.2				
Cy	23.8	20.4				
Pn	30.5	26.6				
Glc			12.4	26.2		
Gal			10.0	29.0		

Table 2. Chromatographic data for blueberry anthocyanins.

	Pigment identified as	r_f in solvent:						
Band		BAW	BuHCl	1% HCl	HAc–HCl	Formic	Forestal	BEW
11112	Dp3Gal	0.20	0.12	0.03	0.15	0.42	0.49	0.16
11111	Dp3Glc	0.23	0.12	0.03	0.15	0.43	0.51	0.18
11131	Pt Gal	0.28	0.13	0.04	0.18	0.56	0.61	0.20
11132	Pt Glc	0.31	0.15	0.04	0.20	0.58	0.65	0.22
31131	Mv Gal	0.34	0.20	0.06	0.28	0.62	0.79	0.39
31132	Mv Glc	0.36	0.20	0.06	0.29	0.64	0.80	0.41
32111	Cy Gal	0.32	0.24	0.07	0.26	0.66	0.64	0.36
32112	Cy Glc	0.34	0.25	0.07	0.26	0.68	0.66	0.38
42131	Pn Gal	0.34	0.29	0.10	0.31	0.73	0.77	0.42
42132	Pn Glc	0.36	0.31	0.10	0.32	0.75	0.79	0.46
	Markers							
	Dp3Glc	0.21	0.11	0.03	0.15	0.44	0.50	0.18
	Pt3Glc	0.30	0.20	0.04	0.21	0.59	0.65	0.23
	Mv Glc	0.35	0.20	0.04	0.29	0.62	0.79	0.38
	Cy Glc	0.33	0.24	0.07	0.27	0.68	0.66	0.38
	Cy Gal	0.31	0.24	0.08	0.29	0.66	0.64	0.36
	Pn Glc	0.37	0.30	0.10	0.33	0.75	0.80	0.47
	Pn Gal	0.34	0.27	0.09	0.31	0.73	0.77	0.43

Table 3. Spectral data for blueberry anthocyanins in 0.01% HCl in methanol.

Band	max (nm)	E_{440}/E_{max} (%)	AlCl$_3$ shift
1111	532	20	+
1113	532	20	+
2111	535	22	+
2113	528	25	+
3111	531	20	+
3113	528	24	+
3211	533	20	−
4111	532	19	+
4123	520	27	−
4211	532	19	+
4213	520	29	−

a mixture of the glucoside and galactoside of each aglycone, but the E_{440}/E_{max} ratios indicate that they were all monoglycosides.

Comparison of the r_f data with authentic pigments suggested that all pigments were monosubstituted in the 3 position.

The glucosides and galactosides of each aglycone were separated with difficulty in Forestal solvent, but sufficient pigment was obtained to get r_f data. In each case the glucoside was present in much larger amounts than the galactoside.

Band 5 in the first BAW separation was very broad and had an r_f value suggestive of a pentoside. This band was rerun in BAW, and three bands developed. Band 51 was obviously similar to the pigments in band 4, 53 appeared to contain degradation products, and 52 was the purified pentoside material. This band was present in only trace amounts. In order to obtain a larger quantity, 50 ml of the original crude extract were neutralized with conc. NaOH solution, buffered to pH 4.0 with sodium acetate buffer, and mixed with 50 mg of anthocyanase (Rohm & Haas, Phil., Pa.) (Harborne, 1965). The mixture was incubated at 30°C for 1 hr, concentrated under vacuum, streaked on No. 3 paper, and run in BAW. The enzyme treatment destroyed most of the glycosides, and the pentosides were easier to recover. However, it was still not possible to recover a sufficient quantity of the pentosides to isolate and purify each band; therefore the pentoside fraction was treated as one pigment. Acid hydrolysis indicated the presence of five aglycones, delphinidin, petunidin, malvidin, peonidin, and cyanidin. Sugar analyses yielded only arabinose. The r_f data indicated that all were monosubstituted; therefore, this fraction was composed of the 3-monoarabinosides of the five aglycones.

Band 10 contained pigments which had r_f values indicative of diglycosides, but the band was grossly contaminated. Attempts to purify these pigments in quantities sufficient for identification were unsuccessful, because of the extremely low concentration. None of the bands showed an absorption maximum at 308-12 nm, indicating that acylated pigments were absent. The r_f data also supported this conclusion.

The occurrence of over fifteen anthocyanins in the berries of *V. angustifolium* is noteworthy, since such complex mixtures of pigments are rare in nature; in fact, no other plant has been recorded as having so many pigments in a single tissue. Berries of other *Vaccinium* species examined apparently have fewer pigments. Hayashi (1949) found only malvidin 3-galactoside in *V. uliginosum*, and Francis and co-workers (Sakamura and Francis, 1961; Zapsalis and Francis, 1965) found four pigments, the 3-galactosides and 3-arabinosides of cyanidin and peonidin, in the American cranberry, *V. macrocarpon*; *V. myrtillus* probably has more than these two species, but cyanidin 3-galactoside is the only pigment completely identified in its berries (Suomalainen and Keranen, 1961). *V. angustifolium* shows many similarities in its anthocyanin pattern with the other three species studied, and it seems that the genus is characterized by the berries containing galactosides and arabinosides of cyanidin, delphinidin, and/or their methyl ethers.

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Lemon Oil Analysis. I. Two-Dimensional Thin-Layer Chromatography

SUMMARY

Recent refinements in thin-layer chromatography (TLC) provide the analytical chemist with an inexpensive, convenient, and rapid method for obtaining basic information on the condition of cold-pressed citrus oil samples. Simple TLC distinguished lemon and lime oils from all other citrus oils. Two-dimensional TLC was used to distinguish between lemon and lime oils. Distilled lemon oils were characterized by their deficiency of UV fluorescence.

INTRODUCTION

Since its earliest development, thin-layer chromatography (TLC) has been employed to analyze citrus oils. The chromatostrips developed by Kirchner *et al.* (1951) and Miller and Kirchner (1953) and later used by Stanley (1959), Stanley *et al.* (1957), Stanley and Vannier (1957a,b), and Vannier and Stanley (1957) have evolved into present day chromatoplates such as those used by Attaway *et al.* (1965a,b) and Fisher and Nordby (1965) to separate typical citrus constituents.

Insofar as lemon oil is concerned, perhaps the most useful TLC application is that reported by Stanley and Vannier (1957b). Among the many separated components in a thin-layer chromatogram of cold-pressed lemon oil, certain compounds were found to fluoresce when viewed under ultraviolet (UV) light. These fluorescent spots, caused by variously substituted natural coumarins (Stanley and Vannier, 1957a) formed a definite pattern on the developed chromatostrip. Stanley (1961) also noticed that certain adulterants that are sometimes added to simulate the UV spectrum of authentic lemon oil give brightly colored TLC spots when exposed briefly to HCl vapor. Advantageous features of the above studies as well as subsequent improvements in TLC technology have been incorporated into the qualitative and semiquantitative procedure described herein to provide analysts with a convenient and informative way of evaluating the condition of cold-pressed lemon oil.

EXPERIMENTAL

Equipment and reagents. Two types of TLC plates were successfully employed: a) home-made 8 × 8-in. chromatoplates coated with a 0.25-mm layer of Silica Gel G prepared in the usual manner on Brinkmann-Desaga apparatus; and b) Eastman Chromagram K301R2 8 × 8-in. precoated sheets cut to 4 × 4 in. All TLC plates were stored in a dry box or desiccator as a precaution against deactivation by moisture. Long-wavelength UV light was provided by a 15-watt, self-filtered, 3660-AU lamp. All chemicals and solvents used were reagent-grade. Other essential TLC equipment included standard development tanks, sample syringes, and a drying oven.

Chromatogram preparation and development. Standard TLC procedures were followed throughout (Bobbitt, 1963). Citrus oil samples to be analyzed were applied in submicroliter increments to the sample origin located in the lower right-hand corner of the plate at least ½ in. from either edge. Plate type *a* received a total sample of 5 μl of citrus oil, and type *b* received 2 μl, except in the case of lime oil, where the sample size was halved. The oils were applied in increments sufficiently small (*ca.* 0.1–0.2 μl) to keep the origin spot diameter at 5 mm. Sample application usually required about 3 min.

The plates were developed by ascending elution with 30% v/v $CHCl_3$ in CCl_4 containing 1% v/v glacial acetic acid, dried briefly in air, then rotated 90° clockwise, and developed in the second dimension with 20% v/v ethyl acetate in cyclohexane containing 1% v/v glacial acetic acid. Plate type *b* required 20 min for completion of solvent ascension in each direction. Plate type *a* took about twice as long. Prior to use, the development tanks were carefully equilibrated with the development solvent vapor. Lining of the tanks with blotter paper helped ensure chamber saturation. The plates were introduced with tongs into the tanks through slots in aluminum foil fitted tightly across the mouth of the tank between it and the lid. In this manner, disequilibration of the solvent vapor during plate introduction was minimized, particularly if the lid was removed by sliding rather than by lifting.

Chromatogram evaluation. A developed plate was first scanned under long-wavelength UV light to record the position and color of the fluorescent spots. To detect certain types of adulteration (Stanley, 1961), the plate was then placed for 1 min in a tank saturated with wet HCl vapor, originating from a container of conc. HCl previously placed inside the tank. Only one faint pink spot should result from such treatment. Changes in the UV fluorescence pattern were then recorded in a second UV scan. Following brief exposure to ammonia vapor, further changes in the visible and UV fluorescent spots were recorded. After the ammonia evaporated, the plate was sprayed with a solution of 5% w/v vanillin and 30% v/v of 85% w/w phosphoric acid in methanol until the plate surface just appeared shiny. Then the plate was heated in an oven for 1 min at 100°, saturated with wet HCl vapor for 15 min and reheated at 100° for 1 min. The colored spots resulting from this treatment were also recorded.

If the plate was immersed for 2 min in a tank saturated with iodine vapor rather than sprayed, brown absorption spots appeared in the same positions as the varicolored spots resulting from spray treatment.

RESULTS AND DISCUSSION

The mixture of non-volatile substituted coumarins naturally present in cold-pressed citrus oils (Stanley and Vannier, 1957b), but either absent or sharply diminished in distilled oils, separated on both types of TLC plates into characteristic patterns of variously colored UV fluorescent spots. Combinations of 15–30% v/v ethanol, dioxane, acetone, ethyl acetate, tetrahydrofuran, or $CHCl_3$ in cyclohexane or CCl_4, with or without 1% v/v acetic acid, served as developing solvent pairs, with success ranging from adequate to good. Every combination separated the fluorescent compounds of bergamot, grapefruit, orange, and tangerine cold-pressed oils into TLC spot patterns distinctly different from that of cold-pressed lemon oils. Only cold-pressed lime oil resembled lemon oil so closely as to require two-dimensional development to differentiate between them. Best suited for this purpose were the solvent pairs of 30% v/v $CHCl_3$ in CCl_4 containing 1% v/v acetic acid, and 20% v/v ethyl acetate in cyclohexane containing 1% v/v acetic acid.

Both the home-made and commercial TLC plates provided essentially the same analytical results when used in the prescribed manner. Developed two-dimensional chromatograms of cold-pressed citrus oils were analyzed successively by UV light, exposure to HCl vapor, exposure to ammonia vapor, and vanillin-phosphoric acid spray. Figs. 1 and 2 indicate the results obtained with lemon and lime oils. The purpose of HCl exposure was to detect the presence of certain compounds added to lemon oil (Stanley, 1961) to simulate the UV spectrum characteristic of natural cold-pressed lemon oil (Sale *et al.*, 1953). No bright colors such as red or yellow should result from the HCl treatment, only a faint pink spot located just above the upper blue-white fluorescent spot, 5-geranoxy-7-methoxycoumarin (GMC) (Fig. 1). After HCl exposure only the two large blue-white fluorescent spots GMC and L, were readily discerned under UV light. When the plate was subsequently exposed to wet ammonia vapor, the pink spot resulting from the HCl treatment disappeared, whereas the lavender fluorescent spot to the right of it and above GMC reappeared under UV light. In lime, the spot due to HCl exposure was blue instead of pink.

Differences between the UV fluorescence patterns of cold-pressed lemon and lime oils were revealed in the first UV scan. For instance, the relative positions of the lavender and purple spots just below and to the left of the lower large blue-white fluorescent

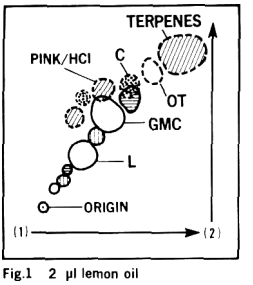


Fig.1 2 µl lemon oil

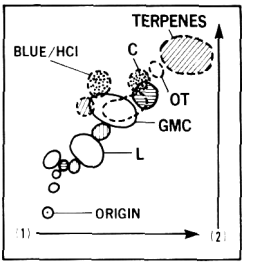
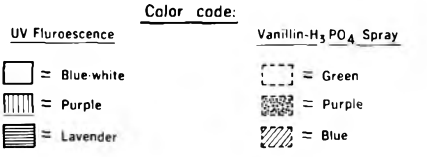


Fig.2 1 µl lime oil

Thin-layer chromatograms of cold-pressed oils, plate type (b), two dimensional development with (1) $CHCl_3$, acetic acid, CCl_4 (30:1:69) and (2) ethyl acetate, acetic acid, cyclohexane (20:1:79).



spot, limettin (L), were reversed (see figures). Between these spots and the sample origin, other differences were evident in the pattern of the small blue-white fluorescent spots. Distilled lemon and lime oils showed only faint fluorescence spots in the positions of the two large blue-white spots, GMC and L.

In the final diagnostic test, the vanillin-phosphoric acid spray reagent (Bobbitt, 1963, p. 102) afforded several colored spots which could be used to distinguish semi-quantitative differences in the terpenoid composition of cold-pressed lemon oils versus other oils. For example, the green oxyterpene (OT) spot (Fig. 1) was slightly more intense than the purple citral (C) spot just to the left and below it, whereas with lime oil (Fig. 2) OT was much less intense than C. Conversely, the lime chromatogram also had a large light-green spot overlapping GMC, which was small and scarcely visible in cold-pressed lemon oil chromatograms.

These TLC procedures provide analysts of lemon oils with a convenient and fairly rigorous series of characteristic diagnostic tests. Within the limits of experimental reproducibility, known authentic samples of natural cold-pressed lemon oil from Argentina, Arizona, Australia, coastal and inland California, Florida, Israel, and Italy exhibited essentially the same patterns when treated as specified. Any unknown sample of lemon oil deviating from the general TLC pattern would probably warrant further examination before it could be accepted as an authentic cold-pressed lemon oil. When analyzing lemon oils with this method, occasional control comparison chromatograms on separate TLC plates should be performed simultaneously with authentic samples to check the operating conditions. The spray reagent should be refreshed daily.

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Lemon Oil Analysis. II. Gas-Liquid Chromatography on a Temperature-Programmed, Long, Open Tubular Column

SUMMARY

Lemon oil was analyzed on a highly efficient modified Apiezon L open tubular column with a single injection. Almost all of the more than forty individual peaks were identified by comparison of mass spectra and retention times with those of known compounds. Lemon oils from throughout the world were found very similar in their gas chromatograms; differences were primarily quantitative rather than qualitative.

INTRODUCTION

With the rapid development of gas-liquid chromatography (GLC) in the past decade, numerous gas chromatograms of lemon oil volatiles have appeared among the publications of Bernhard (1958, 1960), Clark and Bernhard (1960a,b), Stanley *et al.*, (1961a,b), Slater (1961, 1963), Ikeda *et al.* (1962a,b), Ikeda and Spitler (1964), and Hunter and Brogden (1965), as well as in the advertising literature of gas-chromatograph manufacturers. The analytical techniques described therein represented a definite advance over the more cumbersome and less accurate distillation methods formerly used to analyze lemon oil in detail. Nevertheless, the complexity of lemon peel oil presented such a problem in effectively resolving the mixture into its component parts that the GLC columns employed were unequal to the task without some sort of preliminary fractionation of the oil. Mostly, fractionation was accomplished by adsorption chromatography; however, a modification of Girard's procedure (Stanley *et al.*, 1961a; Ikeda and Spitler, 1964) was used twice to separate the aldehydes as a class. Since all these auxiliary steps required extensive precautions to protect the various sensitive lemon oil constituents from acidic, basic, hydrolytic, oxidative, and thermal decomposition, it was obvious that a more direct and efficient method of analysis which minimized such complications would be desirable.

In an intensive examination of delicate volatiles from various fruit sources, Teranishi *et al.* (1960, 1963a,b) found that temperature-programmed capillary GLC efficiently analyzed complex natural volatiles. Their success made it conceivable that the volatiles of whole lemon oil could be largely resolved into individual components in a single sampling, provided a suitable column coating and temperature program could be found. In addition, by initiating the chromatogram at moderate temperatures, the sample could be vaporized under mild conditions and swept into the capillary with minimal sample spreading. After some experimentation, Averill (1965) developed a system which largely fulfilled these needs and provided the basis for our further investigation.

EXPERIMENTAL

The GLC system described by Averill (1965) was used with minor modification. GLC operations were performed on a Perkin-Elmer hydrogen flame ionization gas chromatograph, model 226, equipped with a 300-ft-long 0.010-in.-ID stainless-steel open tubular column coated with a mixture of 5% w/w IGEPAL CO-880 (nonyl phenoxypolyoxyethylene ethanol) and 1% w/w Eastman DOPC (diisooctadecylparacresol) in decolorized Apiezon L (purified by elution from a column of chromatographic alumina in cyclohexane). A special carrier gas mixture, 8.5% v/v hydrogen in helium, obtained from Matheson Scientific, was employed at an inlet pressure of 40 psig. In addition to the normal gas filters, the carrier gas line contained an Engelhard Deoxo catalytic hydrogen purifier cartridge. The injector block containing the annular stream splitter was maintained at 120°, and the initial column temperature was controlled at 60°. A 0.5-μl sample of whole cold-pressed lemon oil was injected into the chromatograph and split 100:1. The smaller portion was swept onto the column which was held at 60° for 1 min, then temperature-programmed at the rate of 1°/min up to 150° and

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maintained there until completion of the chromatogram. A total of 2 hr was required.

GLC operations in conjunction with the Bendix time-of-flight mass spectrometer were carried out essentially in the manner described by McFadden and Teranishi (1963) and McFadden *et al.* (1963). However, in this instance the inlet carrier gas pressure was reduced to 20 psig, the sample size raised to 30 μl (split 100:1), the initial column temperature raised to 80°, and the initial isothermal period extended to 10 min. The entire effluent of the GLC column was introduced directly into the ionization chamber of the mass spectrometer.

RESULTS AND DISCUSSION

Apiezon L, decolorized by liquid-solid chromatography on alumina, has been found by Averill (1965) to be a highly suitable partitioning stationary liquid phase for open tubular (capillary) GLC analysis of cold-pressed lemon oil volatiles when modified with a peak tail-reducer such as IGEPAL CO-880 (nonyl phenoxypolyoxyethylene ethanol) and an antioxidant such as DOPC (diisooctadecylparacresol). The carrier gas mixture used with this column contained 8.5% v/v hydrogen, which could react in the catalytic cartridge with possible traces of oxygen to form harmless traces of water. The remaining hydrogen was available for regeneration of the hindered phenolic antioxidant, DOPC. Although the effectiveness of hydrogen for these purposes has not been conclusively demonstrated, it is neither inconvenient nor dangerous. The efficiency of the column ranked around 80,000 theoretical plates when measured with p-cymene by the formula 5.545 $(V_{R'}/d)^2$, according to Purnell (1962).

Known authentic samples of cold-pressed lemon oils from Argentina, Arizona, Australia, coastal and inland California, Florida, Israel, and Italy were analyzed by the method described and found to have very similar GLC patterns. Differences between the chromatograms were primarily quantitative rather than qualitative. When appropriately programmed, the column was able to resolve the important lemon oil volatiles into individual peaks (Fig. 1) comprising three main groupings: terpene hydrocarbons, oxygenated terpenes, and sesquiterpene hydrocarbons. The straight-chain aldehydes, peaks 3, 14, 19, and 24 (Table 1), were interspersed among the first two groups at regular intervals of increasing molecular weight. The majority of the peaks listed in Fig. 1 and Table 1 were identified by comparison of their GLC retention times with those of known pure standards, and confirmed by comparison of their mass spectra with known spectra. Peaks 8 and 9 were confirmed only as terpenes by the mass spectrometer. The GLC retention time of peak 9 agreed with that of Δ^3-carene. Peaks 29, 30, 33, 34, 35, 36, 37, 38, and 40 were identified by the mass spectrometer only as sesquiterpenes. The retention time of peak 36 suggested that it was ζ-cadinene. No mass spectral data were obtained for peaks 24 and 25; however, their respective retention times correspond to undecanal and nonyl acetate, previously identified in lemon oil (Ikeda *et al.*, 1962; Ikeda and Spitler, 1964). The only known instance of simultaneous elution was peak 17, which, according to both GLC retention times and mass spectra, was a 4:1 mixture of terpinolene and citronellal.

Despite this one drawback, which should be overcome by making the stationary liquid phase either more or less polar, this method

Table 1. Identities of peaks in Fig. 1, as determined by mass spectral and retention time comparisons. Asterisks (*) indicate incomplete mass spectral confirmation.

1. α-thujene	21. neral
2. α-pinene	22. α-terpineol
3. octanal	23. geranial
4. camphene	24. undecanal*
5. sabinene	25. nonyl acetate*
6. myrcene	26. citronellyl acetate
7. β-pinene	27. neryl acetate
8. $C_{10}H_{16}$ a terpene	28. geranyl acetate
9. Δ^3-carene*	29. $C_{15}H_{24}$ a sesquiterpene
10. α-phellandrene	30. $C_{15}H_{24}$
11. α-terpinene	31. bergamotene
12. p-cymene	32. caryophyllene
13. limonene	33. $C_{15}H_{24}$
14. nonanal	34. $C_{15}H_{24}$
15. linalool	35. $C_{15}H_{24}$
16. γ-terpinene	36. ζ-cadinene*
17. terpinolene:citronellal ca. 4:1	37. $C_{15}H_{24}$
18. p,α-dimethylstyrene	38. $C_{15}H_{24}$
19. decanal	39. β-bisabolene
20. terpineol-4	40. $C_{15}H_{24}$

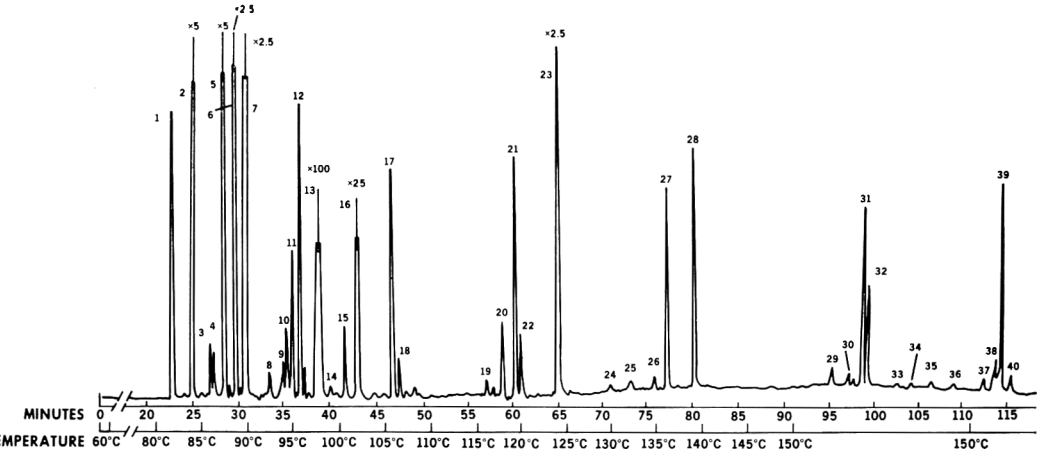

Fig. 1. Temperature-programmed gas chromatogram of inland California cold-pressed lemon oil on an open tubular column. Modified Apiezon L stationary liquid phase, 300 ft long, 0.010 in. ID.

reveals information about the composition of whole cold-pressed lemon oil not previously available from a single injection. Furthermore, it also shows promise of being shortened for rapid routine survey and quality control. It is possible that a routine 30-min GLC run displaying 25–30 principal lemon oil volatiles could be adapted from this method by shortening the column and using a combination of temperature and flow programming (Zlatkis *et al.*, 1965). Efforts are being directed toward this objective.

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Removal of Bacteria from Food Raw Materials by Electrophoresis

I. Factors Affecting the Electrophoretic Mobility of Certain Species of Bacteria

SUMMARY

To determine proper conditions for removal of bacteria from food by electrophoresis, a microscopic study was made of the influences of electrical double layer, intensity of electrical field, electric density gradient, pH of the buffer solution, ionic strength of buffer solution, stage of growth of bacteria, and anion surface-active agent upon the electrophoretic mobility of some species of bacteria. The effect of the electrical double layer between the electrophoretic cell wall and bacterial solution was determined. The observed mobility of the bacteria was smaller at the upper and bottom sides of the cell than the true mobility, and was larger at the center of the cell. The migration velocity (μ/sec) of the bacterial cell is proportional to the intensity of the electric field. The mobility of the bacteria increased at random with increased voltage. The mobility of the bacteria varied directly with the pH value of the buffer solution in which they were suspended. With increase of ionic strength of buffer solution, the mobility of bacterial cells decreased for each species of bacteria used. The mobilities of some sporeforming bacteria varied at various stages of the growth period: mobility increased rapidly after germination and during the logarithmic phase, decreased for vegetative cells in the stationary phase, and decreased much more as the cells entered the sporeforming stage. Bacterial mobility was increased substantially by sodium tetradecyl sulfate.

INTRODUCTION

The region of a biological particle which is accessible to electrophoretic study is its surface. Particles in solution acquire a charge through ionization of their surface and adsorption of ions. Because most of the ionizing groups are weak electrolytes, and because binding energies for adsorption are of the order of magnitude of thermal energies, the surface-charge situation must be described as an equilibrium between ions bound to the surface and ions in solution. Ionizable groups on the particle surface become charged by the gain or loss of hydrogen ions and other ions. Hydrogen ions receive special attention because their concentration is easily controlled and measured, and they readily combine with biological substrates, e.g. the amino, carboxyl, and other groups of proteins (Brinton and Lauffer, 1959).

The surfaces of bacterial cells also have a negative charge. If they are suspended in a solution with a pH higher than their isoelectric point (about pH 3.0), and an electric current is allowed to pass through the solution, they are attracted to the anode. Electrophoresis is used most advantageously in analyzing heterogeneous populations of bacteria, measuring adsorption of charged substances onto the cell surface, and following changes in the surface as a function of time. Other than a way of identification of heterogeneous populations of bacteria, electrophoresis can be applied in the field of the food industry. If the surface of food material or the intestines of shellfish are contaminated with bacteria, electrophoresis would be one good method for removing the bacteria. In removing bacteria by electrophoresis, neither food raw materials nor shellfish are damaged by the operation. However, the electric current to be applied and the design of the instrument of electrophoresis should be determined according to the properties of the food. We have studied it in the removal of bacteria from foods or food raw materials.

This paper reports the influences of some factors upon the mobility of bacteria in the electrophoretic operation.

EXPERIMENTAL

The rate of motion of living bacterial cells in an electric field in water can be designated as mobility (μ). Mobility can be defined as the distance moved by particles (cells) in a unit electric potential in a unit time. Even for a single bacterial strain, the mobility differs according to physiological conditions

of handling before the estimation, i.e. incubation period, pH, ionic strength, etc. When those conditions were held constant, the mobilities of various species of bacteria were found to be specific.

Two methods are available for estimating the mobility of particles by electrophoresis. In the moving-boundary method of Tiselius (1930), the migration of phase boundaries between the aqueous buffered solution of proteins and the dispersion medium in a U-shaped channel caused by the passage of the electric current is observed optically. In another method the migration of charged particles in solution or dispersion is observed directly in a microscope field (Mattson, 1922; Abramson, 1929). The latter was convenient for the present study because the movement of the bacterial cells could be observed directly.

Cell used for electrophoresis. The cell used for electrophoresis by the microscope method was one described by Abramson (1929), available from the Klett Mfg. Co. The authors experimented with three types of cells (Fig. 1a-c).

The simplest cell was a glass slide (25×75 mm) as shown in Fig. 1a. This was easily handled. For

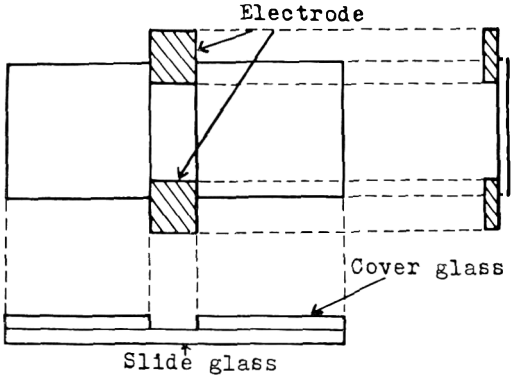

Fig. 1a. A simple electrophoresis cell.

example, the distance between the two electrodes could be easily changed. If buffer solution having a high ionic strength was used for electrophoresis, however, polarization of the electrodes occurred during the passage of electric current, and air bubbles formed near the anode. Hence, mobility estimates with this cell were unreliable. The authors used this cell for experiments with buffer solutions having low ionic strength.

Fig. 1b shows a cell and agar bridge placed between the electrodes and the buffer solution to prevent polarization. The upper part of the cell could not be covered, however, and the buffer solution flowed out, so mobilities could not be determined accurately.

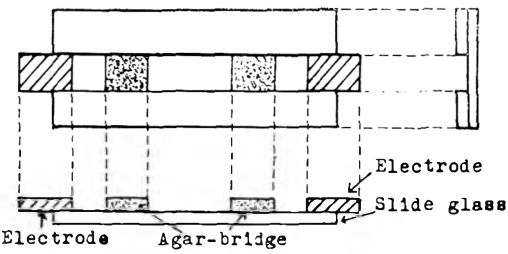

Fig. 1b. An electrophoresis cell having agar bridge.

Fig. 1c shows the cell made according to Abramson (1929). It is essentially a glass tube in which the observation position under the microscope is flat. The cell has the disadvantage that it requires too much buffer solution to fill the cell.

The electrodes of these cells were made of thin carbon plate. As stated above, each cell has some merits and disadvantages. In each case we used the cell most suitable for the particular experiment.

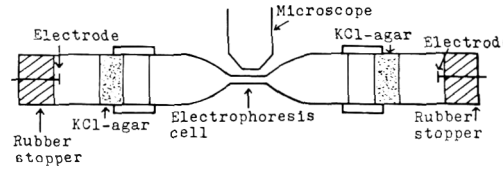

Fig. 1c. An electrophoresis cell according to Abramson (1929).

Calculation of the mobility. If a bacterial cell moves h (μ) in t (second) in the electric field to which E (volt) is given per one cm along the cell of electrophoresis, the mobility, μ, is shown by the following equation:

$$\mu = h/t \cdot E \quad (\mu/\text{sec/volt/cm}) \qquad [1]$$

Unstained bacterial cells were observed under a phase-contrast microscope (×400). Stained cells differed significantly from unstained cells in their properties. The direct-current source (6–24 volts) was a selenium rectifier.

Manipulation of the cell. The electrophoretic cell is filled with a suspension of the bacterium and covered with a cover glass. Excess solution is removed with filter paper to prevent the cover glass from floating. The electrodes are connected with the electric source, and direct current is applied at various voltages. The distance traveled by the bacterial cells in the suspension is accurately measured with the ocular micrometer in a definite time (sec). One graduation of an ocular micrometer is 2.4 μ under ×400 of a microscope. A single bacterial cell is fixed at the zero point of the ocular micrometer, then the electric current is passed through the solution, and the time (sec) required to pass 100 graduations is measured with a stop watch.

The migration of a bacterial cell from anode to cathode is indicated as positive (+), and the opposite as negative (−). The mobilities are obtained by inserting the values (sec) into Eq. 1. For example, with an electrophoretic cell having a 2-cm distance between the electrodes, and with 12 volts, if one bacterial cell requires 12.1 sec to pass 240 μ (100 graduations of ocular micrometer), the mobility is calculated as follows:

$$\mu = 240/12.1/12/2 = 3.31 \ (\mu/\text{sec/volt/cm})$$

Species of bacteria. The bacteria used for the experiments were *Sarcina lutea* ACTT 9341, *Bacillus subtilis* NRRL 558, *Pseudomonas fluorescens* 33F, *Aerobacter aerogenes, Escherichia coli* (O-26), *Bacillus circulans,* and *Bacillus coagulans.* The strains of *B. subtilis* NRRL 558, *Ps. fluorescens* 33F, and *E. coli* (O-26) were received from the Laboratory of Microbiology of our Faculty. The strain of *S. lutea* ATCC 9341 was received from Eli Lilly and Co., Indianapolis, Indiana, U.S.A. The strains of *B. circulans* and *B. coagulans* were originally isolated from spoiled fish sausage and identified by us. The strain of SG-2 (*Bacillus* sp.) was isolated from blackened and swollen canned baby clam meat in our laboratory.

The particular species were selected because these species are usually isolated from various kinds of foods, and are concerned with spoilage; the differences of the shapes and biological characteristics of these bacteria would cause the difference of behavior in electrophoresis; the spores from some of these bacteria are important in their electrophoretical behavior.

Estimation of influence of electrical double layer. The test strains of the bacteria were *A. aerogenes, B subtilis* NRRL 558, *Ps. fluorescens* 33F, *S. lutea* ATCC 9341, and *E. coli* (O-26). Except for *Ps. fluorescens* 33F, the strains were newly cultivated on an agar slant (1% beef extract, 1% peptone, 0.5% NaCl, 2% agar, pH 7.2) at 37°C. After incubation for 24 hr, the bacterial colonies grown on the agar slant were collected and suspended in phosphate–citric acid buffer (*M*/5 Na_2HPO_4 0.17 ml + *M*/10 citric acid 0.02 ml + H_2O 19.8 ml, pH 7.2, ionic strength 0.01). *Ps. fluorescens* 33F was cultivated on an agar slant at 30°C, and a suspension of the bacteria was prepared as described above. The electrophoresis cell (type in Fig. 1a) was filled with the suspension. The distance between the two electrodes of the cell was 1 cm. A direct current of 6 volts and 4.0 ma was applied to the field. First, the cell (depth 0.6 mm) was placed on the stage of the phase-contrast microscope. A focus was adjusted to the under side of the cover glass, and the scale of the fine adjustment was read. Next, a focus was adjusted to the bottom of the cell, and the scale of the fine adjustment was read again. The difference in the reading of the scales is the cell depth ($d = 280$). From the value of d, the stationary level (x) was calculated to be 59 or 221. The migration velocity (μ/sec) of the bacterial cells was estimated under these conditions.

Estimation of influence of the electric field intensity. Bacterial suspensions of *S. lutea* ATCC 9341, *E. coli* (O-26), *B. subtilis* NRRL 558, and *Ps. fluorescens* 33F were prepared from an agar slant in phosphate-citric acid buffer (pH 7.2, ionic strength 0.01) by the method described above. The electrophoresis cell (type in Fig. 1a) was filled with the suspension. The distance between the two electrodes of the cell was varied from 1 cm to 5 cm, and the voltage was varied from 6 to 24 volts, accompanied by variation in amperage from 4.0 to 16.0 ma.

Estimation of influence of the electric density gradient. The newly cultivated strains of *S. lutea* ATCC 9341, *E. coli* (O-26), and *B. subtilis* NRRL 558 were used in the experiment. The bacterial suspensions from these bacteria were prepared in buffer solution of phosphate–citric acid buffer (pH 7.2, ionic strength 0.01). The sectional areas of two carbon electrodes of the electrophoresis cell (type in Fig. 1b) were varied from 0.013 cm^2 to 0.05 cm^2. The direct current of 6 volts was passed through the bacterial suspension in the cell. Thus, the amperages of the electric current were varied from 1.5 ma to 4.0 ma. The mobility of the bacteria was estimated by the same method as described above.

Estimation of influence of pH of the buffer solution. Bacterial cells were *A. aerogenes, S. lutea* ATCC 9341, and *E. coli* (O-26) grown on agar slants to the end of the logarithmic period and washed 5 times in sterile fresh water. Washing was done by centrifugation using 10 cc of sterile fresh water (3000 rpm, 15 min). At last the water was centrifuged off and the cells were resuspended in phosphate–citric acid buffers of differing pH values. The proportions of the composition of the phosphate-citric acid buffers are shown in Table 1. The ionic strength of the buffer solutions was 0.10. The buffer solutions suspending the bacterial cells

Table 1. Composition of buffer solutions for various pH values.

M/5 Na_2HPO_4 (ml)	*M*/10 citric acid (ml)	pH
0.40	19.60	2.2
4.49	15.51	3.2
8.28	11.72	4.2
10.72	9.28	5.2
13.22	6.78	6.2
17.39	2.61	7.2

were transferred into an electrophoresis cell (type in Fig. 1c). The electric current passed through the field was 12 volts and 8 ma. After estimation of the velocity of the bacterial cell in the buffer solution, the mobility of the bacterial cell was calculated.

Estimation of influence of ionic strength of buffer solution. The bacteria employed for this experiment were *S. lutea* ATCC 9341, *E. coli* (O-26), and *B. subtilis* NRRL 558. The strains of these bacteria grown on an agar slant after cultivation for 48 hr were suspended in sterile fresh water. The sterile fresh water suspending the bacterial cells was centrifuged (3000 rpm, 15 min). The centrifugation was repeated 3 times, each time adding a new sterile fresh water. The bacterial cells deposited on the bottom of the tube of centrifuge were then suspended in phosphate–citric acid buffers of various ionic strengths. The constituents of the phosphate-citric acid buffers are shown in Table 2. The buffer solutions suspending the bacterial cells were poured into an electrophoresis cell (type in Fig. 1b). The electric current passed through the field was 12 volts and 8 ma. After estimation of the velocity of the bacterial cell in the buffer solution, the mobility of the bacterial cell was calculated.

Table 2. Composition of buffer solutions with different ionic strengths.

$M/5$ Na_2HPO_4 (ml)	$M/10$ citric acid (ml)	H_2O (ml)	pH	Ionic strength
8.69	1.31	10.00	7.2	0.60
4.35	0.65	15.00	7.2	0.30
2.88	0.44	16.68	7.2	0.50
1.44	0.22	18.36	7.2	0.10
1.20	0.16	18.64	7.2	0.08
0.72	0.11	19.17	7.2	0.05
0.45	0.03	19.52	7.2	0.03
0.17	0.02	19.80	7.2	0.01

Estimation of influence of stage of growth of bacteria. *B. subtilis* NRRL 558 was inoculated on agar slants and incubated at 37°C. Cells at the stages of logarithmic, stationary, decreasing, and bacterial spores were respectively obtained at about 48, 96, 144, and 240 hr. The bacteria at each stage were suspended in separate test tubes containing phosphate–citric acid buffer (1.74 ml $M/5$ Na_2HPO_4 + 0.26 ml $M/10$ citric acid + 18.00 ml H_2O, pH 7.2, ionic strength 0.10) and each bacterial suspension was introduced into the electrophoretic cell for microscopic observation. The electric current was 12 volts and 8 ma. The electrophoresis cell used was the type showing in Fig. 1b.

The strains of *B. circulans*, *B. coagulans*, *A. aerogenes*, and *S. lutea* ATCC 9341 were also incubated on separate agar slants in order to obtain bacterial cells at the stages of logarithmic growth and decreasing growth. The mobilities of these bacteria at the stages of logarithmic and decreasing growth were determined by microscope as described above.

Estimation of effect of anion surface-active agent. The species were *S. lutea* ATCC 9341, *A. aerogenes*, *B. subtilis* NRRL 558, *B. circulans*, *B. coagulans*, *E. coli* (O-26), and SG-2 (*Bacillus* sp). They had been incubated at 37°C for 24–36 hr on agar slant. The colonies grown on the agar slant were washed with sterile fresh water into separate test tubes. Centrifugation (3000 rpm, 15 min) was done to obtain the bacterial cells. The bacterial cells obtained were resuspended in phosphate–citric acid buffers (1.74 ml $M/5$ Na_2HPO_4 + 0.26 ml $M/10$ citric acid + 18.00 ml H_2O, pH 7.2, ionic strength 0.10) containing the anionic surface-active agent (sodium tetradecyl sulfate) (STS). The STS contents of the buffer solution were 5 or 10 ppm. The biological effects of the STS level on the bacterial cells in the buffer solution were none or few. The bacterial suspensions containing STS were poured into the electrophoresis cell (type in Fig. 1c). The electric current passed through the field was 12 volts and 8 ma.

RESULTS

Design of the instrument. In the electrophoresis of biological particles, the effect of the electrical double layer between the electrophoretic cell wall and solution must be considered. The solution near the cell wall flows from anode to cathode, and is counterbalanced by a flow in the opposite direction in the center of the cell, as shown in Fig. 2a.

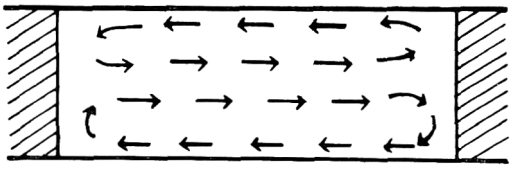

Fig. 2a. Electro-osmosis streaming of the liquid along the glass wall.

The migration velocity of bacterial cells near the cell wall is then obtained as the difference between the electrophoretic velocity and the velocity of streaming of the solution. In contrast, that at the center is the sum of the velocities of electrophoresis of bacterial cells and streaming of the solution. Fig. 2b shows a cross section of a flat electrophoresis cell showing the magnitude and direction of liquid flow as a function of cell depth. *A* and *B* are respectively the top and the bottom of the cell.

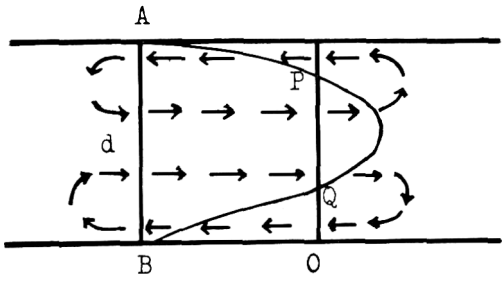

Fig. 2b. Cross section of a flat electrophoretic cell showing magnitude and direction of liquid flow as a function of cell depth. A) Top of the cell; B) Bottom of the cell; O) Point of observation; P,Q) Points of liquid flow; d) Depth of the cell.

The abscissa shows the velocity of bacterial cells. Right of the O, the velocity observed is larger than the true velocity, and left of O, vice versa. At points P and Q, however, there is no liquid flow. Hence, the velocity of bacterial cells observed at P and Q should be the true velocity. The levels of P and Q in an electrophoresis cell can be calculated as follows (Komagata, 1932).

$$x = d\ (1/2 \pm 1/\sqrt{12}) \qquad [2]$$

x = distance from top of the cell (P and Q)
d = depth of the cell

The stationary level x is 0.211 d from the top or the bottom of the cell (Komagata, 1932).

The observed mobility was smaller at the upper and bottom sides of the cell than the true mobility, and was larger at the center of the cell (Table 3, Fig. 3).

Table 3. Mobility at the levels of P and Q in the electrophoresis cell.

Test organisms	Levels	Mobility (μ/sec/v/cm)
Aerobacter aerogenes	P	−1.65
	Q	−1.65
Bacillus subtilis NRRL 558	P	−4.4
	Q	−4.4
Pseudomonas fluorescens 33F	P	−4.0
	Q	−4.7
Sarcina lutea ATCC 9341	P	−4.1
	Q	−3.7
Escherichia coli (O-26)	P	−3.1
	Q	−3.0

This result suggests that they should be controlled by putting the electrodes and sample rack at a definite place in an instrument. There is a streaming of the medium near the cell wall that is without effect on electrophoresis. The streaming of medium is caused by an electrical double layer between medium and cell wall. Thus, if the electrodes and sample rack are placed near the cell wall, the

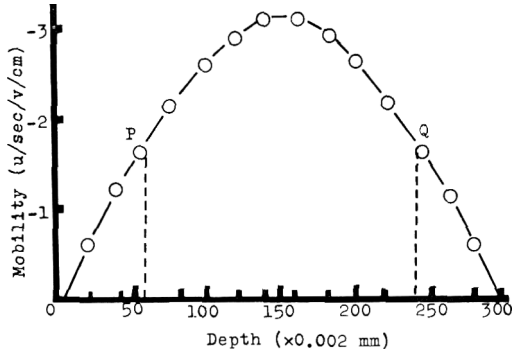

Fig. 3. Changes in the mobility according to change in the depth of electrophoretic cell (*Aerobacter aerogenes*).

streaming of the medium would be concerned with the velocity of bacteria. The size of electrodes and sample rack follow the size of cell of electrophoresis, and can be calculated from Eq. 2.

The migration velocity of bacterial cells could be changed by changing the electric pressure and the distance between the two electrodes. The electric pressure per cm distance along the electrophoretic cell is defined as electric-field intensity, which corresponds to the value obtained when the voltage is divided by the distance between two electrodes. Table 4 indicates the influence of intensity of electric-field upon the velocity of bacteria.

Table 4. Relation between the migration velocity and distance of two electrodes and voltage.

Test organisms	Voltage	Distance between anode and cathode (cm)	Migration velocity (μ/sec)
Sarcina lutea	6	1	21.1
ATCC 9341		2	10.5
		3	6.9
		4	5.5
		5	4.6
	12	1	42.1
		2	20.3
		3	14.2
		4	10.0
		5	10.0
	24	1	—
		2	38.7
		3	29.6
		4	20.0
		5	18.9
Escherichia coli	6	1	14.8
(O-26)		2	8.3
		3	4.1
		4	5.9
		5	2.8

(continued)

Table 4 (continued)

	12	1	36.4
		2	24.2
		3	15.6
		4	14.5
		5	4.7
	24	1	—
		2	43.4
		3	30.0
		4	27.3
		5	12.0
Bacillus subtilis	6	1	19.9
NRRL 558		2	12.6
		3	7.8
		4	7.3
		5	4.9
	12	1	47.6
		2	30.4
		3	18.2
		4	17.4
		5	12.8
	24	1	—
		2	57.1
		3	33.3
		4	29.6
		5	21.6
Pseudomonas			
fluorescens 33F	6	1	16.6
		2	10.2
		3	6.4
		4	6.1
		5	3.9
	12	1	34.3
		2	25.3
		3	14.3
		4	12.8
		5	6.5
	24	1	—
		2	45.3
		3	29.3
		4	25.0
		5	13.2

From Table 4, the following equation can be obtained:

$$H \cdot D = E \cdot b \qquad [3]$$

Here, D is the migration velocity of the bacterial cells (μ/sec), H (cm) is the distance between two electrodes, E (volts) is the electric pressure supplied, and b is a coefficient which depends on the species of bacteria.

As seen in Table 4, the relation between the distance, H, and migration velocity, D, differed with the voltage applied. These results show the migration velocity (μ/sec) of the bacterial cells to be proportional to the electric-field intensity, hence the mobility of the bacteria could be expressed as μ/sec/volt/cm. Further, the results suggest that shortening the distance of two electrodes or increasing the voltages applied is effective in increasing the velocity of bacteria.

In moving-boundary electrophoresis, the mobility of the bacterial cells can be estimated as follows: if q is a cross-section area of the electrophoresis cell, i is the strength of electric current through the electrophoresis cell, K is specific electric conductivity of the particles (bacterial cells), and the particle moved h (μ) for t sec, the mobility of the bacterial cells would be obtained by the following equation (Isemura and Nakauma, 1957):

$$\mu = hqK/i \cdot t \qquad [4]$$

From Eq. 4 the velocity of bacterial cells should depend on the electric density in moving-boundary electrophoresis.

The relation between the mobility of the bacterial cells and electric density by microscopic electrophoretic method is shown in Fig. 4.

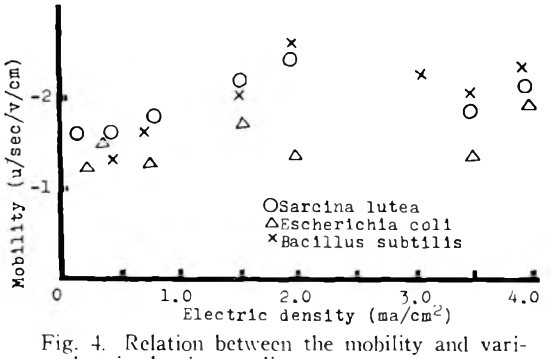

Fig. 4. Relation between the mobility and various electric density gradient.

In general, mobility increased with voltage increased at random. Thus the relationship expressed in Eq. 4 by the moving-boundary method was not the same as that obtained by the microscopic method. In the microscopic method, the mobility could be calculated from the electric-field intensity because the specific electric conductivity of the buffer solution in the electrophoretic cell is uniform.

Larger electric density gradient depends on higher amperage and smaller cross-section area of electrode. Thus, in order to obtain as large a velocity of bacteria as possible a small cross-section area of electrode and high amperage seems to be effective.

Electrophoresis medium. The net number of charges on the surface of a bacterial cell or other biological particle depends upon the number of

ionized acidic and basic groups. The number of charged ions on the surface would vary with the pH of the solution in which such a particle is in contact, and the mobility would vary. We have determined the influence of pH of the buffer solutions upon the mobility of bacterial cells. As seen in Fig. 5, the mobilities of *S. lutea* and *E. coli* increased with increases in pH value. Above pH 2.8, the mobility of *S. lutea* was greater than that of *E. coli*. The mobilities of *A. aerogenes* were different from those of the other two species. Mobility decreased gradually with a decrease in pH, and increased gradually with an increase in pH. The isoelectric points were pH 2.2 for *S. lutea* ATCC 9341 and *E. coli* (O-26), and 1.8 for *A. aerogenes* (Table 5).

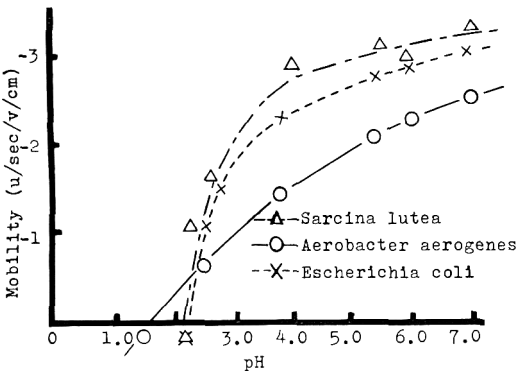

Fig. 5. Variation of the mobilities of some bacteria in buffer solutions at different pH levels.

Table 5. Isoelectric points of various species of bacteria.

Name of species	Isoelectric point (pH)
Aerobacter aerogenes	1.8
Escherichia coli (O-26)	2.2
Sarcina lutea ATCC 9341	2.2

The isoelectric point of *E. coli* (O-26) was close to that found by Moyer (1936) (pH 2.5). Winslow and Upton (1926) reported the isoelectric point of *A. aerogenes* to be pH 3.5, differing from our result. The differences may be due to methods of washing of bacterial cells or to the growth stage of the bacteria used, as pointed out by Moyer (1936).

Thus, a higher pH range above the isoelectric point of bacteria in the electrophoresis medium is effective for obtaining a higher velocity of bacteria.

The mobility of bacterial cells may vary with the ionic strength of the buffer solution as well as the pH value. We have studied the influence of ionic strength of the buffer solution upon the migration velocity of some species of bacteria. The results

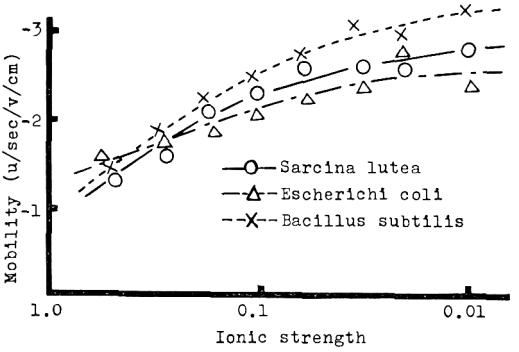

Fig. 6. Relation between the mobility of bacteria and ionic strength of the buffer solution.

are shown in Fig. 6. With increase of ionic strength, the mobility of bacterial cells decreased for each species of bacteria used. This may be due to the following: the pH of the buffer solution (pH 7.2) was higher than the isoelectric point of the bacteria, hence the surface of the cells carried a negative charge. In buffer solutions having a high ionic strength, there are many H^+ ions, so the negatively charged surface of bacteria tends to be decreased electrically. When the effective volume of the charged ion is decreased, mobility will also decrease. In contrast, when ionic strength is low, there is no decrease in effective volume, and mobility increases. By considering practical electrophoresis, the results indicate that higher concentrations of salt solutions as the electrophoresis medium would not be effective in increasing the velocity of bacteria, nor the fitness limit of the instrument.

Conditions of bacteria influencing velocity. Mobility is known to vary with the various stages of growth of bacteria (Moyer, 1936; Watrous, 1937; Stearns and Roepke, 1941; Barry and James, 1952, 1953). However, there are few investigations with sporeforming bacteria through the various growth stages. We studied the influence of the stage of growth of bacteria employing *B. subtilis*. Results are shown in Table 6. The electrophoretic mobility of old spores of *B. subtilis* was —0.08. After germination and during the logarithmic phase the mobility increased rapidly. It decreased for vegetative cells in the stationary phase, and de-

Table 6. The electrophoretic mobilities of *Bacillus subtilis* at various growth stages.

Growing period	Growing stage	Mobility (μ/sec/v/cm)
Decreasing phase	Old spore	—0.80
Logarithmic phase	Germinated bacterial cells	—3.20
Stationary phase	Vegetative cells	—2.78
Decreasing phase	Newly-formed spores	—1.73

creased much more as the cells entered the spore-forming stage. Douglas and Parker (1958) made similar observations on *B. megatherium*. The mobility of the bacteria may be changed because of the thickening of cell wall of spores. The authors have also estimated the electrophoretic mobility of *B. circulans*, *B. coagulans*, *A. aerogenes*, and *S. lutea* in the logarithmic phase and the decreasing phase. Results are summarized in Table 7. Except

Table 7. Electrophoretic mobility of some species of bacteria of two stages of growth.

Strain	Mobility in logarithmic stage (μ/sec/v/cm)	Mobility in decreasing stage (μ/sec/v/cm)
Bacillus circulans	−3.25	−2.42
Bacillus coagulans	−2.18	−1.34
Aerobacter aerogenes	−2.30	−2.52
Sarcina lutea ATCC 9341	−3.47	−2.25

for *A. aerogenes*, electrophoretic mobility was higher in the logarithmic phase. This is similiar to the result with *B. subtilis*.

With *A. aerogenes*, mobility was somewhat larger in the decreasing phase than in the logarithmic phase. The reason is not clear. Barry and James (1952) reported that the electrophoretic mobility of *A. aerogenes* was almost the same at all stages of the growth cycle.

From the result, general determination of the operation time in practical electrophoresis should be based on the velocity of spores or old vegetative cells.

Finally, the electrophoretic mobility of some species of bacteria isolated from foods was estimated. Table 8 indicates that the electrophoretic mobility of *S. lutea*, *A. aerogenes*, *B. subtilis*, and *E. coli* has been measured by many investigators, and their results were almost the same as reported in this paper.

There are few reports on *B. circulans* and *B. coagulans*. The mobility of *B. coagulans* was somewhat lower than the mobilities of the other bacteria. This value, however, is close to that of *B. megatherium* as obtained by Buggs and Green (1935).

The net charges of bacterial cells are affected in the presence of anionic and cationic detergents (Dyar, 1948). An experiment was carried out with 5 or 10 ppm of the anionic surface-active agent (sodium tetradecyl sulfate) (STS) to the electrophoretic medium to determine the changes in the mobilities of several species of bacteria. From the results (Table 9), STS caused a substantial increase in the mobilities of the bacteria. The larger amount of the agent caused the greater increase.

Table 9. Electrophoretic mobility (μ/sec/v/cm) of various species of bacteria with anionic surface active agent (STS).

Strain	Conc. of STS	
	5 ppm	10 ppm
Sarcina lutea	−4.78	−5.32
Aerobacter aerogenes	−4.81	−5.77
Bacillus subtilis	−6.60	−6.75
Bacillus circulans	−4.94	−5.60
Bacillus coagulans	−2.74	−3.12
Escherichia coli	−6.33	−6.44
SG-2 (*Bacillus* sp.)	−5.72	−6.15

DISCUSSION

In order to determine electric current supplied and the design of the instrument, the factors affecting the mobility of the bacteria by electrophoresis were determined by microscopic method. The influence of electrical double layer is important to design of a practical instrument of electrophoresis. The size and area of electrodes and sample rack are calculated from a given size of electrophoresis

Table 8. Electrophoretic mobility of various species of bacteria (present and other findings).

Strain	Mobility (present investigation)	Mobility (other authors)
Sarcina lutea	−2.25	Buggs & Green (1935)
		$\mu = -2.0$ (pH 7.3, I = 0.016)
Aerobacter aerogenes	−2.31	Lowick *et al.* (1956)
	~ −2.51	$\mu = -2.36$ (pH 7.3, I = 0.016)
Bacillus subtilis	−3.60	Buggs & Green (1935)
		$\mu = -3.5$ (pH 7.3, I = 0.016)
Bacillus circulans	−2.42	
Bacillus coagulans	−1.34	
Escherichia coli	−3.38	
		Brinton *et al.* (1954)
		B. coli non-filamented
		$\mu = -3.6$ (pH 7.0, I = 0.018)

cell to increase the effectiveness of electrophoresis.

Shortening the distance between two electrodes or increasing the voltages applied increases the mobility of bacteria. However, the distance between two electrodes cannot be shortened extremely, because the sample rack on which food raw material should be held, is placed between them. The voltages applied also cannot be greatly increased over 30 volts, because the increase may increase the resistance of the electric current through the electrophoresis medium, which would damage the selenium rectifier.

A greater electric density gradient is obtained by larger amperage and smaller cross-section area of electrode. The result in Fig. 4 shows that increased velocity of bacteria depends on a greater electric density gradient. From these, in designing instrument of electrophoresis, as small a cross-section area of electrode as possible is effective. However, when removing bacteria from a large quantity of food raw material in one operation of electrophoresis is considered, an extremely small cross-section area of electrode seems impractical. On the other hand, strength of electric current depends on voltage in a definite electrophoresis medium, and is controlled by an effective voltage applied to electrophoresis. The size of electrode of which cross-section area is small, should be designed by considering the effect of electrical double layer.

The pH value of the electrophoresis medium should be decided by considering the velocity of bacteria and the influence on the quality of food raw material. In general, the velocity of bacteria increases with an increase of pH value in higher pH range from isoelectric point of bacteria (Fig. 5). Leaving food raw material in alkali solution, however, would cause deterioration, and in acid solution would decrease the effect of removing bacteria. Thus, the pH value of the electrophoresis medium is properly neutral or, rather, slightly acid. If living shellfish are held in such a medium for electrophoresis, little effect of pumping activity is found rather than the effect of medium temperature.

A lower amount of salts dissolved in the electrophoresis medium increases the effectiveness of bacteria removal, because the velocity of bacteria decreases with an increase in ionic strength of the medium (Fig. 6). Consequently, in consideration of the influences of pH value and ionic strength of the electrophoresis medium upon the removal of bacteria from food raw material, fresh water is preferred to salt solutions for use in the medium.

It would not be realistic to select representative species and a definite growth stage of bacteria for electrophoresis standards. The velocities of the bacteria used in the experiment were within the range from -0.80 (μ/sec/volt/cm) to -3.60 (μ/sec/volt/cm) (Tables 6, 7, 8). From the results, however, velocity showed no order among different species of bacteria. In representative bacteria, however, spore and old vegetative cells are generally slower than newly-formed vegetative cells. Thus, the time that the electric current should be supplied will be based on the velocity of spores or old vegetative cells.

Surface-active agents have themselves a sterilizable effect, and are utilized in the food industry. The velocity of bacteria increases within increased concentrations of sodium tetradecyl sulfate up to 10 ppm. Thus, usage of this kind of surface-active agent for removal of bacteria from food raw material would be more effective.

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Immunofluorescent Detection of Staphylococcal Enterotoxin B
II. Detection in Foods

SUMMARY

Specific staphylococcal enterotoxin *B* antiserum conjugated with fluorescein isothiocyanate was used successfully for detecting toxin in food smears or food extracts in about 4–5 hr. The fluorescent antibody technique detected minute amounts of enterotoxin—much less than 1 or 0.05 μg/ml, which are the sensitivity limits of single or double gel diffusion tube tests—without involving any extraction procedures.

INTRODUCTION

Staphylococcal enterotoxin *B* has been demonstrated in culture media by the use of antienterotoxin *B* serum conjugated with fluorescein isothiocyanate (FITC) (Genigeorgis and Sadler, 1966). Detection of the enterotoxin in smears prepared from fluid culture media requires a concentration of toxin higher than 15 μg/ml. A technique was developed (drop technique), however, which detected enterotoxin around living staphylococcal cells when its concentration was greater than 1 μg/ml as determined by a quantitative single gel diffusion technique (Hall *et al.*, 1963). Impression smears made from colonies growing on solid agar media gave minimum specific immunofluorescence because of diffusion of the toxin into the agar. With the drop technique, purified enterotoxin *B* in solution was demonstrated and the toxin from the fluid medium was demonstrated in the presence or absence of enterotoxic cells. The possibility of applying the fluorescent antibody technique (FAT) to the detection of enterotoxin *B* in food smears or food extracts was investigated, and the results are reported here.

MATERIALS AND METHODS

Staphylococcal strains. The strains used in this study were the same strains used to investigate the application of FAT to detect enterotoxin *B* in culture media (Genigeorgis and Sadler, 1966). These staphylococcal strains produce types *A*, *B*, *A* and *B*, or no enterotoxin.

Preparation of conjugate. Enterotoxin-*B*-specific antiserum was conjugated with fluorescein isothiocyanate and fractionated through a diethylaminoethyl cellulose column according to a method described by Corstvet and Sadler (1964).

Preparation and inoculation of foods. The following foods were used: commercially vacuum-packed ham, both with and without sterilizing, a variety of hams prepared in the laboratory, commercially canned ham, raw and boiled chicken breasts, and Cheddar cheese. Discs (14-mm diameter and 2-mm thick) of the different food items were prepared as described by Casman *et al.* (1963). The discs were placed in sterile Petri dishes, inoculated with staphylococcal strains, covered with another disc, and then incubated for different periods at 24, 30, and 37°C, either aerobically or anaerobically (hydrogen atmosphere), in a high-humidity chamber.

Immunofluorescent demonstration of enterotoxin B. *In smears.* At the end of the incubation period the two discs of each set were separated, and thin impression smears were prepared on clean coverslips. The coverslips were dried for 30 min at 37°C and then fixed in ethanol for 1 hr at −20°C, dried at the same temperature, and kept there until staining. Frozen sections of the set of the two discs were also prepared with a cryostat and fixed the same way. Six twofold dilutions of the conjugate in phosphate buffer (pH 7.2, 0.02*M*) ranging from 1:5 to 1:160 were made. Six coverslips prepared from the same sample were each stained with two drops of one of the dilutions, placed in a moist chamber for 30 min at 37°C, or for 30 min at 37°C followed by 4 hr at 4°C, and then washed with phosphate buffer and distilled water for 5 min, respectively, air-dried, and mounted on clean slides with 10% buffered glycerol.

In food slurries (living cells). A 50% slurry was prepared by homogenizing 2–3 sets of discs with phosphate-buffered saline (pH 7.2, 0.02*M*) in a graduated centrifuge tube. One drop of the slurry was placed on each of six coverslips and mixed thoroughly with two drops of one of the conjugate dilutions. These coverslips were incubated like those with smears. The washing differed in that the fluid from each coverslip was poured onto wet Millipore filter membranes (0.22-μ pore size and 13-mm diameter), placed on absorbing filter paper, and then washed with 20 drops of buffer. Impression smears were finally made from each membrane on slides (Genigeorgis and Sadler, 1966). Millipore filter membranes have also been used to

catch detached parts of smears made on coverslips or demonstrate enterotoxin in food extracts.

Serological demonstration of enterotoxin B. The same discs used for the preparation of smears or the slurry were also used for the demonstration of enterotoxin *B* by the single-tube gel diffusion (Hall *et al.*, 1963) and microdiffusion (Crowle, 1958) techniques. The toxin was extracted from the food by first making a 50% slurry in phosphate-buffered saline. The slurry was heated for 25 min at 50°C, left at room temperature for 30 min, and then centrifuged at high speed for 30 min at 30,000 × G. The clear supernatant was further analyzed quantitatively and qualitatively as described (Genigeorgis and Sadler, 1966; Hall *et al.*, 1963) without any concentration and with purified enterotoxin *B* as a reference.

Controls. To check for nonspecific immunofluorescence, smears from uninoculated food were first checked for autofluorescence and then stained the same way as smears from inoculated foods. A second control was based on the demonstration of enterotoxin produced in the food, by single or double gel diffusion in tubes or by the double microdiffusion tests (Hall *et al.*, 1965). As a third control, the same foods were inoculated with non-type-*B* enterotoxin-producing strains and then compared with the foods inoculated with type *B* strains. Finally, positive smears were treated first with antienterotoxic antiserum and then the conjugate and checked for positive staining.

All preparations were examined by Zeiss fluorescent microscope with an Osram HBO 200-W maximum-pressure mercury-vapor arc. The UG5 filter was used as an ultraviolet excitation filter, and the combination 0/41 as a barrier filter.

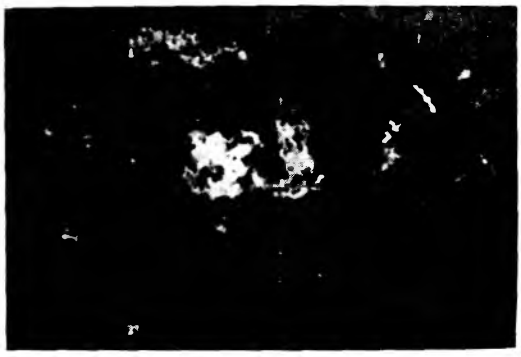

Fig. 1. Positive Reaction. Growth and enterotoxin *B* production by strain S-6 in ham after 48 hr of incubation at 37°C. Impression smear fixed in cold ethanol and stained with 1:20 diluted conjugated antienterotoxin-B. 2500×.

Fig. 2. Positive Reaction. Growth and enterotoxin *B* production by strain S-6 in chicken meat after 48 hr of incubation at 37°C. Impression smear fixed with gentle heat and stained with 1:40 diluted conjugated antienterotoxin-B. 2500×.

RESULTS AND DISCUSSION

Demonstration of enterotoxin B. Enterotoxin *B* was demonstrated in well-fixed thin food smears by the FAT around the bacterial cells producing it. The microscopic appearance of the smears positive for enterotoxin was the same as that described for smears prepared from broth cultures (Genigeorgis and Sadler, 1966). There were halos of amorphous precipitates around the toxin-producing cells, sometimes in the form of clouds covering them completely or precipitates only in the interstitial spaces or completely separated from the cells (Figs. 1, 2). Depending on the dilution of the conjugate used, there was a variation in the morphology of the fluorescent precipitates or clouds. The problem of optimum proportions of the reagents which was found in the studies with broth cultures was not so critical here. Smears prepared from the same sample but stained with different dilutions of the conjugate showed a wide range of positive reactions. Smears from meats with more than 120 μg toxin/ml were stained positively with dilutions of the conjugate ranging from 1:5 to 1:80 and sometimes even to 1:160. Smears from broth cultures with the same toxin concentration were positive only with conjugate diluted 1:5. The results obtained with food smears seem to simplify the method of staining by eliminating the use of many conjugate dilutions to get optimum proportion with the antigen. The wider range of positive reactions would appear to be due to the different amounts of toxin present around the individual cells or colonies. Sugiyama *et al.* (1960) demonstrated a variation in the

amount of toxin produced by different cells. Good positive reactions can, therefore, usually be obtained since some of the bacterial colonies will have toxin in optimum proportions with the conjugated antibody.

With food smears as with broth smears, washing decreased the brilliancy of the fluorescent precipitates. This effect depended greatly on the thickness of the smears and the amount of enterotoxin present. The precipitates were denser in the presence of more enterotoxin, so the effect of washing was minimal. The zone of optimum staining was narrower for smears prepared from foods with small toxin concentration than for smears prepared from foods with higher toxin concentration. Positive reactions were better when there was an additional 4 hr of incubation of coverslips at 4°C.

Frozen section gave the same results as smears. Cross sections of the set of two inoculated discs demonstrated the growth of cells and presence of enterotoxin between the two layers, and a decrease in fluorescent precipitates as the distance increased from the interface.

Sensitivity of the tests. The demonstration of enterotoxin *B* in food smears by the FAT appeared to be more sensitive than the presently applied method of extraction and gel diffusion. A series of sterile ham discs were inoculated with the same number of cells of strain S-6 (producing both *A* and *B* enterotoxins) and checked for toxin production at 3-hr intervals by the FA and gel diffusion (single and double) techniques. Food samples were demonstrated positive for enterotoxin by the FAT much earlier than the time when 1 μg toxin/ml had been produced, the amount needed for its detection by gel diffusion (Genigeorgis, 1965). The FA test showed fluorescent precipitates and clouds around the cells which became denser as the concentration of toxin increased. The FAT showed masses of cells covered with precipitates while the food extract was still negative for enterotoxin by gel diffusion, although enterotoxin could be demonstrated by the latter technique if the food extract was first concentrated 5 times or more. Strongly FA-positive smears were obtained when the extracts were weakly positive by gel diffusion technique. It is considered that the toxin in the extract is the average amount of toxin per unit of volume of the food buffer mixture, and this amount should be many many times smaller than the amount of toxin around the toxic cells per unit volume of food in that area. This may also explain the difference in the sensitivity of FAT when applied to smears prepared from broth cultures or solid foods.

Demonstration in impression smears or drops from toxic food slurries. Smears prepared from slurries (50% in phosphate-buffered saline) were positive for fluorescent precipitates when the concentration of enterotoxin was greater than 5 μg/ml. With smaller concentrations, results were variable. The sensitivity was increased when less than 1:1 buffer was used to prepare the slurry. It appears that, during the preparation of the slurry, the foci of the toxin concentration are destroyed, and so the toxin becomes evenly distributed. It is obvious that if the concentration of toxin around the cells was already small, it would become smaller, and with the added effect of washing, the smear could be negative or be only weakly positive even though the food contained enterotoxin.

Smears made from centrifuged slurry were FA-negative when the supernatant liquid showed small amounts of toxin by gel diffusion. Possibly, homogenization and centrifugation completely removed toxin that was only loosely associated with the cells. This seems to be substantiated by the fact that the cell walls were intensely stained, showing little or no toxin, and the appearance was the same as a nontoxic strain stained as a result of the naturally occurring antibodies in the conjugate reacting with surface antigens (Genigeorgis and Sadler, 1966).

The results were more consistent when the drop technique was used. Since the amount of slurry used is more than can be used to make a smear, more toxin was available to react with the conjugate in an unfixed fluid medium. This aided in the formation of precipitates which were caught later by the filter membrane.

Demonstration in food extracts. Clear food extract from which the fat was removed

by cooling at 4°C was tested for the presence of enterotoxin by the drop technique. Large precipitates were formed with low dilutions of the conjugate. Positive results were more consistent than those obtained with undiluted supernatants from broth cultures. These broths, with over 120 μg toxin/ml, were negative or weakly positive by the drop FAT; however, they proved to be positive when they were diluted 1:3 or 1:5 with buffer, and an increased range of dilutions giving positive reactions was obtained (Genigeorgis and Sadler, 1966). This may be explained by both an excess of antigen and by the effect of constituents in the broth itself. Food extracts gave better reactions by far with the conjugate than did broth cultures. Smaller amounts of toxin were also detected, possibly because there were less soluble solids to affect the antigen-antibody reaction.

Staining of controls. Foods tested for enterotoxin following inoculation with enterotoxin-*B*-producing strains, S-6 and 243, were also tested for toxin after inoculation with non-enterotoxin-*B*-producing strains. The inoculations were heavy to assure good growth. Sterile foods (cheese, ham, and chicken meat) which were inoculated with strains that do not produce type *B* enterotoxin were negative for precipitates or clouds around the bacterial cells by the FAT. Depending on the strain, the cells demonstrated varying classical fluorescence, and the microscopic appearance was the same as that of smears made from broth cultures. Ham, vacuum-packed commercially, showed a variety of rods, cocci, and yeast in addition to the cells of the inoculum. Some of the yeasts were stained intensely green, while the rods and other cocci were blue-violet or gray with an amorphous, thin, uniform cloud of fluorescence which was easily distinguished from specific fluorescent precipitates or clouds around enterotoxin-*B*-producing cells.

Smears were made from uninoculated nonsterile foods and treated the same way as smears made from foods inoculated with strains S-6 and 243. No typical fluorescent precipitates or clouds were demonstrable. A nonspecific fluorescence was obvious in muscle fibers. Natural flora composed of rods and cocci did not show any typical precipitates or clouds around the bacterial cells. With sterile food there was little nonspecific staining, if any, and when present it was weak.

Smears made from foods which had been found to contain large amounts of enterotoxin *B* by the FA and gel diffusion techniques were treated with 2 drops of 1:5 unconjugated antiserum, incubated for 30 min at 37°C, washed once with buffer for 10 min, air dried, and stained with various dilutions of the conjugate (1:5–1:160). Smears so treated were negative for fluorescent precipitates or clouds, but there were cells showing varying fluorescence in the walls. It is believed that surface antigens of these cells reacted with anti-staphylococcal antibodies normally existing in the conjugate after specific precipitates were washed off during the first washing.

Smears made from foods positive for large amounts of enterotoxin were stained with normal conjugated antiserum obtained from a young rabbit never used before for any immunization. The smears were negative for enterotoxin, but individual cells were stained green. It was difficult to demonstrate the presence of enterotoxin in smears made from nonsterile meat products or their slurries previously inoculated with type *B* strains when the toxin was present only in small amounts. In this case, it was difficult to identify fluorescent particles as being specific precipitates rather than autofluorescent meat material. As the concentration of toxin around the cells increased, the morphology of the specific precipitates became characteristic, permitting identification. As with culture media (Genigeorgis and Sadler, 1966) the demonstration of enterotoxin *B* by FAT in foods is based only on the presence of morphologically specific fluorescent precipitates around the bacterial cell and not on the presence of fluorescent cells alone. Efforts to prepare a conjugated antienterotoxin *B* serum which will specifically stain the enterotoxin-producing cells even in the absence of enough toxin to form precipitates, were unsuccessful. Such a conjugated antiserum will, of course, be ideal since precipitates can be washed off while an antigen attached on the cell cannot.

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A New Medium and "mimic" MPN Method for Clostridium perfringens Isolation and Enumeration

SUMMARY

A new medium and "mimic" MPN method for the isolation and enumeration of *Clostridium perfringens* are described. A MPN technique is employed, followed by the confirmation of each MPN tube for the presence or absence of sulfite-reducing anaerobes. The medium and method were compared statistically with the existing sulfite-polymyxin-sulfadiazine (SPS) agar method, and found significantly better by a factor of 2.

INTRODUCTION AND LITERATURE REVIEW

Vegetative cells of certain *Clostridium perfringens* strains, probably arising from germinated spores in cooked food products, have been shown to be the causative agent in an acute gastroenteritis type of food poisoning and have been described by Hobbs (1965), Hall *et al.* (1963), Nygren (1962), Hobbs *et al.* (1953), and others. A sulfite-polymyxin-sulfadiazine (SPS) agar devised (Angelotti *et al.*, 1962) for isolation and presumptive identification of *C. perfringens* has been considered one of the best solid-agar methods for the recovery of sulfite-reducing clostridia (Gibbs and Freame, 1965).

Current recovery methods for clostridia attempt to isolate and often differentiate these anaerobes by use of threshold concentrations of selective agents in the primary medium. Problems involved with these methods and the limitations of solid agar primary recovery media are cited by Gibbs and Freame (1965).

Green and Litsky (1964) proposed a new medium and "mimic" most-probable-number (MPN) method for the isolation of *C. perfringens* vegetative cells. The new medium, described herein, compared equally well with and in most cases better than fluid thioglycollate medium (Difco), RCM (Oxoid), pork-pea-infusion medium (Andersen, 1951), or beef heart infusion medium (Society of American Bacteriology, 1944) for the recovery of *C. perfringens*. The method uses a very selective and confirming secondary medium to identify which tubes of the primary MPN isolation contain sulfite-reducing clostridia. This present investigation further defines this medium and method and determines the statistical validity as compared with existing procedures.

MATERIALS AND METHODS

Organisms. Strains of *C. perfringens* associated with foods and food poisoning were obtained from Drs. Angelotti and Hall (Robert A. Taft Sanitary Engineering Center) and are designated by their code system. The species of *Streptococcus faecalis* and *Proteus mirabilis* used in the simulated food-sampling experiment are from our laboratory collection.

Cultures were grown and maintained on fluid thioglycollate medium with indicator (Difco) at 37°C. Inocula for all experiments were 4–6-hr cultures.

Media and dilution blanks. 1) The primary isolation medium (herein designated as TYD-C) consists of the following: trypticase (Baltimore Biological Laboratories, BBL) 4%, yeast extract 1%, dextrose 0.5%, soluble starch 0.1%, sodium thioglycollate 0.2%, thiamine hydrochloride 1 ppm, Bacto agar 0.15%, and clostrisel broth, powdered, (BBL) 0.3%. The ingredients were added to distilled water, pH adjusted to 7.2 with dilute NaOH, steamed to melt agar, tubed in 16 × 150-mm culture tubes, capped with Bacti-Capall polypropylene closures (Biological Research, Inc., St. Louis, Mo.) and sterilized at 121°C, 15 min. This medium was used within three days of preparation. 2) Sulfite-polymyxin-sulfadiazine (SPS) agar described by Angelotti *et al.* (1962) was commercially prepared by BBL. 3) Ninety-nine-ml dilution blanks contained 0.2% sodium thioglycollate in distilled water.

Experimental methods. *The TYD-C plus "mimic" MPN method.* The MPN technique, using the TYD-C medium, was employed for primary isolation. Tubes for 4–6 tenfold serial dilutions, 5 MPN tubes per dilution, were arranged in a MPN rack. All inoculations of a given dilution were made at one time, and each MPN tube was inocu-

lated deep below the surface of the medium. The MPN racks were incubated for 24 hr at 37°C.

Following incubation, each MPN tube was vigorously mixed for 5–10 seconds (Vortex Jr. Mixer), three loopfuls of culture were transferred into approximately 0.5 ml sterile water blank (16 × 150-mm culture tube), and 5–7 ml of melted SPS agar poured into the blank. These confirmation tubes were arranged in the same order ("mimic") as the primary isolation tubes of the original MPN racks. After 14–18 hr of incubation at 37°C, the appearance of a solid black butt or black colonies, in the SPS tube, confirmed the presence of sulfite-reducing clostridia in the corresponding primary isolation tube. The MPN counts for the primary isolation(s) were obtained from the "mimic" (confirmed) MPN's by use of a standard five-portion MPN table (Hoskins, 1934). *The SPS method.* The SPS agar method described by Angelotti *et al.* (1962) was employed with the following exceptions: illuminating gas was substituted for the N_2 and CO_2 mixture in the Brewer anaerobic jars (BBL), and the motility-nitrate medium was not used, since known *C. perfringens* strains were used in these experiments.

The two methods were compared by employing 10 strains of *C. perfringens*. MPN racks, and Petri plates, six replicas each per strain, were inoculated by appropriate dilutions from the same set of serial dilution blanks and treated as described above. To compare the two methods further, a simulated food-sampling experiment was carried out. A No. 2 can of either chicken, beef vegetable, or clam chowder soup (Campbell Soup Co.) was aseptically opened and mixed with 150 ml of sterile water. In a blender jar, a 10-ml portion of a dilute *C. perfringens* culture was added to 70 ml of the soup. Two 10-ml portions of dilute nonclostridial culture and/or sterile water were added to make a total volume of 100 ml. One experiment consisted of several such jars, each had the same size of inoculum of *C. perfringens* strain, while some jars were inoculated with an estimated excess of a nonclostridial species of 10–1 to 1000–1.

A jar containing soup mix and culture(s) was blended for 1 min in an Oster blender (John Oster Mfg. Co., Milwaukee, Wis.) and samples removed: Four replicas each of MPN racks and Petri plates were inoculated for comparison of the two methods.

Statistical treatment of data. Two statistical methods were employed in these experments: 1) Student's *t*-test was applied to individual observations (MPN's and plate counts) to compare the differences observed between the two methods for either a given strain of *C. perfringens* in the comparison of methods or for a given condition in the simulated food-sampling experiment. 2) For an entire experiment either all strains in the comparison of methods or all conditions in a given simulated food sampling experiment, the comparison of sample means by paired observations method was used. In this method, averages of MPN's, and the plate counts, for a given strain or condition, were considered as a paired observation.

In both statistical treatments, the null hypothesis was assumed, and significant differences (*) considered as probabilities (P) of 0.05 or less, highly significant (**), P = 0.01 or less. Both statistical methods are described by Steel and Torrie (1960).

RESULTS AND DISCUSSION

Table 1 shows the results of the comparison of methods. When the individual strains of *C. perfringens* are statistically treated by Student's *t*-test, the observed differences between the two methods ranged in significance. These varying responses are probably due to individual strain differences. When the entire group of 10 strains are statistically treated by comparison of sample means, paired observations, the difference of twofold greater recovery by the TYD-C plus "mimic" MPN over the SPS method is highly significant (P = 0.0016). This latter statistical comparison, which involves all 10 strains, is more representative of the performance of these methods for the recovering *C. perfringens*.

Similar statistical treatments were applied to the simulated food-sampling experiments. Results of one of these typical experiments are shown in Table 2. In all of these experiments, the TYD-C plus "mimic" MPN showed greater recovery that was significantly different from the SPS method when the over-all comparison of sample means by paired observations was applied. Differences within a given condition, as analyzed by Student's *t*-test, varied in their significance. These latter variations may be due to a combination of strain differences and microbial interactions with nonclostridial species.

In two other simulated food-sampling experiments in which the numbers of viable *C. perfringens* were very low, food debris interfered with the ease of SPS agar plate counting, and plates often contained either no colonies or colony counts of less than 30. The TYD-C plus "mimic" MPN method not only revealed (enumerated) the presence of

Table 1. Statistical comparison of the two methods.[a]

Strain (code)	Av. viable cell counts of *C. perfringens* × 10^8		Statistical treatment: student's t test on $X_1 - X_2$ $df = 6\ (n_1 + n_2 - 2)$	
	Method A X_1	Method B X_2	Statistic t	P
A-19	5.35	3.42	1.81	0.1
#75	9.43	4.13	2.80	0.02*
S-45	9.20	3.35	3.40	<0.01**
A-51	7.60	5.92	1.13	0.3
S-34	7.00	5.93	0.59	0.5
IU-2826	3.77	2.20	2.56	0.02*
S-40	8.52	2.60	2.64	0.02*
E-8	5.70	3.08	2.28	≤0.05*
IU-686	1.11	0.07	6.01	0.001**
#62	1.07	3.68	4.24	>0.01*
Σx	68.24	34.38	comparison of sample means,	
$\bar{x}_2$	6.82	3.44	$X_1 - X_2$: statistic $t = 4.63$,	
Ratio $\frac{\bar{x}_1}{\bar{x}_2} = 2$			$df = 9$, P = 0.0016**	

[a] Method A: the TYD-C plus "mimic" MPN method described in text. Method B: the SPS method (Angelotti *et al.*, 1962).
Statistic t = calculated t distribution, one tail.
df = degrees of freedom.
P = probability of null hypothesis, one-tailed test.
* Significant difference.
** Highly significant difference.

C. perfringens, but would probably have had an even greater advantage over the SPS method in recovering low viable numbers of *C. perfringens*, if greater volumes of TYD-C contained in larger vessels had been used and larger samples (e.g. 10 ml or more) had been taken for the first dilution.

In all of the experiments described, known strains of *C. perfringens* were used. Both methods, as described here, would suffice to presumptively identify and enumerate sulfite-reducing clostridia from a sample with unknown microflora. In unknown samples, additional procedures, such as using the motility-nitrate medium (Angelotti *et al.*, 1962), would be required to confirm the presence of *C. perfringens* or other species of sulfite-reducing clostridia.

The use of approximately 45°C incubation for selective isolation of *C. perfringens* from foods has been reported by Hall and Angelotti (1965), Hobbs (1957), and others. Preliminary experiments in our laboratory, employing the TYD-C plus "mimic" MPN method, indicate that *C. perfringens* are recovered equally well and grow faster with profuse gas formation in TYD-C at 45°C than at 37°C, whereas the nonclostridial microorganisms tested grow sparsely (or not at all) and without gas formation at 45°C. These preliminary experiments suggest that a 45°C incubation be employed in the primary isolation step employing the TYD-C medium, to obtain a presumptive enumeration for *C. perfringens*. Along with the cultural transfer step of the "mimic" MPN procedure we have also streaked solid SPS agar plates, incubated them anaerobically, and obtained isolated black colonies. These isolated colonies could be used for further identification procedures.

We suggest that the above modifications (45°C incubation and SPS agar plate streaking) be incorporated into the TYD-C plus "mimic" MPN method for isolation, enumeration, and identification of *C. perfringens* from samples of unknown microflora.

Our proposed TYD-C plus "mimic" MPN method has the disadvantage of requiring more operational steps, more materials, and more time than the SPS method. However, our method has the advantage of recovering twice the number of viable cells and of being better suited for low population levels of

Table 2. Typical simulated food sampling experiment recovering *C. perfringens*. Strain = A-19, soup = clam chowder.[a]

Condition:	Av. number of viable A-19 cells recovered		Statistical treatment: Student's t test on $X_1 - X_2$ $df = (n_1 + n_2 - 2)$	
	Method A X_1	Method B X_2	Statistic t	P
1. *C. perfringens* only	41	32.8	1.71	0.1
2. plus *P. mirabolis*	62	44.3	5.17	<0.01**
3. plus *S. faecalis*	56.5	34.3	2.34	≥0.05*
Σx	159.5	111.4	Comparison of sample means on $X_1 - x_2$ Statistic $t = 11.3$, $df = 2$, P = 0.01**	
$\bar{X}$	53.2	37.1		

[a] Method A: the TYD-C pluc "mimic" MPN method described in text. Method B: the SPS method (Angelotti *et al.*, 1962).
Statistic t = calculated t distribution, one tail.
df = degrees of freedom.
P = probability of null hypothesis, one-tailed test.
* Significant difference.
** Highly significant difference.

C. perfringens than the SPS method. This advantage of our method has suitable merits for quality control of cooked foods which may initially have low viable numbers of *C. perfringens*.

The tenfold dilution MPN method is less accurate than the equivalent plate counting methods (Cochran, 1950; Taylor, 1962). Some workers have criticized use of the MPN technique for sampling food, etc. These criticisms have been reviewed by Mossel *et al.* (1956). Other workers express a preference for the MPN technique in certain situations, especially for clostridia, which are often difficult to grow anaerobically on agar plates (Gibbs and Freame, 1965). Experiments in our laboratory reveal that the TYD-C medium employing the MPN technique is more conducive to clostridial growth.

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Growth and Sporulation of Smooth and Rough Variants of Bacillus Stearothermophilus in Pea Extract and on Pea Agar

SUMMARY

The influence of rough and smooth variants of *Bacillus stearothermophilus* on growth and sporulation of each was investigated. The smooth variant grew faster from spore inoculum than the rough variant and produced more acid than the rough when grown separately. However, when grown together, the growth was similar to the smooth; and acid production, as measured by pH, was also the same. When the inoculum was vegetative cells, the growth of rough and smooth were similar to the rough both in growth and pH. More spores were produced by the rough variant than by the smooth variant when grown separately on pea agar or in pea extract. When grown together on pea agar or in pea extract, the total number of spores was less than the rough variant but they were of the rough variant type.

INTRODUCTION

Flat-sour spoilage may occur in canned peas and other low-acid foods such as corn, string beans, beets, spinach, pork and beans, hominy, lima beans, asparagus, and succotash. In the canning of peas, the blancher provides an environment in which *Bacillus stearothermophilus* can grow. Knock (1954) used flat-sour counts from water of the blancher, in addition to other line counts, as an index to the level of contamination of the peas. From these counts, he predicted the percentage of infected cans after processing. Knock reported that the temperature of the blancher is above the thermophilic growth range, but the bacteria grow during short shut-down periods. He also noted that the bacteria multiplied during periods of nonoperation if the steam inlet valve was faulty or was not tightly closed.

During blanching, water-extractable materials are removed from the peas, and this material is dissolved in the water and provides nutrients for the growth of spoilage bacteria. As Knock (1954) has pointed out, growth can occur in the blancher during shut-down periods. Assuming that flat-sour organisms entering the processing plant on the peas are a mixture of rough and smooth variants, which variant will gain the ascendancy? Although no data are available on the distribution of these variants of flat-sour bacteria "in nature," one may assume that both are encountered rather frequently since Smith *et al.* (1952) listed both rough and smooth colony types in their description of *B. stearothermophilus*.

The question of which variant will gain the ascendancy and will dominate and produce spores is very important from a practical consideration of flat-sour spoilage. Research in our laboratory showed that there are considerable differences in the heat resistance of the rough and smooth variant of *B. stearothermophilus*. The smooth variant of NCA 1518 is resistant to heat, while the rough is comparatively nonresistant (Fields, 1963). Because of differences in resistance, growth of the variants in food extracts is of interest to the food microbiologist. The research reported herein was designed to study the influence of one variant on another during growth and sporulation in pea extract and on pea agar.

EXPERIMENTAL METHODS

Preparation of pea extract. The pea extract was made by weighing 100 g of frozen peas and adding 400 ml of distilled water in a 1-L beaker. A watch glass was placed over the top of the beaker, and the peas were boiled for 1 hr. Distilled water was used to readjust the volume of the extract to 400 ml after the peas were removed by filtration. The extract was dispensed in screw-top test tubes in 9-ml and 50-ml quantities in flasks for growth studies by turbidimetric methods and plate counts and in 100-ml portions in Roux flasks for sporulation investigations. Pea agar was prepared by adding 15 g of agar to a liter of extract. The pH of the pea extract and agar for sporulation studies was 6.4. The pH's of other extracts were 6.0 and 5.4, respectively.

Spore and vegetative cell inocula. The methods of growing, cleaning, washing, and general preparation of spores used in this study were reported previously (Fields, 1963). Both the rough and the

smooth variant of strain NCA 1518 of *B. stearothermophilus* were used. Both variants conformed to the species pattern of Smith *et al.* (1952) for *B. stearothermophilus*. The number of spores of each variant was adjusted to the same number for the inoculum in the growth experiments by plating in dextrose tryptone agar.

To prepare vegetative cells for inocula in sporulation studies, a loop of cells from nutrient agar slants was used to inoculate test tubes of pea extract which were incubated 18 hr at 55°C. The number of vegetative cells of each variant was adjusted by use of standard curves in which turbidimetric values were plotted against plate counts from previous experiments (Fields, 1964).

Growth measurements. The amount of growth of both variants was measured by taking readings on a Bausch and Lomb Spectronic 20 in test tubes containing 10 ml of pea extract. Readings were made at 650 mμ, with an uninoculated tube as a blank. In the second experiment, the amount of growth was measured by turbidimetric means by removing a 10-ml aliquot from 250-ml Erlenmeyer flasks containing 50 ml of pea extract and by plate counts made at selected intervals during the incubation period from the same flasks.

Measurement of pH and titratable acidity. All pH measurements were made on a Beckman Zeromatic pH meter. The pH of pea agar, after growth of the rough and smooth variants on the surface of the agar, was measured by gently lowering the electrodes down into the agar. Thirty ml of extract were used in the titrations. Samples were titrated to an end point of pH 7.0 using 0.03*N* NaOH.

Sporulation studies. Sporulation was studied in a pea extract and on pea agar. Both the pea extract and the pea agar contained 30 ppm of manganese sulfate to aid in sporulation (Ordal, 1957). One hundred ml of pea extract were added to each Roux flask and sterilized. Three Roux flasks were used for each variant and for the combination of variants.

The Roux flasks were inoculated with 1 ml of an 18-hr culture of each variant and a combination of rough and smooth which had been adjusted to the same number of vegetative cells. An aliquot was removed from each Roux flask after 24 hr, and the amount of growth was measured on the Bausch and Lomb Spectronic 20. The pH was measured on this aliquot. To determine the number of spores, another aliquot of pea extract was removed and boiled for 5 min and then plated in dextrose tryptone agar. The plates were incubated 48 hr at 55°C.

When pea agar was used as the sporulation medium, Petri plates containing the agar were inoculated with 1 ml of an 18-hr culture of each variant, and with a combination of rough and smooth with the same number of vegetative cells. The Petri plates were rotated so that the surfaces of the agar were completely covered with the inoculum. The plates were incubated 24 hr at 55°C.

After the incubation period, each of the five replicate plates were flooded with 10 ml of sterile water and the surface growth removed by scraping with an inoculating needle. The contents of each plate were pooled in a sterile Erlenmeyer flask. The contents were boiled for 5 min and readjusted to the original amount of sterile water and then plated in dextrose tryptone agar.

RESULTS AND DISCUSSION

Growth studies. Pelczar *et al.* (1955) list several methods of determining bacterial growth. Two of these methods were used in this study, namely, by determining the density of a cell suspension by optical measurements and by plate counts. Optical density gives both dead and living cells while the plate count data give only viable counts.

Earlier experiments (Fields, 1964) showed that a higher optical density of the rough variant did not mean that there were more cells of the rough variant but that the longer cells which were present in the suspension were stopping more light than the smooth variant cells. A plot of the optical densities against cell concentration of both of these variant forms when cells were grown in nutrient broth, centrifuged, diluted and optical density measured, yielded a straight line (Fields, 1964). These data agree with Lamanna and Mallette (1965) who stated that if the population is not statistically constant in size, cell shape and chain length, the turbidity measurements will be altered. Such differences in size and length of cells do exist between these variants (rough variant is longer than the smooth, Fields 1963) but these differences do not negate the use of turbidimetric measurements if one is aware of these differences.

Growth studies, as shown in Fig. 1, were repeated three times with each variant being grown in five replicate tubes each run. The data showed that the smooth variant, when grown by itself increased the total optical density rapidly, and the optical density remained at this level throughout the remaining

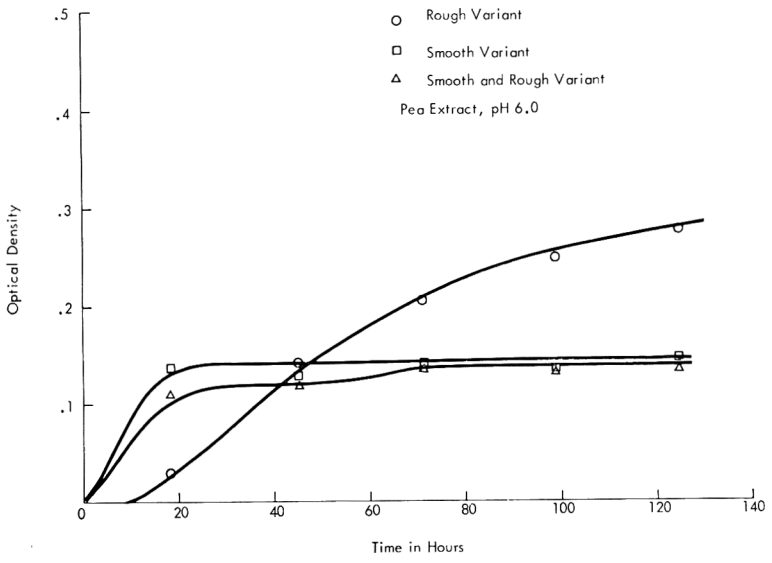

Fig. 1. The growth of rough and smooth variants of *Bacillus stearothermophilus* in pea extract.

times tested. The growth of the rough variant lagged behind the growth of the smooth variant when the tubes were inoculated with the same number of unheated viable spores. When the two variants were mixed and the number of spores of each variant were adjusted so that the tubes contained the same number of rough and smooth variants, as in the individual treatments, the growth curve followed that of the smooth variant after 71 hr of incubation. The optical density in this experiment at 18½ and 44 hr was less for the mixture than for the smooth, while the optical densities of the mixtures in other work (not reported here) were the same as the smooth.

In order to check on the influence of acid production and tolerance of vegetative cells to acid pH values, titratable acidity and plate counts were made at selected time intervals. These results are given in Fig. 2. The shapes of the growth curves of the rough and smooth variant were similar to those in Fig. 1, although a plateau was reached at a lower optical density than in the former experiment. This is probably due to the fact that the original pH of the pea extract in this experiment (Fig. 2) was 5.4 whereas

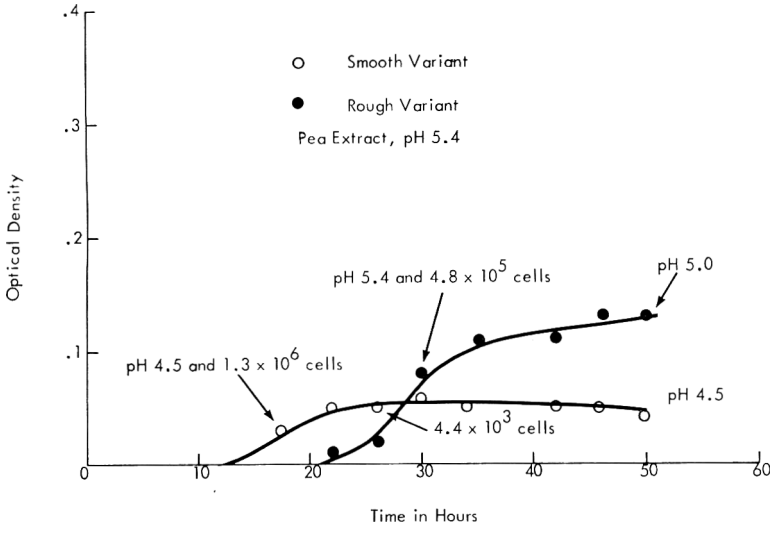

Fig. 2. The growth of rough and smooth variants of *Bacillus stearothermophilus* showing the influence of acid production on pH and viable cells.

the data in Fig. 1 were obtained from an extract with an original pH of 6.0. With a lower original pH of the extract, less growth was needed to produce an inhibitory pH. At 17½ hr after the start of the experiment, the smooth variant changed the pH from 5.4 to 4.5 in the pea extract, and there were 1.3×10^6 viable cells per ml as determined in dextrose tryptone agar. By 26 hr, the vegetative cell count of the smooth variant had fallen to only 4.4×10^3 cells. Titratable acidity was also measured at the same time the pH was taken. The amount of .03*N* NaOH at 17½, 26, and 50 hr for the smooth variant was 1.7, 2.5 and 2.5 ml, respectively. The acidity was probably influencing the recovery of the smooth cells. Not only is the difference in acid production noted here, but it also may be observed when the variants are plated in dextrose tryptone agar.

Based on plate counts, there were 2.4×10^4 rough variant cells per ml at 17½ hr, and at this time the pH of the pea extract was 5.4, with no acid production. At 30 hr, the cell count had increased to 4.8×10^5 cells as determined by plate counts and still no measurable acid production. However, the rough variant had decreased the pH to 5.0 by 50 hr (1.1 ml of 0.03*N* NaOH) as contrasted to a pH of 4.5 (1.7 ml of 0.03*N* NaOH) for the smooth variant at 17½ hr.

These data demonstrate that there is a difference in the lag and generation time of the two variants. The smooth variant because of its acid production and/or other metabolites inhibited the growth of the rough in pea extract. This difference in the rate of growth may be explained on the type of cell division. According to Bisset (1955) the smooth variant types of bacteria divide by the constriction of the cell wall while the rough variant by the formation of a complete cross-wall which subsequently splits.

Sporulation studies. Table 1 shows sporulation of the variant forms when grown by themselves and together in pea extract and on pea agar. The amount of growth of the rough variant either by itself or in combination with the smooth variant was heavy when the inoculum was actively growing vegetative cells. This is in sharp contrast to the growth of the combined variants from spore inocula as given in Fig. 1, in which the growth of the combined variants was similar to the smooth when grown by itself. This greater growth of the rough variant in Petri plates than in test tubes was probably associated with increased oxygen availability.

The largest number of spores was produced by the rough variant when grown by itself either in pea extract or on pea agar. The pH's of the pea extract and the pea agar of the rough variant were also higher than the smooth variant. The next largest number of spores was produced in the mixture of rough and smooth. The pH of these media were similar to the pH of media which supported growth of the rough variant. The least amount of spores was produced by the smooth variant and the lowest pH's of the extract and agar were recorded with this variant.

Table 1. Sporulation of variants of *Bacillus stearothermophilus* in pea extract and on pea agar.

Sporulation medium	Variant	pH of medium in 24 hr [a]	Optical density of pea extract	Av. no. of spores per ml [b]
Pea extract	Rough	5.2	0.39	4,956
Pea extract	Smooth	4.8	0.09	>1
Pea extract	Rough and Smooth	5.2	0.35	910
Pea agar	Rough	5.25	[c]	57,000
Pea agar	Smooth	4.9	[d]	42
Pea agar	Rough and Smooth	5.3	[c]	5,650

[a] pH of extract and of agar: original pH of extract was 6.4.
[b] Spores per ml of extract in Roux flasks and of suspension prepared from five replicate Petri plates.
[c] Heavy growth by visual observations.
[d] Light growth by visual observations.

According to Ordal (1957), environmental factors affecting sporulation are: temperature, pH, oxygen tension, carbon sources, nitrogen source, and growth factors. From the data in Table 1, the increase from 4,956 spores per ml to 57,000 per ml may be attributed to oxygen level. The temperature, carbon source and available growth factors were the same for both variants. There was growth of the smooth variant in the extract and on the agar. Ordal (1957) stated that the optimum for sporulation is similar to that for growth but the range is narrower. The data show that the pH was lower and may be the main reason for the lack of sporulation but a mineral or a vitamin requirement may also be a possibility.

The mixture of rough and smooth produced a pH which was similar to the rough. Apparently some of the rough variant cells were producing basic substances or, since the rough dominated the mixture, lesser amounts of acid were produced. Certainly the growth of a mixture of vegetative cells in extract (Table 1) was different from the growth of vegetative cells from spores (Figs. 1 and 2).

The smooth variant even though it grew rapidly did not sporulate in pea extract and on pea agar even though the number of cells were numerous. When two sporeforming variants are competing for dominance in a food extract, there are two kinds of dominance. The first of these is vegetative dominance and the second is spore dominance. Although one variant may gain the ascendancy in terms of the total numbers of cells in an extract or on an agar, spore dominance may not be correlated with vegetative dominance.

Although the results of this research indicate that the comparatively lower resistant spores predominated in pea extract and on pea agar, studies on the ratio of rough to smooth variants under conditions of actual canning are needed because the environment of the canning plant might allow the smooth variant to dominate.

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Formation of Higher Alcohols During Grape Juice Fermentations at Various Temperatures

SUMMARY

Significant effects of fermentation temperature on the formation of fusel oils in grape juice media were demonstrated. Isoamyl alcohol was formed most abundantly at 70–80°F, and *n*-propyl alcohol least abundantly. Isobutyl alcohol formation seems fairly independent of fermentation temperature. The formation of active amyl alcohol was affected but slightly, in a manner similar to isoamyl. The levels of the sum of the two amyl alcohols formed were 6–131% (averaging 47%) higher with fermentation at 70 or 80°F than with fermentation at 50°F. This effect is considered significant for commercial practice. Possible interpretations of the results are discussed along with pathways of higher alcohol formation.

INTRODUCTION

Studies to influence or control the secondary natural products of wine fermentation have held much interest in these and other laboratories. In particular, effective methods of controlling the levels of the major higher alcohols [*n*-propyl, isobutyl, active amyl (3-methyl 1-butanol), and isoamyl (2-methyl 1-butanol) alcohols] are desirable. From the standpoint of beverage wines, complete elimination of these components of fusel oil cannot be considered wholly advantageous, but any technique which permits a wine maker to control their production within certain limits is potentially advantageous. Distillers of grape brandy, especially in California, are generally interested in minimizing higher-alcohol formation in distilling wines.

Guymon and Heitz (1952) reported a mean fusel oil content (by a colorimetric method not reflecting propyl alcohol contents) of 250 mg per liter (range 162–366 mg) in 120 samples of California white table wines, and a mean of 287 mg per liter (range 140–417 mg) for 130 samples of California red table wines.

In review, Webb and Ingraham (1963) cited several factors which appear to influence fusel oil production. Guymon *et al.* (1961b) compared the fusel oils of aerobically fermented grape juice with those produced under anaerobic conditions. They found that grape juice produces 4-fold more fusel oil when fermented aerobically, contrary to results of Hough and Stevens (1961) with wort. These results are not necessarily in conflict if the probable vast differences of the amino acid contents of the two media and degree of aeration employed are considered.

Dietrich and Klammerth (1941) and Crowell and Guymon (1963) found that the fusel oil produced during fermentation was greater in the presence of suspended solids such as grape pulp. The latter suggested that the increase is due to partially aerobic conditions from oxygen adsorbed by or trapped within the solid material.

The influence of yeast strain has been studied by Guymon *et al.* (1961), Hough and Stevens (1961), and Peynaud and Guimberteau (1962). Commonly employed strains of *Saccharomyces* do not appear to vary excessively, but enough to speculate that proper strain selection might be of industrial value. Peynaud and Guimberteau found fivefold differences in amounts of fusel oil formed by diverse strains of yeasts, but a range of only about 2-fold for *Saccharomyces ellipsoideus*. An intriguing possibility is the possible use of amino-acid-deficient mutant strains of *Saccharomyces cerevisiae* of the type described by Ingraham and Guymon (1960), Guymon *et al.* (1961a), and Ingraham *et al.* (1961) to control or possibly to eliminate fusel oil formation from fermentations. Unfortunately, the mutants employed so far are such slow fermenters that wild types would be expected to rapidly dominate any commercial-type wine fermentation.

The composition of the medium undergoing fermentation has been studied extensively particularly from the standpoint of naturally-occurring or added amino acids.

The implications of these studies have been reviewed by Webb and Ingraham (1963).

Castor (1953) measured the amino acids in seven different varieties of California grape must. The amino acid composition of some French grape juices was reported by Lafon-Lafourcade and Peynaud (1959). Good data are lacking on the naturally-occurring amino acids in grape must as related to various varieties and growing conditions. If amino acid contents were readily available, must composition might be better correlated with fusel oil formation during fermentation.

The influence of fermentation temperature was considered by Hough and Stevens (1961) in conjunction with studies of yeast strains, using wort. Those workers found an optimum temperature of about 75°F for total fusel oil production. Peynaud and Guimberteau reported mean values for diverse species of *Saccharomyces* in various media, including grape musts and synthetic media of different nitrogen composition, for fermentation temperatures ranging from 59 to 95°F. They found maximum amounts of both isobutyl and isoamyl alcohols at 68°F, and the second highest level was at 77°F. Otsuka *et al.* (1963) found that the formation of both isobutyl alcohol and combined amyl alcohols was maximum at 77°F. Webb and Ingraham (1963) stated that the benefits to be derived from fermenting at a temperature lower than 75°F are not enough to be of industrial value.

This study was made to determine the influence of the temperature of fermentation on the production of each individual higher alcohol, using more advanced methods of analysis and carefully controlled fermentation temperatures and standardized fermenting techniques.

MATERIALS AND METHODS

Fermentation. Juices from six different varieties of grapes grown in two different climatic regions—warm (Davis) and cool (Oakville)—were obtained by crushing and pressing fresh-picked fruit. The juice was treated with a standard amount of sulfur dioxide, 100 mg/L, and then yeasted with a pure culture of *Saccharomyces cerevisiae* var. *ellipsoideus*, strain Montrachet. Sufficient culture was added to give about 10^5 to 10^6 viable cells per ml of grape juice. Under these conditions past experience has shown the inoculated culture to remain true.

Two-and-one-half gallons of juice for each variety from each region was divided into five one-gallon containers and fermented in water baths controlled to $\pm 1/2$°F. The temperatures used were 50, 60, 70, 80, and 91°F.

The fermentations were allowed to go to dryness with sufficient °Brix measurements to determine rates of fermentation (Ough, 1964). At the end of fermentation the wines were stored at 70°F for 24 hr, and then the supernatant liquid was siphoned off and stored in full bottles at 32°F until the analyses were made.

Chemical analysis. The routine analyses reported were made by the methods of Amerine (1965). The analysis considered in this paper included measurement of the must for °Brix and of the wines for ethyl alcohol.

Fusel oil analysis. An F & M dual-column gas chromatograph, model 700-12, with dual-flame ionization detectors was employed for fusel oil analysis of 1:1 distillates of each wine. The completeness of the distillation of the fusel oils was verified by direct injection of several of the wine samples. The distillates were found to represent the wine accurately with respect to higher alcohol content. Three-μl samples were injected onto a UCON 50-4B-400, 10% substrate on 60-80-mesh firebrick packing, 6-ft × ¼-inch-OD copper column. Peaks for *n*-propyl, isobutyl, and the combined amyl alcohols (active plus isoamyl) were obtained with respective retention times of about 7.2, 10.4, and 23 min. No peaks were observed under these conditions for other higher alcohols which are usually reported in concentrated fusel oils, such as *n*-butanol, *n*-pentanol, and *sec*-butyl alcohol. The amyl alcohols were separated into the active and isoamyl isomers on a 10% diglycerol column, 6 ft × ¼ inch. For runs with both columns, the oven temperature was maintained at 80°C and the injection ports and detectors at 200°C. The helium carrier gas flow was 60 ml/min in the sample column. Three-μl samples of standard solutions containing increasing amounts of *n*-propyl, isobutyl, and isoamyl alcohols in 10% ethyl alcohol were injected, and the peak areas were plotted versus concentration of each higher alcohol component. From these plots the peak areas resulting from the injection of the 1:1 wine distillate were related to each higher alcohol, expressed as mg per liter. It was found necessary to make at least daily injections of one or two of the standards to ensure against slight differences in instrument response to a given fusel oil concentration.

RESULTS

The initial °Brix and the final ethyl alcohol concentrations are given in Table 1. The range represents what could normally be expected of commercial juices. The decline of the alcohol with increasing temperature is expected and normal.

A complete report of the individual higher-alcohol analysis of the wines is shown in Table 2 for the results of the 60 fermentations. Total fusel oil formation seems to be maximum at an intermediate temperature of about 75°F, in general agreement with most of the previously cited literature. However, exceptions were noted. Several individual wine series had maxima at lower temperatures or showed very little change over the temperature range studied. In order to assess the general effect, the levels of the individual fusel oils were averaged for each temperature value for the twelve fermentations. The results of the averaging for both amyl alcohols are given in Fig. 1. The values do change with temperature, and have maximums at about 75°F. The averaged results for *n*-propyl alcohol and isobutyl alcohol (Fig. 2) show a different pattern. The *n*-propyl forms a minimum value at about 75°F, and it appears in general that the temperature response is opposite to that of the amyl alcohols. The isobutyl changes very little with temperature, and what small changes there are seem to be erratic.

A rather complete chemical analysis was done on these wines, and the results are reported elsewhere (Ough and Amerine, 1965). These data were investigated for correlation with the fusel oil data.

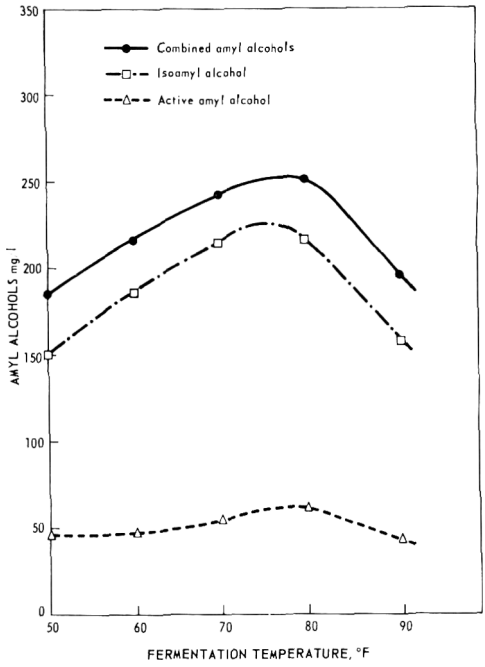

Fig. 1. The distribution of over-all values of active amyl and isoamyl alcohols by temperature for six wines, and the distribution of average values of the combined amyl alcohols by temperature for 12 wines.

Table 1. Initial °Brix of juice and ethyl alcohol in wines from six grape varieties from two climatic regions.

Variety and source	° Brix	Ethyl alcohol (vol/v) Fermentation temperatures 50°F	60°F	70°F	80°F	91°F
Sauvignon blanc						
Davis	21.5	12.5	12.3	12.1	11.9	11.7
Oakville	26.0	15.7	15.2	15.0	14.9	14.5
White Riesling						
Davis	18.8	10.7	10.6	10.2	10.4	10.3
Oakville	21.6	12.7	12.0	12.4	12.4	12.0
Semillon						
Davis	22.4	13.2	13.1	12.8	12.8	12.5
Oakville	23.2	13.9	13.8	13.5	12.9	13.2
Chenin blanc						
Davis	23.2	13.8	13.4	13.3	13.0	12.9
Oakville	22.8	13.8	13.3	13.3	13.3	12.6
French Colombard						
Davis	21.0	11.0	11.6	11.3	11.1	11.0
Oakville	23.0	13.4	13.2	12.3	12.7	12.7
Pinot blanc						
Davis	23.6	14.5	14.3	14.4	14.0	13.5
Oakville	21.6	12.5	12.5	12.3	12.4	12.2

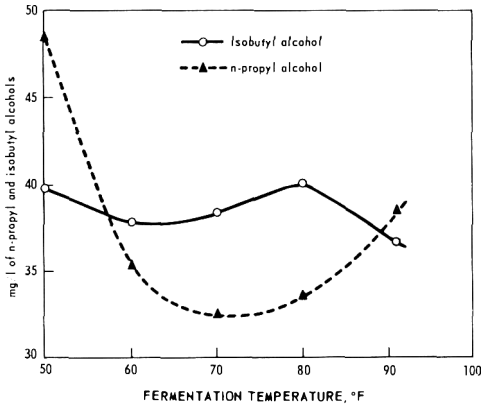

Fig. 2. The effects of fermentation temperature on the average value of the isobutyl and *n*-propyl alcohols in twelve wines.

DISCUSSION

Fig. 3 shows an abbreviated outline of the known pathways for amino acid and higher-alcohol formation via α-ketoacids formed from carbohydrate. In light of this scheme, certain possible conclusions may be derived from the data. Since isoamyl alcohol is formed during grape juice fermentation from an intermediate, α-ketoisocaproate, via the pathway for leucine synthesis, the enzymes responsible for the production of this ketoacid must be at optimum activity at 75°F. Similarly, active amyl alcohol, also formed in greatest amount at this temperature, is derived from α-keto-β-methylvalerate, and the same explanation could be made.

Yeast growth, which generally reaches a maximum at 70–80°F in fermenting wines, would logically be accompanied by an increased demand for amino acid synthesis for protein formation at these same temperatures. If higher alcohols are viewed as spill-over products of amino acid synthesis, as suggested by Lewis (1964) and others, the data presented for the amyl alcohols appear reasonable. That is, the highest levels for these alcohols are formed at the same temperatures as those at which maximum yeast growth occurs and, hence, maximum synthesis of leucine and isoleucine.

Referring to Fig. 2, no such explanation for isobutyl or *n*-propyl alcohol formation can be advanced. The isobutyl alcohol values show no clear relationship to fermentation temperature. Lacking information on the yeast cell's need for individual amino acids, one cannot relate isobutyl alcohol formation to valine synthesis. Furthermore, Fig. 3 shows that α-ketoisovalerate, a precursor of isobutyl alcohol, is also an intermediate for isoamyl alcohol synthesis via α-ketoisocaproate. Perhaps cellular demand for leucine prevents any large accumulation of α-ketoisovalerate.

Äyräpää (1965), testing *S. carlsbergensis* in a synthetic medium, found that the concentration of the nitrogen source regulated the production of the fusel oils. If the medium contained more than a minimum amount of leucine or isoleucine, the formation of isoamyl, active amyl, and isobutyl alcohols decreased with increasing amino acid concentration. However, using several nitrogen sources in the medium, he showed that *n*-propanol concentration increased with increasing nitrogen concentration.

The *n*-propanol-fermentation temperature relationship (Fig. 2) can be partially explained. As the natural amino acid source is depleted and the demand for isoleucine for cellular protein synthesis increases, the reactions along the isoleucine synthesis pathway toward isoleucine are favored (see Fig. 3). In the fermentation temperature range where yeast cell multiplication is greatest (70–80°F), demand for the amino acid syn-

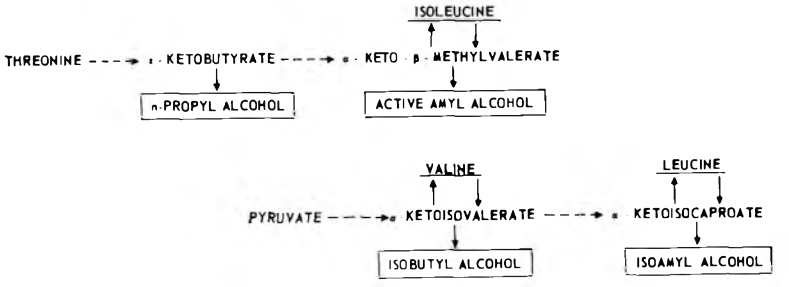

Fig. 3. Condensed pathways for amino acid and higher-alcohol formation from α-ketoacids.

Table 2. Fusel oil alcohols produced at five fermentation temperatures for six grape varieties from two climatic regions.[a]

		mg of alcohol per liter of wine									
		Fermentation temperature									
		50°F		60°F		70°F		80°F		91°F	
Variety	Alcohol	D	O	D	O	D	O	D	O	D	O
Sauvignon blanc	*n*-Propyl	43	80	28	62	29	62	28	63	43	60
	Isobutyl	36	42	31	36	37	32	41	30	29	28
	Comb'd. amyls [b]	174	210	210	240	250	240	250	220	160	176
White Riesling	*n*-Propyl	23	52	18	24	18	26	20	29	21	32
	Isobutyl	42	30	37	22	47	27	57	28	53	28
	Comb'd. amyls	162	176	205	175	285	218	310	298	220	185
	Fraction active amyl	0.23		0.19		0.20		0.19		0.21	
Semillon	*n*-Propyl	23	53	24	32	24	27	24	28	32	35
	Isobutyl	46	42	40	37	44	33	43	33	36	38
	Comb'd. amyls	196	190	250	208	272	222	290	210	200	185
	Fraction active amyl	0.25		0.22		0.23		0.30		0.24	
Chenin blanc	*n*-Propyl	52	22	33	20	29	16	32	16	39	22
	Isobutyl	56	48	45	41	56	48	57	54	53	41
	Comb'd. amyls	230	268	260	280	296	340	297	384	230	244
	Fraction active amyl	0.22	0.25	0.17	0.25	0.18	0.24	0.19	0.25	0.22	0.23
French Colombard	*n*-Propyl	64	58	65	40	64	35	60	44	68	40
	Isobutyl	26	33	30	35	32	30	32	33	26	31
	Comb'd. amyls	124	170	180	170	175	170	165	180	128	165
	Fraction active amyl	0.18		0.16		0.15		0.16		0.16	
Pinot blanc	*n*-Propyl	62	50	41	36	30	30	31	31	34	34
	Isobutyl	33	44	53	48	35	40	39	38	40	38
	Comb'd. amyls	130	193	195	224	200	245	235	192	214	180
	Fraction active amyl		0.22		0.20		0.19		0.21		0.20

[a] D = Davis; O = Oakville.
[b] Isoamyl plus active amyl alcohols.

thesis is highest. Less α-ketobutyrate would accumulate, and therefore smaller amounts of *n*-propanol would be formed. At the fermentation temperatures where there is a smaller demand for amino acid synthesis, then a larger pool of α-ketobutyrate could exist and a greater amount of *n*-propanol formed. This agrees with observations of Guymon and Kunkee (1966), that *n*-propanol is formed earlier in fermentation whereas the other three fusel oils are accumulated up to the end of the fermentation.

From the standpoint of applications, these data show that levels of fusel oil alcohols in wines may be partially controlled by limiting the temperature of fermentation. Affected most are the combined amyl alcohols, the most significant components of fusel oils from a sensory standpoint. Comparing the wines fermented at 50°F with those fermented at 70–80°F, there is an increase of 6–131% (average 47%) at the higher temperatures. Aside from the obvious economic disadvantage of fermenting wines more slowly, because of the effect of lower temperatures, this practice provides a positive reduction in the formation of fusel oil alcohols.

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All-Vegetable Protein Mixtures for Human Feeding. The Development of INCAP Vegetable Mixture 14 Based on Soybean Flour

SUMMARY

Study of the complementary value of soybean and corn proteins in young growing rats resulted in the formulation of INCAP Vegetable Mixture 14: 58% corn flour, 38% soybean flour, 3% torula yeast, 1% calcium phosphate, and 4,500 I.U. vitamin A per 100 g. The mixture contains approximately 27% protein. Formula 14, evaluated in young growing dogs, was slightly lower in protein quality than casein. Its main limiting amino acid is methionine, but only when fed at a low level of protein. The addition of lysine and threonine in the presence of methionine caused a highly significant increase in protein efficiency ratio. Only a small response was obtained when milk protein replaced Formula 14 protein in isoproteic diets.

INTRODUCTION

The widespread prevalence and great public-health significance of protein-calorie malnutrition among young children in protein-poor areas of the world is now well recognized. During the last few years, several laboratories throughout the world have developed vegetable-protein mixtures for supplementary feeding of children and adults as a means of controlling protein-calorie malnutrition in underdeveloped areas (National Academy of Sciences, 1961). These mixtures usually consist of various combinations of a cereal grain with a vegetable protein concentrate. The vegetable protein concentrates that have received the greatest attention are soybean, cottonseed, peanut, and sesame flour (Altschul, 1958).

In our laboratories, a great deal of attention has been given to vegetable-protein mixtures based on cottonseed flour and corn (Bressani *et al.*, 1961). One of the mixtures developed was tested extensively in experimental animals (Bressani *et al.*, 1962) and children (Scrimshaw *et al.*, 1961) and is now being produced on an industrial scale under the commercial name of Incaparina (Béhar, 1963). Emphasis was placed on using cottonseed flour because of its availability in the Central American countries (Bressani, 1965) and other parts of the world (USDA, 1963) where protein-rich foods are needed.

Vegetable-protein mixtures containing other vegetable-protein concentrates are highly desirable because the availability of such concentrates varies from one area to another. Therefore, soybean flour was tested as the main vegetable-protein concentrate to develop mixtures with cereal grain protein.

The literature on the nutritive value of soybean protein has been reviewed in an excellent manner by several workers (e.g. Cicle and Johnson, 1958). Also, a considerable amount of work has been reported on the supplementary effect of soy flour on the protein of wheat flour (Jarquín *et al.*, 1966) and other cereal grains (Dean, 1958). In this report, soybean flour protein was tested for its complementary value to corn proteins, resulting in the development and testing of INCAP Vegetable Mixture 14.

MATERIALS AND METHODS

The experiments were done with a toasted soybean flour (kindly supplied by General Mills) containing 50% protein and local whole corn flour. On the basis of the nitrogen content of both ingredients, the complementary and supplementary value of soybean protein to corn protein was studied. For the experiments on the complementary value of soybean flour protein, a series of diets were prepared in which protein level was kept at approximately 9%. In one of the diets, all the protein was derived from corn flour, while in remaining diets, corn protein was replaced progressively by soybean flour protein, so that the protein of the last diet of the series was all derived from soybean flour and all contained equal amounts of total protein. The partial composition of the diets fed is shown in Table 1.

The supplementary value of soybean flour to corn protein was studied by adding increasing levels of soybean flour, from 0 to 20%, to a fixed level of corn in the diet. In this case, the protein content of the diet increased with increasing levels of soybean protein. As before, the partial composition of the diets used is shown in Table 2.

Table 1. Optimum combination between the proteins of corn and of soybean flour.

Percentage protein distribution in diet		Protein distribution in diet		Weight distribution in diet[a]		Weight gain[b]	
Corn	SBF	Corn	SBF	Corn	SBF	(g)	PER
100	0	8.0	0	90.0	0	33	1.43
80	20	6.4	1.6	72.0	3.2	57	2.15
60	40	4.8	3.2	54.0	6.4	73	2.53
40	60	3.2	4.8	36.0	9.6	83	2.71
20	80	1.6	6.4	18.0	12.8	83	2.61
0	100	0	8.0	0	16.0	68	2.33

[a] The other ingredients of the diets were: 4.0% mineral mixture (Hegsted *et al.*, 1941), 5% refined cottonseed oil, 1% cod liver oil, and cornstarch to adjust to 100%. All diets were supplemented with 4 ml of a complete vitamin solution (Manna and Hauge, 1953) per 100 g.
[b] Average initial weight: 48 g (12 animals per group).

A procedure similar to that described above was followed in studies to find the complementary effect between skim milk and the best corn-soybean flour mixture. These experiments were done at the 10 and 20% protein levels in the diet. The partial composition of the diets used is described in Table 3.

Finally, the limiting amino acids in Mixture 14 were determined in young growing rats with diets containing 10 and 20% protein. The amino acids added, as well as the amounts, are shown under "Results." The other components of the diets are also shown in Table 4.

In all these studies, assays were carried out with young growing rats of the Wistar strain of the INCAP colony. Six to 12 rats were assigned at random to each experimental diet. The rats were placed in individual all-wire cages, with raised screen bottoms. Both feed and water were provided *ad libitum*, and the experimental period lasted 28 days. Weight of the rats and consumption of food was recorded every 7 days. Protein efficiency ratios (PER) were used to assess the effect of the different treatments. Partial description of the diets used is given in the results section, together with the performance of the experimental animals.

The best protein combination between corn and soybean flour served as the basis for the formulation of Mixture 14, which was then fed to 4 young growing dogs to determine its nitrogen balance index (NBI).

The animals were fed decreasing levels of protein from a diet made of: 87% Formula 14; 10% hydrogenated vegetable fat, 2% mineral mixture (Hegsted *et al.*, 1941), 1% cod liver oil, and 3 ml of a complete vitamin solution (Manna and Hauge, 1953) per 100 g. The intake of calories was maintained at 135 Kcal/kg/day throughout the study. When needed, the intake of calories was adjusted with an N-free diet made of: 20% cornstarch, 40% dextrose, 24% sugar, 3% cellulose, 10% hydrogenated vegetable fat, 2% mineral mixture (Heg-

Table 2. Effect of complementing and supplementing corn protein with soybean flour protein in rats.

Percentage protein distribution in diet		Protein distribution in diet		Protein in diet	Weight distribution in diet		Weight	
Corn	SBF	Corn	SBF	(%)	Corn	SBF	gain[a]	PER
100	0	7.82	0	7.82	90.0	0	36	1.60
80	20	6.41	1.60	8.01	72.0	3.2	58	2.29
60	40	4.70	3.13	7.83	54.0	6.3	74	2.72
40	60	3.20	4.79	7.99	36.0	9.5	88	2.91
20	80	1.63	6.54	8.17	18.0	12.7	79	2.67
0	100	0.00	8.07	8.07	0.0	16.0	77	2.56
100	0	6.99	0.00	6.99	70.0	0.0	22	1.31
76.0	24.0	6.32	2.00	8.32	70.0	4.0	62	2.30
59.8	40.2	5.96	4.00	9.96	70.0	8.0	101	2.61
48.4	51.6	5.63	6.00	11.63	70.0	12.0	129	2.59
39.6	60.4	5.24	8.00	13.24	70.0	16.0	158	2.69
33.2	66.8	4.96	10.00	14.96	70.0	20.0	154	2.55
57.9	42.1	5.64	4.10	9.74	70.2	8.2	100	2.63
15.8	84.2	2.14	11.39	13.53	32.4	22.9	141	2.40
15.1	84.9	2.26	12.70	14.96	36.0	25.4	139	2.21

[a] Average initial weight: 47 g (6 animals per group).

Table 3. Complementation between the proteins of Formula 14 and skim milk at two levels of protein in the diet.

Percentage protein distribution in diet		Protein distribution in diet		Weight distribution in diet[a]		Protein in diet (%)	Weight gain (g)	PER
Formula 14	Skim milk	Formula 14	Skim milk	Formula 14	Skim milk			
100	0	9.65	0.00	36.40	0.0	9.65	123	2.79
80	20	8.00	2.00	29.12	6.06	10.00	135	2.88
60	40	6.12	4.08	21.84	12.12	10.20	122	2.84
40	60	4.20	6.30	14.56	18.18	10.50	137	2.95
20	80	2.23	8.92	7.28	24.24	11.16	131	2.82
0	100	0.00	12.00	0.00	30.30	12.00	129	2.73
100	0	19.10	0.00	72.80	0.00	19.10	143	1.77
80	20	15.92	3.98	58.24	12.12	19.90	151	1.76
60	40	12.00	8.00	43.68	24.24	20.00	154	1.83
40	60	8.12	12.18	29.12	36.36	20.30	157	1.81
20	80	4.00	16.00	14.56	48.48	20.00	146	1.84
0	100	0.00	20.80	0.00	60.60	20.80	138	1.67

[a] The diets were supplemented with: 4.0% mineral mixture (Hegsted *et al.*, 1941), 5% refined cottonseed oil, 1% cod liver oil, and cornstarch to adjust to 100%. All diets were also supplemented with 4 ml of a complete vitamin solution (Manna and Hauge, 1953) per 100 g.

sted *et al.*, 1941), 1% cod liver oil, and 4 ml of a complete vitamin solution (Manna and Hauge, 1953) per 100 g of diet. The Formula 14 diet contained 3.62% nitrogen and 435 calories per 100 g. The N-free diet contained 435 calories per 100 g.

Each level of protein was fed for 8 days, providing two 4-day balance periods. Feces and urine were pooled every 4 days and homogenized before obtaining a subsample for nitrogen analysis. From the nitrogen intake and fecal and urinary nitrogen excretion, nitrogen absorption and balance were calculated.

RESULTS

Complementation between corn and soybean proteins. Table 1 shows the results of the first study designed to determine the best protein combination between corn and soybean protein. From

Table 4. Limiting amino acids in mixture 14 at two levels of protein in the diet.[a]

Amino acid supplement	Protein in diet (%)	Weight gain (g)	PER
None	10.10	110	2.56
+ 0.20% L-lysine HCl	10.30	93	2.35
+ 0.20% L-threonine	10.80	102	2.31
+ 0.20% DL-methionine	10.10	139	2.93
+ 0.20% L-lysine HCl + 0.20% L-threonine	10.10	121	2.69
+ 0.20% L-lysine HCl + 0.20% DL-methionine	10.40	126	2.74
+ 0.20% L-threonine + 0.20% DL-methionine	10.00	142	3.05
+ 0.20% L-lysine HCl + 0.20% L-threonine + 0.20% DL-methionine	10.10	164	3.39
None	18.04	145	2.01
+ 0.20% L-lysine HCl	18.36	156	1.99
+ 0.20% L-threonine	17.80	169	2.11
+ 0.20% DL-methionine	18.00	171	2.20
+ 0.20% L-lysine HCl + 0.20% L-threonine	17.34	162	2.11
+ 0.20% L-lysine HCl + 0.20% DL-methionine	17.00	168	2.25
+ 0.20% L-threonine + 0.20% DL-methionine	17.56	178	2.30
+ 0.20% L-lysine HCl + 0.20% L-threonine + 0.20% DL-methionine	18.05	164	2.19

[a] All diets were supplemented with: 4.0% mineral mixture (Hegsted *et al.*, 1941), 5% refined cottonseed oil, 1% cod liver oil, and cornstarch to adjust to 100%. All diets were also supplemented with 4 ml of a complete vitamin solution (Manna and Hauge, 1953) per 100 g.

both weight gained and PER values, it is evident that the proteins complement each other when corn provides 20–40% of the protein of the diet and soybean flour 60–80%. From these results, and by selecting the higher level of soybean flour and the lower of corn, Mixture 14 was formulated to consist of a mixture of 58 g of corn and 38 g of soybean flour per 100 g of mixture.

Table 2 shows the supplementary effect of soybean flour to corn. Complementation of soybean and corn proteins occurred as before when 60% of the dietary protein was derived from soybean and 40% from corn. It can be seen from the PER values that supplementation was optimum when 8.0% soybean flour was added to 70% corn. Larger amounts of soybean flour did not increase PER, although weight gain increased up to a level of 16% soybean flour in the diet.

Fig. 1A shows the relation between nitrogen intake and nitrogen retained, while Fig. 1B shows the relation between nitrogen absorbed and nitrogen retained in four days as carried out in dogs. From the slope of the lines, the nitrogen balance index was found to be 0.62 for the mixture of 56 g of corn and 38 g of soybean flour. Nitrogen equilibrium is obtained at a nitrogen intake of 230 mg/kg body weight/day. The biological value of Formula 14 was 73.1 calculated from the NBI, and 73.3% from the direct determination. Apparent and true protein digestibilities were respectively 78.6 and 80.2%.

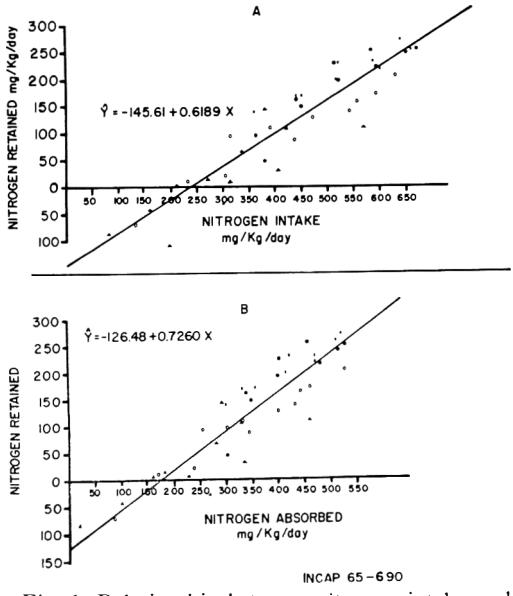

Fig. 1. Relationship between nitrogen intake and nitrogen balance and between nitrogen intake and nitrogen absorbed in dogs fed Mixture 14.

● dog No. 36; × dog No. 37; o dog No. 38; ▲ dog No. 39.

A) $\hat{Y} = -145.61 + 0.6189\ X$

B) $\hat{Y} = 126.48 + 0.7260\ X$

Dogs fed decreasing levels of a casein diet gave a regression equation equal to $\hat{Y} = -90.28 + 0.64\ X$ when nitrogen intake was plotted against nitrogen balance, and of $\hat{Y} = -82.43 + 0.69\ X$ when nitrogen absorbed was plotted against nitrogen balance ($\hat{Y}$ = nitrogen retention; X = nitrogen intake). On this basis, the protein value of Formula 14 was found to be very close to the nutritive value of casein.

Table 3 shows results based on the best protein combination between Formula 14 and skim milk at two levels of protein in the diet. It appears, from both weight gained in 28 days and PER values, that all combinations between Formula 14 and skim milk have a high nutritional value, although slightly higher values for both parameters were found when Formula 14 provided 40% of the dietary protein and skim milk 60%.

Table 4 presents the results on the amino acid supplementation of Formula 14 at two levels of dietary protein. The addition of methionine caused a significant increase in both weight gained and PER at the lower level of protein in the diet, while the addition of lysine and of threonine alone caused a slight decrease in PER and weight gained. The addition of the three amino acids together brought about a highly significant response in both weight gained and PER over the unsupplemented diet and the diet supplemented with methionine. Amino acid supplementation at the higher level of dietary protein brought about only a small increase in weight gained and PER.

DISCUSSION

The findings of the present investigation show that soybean flour protein and corn proteins complement each other, yielding a mixture having a nutritive value higher than that of either component fed alone. The type of response obtained when the protein of one component is replaced by the protein of the second component in isoproteic diets is different from the response obtained from mixtures of cottonseed flour and corn proteins (Bressani, 1965). In this case, replacement of cottonseed protein by corn protein results in a sharp decrease in the nutritive value of the mixture where corn protein contributes more than 60% of the protein in the diet.

Soybean protein supplements corn protein, as has been shown by several workers (Gilbert and Gillman, 1959), but the sup-

plementary effect is not as great as the complementary value between the two proteins, as indicated by the PER values. There is, therefore, a basic difference between the two methods of combining soybean and corn proteins. In one case, that of supplementation, soybean protein is supplying the limiting amino acids in corn protein; however, at the same time other amino acids are added in the form of protein, which may limit the effect of the beneficial amino acids, which in this case are lysine and tryptophan. The situation is similar to that taking place when a fixed level of casein is supplemented with increasing levels of gelatin (Harper, 1959), although the effect is not as marked. In the case of complementation, the amino acid pattern of one protein component adapts with the amino acid pattern of the other component, resulting in a more efficient mixture of amino acids in the form of protein.

A further difference between the two types of protein enrichment is that, with complementation, mixtures ranging in protein content of equal nutritive value from 8 to 30% can be formed, whereas with supplementation the protein content is limited by the amount of the supplement added, to give the highest PER.

From the results on complementation, Mixture 14 was developed. It consists of a mixture of 56% corn and 38% soybean flour. Since these mixtures are being developed for the supplementary feeding of infants and young children whose diets are also deficient in vitamins and minerals, 3% torula yeast is added, as with previous mixtures (Scrimshaw *et al.*, 1962), and 1% calcium phosphate as source of Ca and P. Since vitamin A has also been found deficient in rural diets (Flores *et al.*, 1964), 4,500 I.U. per 100 g are also added.

The NBI of this mixture is superior to that of the Vegetable Mixture 9 (Bressani, 1966) and very similar to that of casein. Furthermore, its true protein digestibility is slightly higher, which may be the reason that its nutritive value is higher than that of Vegetable Mixture 9.

Even though the PER of the mixture between corn and soybean is superior to that of either component fed alone, the mixture is deficient in methionine at low levels of protein in the diet. However, the cost of this amino acid is low, and if its addition is considered practical, it will have only a slight effect on the cost of the final product. Lysine and threonine increased the nutritive value of Mixture 14 only after supplementation with methionine to about the same degree. The addition of three amino acids which appear to be deficient in the mixture of soybean flour and corn, produced a blend of a relatively high protein quality.

From the complementation experiments between milk and Mixture 14, protein mixtures containing some animal protein may be developed. It was of interest to learn that only a small improvement in nutritional value was obtained when skim milk replaced the protein of Formula 14 in isoproteic diets. This may be due to the fact that, at low levels of protein in the diet, methionine would be also the first-limiting amino acid in skim milk, although it contains slightly more of it than in Formula 14. The use of milk has several advantages, and allows the larger distribution of more milk to more people suffering from protein malnutrition.

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Further Study on the Immunogenicity of Farm-Processed Cocoa

SUMMARY

Beans and pulp from cocoa were subjected to laboratory fermentation reproducing farm-processing conditions. A parallel was noted between the gradual decrease of the sugars content and the progressive disappearance of immunological activity when unfermented, incompletely-fermented, and fully-fermented bean extracts were compared. The saccharidic nature of the antigens was evidenced through staining with Schiff's reagent on gel electrophoretogram, paper chromatography, and acid hydrolysates.

One of the two antigens, Fraction A, is present in unfermented bean extracts; both antigens yield glucose only when they are subjected to an acid hydrolysis.

Dialysis showed that the only antigen (Fraction E) found in incompletely fermented bean extracts is a molecule smaller than that of the Fraction B found in unfermented extracts. Beans adequate for the chocolate industry did not show an immunogenic component. A rapid and simple ring test is proposed for determining the degree of fermentation (quality of the farm product) of cocoa beans.

INTRODUCTION

Levanon and Martelli (1964) noted that extracts of unfermented cocoa beans were immunogenic, while beans adequately fermented for the chocolate industry were not. Upon this basis, Levanon (1965) proposed a serological method for determining the degree of fermentation of cocoa beans. This method represents a test for cocoa bean classification which is influenced by personal bias less than in previous classification, and the basic test offers an accurate and reproducible procedure for controlling the quality of the farm product. In this respect, early classification of cocoa beans for industrial use was based upon the physical aspect of the whole beans or upon internal color determined after sectioning. The present paper records a further study of the immunogenicity of farm-processed cocoa beans.

MATERIALS AND METHODS

Beans were collected from the "Forastero," "Catongo," and "Criollo" varieties of *Theobroma cacao* plants grown at the Cocoa Experimental Station, Uruçuca, State of Bahia, Brazil. Beans of each variety were collected separately, rinsed with tap water to eliminate the pulp, then dried at 37°C, and finally stored at room temperature. In addition, commercial samples of cocoa beans were collected from export dealers in the cities of Itabuna and Ilheus (State of Bahia). In this respect, commercial samples are mixtures of beans from plants of several varieties. The estimation of bean quality was done according to De Witt (1953). Fruit pulp (cocoa honey) was collected separately from fruits of each variety in Erlenmeyer flasks and frozen at −45°C. Thawed pulp showed a pH of approximately 3.8.

A strain of *Saccharomyces* sp. isolated from fermentation boxes in the Cocoa Experimental Station at Uruçuca and maintained in Sabouraud medium was used in the laboratory fermentation. Portions of 200 ml of cocoa pulp were sterilized by filtration through a Seitz filter, placed in 1000-ml Erlenmeyer flasks, and incubated 72 hr at 37°C to disclose an eventual turbidity due to undesirable microbial growth. Two hundred unfermented cocoa beans, previously washed with a solution of 1% merthiolate and then with sterile distilled water, were added to each flask. These flasks were incubated with 1 ml of yeast-cell suspension whose turbidity was equivalent to tube no. 3 of the McFarland scale, and the flasks were incubated at room temperature. During fermentation, samples were withdrawn every 24 hr and the antigen was prepared and tested by an immunoprecipitation technique (see below). When advisable, bean extracts were concentrated by freeze-drying after being dissolved in the required volume of saline.

Laboratory preparations from fully fermented batches of cocoa beans were marked *F*; those from incompletely (partially) fermented batches, *PF*; preparations from unfermented batches, *NF*; and those from over-fermented batches, *OF*. Besides, the percentage of well fermented ("good-quality") beans contained in the batch was represented by a superscript number, while the duration of fermentation in hours was represented by a subscript number. For instance, PF_{72}^{50} indicates that this prep-

aration was made from a batch of beans fermented for 72 hr (partial fermentation, incomplete fermentation) and contained 70% good-quality cocoa beans.

Total polysaccharides were determined by the anthrone method (Trevelyan and Harrison, 1952), and polyphenols by the permanganate method (Forsyth, 1926). Fat substances were determined in 10-g samples by Soxhlet extractions and direct weighing.

Ascending chromatography was run on a strip of 36 x 11-cm Whatman no. 1 filter-paper using phenol-water (8+2) solvent to which 0.01% of Merck Triplex (sodium ethylenediaminetetraacetate) was added to avoid oxidation. Of the antigen solution tested, 0.01 ml was placed at the chromatography origin and processed for 24 hr. After drying at 90°C, the chromatogram was developed with the anthrone reagent (Sunderwirth *et al.*, 1964) or the anilin-diphenylaminophosphate reagent (E. Merck A.G.). The places corresponding to the previously developed spots on 25 paper strips were cut and eluted with 10 ml of distilled water by mechanical shaking overnight, then freeze-dried, and finally dissolved in 0.5 ml of saline solution.

A 30-cm-high 2.7-cm-diameter chromatographic glass column was filled with DEAE-cellulose (diethylaminoethylcellulose). This material was repeatedly washed with a 0.02*M* phosphate buffer until the washing had a pH of 7.4. Then, 5 ml of cocoa-pulp preparation were poured on the top of the column. This was adapted to a fraction collector, and samples of 6 ml were collected by adding 0.02*M* phosphate buffer of pH 7.4. Each tube was tested with the anthrone reagent. The tubes showing a positive reaction were pooled and labeled "Fraction *C*." The column was then eluted with 0.02*M* citrate buffer (pH 3.8), and the fractions which gave a positive anthrone test were pooled and labeled "Fraction *D*." Fractions *C* and *D* were dialyzed separately against distilled water, freeze-dried, and dissolved in 5 ml of saline. The fractions isolated from *PF* samples were labeled *E*.

Samples of 10 ml of the purified fractions containing 500 mg of polysaccharide were hydrolyzed by adding 4*M* HCl, to make a final two-molar concentration. The tubes were incubated for 6 hr in a boiling-water bath. After cooling, the pH of the solution was adjusted to 7.0 with 40% NaOH. Phenylosazones were prepared by adding 0.5 ml of phenylhydrazine and 0.5 ml of glacial acetic acid to 2 ml of the solution tested, followed by incubation in a boiling-water bath for 30 min. The crystals were tested for their specific melting point and form.

Antigens for injection of animals were prepared from *F* 144, *PF* 72 and *NF* by adding 90 ml of 0.15*M* solution of sodium chloride to 1 g of washed beans, and the mixture was homogenized in a Waring blender. It was then spun in a centrifuge for 20 min at 3000 rpm. The supernatant was separated and made up to 100 ml (1% solution). Antigens from fruit pulp were obtained by centrifugation of unfermented pulp for 30 min at 3000 rpm and separation of the clear, fiberless supernatant. Equal parts of this supernatant and Freund's adjuvant (1947) were emulsified for injection into animals. Each rabbit was injected with 0.5 ml, and each guinea pig with 0.25 ml of the emulsion in each foot pad at intervals of seven days during six weeks. Ten days after the last injection, the animals were bled and the serum was separated and stored in a refrigerator, after addition of merthiolate at the rate of 1 to 10000.

Specific precipitates were determined, according to Heidelberger and MacPherson (1943), in 0.5-ml portions of antigen preparation by adding anti-*NF* antiserum, followed by incubation at 37°C for 2 hr and later at 2°C for 18 hr. The precipitates were separated by centrifugation, and a ring test (see below) was carried out with various dilutions of antiserum. If antigen was still detectable, the absorption was repeated. The precipitates were washed three times with cold saline solution and dissolved in 0.1*N* NaOH. The nitrogen content of the antigen-antibody complex was determined by a micro-Kjeldahl method (Elec and Sobotka, 1926).

Absorption of immune serum was done by adding PF_{72}^{0} extracts to immune (anti-*NF*) sera followed by incubation for 30 min at 37°C and 18 hr at 2°C. The precipitate was separated by centrifugation, and a ring test was carried out with various dilutions of extract as antigen. If antibody was still detectable, the absorption was repeated.

Immunoprecipitation was done according to Ouchterlony (1949), and the immunoelectrophoresis and gel electrophoresis according to Grabar and Williams (1955) using veronal buffer of pH 8.6 $\mu = 0.05$. Bands were stained with the Schiff reagent (Uriel and Grabar, 1956).

RESULTS

Chemical analysis of beans. Table 1 shows nitrogen, fats, glycide, and polyphenols in beans of the Forastero variety. Data were essentially similar for the other varieties. It can be seen that the fermentation process does not modify the nitrogen content appreciably until it reaches the overfermentation stage, when there is a significant decrease in nitrogen. On the other hand, fermentation determines a progressive decline of the content of glycide and polyphenols (Table 1).

Chromatographic analysis of bean extract and fruit pulp. The paper chromatography of *NF*

Table 1. Chemical composition (g per 100 g) of cocoa beans extracts.

Sample	Nitrogen	Fats	Glyceride	Polyphenols	Others
OF_{102}	7.2	47.7	6.3	2.0	36.8
F_{144}	12.0	49.0	10.8	2.4	25.8
PF_{72}	12.0	50.7	15.3	7.2	14.8
NF	12.0	51.7	19.7	9.8	6.8

bean extracts and unfermented cocoa fruit pulp revealed two spots after treatment with the anthrone reagent: one, with $R_f = 0.37$, was denominated "Fraction B," and the other, with $R_f = 0.50$, was denominated "Fraction A." After staining with Merck's aniline-diphenylamine-phosphate reagent, Fraction A exhibited blue spots and Fraction B showed brown spots. According to the monography (E. Merck A.G.), this would indicate the presence of 1-4 linkages in polysaccharide A and of 1-6 linkages in polysaccharide B, though these results must be verified by other physicochemical methods. After hydrolysis, both purified Fractions A and B exhibited only one spot, with $R_f = 0.41$, corresponding to d-glucose. The eluates of the chromatographic spots at the level of Fractions A and B showed a neat positive "ring test." When these eluates were tested against the antisera on Ouchterlony plates, they yielded the corresponding precipitation lines. Similar tests of the hydrolysates did not yield precipitations.

Separations of antigens from fruit pulp. Because fruit pulp contains a larger proportion of Fraction A and B than beans, these antigenic fractions were separated in purified form by using the raw pulp as starting material. Preparations of fruit pulp yielded two antigenic fractions in chromatographic columns of DEAE-cellulose. The first fraction was eluted with phosphate buffer pH 7.4 and showed, in paper chromatography, an $R_f = 0.50$, equal to that of Fraction A. The second fraction was eluted with citrate buffer pH 3.6, and yielded a spot with $R_f = 0.37$, corresponding to Fraction B. Fractional precipitation with ethanol allowed separations of these fractions. With 1.5% ethanol, a fraction with $R_f = 0.50$ precipitated, while with 15% ethanol a fraction of $R_f = 0.37$ precipitated. This fractionation procedure did not yield results as neat as those of chromatographic separations.

When osazones were obtained from Fractions C and D, two different crystalline forms were observed. Fraction C osazone showed small square crystals whose melting point was 183.0°C, while Fraction D showed round crystals with wrinkled borders whose melting point was 198.5°C. The hydrolysates of Fractions C and D yielded needle-shaped osazones corresponding to glucosazone, and they had a melting point of 204.5°C. Fractions C and D from NF bean extracts or fruit pulp did not dialyze against saline solution or distilled water, while Fraction E separated from PF_{72}^{70} extracts dialyzed and showed an $R_f = 0.63$ in chromatograms.

The antibody-nitrogen content of specific precipitates. The antibody-nitrogen content of specific precipitates of unfermented bean extracts decreased in approximately linear relationship as fermentation time increased (Fig. 4). Precipitates of extracts fermented for 144 hr did not show detectable antibody-nitrogen.

Immunochemical analysis of bean extracts and fruit pulp. Rabbits injected with NF extracts yielded antisera (anti-NF) showing two precipitation lines on Ouchterlony plates when tested against NF extracts, one line against PF extracts, and no line against F or OF extracts. The PF_{72}^{70} extract yielded one precipitation line (Fraction E) when tested against anti-NF antiserum. This line corresponds to one of the components (Fraction B) of the NF extract or of cocoa fruit pulp (Fig. 3). The Fraction E in PF_{72}^{70} bean extract does not differ immunologically from the Fraction B present in NF extracts, in spite of these two fractions differing in immunologic properties, in R_f, and in behavior during dialysis. Determination of anti-NF antibody nitrogen yielded 0.8–1.0 mg of nitrogen per ml of antiserum, when a mixture of purified polysaccharides isolated from cocoa fruit pulp was used as antigen. Immunization of rabbits with a cocoa pulp preparation yielded an anti-M (anti-fruit pulp) antiserum behaving similarly to the anti-NF antiserum. The anti-M antiserum showed 0.8 mg of antibody nitrogen per ml. Finally, F and PF_{72}^{70} extracts dit not provoke response in precipitating antibody.

Immunoelectrophoresis yielded results (Fig. 2) in agreement with those of immunodiffusion on Ouchterlony plates (Fig. 3). In the presence of anti-NF antiserum, the NF extract showed one line, and the F extract exhibited no line. The two precipitation arcs observed in immunoelectrophoresis

corresponded to two bands stainable by the Schiff reagent in parallel tests on agar-gel electrophoresis. One of the bands was localized at the level of the origin, and another band was oriented toward the anode (Fig. 1).

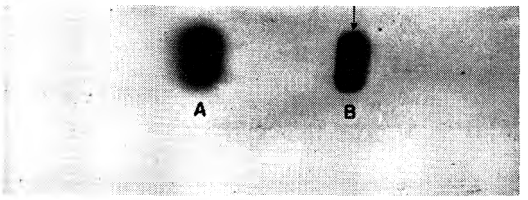

Fig. 1. Electrophoresis of cocoa immunologically active substances developed with Schiff's reagent. *A*) Fraction *A*; B) Fraction *B*. Electrophoretic migration 120 min, 8 V per cm.

Ring test for determining degree of fermentation. Ring tests (immunoprecipitation in a capillary tube) were done by carefully layering an antigen solution over Anti-fraction *A* antiserum in capillary tubes. The appearance of a definite turbidity in the interphase was taken as a positive result. A negative ring test indicated absence of Fraction *A*, and this result was interpreted as corresponding to "good-quality" cocoa beans in the classification of De Witt (1953), because of the findings made in the immunochemical analysis of bean extracts and fruit pulp. Anti-*NF* antiserum absorbed with purified Fraction *B* yielded essentially the same results, but the procedure was more complicated. Anti-fraction A may be obtained by injecting purified Fraction *A*, in which case the absorption of the antiserum would not be necessary. Anti-*NF* antiserum might also be absorbed with extracts of incompletely fermented (*PF*) beans that previously demonstrated only one line on Ouchterlony plates.

DISCUSSION

The amount of sugars and polyphenolic compounds decreased as the degree of fermentation increased. The parallelism between the gradual decrease of the glycides and the progressive disappearance of the immunological activity is explainable by the polysaccharide nature of the antigens. This was evidenced through specific staining with Schiff's reagent in the gel-electrophoretogram, paper chromatography, and in the hydrolysate.

The absence of cross-reaction between the two antigens evidenced in unfermented cocoa bean extracts or fruit pulp may be attributed to the fact that one of the antigens, Fraction *A*, could perhaps contain 1-4 linkages while the other antigen, Fraction *B*, could contain 1-6 linkages; these two polymers are formed only by glucose units as shown by acid hydrolysis, osazone formation, chromatographic and electrophoretic analyses.

Dialysis evidenced that the only antigen (Fraction *E*) found in incompletely fermented cocoa bean extract is a molecule smaller than that of Fraction *B* found in unfermented extracts. This would explain the failure of Fraction *E*, present in incompletely fermented extracts, to provoke precipitating antibody response in rabbits. Since fermentation of cocoa beans and cocoa fruit pulp results from enzymatic action, it might be presumed that at first there is a depolymerization of a macromolecule, and, at the beginning of this depolymerization, the resulting product could not stimulate production of precipitating antibodies

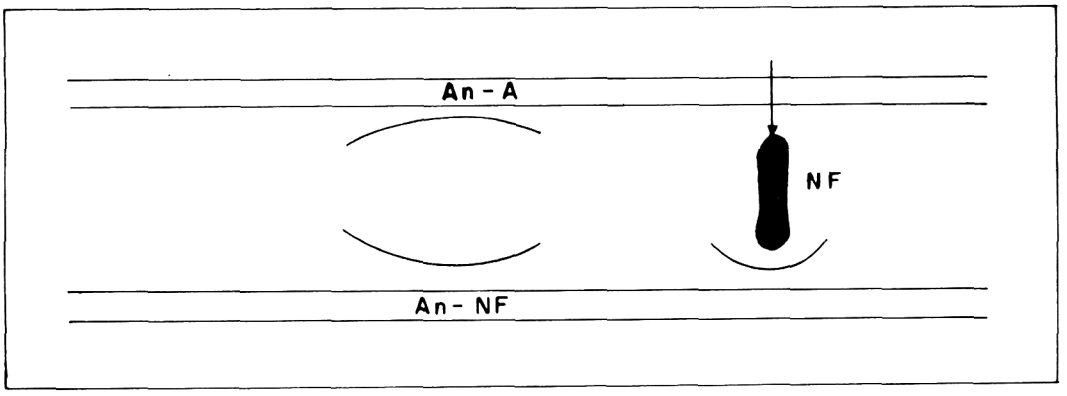

Fig. 2. Immunoelectrophoresis of *NF* extract of cocoa beans. *NF*: unfermented extract. *An-A*: antiserum anti-*NF* previously absorbed with incompletely fermented (*PF*) extract. *An-NF*: antiserum anti-unfermented extract. Electrophoretic migration 120 min, 8 V per cm.

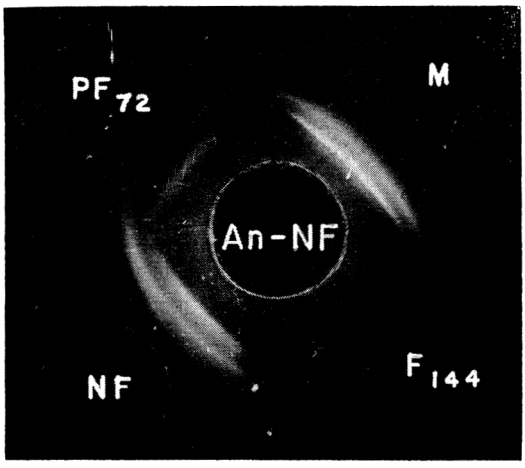

Fig. 3. Comparative double-diffusion test of cocoa fruit pulp and completely fermented, incompletely fermented, and unfermented cocoa bean extracts. *M*: cocoa fruit pulp; *NF*: unfermented cocoa bean extract; PF_{72}: incompletely fermented cocoa bean extract; F_{144}: Fully fermented cocoa bean extract; *An-NF*: antiserum anti-unfermented cocoa beans.

although it would provoke a precipitation reaction. Later, when fermentation is fully accomplished, no immunoprecipitation could be observed. It seems clear that fermentation of cocoa beans destroys the antigenic molecules and also modifies various components of the cocoa bean and cocoa fruit pulp. These components are necessary for the growth of microorganisms responsible for the production of the enzymes yielding the chocolate flavor and the acetic acid permeating the tegument (Levanon and Rossetini, 1965).

It is known that excessive fermentation ("overfermentation") of cocoa beans yields NH_3. This probably happens because the main factors for microbial growth are carbohydrates (fermentation), and when these substances are exhausted the adoptive enzymes begin to metabolize the nitrogen-containing compounds (putrification). This hypothesis is supported, in the present study, by a decrease of nitrogen content in the "overfermented" bean extracts. Besides, the antigen of bean extracts decreased as the fermentation progressed. First, Fraction *A* was inactivated from the standpoint of immunoprecipitation, and, later, Fraction *B* was inactivated. It may be noted that well fermented extracts of commercial batches of cocoa beans do not contain Fraction *A*, thus differing from insufficiently fermented samples (Levanon and Martelli, 1964).

It seems that the disappearance of the antigen here called Fraction *A* indicates the end of the

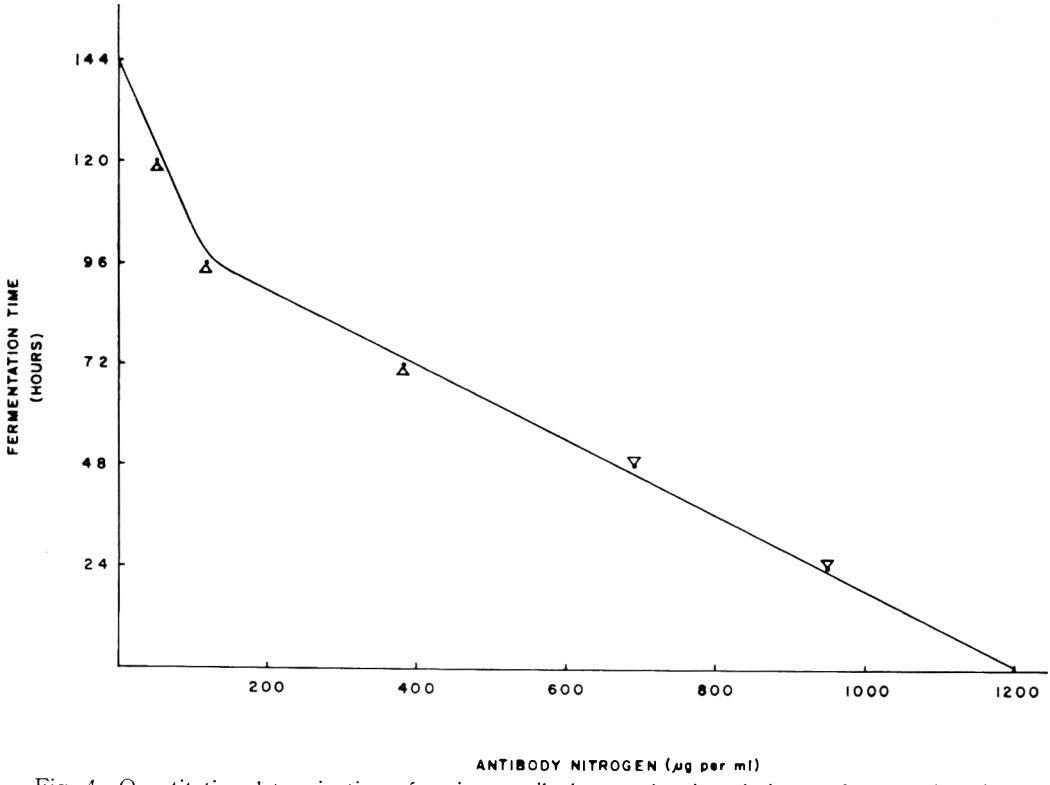

Fig. 4. Quantitative determination of antigen-antibody complex in relation to fermentation time.

biofermentation stage described by Levanon and Rossetini (1965). As this moment, the beans are ready to be withdrawn from the fermentation boxes or piles, and the "curing" begins. It thus appears that the presence or absence of Fraction *A* may be used to determine the degree of fermentation of cocoa beans. Levanon and Martelli (1964) tested commercial batches of "good-quality" beans and evidenced only one precipitation line on Ouchterlony plates. The corresponding antigen (Levanon and Martelli's Fraction *B*) did not stimulate antibody production and was dialyzable. Even if concentrated twenty times, the well-fermented extracts of bean batches tested in the present work failed to show the antigen corresponding to Fraction *A*. It is worthy of mention that commercial batches used by Levanon and Martelli (1964) were composed generally of 70–90% good fermented cocoa beans and that the samples used in the present study were fermented in the laboratory, this yielding samples of known uniform degree of fermentation.

The gel immunoprecipitation technique used by Levanon and Martelli (1964) requires a relative long time, some 24 hr, while the ring test employed in the present work requires only a few minutes. The latter test may be useful in two steps of the cocoa bean preparation for industrial use. When the beans are still in the fermentation boxes or piles, the ring test may determine the end of the required biofermentation step. "Cured" cocoa beans may also be tested to determine if the previous fermentation was sufficient, since the chemofermentation does not destroy the antigens (Levanon and Martelli, 1964).

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Direct Olfactory Demonstration of Fractions of Chicken Aroma

SUMMARY

A general method of dividing and subjectively evaluating the fractions of cooked chicken aroma was developed. The fractions were separated by passing nitrogen gas laden with the freshly formed aroma through solid absorbents or solutions of metal salts. The unabsorbed effluents were then characterized or identified in terms of the dominant odor by a trained panel. Absorbents were selected for their ability to permit passage of easily identified aroma fractions and for their general inability to form, react with, or concentrate on a volume basis the unabsorbed fractions of interest. The role of hydrogen sulfide was established in two ways: 1) when essentially all aroma constituents except hydrogen sulfide were removed from the nitrogen stream by anhydrous calcium sulfate (or calcium chloride or calcium carbonate), the residual aroma was easily recognized as hydrogen sulfide; and 2) when hydrogen sulfide was removed by any of a variety of heavy metal salts, a completely foreign disagreeable aroma remained that indicated the blending or masking effect of the hydrogen sulfide. When hydrogen sulfide and other components were removed by magnesium oxide, an ammoniacal odor characteristic of ammonia or aliphatic amines was exposed. Many absorbents or combinations of absorbents were found that trapped all fractions of the cooked chicken aroma.

INTRODUCTION

In an editorial, Stewart (1963) called attention to the critical challenge in flavor research—to balance chemical identification studies with tests of sensory significance. His remarks are particularly pertinent to the field of research on poultry flavor, where more than 100 chemical constituents of cooked poultry volatiles have been separated (Pippen, 1965), but very few if any have been unequivocally shown to contribute significantly to the blend that we recognize as cooked-chicken aroma. Many investigators have concentrated, trapped, and identified volatiles from cooking chicken, but none has clearly demonstrated the importance of any volatile at its concentration in the vapors over cooking or cooked chicken.

Minor *et al.* (1965a,b), in a detailed study of the volatile constituents of cooking chicken, subjectively characterized a "meaty odor" that was eliminated by either mercuric chloride or cyanide solution traps, and a "chickeny aroma" that was removed by dinitrophenyl hydrazine traps, and which they attributed to carbonyls. Since the dinitrophenyl hydrazine is used in fairly strong acid media, it would also remove amines and other bases present.

In the present study, certain gross chemical fractions from aroma-laden nitrogen in equilibrium with cooking chicken were absorbed by solid absorbents or solutions, and panelists then smelled the unabsorbed effluent gaseous mixtures. In general, solid absorbents were used that would neither chemically modify the unabsorbed components nor concentrate them on a volume basis. Hence, we believe that familiar simple odors such as that of hydrogen sulfide or ammonia that were easily recognized in the unabsorbed fractions of the aroma make a contribution to the unfractionated whole aroma, although they could not be identified in the original complete mixture. This approach to the analysis of complex aromas is in some respects fundamentally different from previous and current ones in which gas chromatography, infrared and mass spectrophotometry, and odor threshold tests have been used, first to identify and measure chemical constituents, and subsequently to attempt to determine quantitative relationships between amount present and minimum amount detectable under some arbitrary condition of smelling. In the first place, the present approach utilizes the nose immediately after the aroma fractionation to characterize gross fractions in terms of known odors of compounds previously established to be present in the total volatiles [hydrogen sulfide and ammonia (amines) in cooked chicken volatiles]. Secondly, conditions are maintained in the fractionation that

will minimize the formation of artifacts such as might develop under the high temperatures of some gas chromatographic analyses. Fractionations are carried out at room temperature on relatively inert solids such as calcium carbonate and calcium sulfate. Finally, under the present approach, the unabsorbed aroma fractions of interest come through the absorbent immediately, without a time lag that might indicate absorption and then elution at a higher concentration on a gas volume basis. Hence, on a gas volume basis, the concentration at which the unabsorbed aroma fraction is smelled is no greater than the concentration at which it existed in the original complete aroma blend. In contrast, gas chromatography, through fractional absorption and elution, is almost sure to drastically modify the concentration of a particular component on a gas volume basis.

PROCEDURES

The general procedure is illustrated in Fig. 1, and a typical arrangement of the apparatus is shown in Fig. 2. Diced breast or thigh meat, free of gross fatty tissue, from commercial cut-up broiler chickens held at −10°F 1–2 months prior to use, is added to twice its weight of cold tap water in a 1-L round-bottomed flask. Very pure water-pumped nitrogen gas, further treated by successive passages through activated alumina and water, bubbles through the mixture of meat and water for about 5 min. The temperature of the mixture is then raised to boiling, by a heating mantle. The mixture is heated intermittently for about 10 min, until the foaming stops. Heat is then reduced by means of a variable transformer to maintain a gentle simmer throughout the remainder of the experiment. The nitrogen gas passes through the simmering mixture of meat and water, where it picks up a burden of volatiles that represents a characteristic cooking chicken-broth aroma, and then the aroma-laden nitrogen flows through a short, tap-water-cooled reflux condenser, then through a sintered disc to a column of the selected absorbent, and finally out through a cup from which the effluent can be smelled at intervals by removing a glass cap. During a sensory evaluation of the effluent the entire apparatus except the smelling cup is shielded from the panel's view. Rate of flow of nitrogen through the system is regulated by a needle valve at the tank and is measured by a ball flow meter inserted between the initial alumina column and the water scrubbing tower.

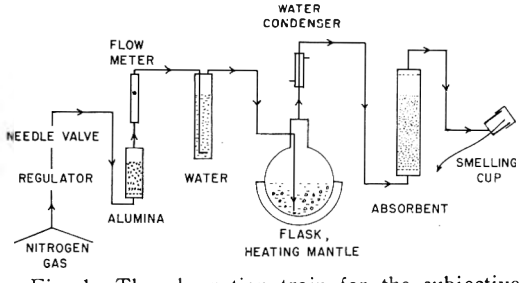

Fig. 1. The absorption train for the subjective evaluation of effluent aroma fractions from cooking chicken. Flask contains simmering mixture of 1 part chicken muscle and 2 parts water.

We chose nitrogen, rather than air, as a carrier gas, to limit the aroma to those compounds that would form under relatively non-oxidative conditions. Preliminary trials, in which nitrogen and air were compared, indicated very little difference in effluent aroma during the first 1–2 hr of cooking. The aroma that formed in the presence of nitrogen had a characteristic chicken broth odor.

The odor panel was 8–10 laboratory workers unselected for acuity in detection or identification of odors. Each panel member was made familiar with the following odors by repeated exposures to controlled concentrations of known identified materials. The panel was acquainted with the odorless nitrogen stream, and the positive control, that is, the aroma carried by the nitrogen stream after passing through the simmering meat and a short condenser but through no traps. Recognition of low concentrations of hydrogen sulfide and ammonia was established by introducing, from condensed sources in liquid nitrogen (Fig. 3), known partial pressures of these gases into a 5-L bulb and making the total pressure up to atmospheric with highly pure nitrogen. The final gaseous mixture was displaced from the 5-L bulb to a smelling cup by highly pure nitrogen. Based on the nitrogen flow rate (about 100 ml/min) and the total elapsed time during which the nitrogen was flowing and the panel was smelling the displaced gas (5–8 min), the maximum dilution of the displaced mixture by the propelling gas would amount to less than a 20% decrease in concentration of hydrogen sulfide (or ammonia) on a volume basis. Panel members detected and identified concentrations ranging from 0.1 to 0.3 μ pressure of hydrogen sulfide, and 100 to 200 μ of ammonia. Although no attempt was made to establish individual thresholds, thresholds evidently varied widely between members within the panel.

Unknown vapor samples were submitted to the panel of 9 members in random order and with at least 4 replications per treatment.

Chemicals used were reagent grade. The calcium sulfate was an anhydrous type used as a desiccant, and was 8-mesh.

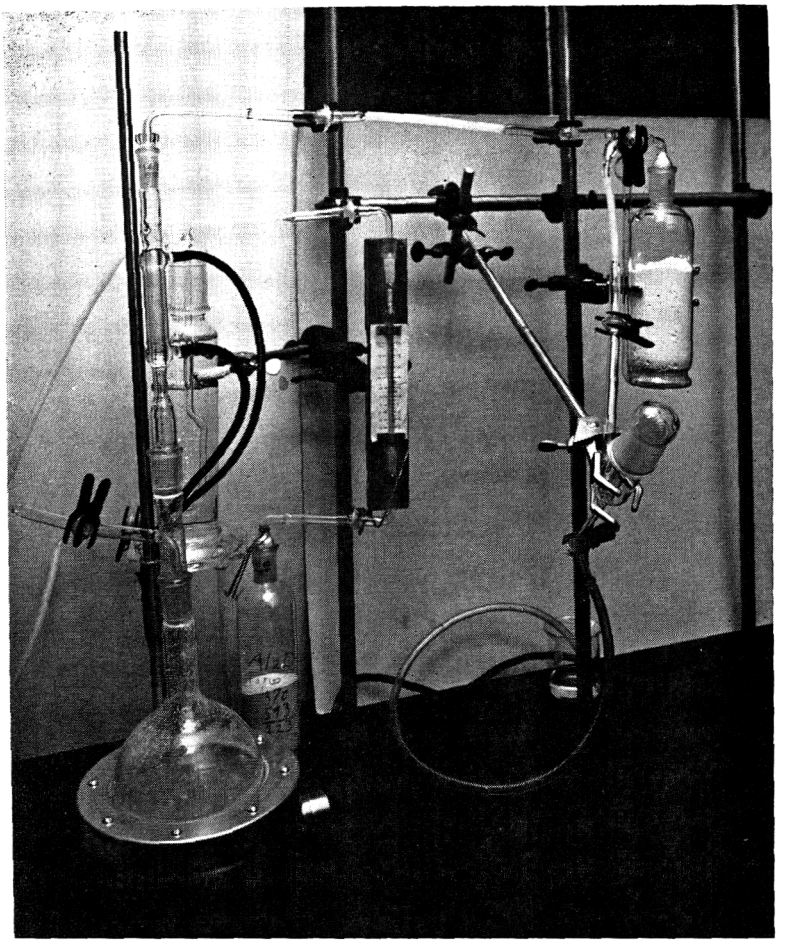

Fig. 2. Photograph of apparatus diagrammed in Fig. 1. Flask containing cooking chicken is on extreme left; absorbent column and smelling cup are on extreme right.

RESULTS AND DISCUSSION

Fractionation of a hydrogen-sulfide-like aroma and an ammoniacal aroma. Panel results which established the contribution of these two fractions to cooked chicken aroma are in Table 1. Panel members had no difficulty in recognizing the typical hydrogen sulfide odor of the effluent from the calcium sulfate column. When the calcium sulfate column was connected to the condenser exit from which the nitrogen-chicken aroma was flowing, the time lag before hydrogen sulfide could be detected in the effluent stream from the calcium sulfate (about 2–3 min) corresponded roughly to the time required to displace the air in the trap, smelling cup, and connecting lines. Thus there was no opportunity for the hydrogen sulfide to be concentrated on a volume basis before smelling. The calcium sulfate column had a great capacity for the fraction that it absorbed, so that the effluent hydrogen-sulfide-like aroma fraction flowed continuously for many hours. Dark meat produced a higher concentration of hydrogen sulfide than light meat, in correspondence with results of Mecchi *et al.* (1964). Recognition of an ammoniacal aroma in the effluent gas from the magnesium oxide column was somewhat difficult for those members of the panel who had higher threshold values for pure ammonia. Here again, the dark meat produced higher concentrations of ammoniacal components than did the light meat. The panel's recognition of an ammoniacal aroma could involve many aliphatic amines in addition to ammonia.

Several compounds absorbed aroma fractions in the same way as anhydrous calcium sulfate: anhydrous calcium chloride, anhydrous calcium carbonate, and, to a limited

Table 1. Identification of aroma fractions from chicken cooking in water.

Sample	No. of judgments	Identification
Unfractionated control		
Dark meat	33	28 as control
Light meat	43	43 as control
Fraction through calcium sulfate		
Dark meat	24	24 as hydrogen sulfide
Light meat	44	44 as hydrogen sulfide
Fraction through magnesium oxide		
Dark meat	51	38 as ammonia
Light meat	33	22 as ammonia

extent, phosphorus pentoxide and 0.5*N* hydrochloric acid. This list suggests that the fraction which blends with and masks the hydrogen sulfide in the total aroma, and which is absorbed by all of these reagents, is predominantly basic. However, it is well known that calcium can complex many compounds other than basic amines, including hydroxyl and carbonyl compounds. A large number of carbonyl compounds have been found in volatiles from cooking chicken (Pippen, 1965).

Effect of heavy metal ions. To provide supporting evidence for the contribution of hydrogen sulfide, a series of informal odor tests were made on the effluents obtained by passing the total aroma-laden nitrogen through aqueous solutions of heavy metal ions that have small solubility products for their sulfides. The salts included manganous chloride, ferrous chloride, nickelous chloride, cobaltous chloride, zinc chloride, cadmium chloride, lead chloride, cupric sulfate, mercurous nitrate, cuprous chloride, and silver nitrate, all listed in decreasing order of sulfide solubility products. Concentrations ranged from 10^{-1} to 10^{-4} Molal. In general the effluent fraction coming through the solution of metal salt had a characteristic unpleasant aroma not identified with any compound or type of compound, and therefore herein referred to as aroma fraction *X*. The metal salts of low sulfide solubility product produced an easily detectable modification of the aroma at considerably lower concentration than did the salts of metals with high solubility product (10^{-3} to 10^{-4} *M* for cuprous, compared to 10^{-1} to 10^{-2} *M* for manganous). Many of the ions can both remove hydrogen sulfide and also complex ammonia, amines, and other compounds. Only one, cuprous chloride in complete equilibration with the entering aroma constituents, could remove essentially all of the aroma components.

Effects of combinations of absorbents. The two groups of absorbents discussed above had somewhat opposite effects: the

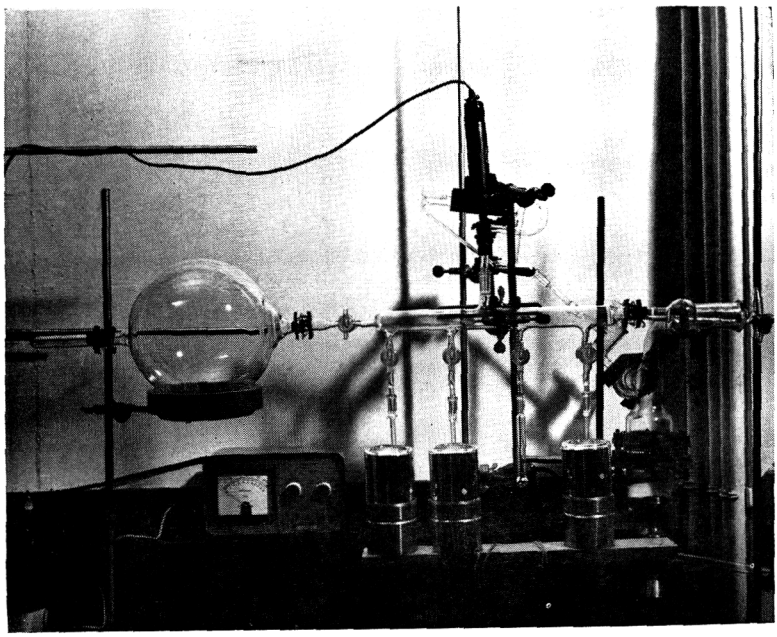

Fig. 3. Vacuum system designed to introduce known partial pressures of compounds condensable in liquid nitrogen into 5-L bulb for odor identification. Partial pressure was reduced, step by step, below range of pirani vacuum gauge by first establishing pressure in manifold above condensed sources in Dewar flasks, then equilibrating contents of manifold with evacuated 5-L bulb.

calcium salts and acids removed enough of certain aroma components so that hydrogen sulfide could be easily identified; the metal salts removed enough hydrogen sulfide (and possibly other components) to expose a completely foreign aroma, fraction *X*. Magnesium oxide does not fit into either category: it appears to absorb hydrogen sulfide and some of *X*, to expose a clearly ammoniacal aroma fraction. It was of interest at this stage to determine what absorbents or combinations of absorbents in series would be able to absorb all of the aroma components from the complete cooked chicken aroma. These are listed in Table 2, in three groups. First are the single solid absorbents which have both the basic quality to hold back the acidic, hydrogen sulfide, fraction, and a complexing quality necessary to trap the amine-type fraction. The second group represents the combinations of two solid absorbents, one metallic or fairly basic to trap the hydrogen sulfide fraction, and the other to complex the amine fraction. The third group is essentially the same as the second, except that the heavy-metal salts were in the form of a dilute solution. It is of interest that cuprous chloride, when permitted to thoroughly equilibrate with the effluent aroma, can hold back essentially all of the aroma. This suggests that cuprous ion forms a weak complex with fraction *X*.

Table 2. Absorbent systems that remove all aroma.

Alumina (activated, powder)
Calcium hydroxide (powder)
Barium oxide (powder)
Zinc oxide (powder) + calcium sulfate (anhydrous, granular)
Calcium sulfate (anhydrous, granular) + cupric oxide (granular)
Magnesium oxide (powder) + calcium sulfate [a] (anhydrous, granular)
Ferrous chloride (0.01*M*) + calcium sulfate anhydrous, granular)
Zinc chloride (0.01*M*) + calcium sulfate (anhydrous, granular)
Calcium sulfate (anhydrous, granular) + cobalt chloride (0.01*M*)
Cuprous chloride (0.01 mole/L) [b] + calcium sulfate (anhydrous, granular)
Cuprous chloride (0.01 mole/L) [b] + calcium chloride (anhydrous, granular)
Cuprous chloride (0.01 mole/L) [b] with slow gas flow rate to give complete equilibration

[a] A faint cabbage odor, suggestive of methyl mercaptan, came through this system after a period of several hours.

[b] 0.01 mole of cuprous chloride added to 1 L of water, not all soluble.

Recombination. Although the various fractions developed in these studies have been recombined and smelled, to a limited extent the subjective estimates of the recombinations in terms of original aroma are too indefinite to warrant discussion at this time. Efforts will continue in this direction.

The trapping reagents were smelled by the senior author when the absorption train was dismantled after each experiment. Reagents which were absorbing all of the influent coming to them, of course, had no odor. Reagents which were allowing a certain fraction to pass through them always had a trace odor of this fraction. Absorbents carrying a load of the absorbed fraction *X* below their saturation point had no odor corresponding to this fraction; however, the odor could be developed at high concentrations by heating the absorbent with a stream of nitrogen passing through, or by adding water to the absorbent and then heating to boiling with a stream of nitrogen passing through. Examination of these recovered fractions is in progress.

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