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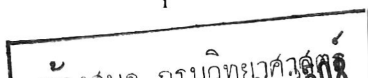

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University of Wisconsin, Madison, Wisconsin 53706

Porcine Muscle Properties. A. Alteration of Glycolysis by Artificially Induced Changes in Ambient Temperature

SUMMARY

Relatively mild preslaughter temperature treatments, involving warm air, cold air, and cold air with an ice-water spray, were administered, singly and in various combinations, to simulate extremes in environmental conditions and fluctuations. Cold-air treatment improved mean 24-hr color-structure scores and expressible-juice ratios, although a few animals responded adversely to this treatment given singly. Improvement in these parameters was the most significant and consistent in the muscles of pigs subjected to a change from a warm-air to cold-air environment. Rate of pH decline was significantly slower ($P < .05$) in muscles of pigs from groups involving warm-to-cold treatments than in control animals. Although glycogen and lactic acid levels at death were altered by treatment, glycogen levels were decreased the most by the warm-to-cold treatments. These improvements resulting from a change in environment from warm to cold, were observed even though the treatments were not severe enough to cause a decrease in muscle temperature. Thus, a sudden change from a warm-air to a cold-air antemortem temperature, even when of short duration and not severe enough to reduce muscle temperature at death to levels below normal, altered the post-mortem glycolytic rate and associated properties of porcine muscle, and improved meat quality.

INTRODUCTION

The rate of post-mortem glycolysis has been shown to be a major factor in determining the ultimate properties of porcine muscle (Briskey and Wismer-Pedersen, 1961a; Sayre *et al.*, 1963b; Sayre and Briskey, 1963). A rapid rate of post-mortem glycolysis, with the onset of rigor mortis occurring at a relative high temperature and low pH, resulted in pale, soft, exudative (PSE) muscle (Briskey and Wismer-Pedersen, 1961a; Briskey, 1963; Sayre and Briskey, 1963). When certain strains of pigs were exposed to a warm environment immediately prior to death, the post-mortem glycolytic rate was markedly accelerated and the musculature became extremely PSE (Sayre *et al.*, 1963b). Sybesma (1963) also reported a more rapid post-mortem pH decline in the musculature of pigs reared under conditions of high humidity.

Many workers (Wismer-Pedersen, 1959; Ludvigsen, 1954; Judge *et al.*, 1959; Osinka *et al.*, 1960) have noted higher incidence of PSE muscles in pigs exsanguinated during warm summer months than during winter months. Forrest *et al.* (1963) conducted a survey of incidence of PSE muscle over a 12-month period and reported a high incidence of PSE muscle during periods of large daily environmental change. Sayre *et al.* (1961) have also observed that subjecting pigs to a cold environment (ice-water bath) immediately before slaughter depleted initial muscle glycogen and resulted in higher post-mortem pH and darker color. Part of this response may be due to the fact that unacclimatized animals reportedly show a larger response to hypothermia than acclimatized animals (Guseva, 1962).

In more recent work, Kastenschmidt *et al.* (1964) found that a single severe, abrupt change in the pig's environment from warm (air) to cold (ice water) prior to slaughter retarded post-mortem glycolytic rate and greatly improved the gross morphology (color-structure), protein solubility, and water-binding capacity of the musculature. The present studies were conducted: 1) to evaluate the effects of mild temperatures and temperature changes on the concentration of glycogen and lactic acid in the muscles at death; and 2) to determine if a relatively mild change from a warm (air) to a cold (air) environment could, without decreasing the initial muscle temperature, alter the rate of post-mortem glycolysis.

EXPERIMENTAL

Forty-two market-weight Poland China pigs were allotted randomly among groups subjected to seven temperature treatments just prior to slaughter: I, control (no treatment); II, (warm)

45°C environment (30 min); III, (cold) −29°C environment (30 min); IV, (cold) −23°C environment plus intermittent cold (0–4°C) water spray (30 min); V, (treatment II followed immediately by III); VI, (treatment II followed immediately by IV); VII, 50°C environment (5 min) followed by −23°C environment plus cold spray (5 min).

Warm-air treatment was administered with the warm-temperature chamber described by Sayre *et al.* (1963b). All warm-temperature treatments were carried out at approximately 100% relative humidity. Cold treatment was administered with the cold-temperature chamber shown in Fig. 1. The cooling unit was a thermostatically controlled heat exchanger which was cooled by pumping ethanol, cooled with solid CO_2 to −55°C, through the coils. This device facilitated the maintenance of air temperature at −29°C for the duration of the treatment. A small hole in the door was used to deliver the cold (0–4°C) water spray.

Separate samples were excised, immediately post-mortem, from the left longissimus dorsi (LD) for determination of the time course of rigor mortis (Briskey *et al.*, 1962) and for determination of lactic acid and glycogen concentrations. The muscle samples for chemical analyses were frozen in liquid nitrogen immediately after excision, powdered with the Waring blender technique described by Borchert and Briskey (1965), and stored in liquid nitrogen until the analyses were completed. Lactic acid was determined by a slight modification of the method of Barker and Summerson (1941). Initial levels of glycogen were measured by the phenol-sulfuric acid method of Dubois (1956).

The temperature and pH of the right LD muscles were recorded at 0, ¼, ½, 1, 2, 3 and 24 hr post-mortem. The temperature measurements were taken with a probe thermometer at 0, ¼, and ½ hr. Subsequent measurements were made with thermocouples placed in the LD muscle at the area of the 12th thoracic vertebra. The pH values were obtained by placing a combination electrode directly on the freshly cut cross-sectional surface of the muscle fibers.

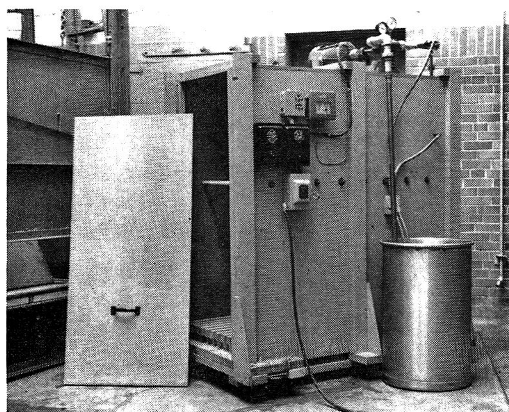

Fig. 1. Cold-temperature environmental chamber. Cooling system consisted of ethanol and dry ice.

Water-binding capacity of the LD was determined by the filter-paper method of Grau and Hamm as modified by Briskey *et al.* (1959). Values are expressed as the ratio of water area to meat film area. Thus, a larger ratio indicates an increase in the "exudative" condition of the muscle or a decrease in its water-binding capacity.

Objective color measurements were made at 24-hr post-mortem with a Bausch and Lomb "Spectronic 20" colorimeter with reflectance attachment. The color reflectance values (485 mμ) are reported as percent reflectance based on a magnesium carbonate block as a standard for 100% reflectance. Gross morphological or color-structure subjective scores were made by two independent judges on the 5-point scale of Forrest *et al.* (1963). A low score (0.5–2) indicated a PSE muscle; a score of 3 was considered normal; and 5 represented a dark, firm, dry muscle.

The thyroid and adrenal glands were removed within 30 min post-mortem, trimmed of excess membranes, blotted dry, and weighed.

All of the data were analyzed by Duncan's new multiple-range test (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Muscle temperature. Table 1 shows the muscle (LD) temperatures immediately after death of the animals. These data indicate, as shown in previous studies (Sayre *et al.*, 1963; Kastenschmidt *et al.*, 1964), that pigs are extremely sensitive to elevations in environmental temperatures. Animals in Lot II (warm) had a mean muscle temperature of 42°C, compared to 39.8°C for controls. The fact that these temperatures were lower than the mean of muscle temperatures from previously described ante-mortem temperature treatments (Kastenschmidt *et al.*, 1964) is not surprising, because the period of exposure was shorter. It is also appreciated that heat may affect animals in different ways. For example, within the warm-temperature treatment group it was observed that a visibly nervous pig developed a temperature of 43.9°C at death whereas a calm pig receiving the same treatment developed a muscle temperature of only 40.5°C. Of significance to the present work, Frankel *et al.* (1963) implied that the development of

Table 1. Treatment effect on post-mortem muscle temperature.

Post-mortem time period		Treatment						
		I	II	III	IV	V	VI	VII
0 hr	$\bar{x}$	39.8_{bc}	42.0_{a}	39.2_{c}	40.1_{b}	39.3_{bc}	39.6_{bc}	40.2_{b}
	$s_{\bar{x}}$	.44	.44	.49	.50	.72	.31	.61
¼ hr	$\bar{x}$	40.7_{b}	42.9_{a}	39.3_{d}	39.8_{c}	39.9_{c}	39.6_{c}	40.2_{bc}
	$s_{\bar{x}}$	.22	.50	.39	.22	.67	.24	.56
½ hr	$\bar{x}$	40.1_{b}	42.1_{a}	39.1_{c}	38.9_{c}	40.0_{b}	40.1_{b}	40.2_{b}
	$s_{\bar{x}}$	.28	.61	.56	.33	.39	.39	.56
1 hr	x	37.6_{b}	37.5_{a}	37.0_{b}	35.4_{c}	37.5_{b}	36.9_{b}	36.6_{b}
	$s_{\bar{x}}$	.56	1.22	.17	1.17	.67	.61	.56
2 hr	$\bar{x}$	30.4_{a}	29.9_{a}	30.2_{a}	27.0_{b}	29.4_{a}	29.6_{a}	29.3_{a}
	$s_{\bar{x}}$	.67	1.95	.94	1.11	1.39	.61	.78
3 hr	$\bar{x}$	24.3_{a}	25.1_{a}	24.2_{a}	22.2_{c}	23.5_{b}	23.2_{bc}	23.7_{ab}
	$s_{\bar{x}}$	.94	2.44	1.00	.94	1.05	.39	1.05

Explanation of table. Means having the same letter subscript are not significantly different at the 5% level of probability. The lower figure in each horizontal row ($s_{\bar{x}}$) is the standard error of the mean.

Treatments I, Control; II, warm; III, cold; IV, cold plus spray; V, warm to cold; VI, warm to cold plus spray; VII, short warm-cold.

tissue hypoxia, rather than external respiration failure, was the primary physiologic failure in progressive hyperthermic dogs. Temperature treatments have been shown by Forrest *et al.* (1964) to have a pronounced influence on respiration and heart rates in pigs. It is also pertinent that Ingram *et al.* (1963) observed marked increases in body temperature, heart rate, respiration, and blood pressure during high-humidity hyperthermia in Ayrshire bull calves.

Initial mean temperatures of LD muscles of the pigs from the other temperature-treatment groups were not significantly (P > .01) different from those of the control group. It is important to note that the muscles of animals which had elevated muscle temperatures prior to cold treatments (i.e., Lots V, VI, VII) were returned to, but not below, mean muscle temperatures of the control lot. The treatments involving cold air plus cold spray (VI), which were initially expected to be most severe, failed to lower mean muscle temperatures to levels found in the musculature of pigs subjected to treatments involving only cold air (III). Perhaps the increased excitement evoked by the spray resulted in more extensive thermogenesis in these animals. That excitement could have resulted in more extensive thermogenesis was suggested by the fact that one animal in treatment IV (cold + spray) which resisted confinement in the cold chamber had a muscle temperature of 42.2°C at death, compared to the mean of 40.0°C for the other animals in this treatment.

Initial glycogen levels. Fig. 2 shows the marked influences of preslaughter temperature treatment on the initial levels of glycogen in the musculature at time of death. The muscles from the pigs subjected to the warm-to-cold treatment (V) had a significantly lower glycogen level than the muscles of pigs from all other treatments except the warm-to-cold-plus-spray (Lot VI) treatment. This is similar to the effects of severe-warm-to-cold treatments reported by Kastenschmidt *et al.* (1964). The control animals (I) had the highest muscle glycogen levels, but they did not differ significantly (P > .05) from the cold (Lot III) or short warm-cold (Lot VII) treatment groups. These

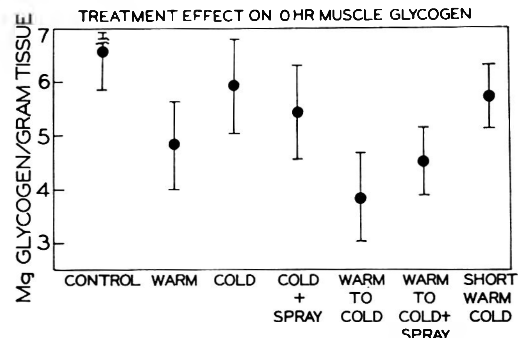

Fig. 2. Treatment effect on initial (0-hr) muscle glycogen.

findings agree with work of Kastenschmidt *et al.* (1964) and Sayre *et al.* (1961), who found decreased levels of muscle glycogen in pigs subjected to severe cold (water) treatments, although, as in the present work, glycogen levels were not significantly lower than in the controls. Animals subjected to warm treatment (II) had significantly less initial muscle glycogen than those in the cold (Lot III) or cold-spray (Lot IV) treatment groups. The severity of these changes is undoubtedly highly influenced by humidity in the chamber, which can markedly affect respiration and circulation changes (Ingram, 1963) as well as alter the rate of post-mortem pH decline in the muscle (Sybesma, 1963). The extent of anoxia in these tissues is not known, but it is pertinent that Morgan *et al.* (1963) observed an increased glucose uptake and phosphorylation of glucose in the anoxic isolated perfused rat heart.

It is of further interest that the mean level of glycogen in the LD muscle of the control pigs (Lot I) in the present study is in close agreement with the value obtained previously for cold-acclimated animals, while it is higher than the value obtained for unacclimated pigs (Kastenschmidt *et al.*, 1964). This is in further support of work of Guseva (1962), who noted higher glycogen levels in cold-acclimated animals. The mean muscle glycogen levels were not decreased as markedly by treatment as in previous work, which can be explained only on the basis that the animals were sensitive to the treatments used in the present study.

Initial lactic acid concentrations. Lactic acid values, expressed as mg/g fresh tissue, are shown in Fig. 3. At death, the lactic acid concentrations were significantly ($P < .01$) higher in the control (I), cold (III), and cold-spray (IV) treatment groups than in the warm (II) and warm-to-cold (V) treatment groups. Warm-to-cold-plus-spray (VI) and short warm-cold (Lot VII) groups also tended to have lower muscle lactic acid levels than Lots I (control), III, (cold) and IV (cold-spray). While lactate accumulation is the commonly accepted measure of the extent of glycolysis, it should be noted, as emphasized by Peterson *et al.* (1964), that α-glycerol phosphate formation may also be of importance in this evaluation.

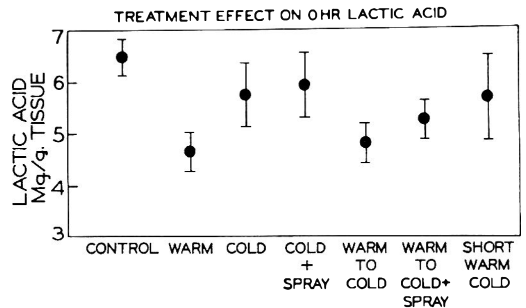

Fig. 3. Treatment effect on initial (0-hr) lactic acid.

Since all treatments involving heat-cold treatments tended to lower initial muscle lactic acid concentration, it is reasonable to postulate that the increased circulation presumably brought about by the humidity and heat allowed lactic acid to be removed from the muscle, resulting in lower initial lactic acid concentrations. This observation is supported, in part, by work of Forrest *et al.* (1965), who demonstrated major changes in respiration and heart rates on these same animals after heat treatment. One may also explain the low levels of lactic acid in the musculature of the pigs subjected only to the warm treatment on the assumption that the treatment was not severe enough to make the pigs anoxic or change the membrane permeability, whereas it may have been sufficiently severe in previous work of Sayre *et al.* (1963) and Kastenschmidt *et al.* (1964). The low initial lactic acid levels in the warm-to-cold and warm-to-cold-spray groups might also be due to a longer period of treatment, during which the lactic acid formed in the muscle could be transported to the liver.

The high initial concentration of lactic acid in the control group may be related to the rapid post-mortem changes in these muscles, since Briskey and Wismer-Pedersen (1961b) have observed that pig muscles characterized by a rapid post-mortem glycolytic rate also contained high levels of lactic acid in biopsy samples.

Rate of glycolysis. Fig. 4 shows the pH changes with time during the first 3 hr post-mortem. Ultimate pH values are shown in Table 2. The rate of post-mortem pH decline was most rapid in muscles of pigs in the control group (I), while treatments in-

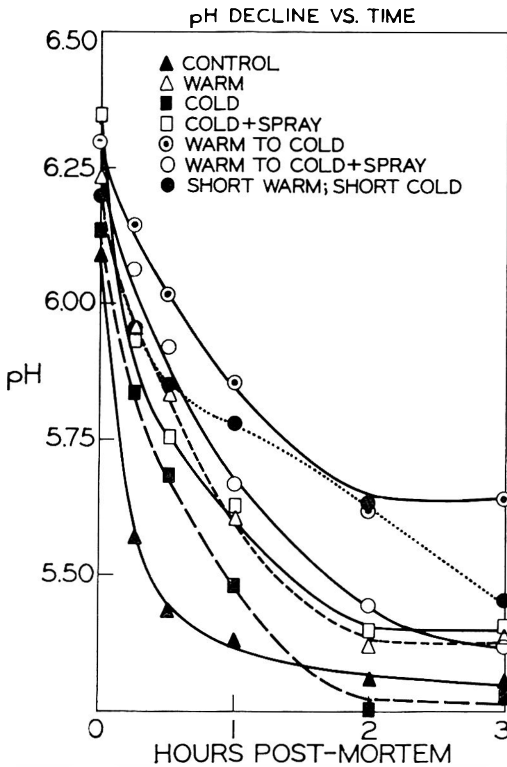

Fig. 4. Treatment effect on the rate of pH decline.

volving a change from warm to cold (V, VI, VII) produced a more gradual muscle pH decline. Differences in pH at 15 min were especially evident; Lots V and VI (warm to cold; warm to cold plus spray) were significantly ($P < .01$) higher in pH than all other lots. At 24-hr post-mortem only the pigs from Lot V (warm to cold) were significantly higher in muscle pH than pigs from all other treatments. This was expected on the basis of initial glycogen data.

Fig. 5 shows the frequency distribution of pigs having muscles with "fast," "intermediate," or "slow" types of glycolysis within each treatment. This classification is based on rate of pH decline. A pH of 5.4 or below at 30-min post-mortem was considered to be a "fast" glycolysis; a pH of 6.0 or above at 1 hr was considered to be a "slow" glycolysis; and those fitting neither group were considered "intermediate" in glycolytic rate. A majority of the animals in the control group possessed muscles which were characterized by "fast" glycolysis. Conversely, pigs in the warm-to-cold and warm-to-cold-plus-spray treatments had a higher incidence of "slow" and "intermediate" types of glycolysis. It should be further noted that animals having a fast glycolysis had significantly higher levels of lactic acid in their muscles at death than animals which had intermediate or slow types of glycolysis.

Onset of rigor mortis (Fig. 6) was most rapid in the control lot, as one would predict from rate of pH decline. Because of a large standard deviation of rigor mortis measurements in muscles from pigs subjected to other treatments, the other differences were not significant.

Thyroid and adrenal weights. No significant differences were noted between treatment groups with respect to thyroid weights, adrenal weights, and thyroid/adrenal weight (T/A) ratios (Table 3). Nevertheless it is of interest to note that animals which ultimately showed a rapid post-mortem glycolysis tended to have a larger T/A ratio, due primarily to lighter adrenals (Table 4). If one can equate lighter adrenals with decreased adrenal corticoid production, this finding is then in agreement with the report of Ludvigsen (1960), who postulated an adrenal insufficiency in animals which event-

Table 2. Ultimate pH values.

Treatment	pH (24 hr post-mortem) $\bar{x}$	$s_{\bar{x}}$
I Control	5.27_c	.04
II Warm	5.38_b	.09
III Cold	5.23_c	.03
IV Cold plus spray	5.26_c	.03
V Warm to cold	5.50_a	.10
VI Warm to cold plus spray	5.33_c	.05
VII Short warm-cold	5.40_b	.03

Means having the same letter subscript are not significantly different ($P > .05$).

TREATMENT EFFECT ON THE TYPE OF GLYCOLYSIS

TYPE OF GLYCOLYSIS	TREATMENT: CONTROL	WARM	COLD	COLD + SPRAY	WARM TO COLD	WARM TO COLD+ SPRAY	SHORT WARM COLD
FAST	XXXX	XX	XX	XX			XX
INTER-MEDIATE	XX	XXX	XXXX	XX	XX	XXXX	XX
SLOW		X		XX	XXX	XX	XX

Fig. 5. Treatment effect on type of glycolysis. Animals within each treatment were classified as to the type of glycolysis on the basis of pH decline (see text).

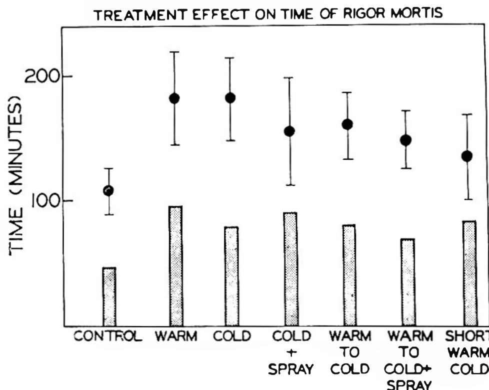

Fig. 6. Treatment effect on time course of rigor mortis. The lower solid bars represent the onset phase of rigor mortis. Total time to completion of rigor mortis, shown with brackets indicating two times the standard error of the mean, is shown by the top set of points.

ually displayed the "muscular degeneration" syndrome.

Simple overall correlation coefficients also support this idea. The combined weight of right and left adrenals was positively correlated (.32*) with the color-structure (gross morphology) score of the gluteus medius and light portion of the semitendinosus.

This relationship was also observed in the thyroid. Thyroid gland weight was significantly and negatively correlated with ultimate color-structure (gross morphology) score in the gluteus medius (−.36*), biceps femoris (−.36*), and dark portion of the semitendinosus (−.40**). In addition, thyroid gland weight was positively correlated with expressible-juice ratio of the longissimus dorsi muscle (.31*). Thus, on the basis of correlations, one can conclude that thyroid and adrenal relationships may influence the animals' response to treatment and consequently influence the effect of treatment on ultimate muscle properties.

Table 3. Thyroid and adrenal gland weights.

Treatment		Gland weights		
		Adrenal (g)	Thyroid (g)	Thyroid/adrenal [a]
I	Control	3.99	8.78	2.62
II	Warm	4.20	9.43	2.22
III	Cold	3.68	8.05	2.26
IV	Cold plus spray	3.89	8.06	2.24
V	Warm to cold	3.91	9.58	2.48
VI	Warm to cold plus spray	4.22	7.70	1.88
VII	Short warm-cold	4.21	8.28	2.05

[a] Weight ratio.

Table 4. Relation of thyroid and adrenal weights to type of post-mortem glycolysis.

Type of glycolysis [a]	Gland weights		
	Adrenal (g)	Thyroid (g)	Thyroid/adrenal [b]
Fast (12) [c]	3.82	7.77	2.32
Slow (10)	4.23	8.81	2.19
Intermediate (17)	4.13	8.38	2.01

[a] Classification based on rate of pH decline. A pH of 5.4 or below at 30 min post-mortem was considered "fast"; 6.0 or above at 1 hr was considered "slow"; those fitting neither group were "intermediate."

[b] Weight ratio.

[c] ( ) refers to number of animals in each particular type.

Gross morphology, color, and juice retion. Figs. 7 and 8 show the gross morphological (color-structure) scores and the reflectance color measurements for the LD. The color-structure scores for the gluteus medius, biceps femoris, and quadriceps femoris are listed in Table 5. The animals in the control (I) and warm treatments (II) possessed PSE LD muscles, while the darkest and firmest muscles were observed in the pigs from treatments V and VI (warm to cold). These differences were significant at the 1% level of probability. Color reflectance measurements of the LD support these subjective observations.

Inferior muscle water-binding capacities (Fig. 9) were clearly shown by high expressible-juice ratios in the control and warm-treatment groups. Even though all treatments involving cold improved expressible-juice ratios, the most desirable muscles

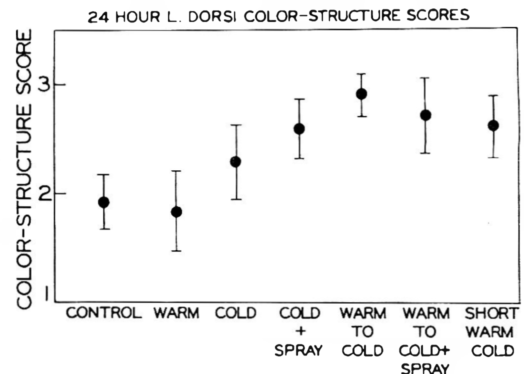

Fig. 7. Treatment effect on LD color-structure scores. Based on a 5-point scale; a low score indicates a PSE muscle; a score of 3 is considered normal; and a high score (4–5) indicates a dark, firm, dry muscle.

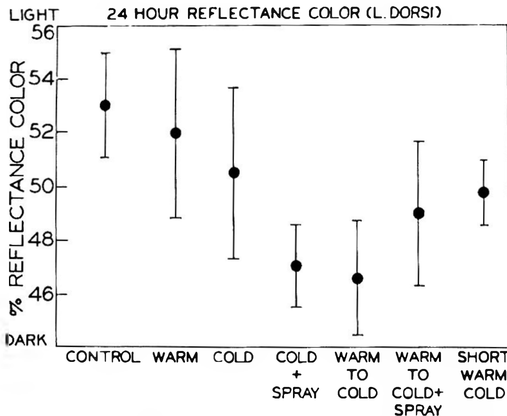

Fig. 8. Treatment effect on reflectance color. A high score indicates a lighter muscle, while a lower score represents a darker muscle.

in this respect were produced by the warm-to-cold-spray treatment.

These observations clearly show that a relatively mild ante-mortem temperature treatment involving a change from a warm to a cold environment prior to slaughter can markedly reduce both muscle glycogen and lactic acid levels. These changes ultimately lead to a superior quality of muscle from the standpoint of both gross morphology (color-structure) and water-binding capacity. This was true even though the animals in the warm-to-cold-spray treatment (Lot VI) were the most muscular, as indicated by loin eye size, and, according to Ludvigsen (1954), may have been more inclined to have had the highest incidence of PSE muscle.

ABBREVIATIONS USED IN THIS PAPER

$\bar{x}$ = mean
$s_{\bar{x}}$ = standard error of mean

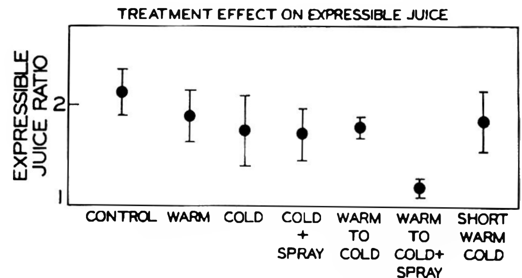

Fig. 9. Treatment effect on expressible-juice ratio.

PSE = pale, soft, exudative
LD = longissimus dorsi
GM = gluteus medius
BF = biceps femoris
Quad = quadriceps femoris
|— on graphs is standard error of the mean

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Table 5. Effect of treatment on color-structure scores (24 hr) of various muscles.

Muscle		Treatment I	II	III	IV	V	VI	VII
GM	$\bar{x}$	0.90_{e}	1.45_{d}	1.28_{de}	2.13_{bc}	2.44_{ab}	2.93_{a}	1.63_{cd}
	$s_{\bar{x}}$	.17	.43	.37	.39	.49	.28	.46
BF	$\bar{x}$	1.97_{e}	2.06_{de}	2.40_{cd}	3.08_{ab}	3.24_{a}	3.32_{a}	2.81_{bc}
	$s_{\bar{x}}$	.28	.33	.19	.30	.31	.24	.44
Quad	$\bar{x}$	2.90_{e}	3.20_{de}	2.93_{e}	3.98_{ab}	4.34_{a}	3.80_{bc}	3.40_{cd}
	$s_{\bar{x}}$	.37	.52	.24	.14	.10	.23	.49

Explanation of table. Color-structure scores (24 hr) for gluteus medius (GM), biceps femoris (BF), and quadriceps femoris (Quad). Means having the same letter subscript are not significantly different ($P > .05$).

Treatments I, control; II, warm; III, cold; IV, cold plus spray; V, warm to cold; VI, warm to cold plus spray; VII, short warm-cold.

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A. L. SHEWFELT
Department of Food Science and Biochemistry
Clemson University, Clemson, South Carolina 29631

Changes and Variations in the Pectic Constitution of Ripening Peaches as Related to Product Firmness

SUMMARY

Samples of 6 commercial varieties of peaches—5 freestone (Ranger, Triogem, Sunhigh, Elberta, and Afterglow), and 1 clingstone (Ambergem)—were examined at 4 stages of postharvest ripeness. Fresh fruit samples were tested for firmness and analyzed for pectinesterase activity and for three pectin fractions (water-soluble, Versene-soluble, and Versene-insoluble). Shear press readings were taken on the corresponding canned samples as well as subjective firmness ratings for each variety at each stage of ripeness.

In Ambergem, the proportions of the three pectin fractions remained relatively constant in fruits held 4 days at 25°C. In freestone peaches, the proportion of water-soluble pectin increased rapidly at the expense of the other two fractions. There were significant statistical differences between the freestone varieties in the proportions of Versene-soluble pectin retained with advancing ripeness. The differences in pectin constitution were related to the firmness of the fresh and canned products. Pectinesterase activity was markedly lower for Elberta than for the other freestone varieties. The level of pectinesterase activity was not in itself related to firmness retention but may be involved indirectly in demethylation for cationic-binding.

INTRODUCTION

Ripening clingstone peaches retain firmness longer than freestone types. For this reason, they are better adapted to handling and processing operations. Freestone varieties make up most of the peach acreage east of the Rocky Mountains in the United States. They continue to supply a large proportion of the fresh market and substantial amounts are processed commercially. There is sustained interest in improving the firmness characteristics of freestone peaches in order that spoilage losses incurred in marketing fresh fruit may he reduced and also that more desirable and uniform texture in processed products can be achieved.

The transformation of protopectin to water-soluble pectin was demonstrated by Appleman and Conrad (1926). Nightingale *et al.* (1930) associated the "non-melting" character of clingstone peaches with a high content of insoluble protopectin. Postlmayr *et al.* (1956) investigated the difference in pectic constitution of freestone and clingstone peaches. They compared three pectic fractions of Elberta freestone and Halford clingstone at four stages of ripeness and found the changes in pectic constitution to be very small in the clingstone in relation to those in the freestone. Sterling and Kalb (1959) noted a decrease in the methyl ester content of the pectic substances during the ripening of Elberta peaches in addition to the change from protopectin to water-soluble pectin. DeHaan (1957) showed that peaches having high protopectin developed a "woolly" texture in refrigerated storage.

The present work was undertaken to determine variations in the pectic constitution of some of the more common commercial varieties of peaches grown in southeastern United States. Particular consideration was given to changes in water-soluble, Versene-soluble, and Versene-insoluble fractions, and in pectinesterase activity, and the relationship of these changes to firmness retention of the canned peaches.

EXPERIMENTAL METHODS

Fruit samples. Six commercial varieties were included: Ranger, Triogem, Sunhigh, Ambergem, Elberta, and Afterglow, all freestone except the Ambergem, a clingstone. A 3-bushel lot of each variety was collected from a commercial packing house on the day the fruit was harvested for regular shipping, but after the normal sorting, grading, sizing, and packing steps. Each lot was divided in the laboratory into 8 uniform subsamples, duplicates of which were held for 1, 2, 3, and 4 days at 25°C.

Firmness measurement and analyses of fresh peaches. Ten peaches from each subsample were subjected to a firmness test using an Asco Firmness Meter. This instrument provided a non-destructive means of measuring the firmness of an individual fruit by applying stress to a flexible band positioned around the lateral circumference. High resistance of the fruit tissue to the applied stress

resulted in lower instrument dial readings. The instrument was used with a pre-stress weight of 100 g and a test weight of 1500 g. The test time was 10 sec. These ten peaches were then peeled and pitted and the combined peach tissue analyzed for total solids, soluble solids, and total acidity according to methods described by Postlmayr *et al.* (1956).

The alcohol-insoluble solids fraction from each subsample was prepared and determined by blending 200 g of fresh slices with 600 ml of 95% ethyl alcohol. The blended mixture was simmered for 20 minutes on a steam bath, then cooled rapidly to room temperature. The mixture was then filtered through Whatman No. 2 paper in a Buchner funnel with light suction. The alcohol-insoluble residue was washed with four separate 200-ml portions of 95% ethyl alcohol followed by one 200-ml portion of absolute ethyl alcohol and two 100-ml portions of acetone. The residue was dried overnight at 48°C, weighed, then ground in a Wiley mill with a 60-mesh screen. The dried residue was used in determining the pectic constitution.

For pectinesterase activity, a 50-g portion of freshly blended peach tissue and 10 grams of sodium chloride were transferred to a blender jar with 140 ml of distilled water. The mixture was blended at high speed for 2 min, and a 25-ml portion was used to determine pectinesterase activity by the method of Rouse and Atkins (1955).

Canning. After removal of the fruits from each subsample for firmness measurement and fresh product analyses, the remaining fruits were canned by a procedure described by Postlmayr *et al.* (1956) except that cooking and cooling were performed with a continuous agitating cooker and cooler, details of which have been given by Van Blaricom (1955). Cooking time was 4½ min and cooling time was 10 min. The canned samples were stored at 25°C for four months prior to evaluation of product firmness.

Determination of pectin fractions. The procedure outlined by McCready and McComb (1952) was used to determine the total pectin in the dried alcohol-insoluble residues. The results were calculated as percentage of anhydrogalacturonic acid (AGA) in the dry solids of the fresh tissue.

The water-soluble pectin was determined in duplicate 0.1-g aliquots of the alcohol-insoluble solids residue. Each aliquot of powder was suspended in 40 ml of deionized water, agitated for 1 hr, made up to 100 ml, and then filtered through Whatman No. 1 paper. A 5-ml portion of the filtrate was de-esterified in 0.05N NaOH as described by Rouse and Atkins (1955), and then analyzed for AGA by the method applied to the filtrate for total pectin.

The Versene-soluble pectin was determined in the same manner as water-soluble pectin except that 0.5% Versene was used instead of water for extraction, and the pH was adjusted to 6.0. The resulting extract included both the water-soluble and Versene-soluble fractions. The amount of water-soluble pectin was subtracted from the Versene-extracted fraction, and the remainder reported as Versene-soluble pectin.

The Versene-insoluble pectin was calculated as the difference between the total pectin and the combined water-soluble and Versene-soluble fractions. As a check, the Versene-insoluble fraction was also determined for each residue by demethylating the Versene-extracted sample with 0.05N NaOH prior to extraction.

Firmness evaluation of canned product. The firmness of the canned halves from each subsample was measured with an Allo Kramer Shear Press having an electronic recording attachment. Measurements were made on duplicate 200-g portions using a 3000-lb ring, a 300-lb range, and a time of 40 sec. Subjective evaluation of the canned samples was conducted with a panel of 10 judges. A 7-point scale was used, from −3 (much softer than desirable) to 0 (desirable) to +3 (much harder than desirable). Comparisons were made by each person daily on four random subsamples.

RESULTS AND DISCUSSION

Table 1 shows data for firmness, solids content, total acidity, and pectinesterase activity in the fresh peach samples and shear press values for the corresponding canned samples. The Table includes least significant difference values between means for variety and for stage of ripeness where statistical significance at the 5% level was indicated. The interactions between variety and stage of ripeness were not statistically significant for these measurements.

Significant differences between varieties occurred for firmness of the fresh and canned fruit and for total solids, soluble solids, alcohol-insoluble solids, and total acidity. Solids content was higher for the later varieties. Differences between stages of ripeness were, with the exception of the firmness measurements, much smaller than varietal differences and showed no distinct trends with advancing ripeness under the conditions of this experiment.

Pectinesterase. Pectinesterase activity was highest for Ambergem, Sunhigh and Triogem. Elberta was notably low in pectinesterase activity. The differences between

Table 1. Firmness, solids, acid and pectinesterase levels of 6 varieties of fresh peaches at 4 stages of ripeness.

Variety	Date of harvest	Stage of ripeness	Firmness meter (dial reading)	Total solids (%)	Soluble solids (%)	Alcohol insoluble solids (%)	Total acid (%)	Pectin-esterase (P.E. u/g × 10⁴)	Shear press reading of canned (lb)
Ranger	July 2	1	59.6	10.14	9.82	1.601	0.88	6.24	90.6
		2	74.4	10.22	9.18	1.612	0.89	6.24	45.4
		3	80.6	10.30	9.40	1.588	0.90	4.72	50.0
		4	82.8	10.12	9.30	1.664	0.86	5.68	32.4
Triogem	July 9	1	53.2	12.64	10.00	1.642	0.80	8.06	88.2
		2	75.4	12.02	10.12	1.706	0.74	7.20	78.2
		3	83.8	11.67	10.08	1.886	0.74	7.72	42.2
		4	83.8	11.69	10.88	1.891	0.66	6.36	52.8
Sunhigh	July 16	1	46.6	11.42	10.30	1.752	0.74	8.40	198.2
		2	62.3	12.32	10.40	1.858	0.78	7.60	67.4
		3	71.6	12.22	10.70	1.920	0.83	8.12	80.9
		4	72.6	12.37	10.70	2.012	0.86	6.96	62.4
Ambergem	July 23	1	44.8	13.48	11.42	1.762	0.82	8.04	204.0
		2	43.8	13.20	11.00	1.750	1.00	8.08	137.4
		3	48.1	12.60	11.00	1.737	0.83	7.76	155.2
		4	50.7	13.38	11.20	1.831	1.04	9.12	160.0
Elberta	July 30	1	47.8	14.38	12.02	2.093	0.90	3.66	178.2
		2	67.8	13.44	11.80	2.084	0.72	2.80	120.9
		3	77.7	13.26	12.20	1.990	0.73	3.86	57.0
		4	81.8	13.92	11.78	2.044	0.73	3.40	60.0
Afterglow	August 6	1	49.3	15.16	13.28	2.267	1.00	7.62	159.5
		2	62.4	15.80	14.02	2.316	0.82	5.88	143.7
		3	66.0	15.63	13.90	2.607	0.83	8.26	109.9
		4	78.6	15.87	14.30	2.354	0.86	6.66	106.0
LSD between ripeness means (5% level)			3.6	0.34	0.25	0.085	0.04	0.62	39.4
LSD between variety means (5% level)			2.6	ns	ns	0.068	ns	0.50	32.2

LSD, least significant difference.
ns, not significant.

stages of ripeness for pectinesterase activity, though significant, were, again, much smaller, and showed no distinct trend with advancing ripeness. While it is believed that pectinesterase is the most likely pectic enzyme to be concerned with softening of peaches, other pectic enzymes may well be involved. Further investigation is needed in this area.

Pectin fractions. Fig. 1 shows changes in the three pectin fractions (water-soluble, Versene-soluble, and Versene-insoluble) for each variety and each stage of ripeness. The difference between Ambergem and the freestone varieties conforms with differences found by Postlmayr *et al.* (1956) between Halford clingstone and Elberta freestone. In the freestone varieties, the proportion of Versene-soluble pectin decreased to a lower level in Ranger, Triogem, and Elberta than in Sunhigh and Afterglow. The apparent increase in total pectin with stage of ripeness in some varieties was attributed largely to the manner in which the pectin was bound in the green fruit. The extraction methods used in this study were not considered exhaustive but were chosen because they could be repeated satisfactorily with minimum change in the natural pectic substances. A comparison of Fig. 1 (pectin fractions) and Fig. 2 (texture ratings) indicates a relationship between changes in pectic constitution of the fresh fruit samples and texture ratings of the canned products prepared from these samples. Shear press readings for the canned samples were less closely related to the

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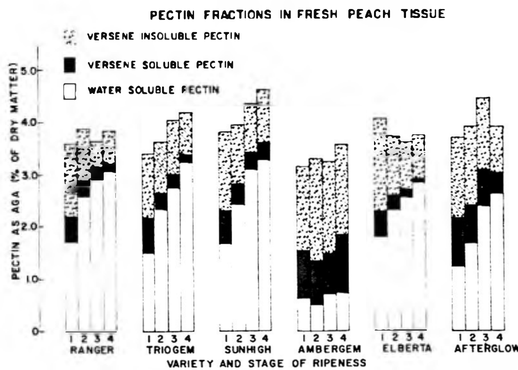

Fig. 1. Pectic fractions in fresh peach samples expressed as percentage anhydrogalacturonic acid (AGA) in dry matter. (LSD at 5% level of significance for treatments: water-soluble pectin, 0.12; Versene-soluble pectin, 0.16; total pectin, 0.15).

texture ratings than were the firmness readings of the fresh peaches.

Sunhigh had a somewhat higher proportion of Versene-soluble pectin retained with advancing ripeness than did the other early-season freestone varieties. It also had a relatively high pectinesterase activity. Triogem and Elberta retained a relatively low proportion of Versene-soluble pectin with advancing ripeness and this appeared to be closely associated with rapid fruit softening. Pectinesterase activity, however, was much higher for Triogem than for Elberta. Theoretically, a high pectinesterase activity could result in increased retention of Versene-soluble pectin as long as bivalent cations such as calcium and magnesium are present to stabilize the demethylated pectin. Study of such a relation is planned. The results reported here indicate the variations that occur in pectic constitution between different commercial varieties of freestone peaches.

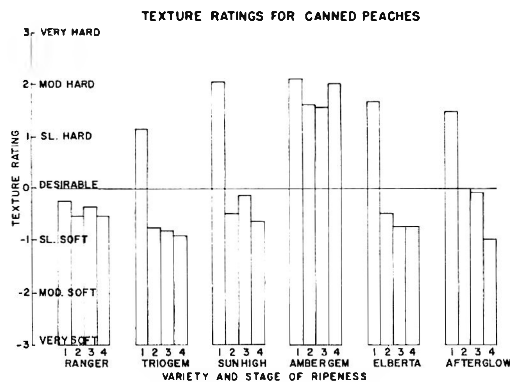

Fig. 2. Subjective texture ratings of canned peach samples (LSD at 5% level of significance for treatments, 0.65).

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R. D. DAFTARY and Y. POMERANZ

Department of Flour and Feed Milling Industries, Kansas State University, Manhattan, Kansas 66504, and Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture

Changes in Lipid Composition in Maturing Wheat

SUMMARY

Changes in lipid composition during wheat development were followed by qualitative and quantitative thin-layer chromatography (TLC) and by fractionation on silicic acid columns. Development of the wheats was accompanied by a slight decrease of lipid content on an as-is basis, and by almost doubling of lipids on a kernel basis. Free fatty acids in mature wheat were less than half the amount in wheat 21–23 days preceding ripeness. A similar decrease was found in the levels of mono- and diglycerides. No consistent changes were found in phospholipid fractions of lipids extracted from wheat at various stages of maturity. Carotenoid pigments disappeared as the wheat kernel developed and matured.

INTRODUCTION

The biochemical transformations during plant growth and development are highly complex. Available literature data suggest that analyses of seeds at various stages of maturity might show major differences and changes in composition and structure of lipids. Our knowledge of such changes is very limited. A survey of literature (McNair, 1945; Zeller, 1957) indicates relatively few studies concerning variations in lipids during the developmental stages of seeds and fruits. Available data, mainly from analyses of oil seeds, show that in all tested samples four stages were observed: 1) rapid fruit or seed growth, accompanied by little fat increase; 2) rapid fat increase; 3) stationary fat level; and 4) slight fall in fat levels (in fruits but not in seeds) shortly before maturity. Guerrant (1927) found that lipids in oat seeds increased during maturation from 1.95 to 3.10% of dry matter. Pulkki and Puutula (1941) reported that the increase in thiamin was correlated in an almost linear manner with increase in total lipid of maturing wheat. According to Evans (1941), the ether extract of corn increased from 3.78–4.17% of dry matter, 15–57 days after silking. Ether extractives in Bengal gram increased from 1.6–5.1% on an as-is basis, but the increase was very small (4.7–5.4%) when calculated on a dry-matter basis (Verma *et al.*, 1964). Most of our knowledge on qualitative changes during seed ripening is centered on the increase of unsaturated fatty acids and the decrease of free fatty acids during maturation. Evans (1941) found that the iodine value in maturing corn increased from 100.8 to 127.1, and the acid value and free fatty acids respectively decreased from 67.2 to 13.4, and from 33.7 to 6.8. Results indicate that oil formation in corn begins at an early stage, and that the nature of the oil changes progressively, becoming less and less saturated in character.

The introduction of new methods to fractionate and characterize lipids makes it possible to obtain a more thorough insight into the changes in lipids of maturing seeds. Our study attempted to determine the relative proportions of the various lipids of the wheat kernel at different stages of development. We employed silicic acid column chromatography and qualitative and quantitative thin-layer chromatography (TLC).

MATERIALS AND METHODS

Wheat samples. Kaw and Pawnee wheat at 9 stages of maturity from 23 days before ripe to ripe were harvested at Manhattan, Kansas, in 1961. It is possible that the results are affected by secondary phenomena such as differences in enzymatic activity between unripe and dry grain. Every care was therefore taken to reduce these changes to a minimum by rapid drying the unthreshed samples at about 20°C and storing at 4°C. The wheat samples separated from the chaff and straw were air-dried in thin layers in the shade with the aid of electric fans and stored in closed tin containers at 4°C until analyzed. The first sample in the series was taken at as immature a stage as practical to collect. Sampling was continued at 2–3-day intervals until the growing wheat was ripe.

Extraction procedures. Analytical determinations were made according to Cereal Laboratory Methods (AACC, 1962). Wheat was ground to pass a 20-mesh sieve on a micro-Wiley mill and

extracted with benzene by the AACC method for free fatty acid determination.

Additionally, 15-g samples were extracted with 100, 50, and 50 ml of water-saturated 1-butanol, allowing 4, 2, and 2 min after the extraction steps. Water-saturated butanol does not extract all the lipids and does extract some of the non-lipid material. The non-lipid material was eliminated by redissolving the butanol extract with petroleum ether. The combined extracts were decanted, filtered, and evaporated almost to dryness under vacuum in a glass apparatus at about 45°C. The extracts were kept under vacuum in a desiccator for 40 hr over P_2O_5 at 4°C and extracted three times with Skellysolve B, and the combined extracts were evaporated under vacuum. The lipids were dissolved in 80 ml of a chloroform-methanol mixture (2:1), washed with 17.5 ml of 0.04% calcium chloride, followed by two washings with 10 ml each of aqueous 0.02% calcium chloride solution, and the volume of the washed lipids was made to 50 ml with chloroform. Total lipids were determined by drying two 5-ml portions to constant weight; the remaining 40 ml were concentrated under vacuum to about 2 ml for separation on silicic acid columns.

Silicic acid columns were 15 cm long and 2 cm in diameter. Twenty-g lots of silicic acid for chromatography of lipids from Mallinckrodt, N.Y., were washed twice with 60 ml of a 7:1, and once with 60 ml of a 15:1 chloroform-methanol mixture, and finally with 80 ml chloroform. The slurry was transferred to columns, and the neutral lipids were eluted with 120–150 ml chloroform, and the polar lipids with 120–150 ml methanol. Completion of elution was checked in each case by TLC. Each of the two fractions was concentrated to 100 ml under vacuum, and two 10-ml aliquots were drawn from each, to determine neutral and polar lipid content, respectively. The remaining fractions were freed of solvent under reduced pressure, dissolved in a 2:1 mixture of chloroform-methanol, and used for TLC.

Fractionation of neutral and polar lipids by silicic acid column chromatography. The water-saturated butanol extract was separated into ten fractions by elution from silicic acid (Bio-Rad Lab., Richmond, Calif.) employing the apparatus described by Hirsch and Ahrens (1958). The neutral lipids were eluted by the solvents given by Barron and Hanahan (1958), followed by elution of polar lipids according to the method of Hanahan *et al.* (1957). The fractions collected contained as predominant lipid components: 1) hydrocarbons; 2) sterol esters and esters of higher alcohols; 3) triglycerides; 4) sterols and higher alcohols; 5) diglycerides; 6) monoglycerides; 7) polyglycerolphosphatides and glycolipids; 8) phosphatidyl ethanolamine and phosphatidyl serine; 9) inositol phosphatide, lecithin, and lysophosphatidyl ethanolamine; and 10) lysolecithin. The fractions were evaporated under reduced pressure; a portion of each was dried to constant weight, and another portion was dissolved in a 2:1 mixture of chloroform-methanol. TLC indicated incomplete separation by silicic acid column chromatography.

Thin-layer chromatography. Glass plates (20 × 20 cm) were coated with a 250-mμ layer of silica gel G (E. Merck, A.G., Darmstadt, Germany) in a conventional manner using a commercial spreader (C. A. Brinkmann and Co., Great Neck, N.Y.). The plates were dried 3 hr at 130°C. The most useful solvents for ascending development of lipid spots (20–40 μg) were: chloroform, a mixture of petroleum ether-ethyl ether-acetic acid (80:20:1) (ether mixture), or a mixture of chloroform-methanol-water (65:25:4) (chloroform mixture). Two-dimensional TLC was performed according to Abramson and Blecher (1964). All solvents were of analytical grade, redistilled from glass; ether was redistilled from above metallic sodium.

The spots were located and visualized by exposure to iodine vapor, by spraying of dried plates with sulfuric acid and heating for 15 min at 130°C, by spraying with a 0.2% solution of ninhydrin in butanol containing 1% pyridine (Lepage, 1964) as a spray specific for free amino acids, with a modified Dragendorff reagent (Mangold, 1961) for choline phosphatides and glycolipids, or with a molybdenum spray (Dittmer and Lester, 1964) for the detection of phospholipids. Lipids separated by TLC were tentatively identified by comparing R_f values with literature data (Mangold, 1961; Blank *et al.*, 1964; Lepage, 1964; Fisher *et al.*, 1964), use of specific sprays and comparing R_f values with those of pure compounds. Among the neutral lipids used were lauric acid, myristic acid, 1-monopalmitin, 2-monopalmitin, 1,2-dipalmitin, 1,3-dipalmitin, tripalmitin, and tristearin (H. L. Mitchell, Biochemistry Dept., Kansas State University). Among the phospholipids (phosphoglyceride standards from Applied Science Labs., Inc., State College, Pa.) were plant lecithin, phosphatidyl ethanolamine, and phosphatidyl serine. Plates were viewed under ordinary light. Additionally, plates were observed under ultraviolet light (long wave, 3660 Å) prior to and after being sprayed with sulfuric acid.

Quantitative analysis of lipids by TLC. Lipid fractions from silicic acid columns and lipids separated by TLC were measured quantitatively (Blank *et al.*, 1964) by photodensitometry of spots charred by heating after spraying with sulfuric acid. Color intensity was determined with a densitometer with scanning stage and varicord recorder.

Table 1. Lipid (benzene extract) and free fatty acid (FFA) contents of maturing wheat (on as-is basis).

Pawnee variety						Kaw variety					
Days pre-ripe	Moisture (%)	1000 kernel wt. (g)	Lipids (%)	Lipids/kernel (mg)	FFA (mgKOH/100 g wheat)	Days pre-ripe	Moisture (%)	1000 kernel wt. (g)	Lipids (%)	Lipids/kernel (mg)	FFA (mgKOH/100 g wheat)
21	12.6	11.92	1.85	0.221	31.2	23	13.2	10.56	......	......	......
19	13.1	15.80	1.62	0.256	19.7	21	12.0	14.30	1.62	0.232	32.0
17	12.8	17.08	1.58	0.270	18.5	19	11.2	17.32	1.58	0.274	20.5
15	14.3	20.26	1.66	0.336	18.5	17	11.9	22.30	1.52	0.339	19.7
12	12.3	20.77	1.76	0.366	17.7	14	15.2	23.83	1.46	0.348	19.3
9	12.2	20.46	1.58	0.323	14.9	11	13.2	26.04	1.50	0.391	15.6
6	12.6	22.57	1.64	0.370	14.9	8	12.9	28.71	1.35	0.388	15.6
3	12.4	21.54	1.60	0.345	14.9	5	14.4	30.42	1.35	0.411	15.2
0	13.0	22.78	1.64	0.374	15.3	0	12.9	27.88	1.34	0.374	15.6

Table 2. Distribution (percent) of total, neutral, and polar lipid fractions (as-is basis) in water-saturated butanol extract of maturing wheat.

Pawnee variety						Kaw variety					
Days pre-ripe	Moisture	Total lipids	Neutral (in total)	Polar (in total)	Recovery from column	Days pre-ripe	Moisture	Total lipids	Neutral (in total)	Polar (in total)	Recovery from column
21	12.6	2.76	69.0	31.0	96.5	23	13.2	2.48	64.8	35.2	93.4
19	13.1	2.30	64.3	35.7	99.4	21	12.0	2.32	65.4	34.6	93.7
17	12.8	2.32	64.0	36.0	96.7	19	11.2	2.27	69.6	30.4	98.7
15	14.3	2.24	67.3	32.7	101.8	17	11.9	2.21	66.4	33.6	92.8
12	12.3	2.33	66.9	33.1	98.0	14	15.2	2.18	65.7	34.3	95.0
9	12.2	2.18	65.3	34.7	99.2	11	13.2	2.12	70.5	29.5	98.3
6	12.6	2.24	65.1	34.9	99.0	8	12.9	1.98	69.7	30.3	102.2
3	12.4	2.19	63.9	36.1	94.8	5	14.4	2.02	69.7	30.3	97.7
0	13.0	2.19	67.3	32.7	102.6	0	12.9	1.99	69.7	30.3	95.6

RESULTS AND DISCUSSION

Total lipid content and free fatty acids in benzene extract of maturing wheat are summarized in Table 1. Pawnee wheat contained consistently higher total lipid levels than Kaw wheat. On a percentage basis the lipid content decreased in both wheat varieties during maturity, but on a kernel basis the lipid content almost doubled during maturation in both wheat varieties. Free fatty acids decreased during grain development and maturity. Water-saturated butanol extracted more lipids than did benzene, as a result of extracting "bound" lipids by the more polar solvent (Table 2). Again, the lipid content was higher in Pawnee than in Kaw wheat, and in both it decreased on an as-is basis during grain development. Fractionation into neutral and polar lipids by silicic acid chromatography showed no consistent differences in lipids between wheat varieties at various maturity stages. That the decrease in lipid content is apparent only during maturation is shown in Table 3. The water-saturated butanol extract contained larger amounts of nonlipid extracts in immature than in mature wheat. The "true" lipids, petroleum-ether-soluble fraction of water-saturated butanol extract, were present in nearly identical amounts in immature and ripe wheat.

No consistent differences were found during fractionation of lipids from wheat samples of both varieties varying in maturity and development. Triglycerides constituted

Table 3. Water-saturated butanol extract of maturing wheat.

	Kaw variety		Pawnee variety	
Days preripe	23	0	21	0
Total water-saturated butanol extract (%)	2.30	2.24	2.40	2.26
Petrol-ether-insoluble (5)	0.45	0.38	0.38	0.24
Petrol-ether-soluble (%)	1.85	1.86	2.02	2.02

the largest fraction (35.9%) and phospholipids 37.3% of the butanol-extracted lipids. Other silicic-acid-column-separated lipid fractions, when tested by TLC, showed incomplete separation.

Separations of butanol extracts of Kaw wheat at various stages of maturity, by TLC are shown in Fig. 1. A mixture of hydro-

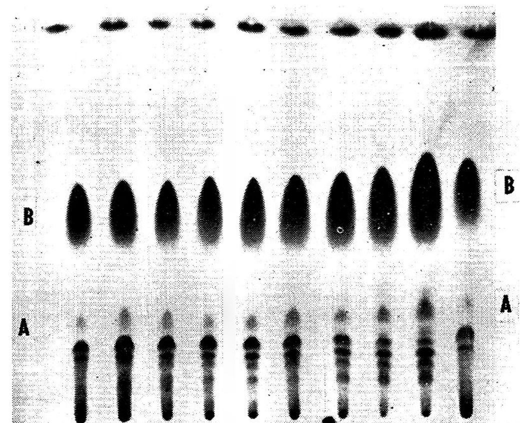

Fig. 1. TLC of butanol extracts of Kaw wheat at various stages of maturity. From left to right: 0, 23, 21, 19, 17, 14, 11, 8, 5, and 0 days preripe. Developed with ether mixture; spots visualized by charring with sulfuric acid. Picture taken under UV light. A) free fatty acids; B) triglycerides.

carbons and sterol esters migrated to the solvent front line. Mono- and diglycerides have R_f values lower than those of free fatty acids. Similar chromatograms were obtained by fractionation of extracts from Pawnee wheat. Fractionation of nonpolar lipids of both varieties by either ether mixture or chloroform shows disappearance of free fatty acids, mono- and diglycerides during wheat maturation and development. Quantified results of the TLC are summarized in Table 4. The lowest levels of free fatty acids, mono-, and diglycerides were found in wheat about 3–6 days preripe. Such wheat is considered to be in a stage of physiological maturity (Scott *et al.*, 1957). Finney (1954) showed that proteolytic activity and water-soluble nitrogen contents of wheat were highest in samples harvested at the earliest stages of maturity. As the wheat ripened, values decreased sharply, reached a minimum prior to maturity, and thereafter increased somewhat. Maximum loaf volumes and superior physical flour properties

Table 4. Neutral lipid fractions (mg per 100 g wheat) of maturing Pawnee wheat as determined by quantitative thin-layer chromatography.

Days preripe	Monoglycerides	Diglycerides	Free fatty acids
21	275	217	338
17	108	157	204
15	74	161	190
12	122	162	145
9	41	142	116
6	22	95	53
3	22	126	79
0	132	190	106

were obtained with wheat that was harvested slightly preripe.

TLC with chloroform mixture showed no consistent or significant differences in amounts of phospholipid fractions in extracts of wheat from various maturity stages. Fractions in Fig. 2 were visualized by charring with sulfuric acid. Phosphatidyl ethanolamine had an R_f value intermediate between digalactosyl glyceride and monogalactosyl glyceride. Two-dimensional TLC indicated that monogalactosyl glyceride, separated by the one-dimensional method, contained also phosphatidic acid. This was confirmed by spraying polar lipids separated

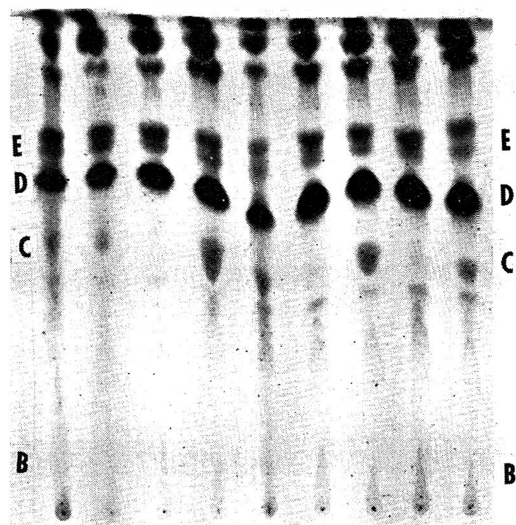

Fig. 2. TLC of butanol extracts of Kaw wheat at various stages of maturity. From left to right: 23, 21, 19, 17, 14, 11, 8, 5, and 0 days preripe. Developed with chloroform mixture; spots visualized by charring with sulfuric acid. Pictures taken under UV light. Tentatively identified as: B) phosphatidyl serine; C) phosphatidyl choline; D) digalactosyl glyceride; and E) monogalactosyl glyceride.

by the chloroform mixture with the molybdate reagent. Additional plates were exposed to iodine vapor or sprayed with ninhydrin, Dragendorff, and molybdate reagents. With ninhydrin, an unidentified component having an R_f value about 0.9, was present in mature wheat or wheat 3–17 days preripe, but absent in wheat 19–23 days preripe. At least 4 carotenoid pigments were found in benzene and butanol extracts of immature wheat, during separation by TLC. Fig. 3 gives a diagrammatic picture of carotenoid pigments in wheat and their disappearance as maturity progressed. Carotenoid pigments were identified only by red fluorescence under ultraviolet light. Comparison with crystalline beta-carotene (from Nutritional Biochem. Corp., Cleveland, Ohio, and Hoffman-La-Roche, Inc., Nutley, N.J.) indicated that immature wheat contained only traces, if any, of beta-carotene.

This study summarizes some of the transformations of lipid and lipid-soluble components of the developing wheat grain. TLC is best exploited when used in combination with other fractionation methods. Silicic acid column chromatography was used in our study. We are now investigating fractionation of wheat lipids in maturing wheat by gas-liquid chromatography, combined with modified and refined methods of qualitative and quantitative preparative TLC.

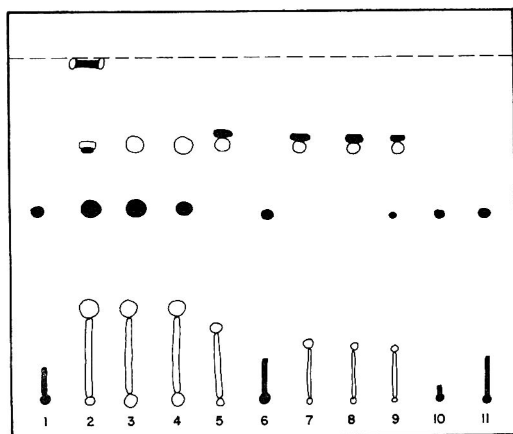

Fig. 3. TLC of butanol extracts of Kaw wheat at various stages of maturity. From left to right: 0, 23, 21, 19, 17, 0, 14, 11, 8, 5, and 0 days preripe. Developed with chloroform; unsprayed plates viewed under UV light. Full circles, bluish fluorescence of lipids; empty circles, red fluorescence of carotenoids.

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CECILIA P. HSU,[a] S. N. DESHPANDE,[b] and N. W. DESROSIER [c]
Department of Horticulture, Purdue University, Lafayette, Indiana 47907

Role of Pectin Methylesterase in Firmness of Canned Tomatoes

SUMMARY

Four sets of eight tomatoes were subjected to four blanching times of 30, 60, 90, and 120 sec at 100°C. Two fruits in each set were also subjected to holding times of 5, 10, 15, and 20 min between the blanching and exhausting operations. Pectic substances were determined as water-soluble, ammonium-oxalate-soluble, and dilute-hydrochloric-acid-soluble fractions. The methoxyl content of each fraction was also determined. Firmness was determined by objective measurements. The 30-sec blanching treatment yielded the firmest tomatoes. These fruits were also characterized by the highest content of ammonium-oxalate-soluble pectin. The activity of pectin methylesterase was highest in treatments consisting of 30 sec of blanching and a 10-min holding time. The activity of pectin methylesterase seemed to be greatly suppressed after 90 sec of blanching.

INTRODUCTION

The hardening of fruits in canned tomatoes is usually accomplished by the addition of calcium salts. The firming effect of this treatment is believed to be due to the combination of calcium ions with pectic acid or low-methoxyl pectinic acid to form calcium pectate or calcium pectinate (Hamson, 1952; Kertesz, 1939a,b, 1951). Also of interest is the fact that during the calcium treatment of tomatoes, canned tomatoes react better to calcium firming than do raw tomatoes (Appleman, 1927; Kertesz, 1939a; Locanti and Kertesz, 1941). This is probably due to enzyme action, resulting in some de-esterification during the transient period of heating (Deshpande, 1962; Kertesz, 1951). However, no evidence was available relating the effect of enzyme activity to the firmness of canned tomatoes. De-esterified pectin is desirable in hardening canned tomatoes because of the increased supply of free carboxyl groups to form a supporting structure of calcium pectate (McColloch and Kertesz, 1949; McColloch *et al.*, 1952; McCready and McComb, 1954). It appeared possible that a holding time between blanching and heating processing would permit more demethylation (Deshpande, 1962). Commercially, blanching is necessary to facilitate peeling. The time of blanching is probably critical to induce pectin methylesterase activity. Prolonged blanching will inactivate the enzyme completely, while insufficient blanching will hinder peeling and fail to liberate the enzyme. This experimental work was designed to investigate the effect of blanching time and holding time on the concentrations of the three pectic fractions, the activity of pectin methylesterase, and their relationship to tomato firmness.

On the basis of their solubility, pectins can be classed in three broad groups: pectic acid, pectinic acid, and protopectin. Pectic acid is essentially free of methyl ester groups. The salts of pectic acid are either normal or acid pectates. Because of the complete demethylation, pectic acid is insoluble in water. This insolubility of pectic acid is probably due to the presence of electrolytes, which render it insoluble in water. Pectinic acid is also composed of polygalacturonic acid units, but contains more than a negligible proportion of methoxyl groups. Generally, the solubility in water increases with an increase in the proportion of methyl ester. In the presence of even very minute quantities of certain ions, especially polyvalent cations, the pectinic acid of less than 7% methoxyl content may become entirely insoluble in water. The low-methoxyl pectins constitute a further special group of pectinic acids which possess some 3–7% methoxyl and are consequently greatly influenced by the presence of polyvalent cations such as calcium. Because of their ability to form calcium salts, low-methoxyl pectins are regarded as important in the hardening of canned fruit. Protopectin is the insoluble parent pectic compound of natural occurrence. In the ripening of fruits, the insoluble protopectin is changed gradually to soluble

[a] Present address: Beech-Nut Life Savers, Inc., Cedar Lane, Knoxville, Tennessee.
[b] Present address: School of Agriculture, Tuskegee Institute, Tuskegee Institute, Alabama 36088.
[c] Present address: Beech-Nut Life Savers, Inc., 477 Madison Ave., New York, N. Y.

pectin by hydrolysis and depolymerization (Woodmansee and McClendon, 1959). Appleman and Conrad (1927) studied transformations in pectic substances during maturation and during canning. They found that pectinic acid content increased while the content of protopectin decreased as the fruit passed through the green-mature to the pink and red-ripe stages of development. Some transformations of protopectin into more easily soluble pectinic acid were observed during the canning process.

Evidence that calcium pectate formation is responsible for the increased firmness is provided by the fact that calcium-chloride-treated tomatoes lose their firmness upon immersion in ammonium citrate solution. When these tomatoes are again treated with calcium chloride, they do not regain their initial degree of firmness. Evidently the compound responsible for the stronger texture is dissolved in the ammonium citrate solution. Analysis of this solution reveals the presence of a pectic material (Deshpande, 1962; Kertesz, 1939b; Locanti and Kertesz, 1941).

MATERIALS AND METHOD

Locally grown tomatoes of the Roma variety were used in this study. To reduce variability, fruits from only one location were used. Four sets of 8 tomatoes were subjected to blanching times of 30, 60, 90, and 120 sec in boiling water. After blanching, fruits were peeled and packed in 300 × 211 cans. The tomatoes were then held for 5, 10, 15, and 20 min prior to the addition of 0.05% $CaCl_2$ in pH 4.4 buffer at 212°F. Immediately after the holding periods and the addition of the $CaCl_2$ the cans were sealed and processed for 45 min at 212°F. The different holding periods permitted different degrees of demethylation by pectin methylesterase activity.

The tomatoes were drained for 2 min on a 0.5-inch-mesh screen and their weights were recorded. The contents were packed again in fresh cans, agitated on a mechanical shaker for 2 min, drained as before, and reweighed. An objective index of firmness to canned whole tomatoes was assigned by the following formula (Deshpande, 1962).

$$\frac{\text{Weight of strained fruit after shaking, in g}}{\text{Weight before shaking, in g}} \times 100$$

After the determination of firmness, a 20-g sample of canned tomatoes was homogenized in a Waring blender with 95% EtOH to remove soluble sugars and free uronic acid. The pectin was extracted in three fractions as follows: The water-soluble extract. Twenty grams of the blended tomatoes were transferred to a 200-ml Erlenmeyer flask with 60 ml of distilled water and extracted for 2 hr at room temperature with constant agitation. The mixture was filtered following extraction, the supernatant liquid was removed and saved, and the residue extracted with water as before. The combined filtrates contained the water-soluble pectic substances. The same procedure was repeated with 0.2% ammonium oxalate solution and 0.95*N* HCl. The extracts were designated as oxalate-soluble and acid-soluble extracts.

The method of McCready and McComb was used for determining total pectic content (McComb and McCready, 1952; McCready and McComb, 1952).

The saponification method described by Gould and Davis (1954) was used for determination of the methoxy content. With the aid of a pH meter, the free acidity of the pectin extracts was neutralized to pH 7.5 with 0.05*N* NaOH. Then an extra 10 ml of 0.05*N* NaOH was added in order to completely saponify all the methoxyl groups; the mixture was agitated vigorously and allowed to stand for 1 hr at room temperature. Following saponification, the excess alkali was neutralized (to pH 7.5) by titrating with 0.01*N* HCl. A distilled-water blank was treated exactly like the experimental sample. The difference between the amount of HCl required to back-titrate the blank and the sample was equivalent to the base consumed in the saponification of the methyl ester groups.

The results of analysis are reported in Tables 1–5.

The effect of holding time on firmness varied with blanching time. In this study the 30-sec blanch with a 10-min holding period produced the firmest canned whole tomatoes. With the 30-sec blanch, holding periods of less than 10 min or more than 10 min resulted in a firmness loss. The 60-sec blanch with the 20-min holding period gave the next-firmest fruit. Unexpectedly, there was a progressive increase in firmness as holding time was increased from 5 to 20 min. Following the 90- and 120-sec blanch the length of holding period did not influence fruit firmness. Blanching times of less than 30 sec are not desirable, because it causes difficulty in peeling tomatoes. The canned tomatoes may be damaged during peeling if blanching time is not sufficient.

The activity of pectin methylesterase was measured by the saponification method and expressed as mg% of CH_3O on a fresh-weight basis. This measurement represents an inverse relationship since the methoxyl content of the pectic substances varies inversely with the enzyme activity. The highest pectin methylesterase activity was obtained

Table 1. Relationship of treatment to firmness and methoxyl content.

Sample			Methoxyl content[a]			
Time of blanching (sec)	Holding period (min)	Index of firmness	Water-soluble fraction	Ammonium-oxalate-soluble fraction	Acid-soluble fraction	Total methoxyl fraction
30	5	80.5	3.10	2.28	4.50	9.88
		85.2	2.92	2.72	4.68	10.32
	10	95.4	1.25	3.40	4.10	8.75
		93.6	1.40	3.00	4.27	8.67
	15	80.6	1.20	2.88	4.53	8.61
		80.0	1.10	3.75	4.60	8.45
	20	75.4	1.06	2.45	4.82	8.33
		68.8	1.18	2.21	4.67	8.06
60	5	74.5	4.27	2.48	4.86	11.61
		69.7	4.01	2.78	5.12	11.91
	10	70.2	3.77	2.95	5.84	12.56
		74.6	3.58	2.87	5.42	11.87
	15	30.2	3.72	2.48	4.45	10.65
		77.6	3.04	2.58	5.22	10.85
	20	88.7	2.60	3.40	5.44	10.44
		85.6	1.79	2.74	5.76	10.29
90	5	72.4	8.14	2.25	4.85	15.24
		68.5	6.45	2.48	5.12	14.05
	10	65.5	5.72	2.00	4.47	12.19
		70.2	6.25	1.84	5.67	13.76
	15	69.4	8.44	1.74	6.60	16.80
		75.5	7.16	2.30	6.77	16.23
	20	64.2	5.43	1.78	6.58	13.79
		70.5	5.84	2.59	5.41	13.84
120	5	64.4	7.72	1.48	5.64	14.84
		70.2	8.28	2.52	5.08	15.88
	10	70.8	8.45	2.44	4.79	15.68
		68.2	9.22	2.20	4.67	16.09
	15	64.4	7.45	2.16	4.60	14.21
		65.5	8.22	2.88	4.12	15.22
	20	70.5	7.45	2.54	4.40	14.39
		68.9	9.00	2.87	4.52	16.39

[a] Mg% on fresh-weight basis.

in the 30-sec blanch. The total methoxyl content of the 3 major fractions increased from 9.00 mg% to 15.34 mg% when blanch time was increased from 30 to 120 sec. The water-soluble fraction, which is the main substrate of pectin methylesterase, showed a significant increase of methoxyl content from 1.67 mg% to 7.22 mg% when blanching time was increased from 30 to 120 sec; however, in the ammonium-oxalate-soluble fraction the methoxyl content tended to fall off with the length of blanch. Methoxyl content in the acid-soluble fraction did not show much change, regardless of the length of blanching time.

Within each fraction, methoxyl content roughly reflected the magnitude of uronide content present in the respective fractions. For example, the water-soluble pectic substances were lowest in the 30-sec blanch and highest in the 120-sec blanch. Correspondingly, the mg% methoxyl content was lower in the water-soluble fractions of the 30-sec blanch. This raised the question whether the lower methoxyl content in the 30-sec blanch may simply be due to a lower content of pectic substances. In order to clear up this uncertainty the ratio of total methoxyl content to total pectic content was calculated. The increase in this ratio gives support to the concept that the shorter blanching time results in a higher pectin methylesterase activity.

It would be expected that prolonged blanching would encourage deeper heat penetration and thus result in reduced pectin methylesterase activity. On the other hand, blanching for a shorter period

Table 2. Relationship of treatments to firmness and pectic substances.

Sample			Total pectic substances[a]			
Time of blanching (sec)	Holding period (min)	Index of firmness	Water-soluble fraction	Ammonium-oxalate-soluble fraction	Acid-soluble fraction	Total pectic substances
30	5	80.5	33.0	83.3	90.0	206.3
		85.2	31.0	93.3	88.3	192.6
	10	95.4	28.7	103.5	78.2	210.4
		93.6	25.6	106.7	82.0	211.3
	15	80.6	24.0	91.1	70.0	180.1
		80.0	32.9	83.0	88.8	214.7
	20	75.4	19.1	71.1	87.1	188.3
		68.8	20.0	77.2	92.5	189.7
60	5	74.5	55.8	69.0	99.0	226.8
		69.7	45.9	62.0	98.5	206.4
	10	70.2	45.3	69.5	103.4	218.2
		74.6	42.6	65.6	95.4	203.6
	15	80.2	43.7	70.8	86.6	201.1
		77.6	47.8	75.8	108.8	232.4
	20	88.7	40.2	80.6	105.2	226.0
		85.6	42.0	80.3	98.5	220.8
90	5	72.4	88.6	50.0	105.1	243.7
		68.5	73.3	52.1	115.1	240.5
	10	75.5	64.4	45.6	90.8	200.8
		70.2	72.7	32.7	123.9	229.3
	15	69.4	92.8	38.8	113.1	244.7
		75.5	77.8	55.4	120.6	252.7
	20	64.2	88.5	40.4	115.6	244.5
		70.5	64.3	60.8	98.8	223.9
120	5	64.4	111.6	34.5	95.5	241.6
		70.2	95.5	55.0	93.8	244.3
	10	70.8	88.0	60.0	88.7	236.7
		68.2	99.1	48.5	90.8	238.4
	15	64.4	88.0	45.0	94.7	227.7
		65.5	98.6	40.0	84.3	222.9
	20	70.5	85.4	47.0	95.6	228.0
		68.9	100.0	40.0	87.0	227.0

[a] Mg% on fresh-weight basis.

in boiling water probably inactivated only the surface-located pectic enzymes and at the same time caused disruption of cells within the fruit, where the temperature would not be sufficient to kill the enzymes but would encourage enzyme-substrate contact.

The most significant observation was the change in the proportion of the pectic substances between the ammonium-oxalate-soluble fraction and water-soluble fraction with the length of blanch. As blanching time was increased the proportions of pectic substances in the ammonium-oxalate-soluble fraction decreased and the water-soluble fraction increased. The low water-soluble pectin content in the 30-sec blanch was probably due to high pectin methylesterase activity which de-esterified the methoxyl groups to such an extent that the pectin in this fraction became water-insoluble. In the 90- and 120-sec blanch the high retention of water-soluble pectin was probably due to decreased pectin methylesterase activity.

As shown in Fig. 1, the highest ammonium-oxalate-soluble content was attained during the 10-min holding period following the 30-sec blanch. Beyond 10 min this fraction decreased. This decrease could be due to an increased activity of pectin polygalacturonase beyond the 10-min holding period. Fig. 1 also shows the effect of holding period following the 60-sec blanch. Following the 60-sec blanch the ammonium-oxalate-soluble fraction increased with increased holding time. This increase was probably due to the complete or par-

Table 3. Relation between blanching time, firmness, total pectic substances, and methoxyl contents expressed as averages of 4 holding times.

Time of blanching (sec)	Index of firmness	Total pectic substances (mg% on fresh-weight basis)			Total pectic substances	Methoxyl-content (mg% on fresh-weight basis)			Total methoxyl content
		Water-soluble fraction	Ammonium-oxalate-soluble fraction	Acid-soluble fraction		Water-soluble fraction	Ammonium-oxalate-soluble fraction	Acid-soluble fraction	
30	82.4	26.8	88.7	84.3	199.8	1.65	2.83	4.52	9.00
60	77.5	45.4	70.3	98.2	213.9	3.97	2.28	4.26	11.32
90	69.5	77.8	47.0	100.8	225.6	6.68	2.12	5.68	14.48
120	67.2	97.0	47.3	93.8	241.1	7.22	2.40	4.93	15.34

Table 4. Changes of pectic substance constituents during various blanching treatments expressed as percentage of total pectic substances.

Time of blanching (sec)	Water-soluble fraction	Oxalate-soluble fraction	Acid-soluble fraction
30	13.4	44.4	42.2
60	21.2	32.6	46.2
90	34.4	21.2	44.4
120	40.7	19.3	40.0

Table 5. Effect of blanching time on the ratio of total methoxyl to the total pectic content.

Time of blanching (sec)	Total methoxyl content / Total pectic content
30	4.50
60	5.29
90	6.42
120	6.36

tial inactivation of pectin polygalacturonase by the 60-sec blanch.

Firmness of canned whole tomatoes was positively correlated with ammonium oxalate fraction, i.e. Ca pectates and low methoxyl pectin (Fig. 2). As shown in Fig. 3, firmness was also positively correlated with methylesterase activity. This was in agreement with the observation that increased

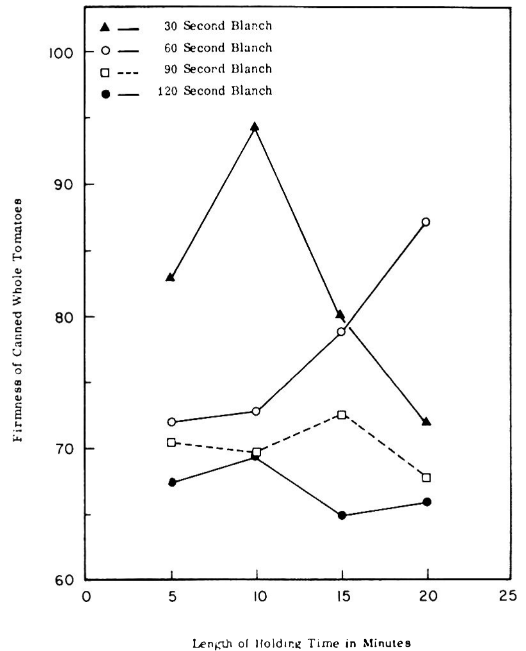

Fig. 1. Effect of blanching and holding time on firmness of canned whole tomatoes.

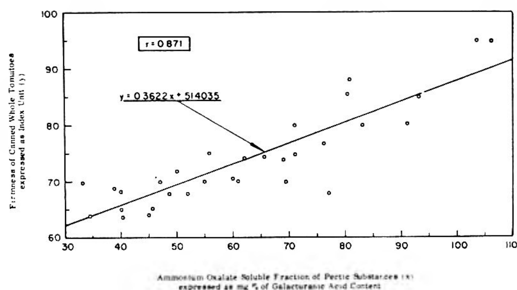

Fig. 2. Relationship of firmness of canned whole tomatoes to the ammonium-oxalate-soluble fraction.

firmness was positively correlated with the magnitude of the ammonium oxalate fraction (Fig. 2). The greater activity of pectin methylesterase resulted in an increase of low-methoxyl pectin for calcium pectate formation. This increased pectate formation, in turn, resulted in a higher firmness retention.

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J. W. KITCHEN[a] **and E. E. BURNS**
Department of Soil and Crop Sciences, Horticulture Section
Texas A & M University, College Station, Texas 77843

The Effect of Maturity on the Oxalate Content of Spinach (Spinacia oleraceae L.)

SUMMARY

Dark Green Bloomsdale spinach was grown in the fall and in the spring to study the effect of stage of development upon the distribution of total and soluble oxalates in the plant. The total oxalate content of fall-grown spinach was highest 32 days after planting, with the amount decreasing as the plant developed vegetatively. Spring-grown spinach was highest in total and soluble oxalates 32 days after planting, with the amounts decreasing as the plants developed vegetatively. Leaves harvested earlier in growth were higher in total oxalates than leaves harvested when older. The concentration of total and soluble oxalates was highest in the leaves, petioles, and roots, in that order. The total oxalates were closely correlated between the leaves, petioles, and roots. Soluble oxalates were closely correlated between leaves and petioles, but not in the roots. The oxalates in all plant parts were predominantly in the soluble form. Analysis of leaf tissues for total oxalates could indicate the level to be expected in the petioles and roots. The level of soluble oxalates in the leaves and petioles could also be predicted.

INTRODUCTION

The study of oxalate composition of plant material in relation to the diets of animals and humans has gained impetus from the increase in knowledge of the physiological effects of oxalates after ingestion. The principal factors in the metabolism of oxalates in humans and animals are the effects on the availability of dietary calcium, causation of urinary calculi, and the increase in the incidence of oxaluria and oxalemia. The oxalate content should therefore be considered as a quality attribute of spinach.

The stage of development of the spinach plant has been recognized as a factor contributing to variation in the oxalate content of the plant. Researchers are in agreement that oxalates are higher in the leaves than in the petioles. There are divergent opinions in the literature as to the influence of stage of maturity of the plant on the accumulation of oxalates. Recently, researchers have noted the distinction between soluble and insoluble oxalates in spinach. The literature contains little information concerning the difference in oxalates and their distribution in the plant as it matures.

This study was made to investigate the effect of stage of maturity of the spinach plant on total and soluble oxalate contents and their distribution.

REVIEW OF LITERATURE

Oxalic acid production and accumulation in spinach has been demonstrated to be a response of several physiological factors, especially the stage of development of the plant. According to Maximov (1938), calcium oxalate crystals usually appear in cells approaching senescence and are especially abundant in drying tissue. The toxic action may kill cells in which they are deposited.

DeVilmorin and Bilques (1957) observed that the ash content as well as the oxalic acid content of spinach increased regularly with the age of the plant. Conversely, Ryder (1930) noted that small leaves of spinach contained the highest percent of total solids and oxalic acid; leaves over four inches contained smaller quantities. He also noted that leaf petioles contained less oxalic acid than the leaf blade. Srivastava and Krishman (1959) and Pierce and Appleman (1943) similarly observed differences in the oxalates present in the petioles and leaf tissue. Grutz (1954) found that old leaves were higher in oxalates than young leaves.

The oxalate content in relation to the development of the plant has been reported for other species. In the leaves of bathua (*Chenopodium album* L.), Singh and Sur (1962) observed an increase in both total and soluble oxalates until the flowering stage. Zarembski and Hodgkinson (1962) attributed differences in the oxalates of forced rhubarb and that of rhubarb harvested at the end of the growing season to the age of the plant. Victoria rhubarb (*Rheum* L. var.) contained 260 mg of oxalates per 100 g of fresh weight when forced; at the end of the season it contained 620 mg per 100 g of fresh weight. Schuphan and

[a] Present address: Department of Park Administration, Horticulture and Entomology, Texas Technological College, Lubbock, Texas.

Weinmann (1958) found that certain climatic conditions caused increases in oxalic acid from an average of 400 mg of oxalate per 100 g of fresh material to levels as high as 1500 mg per 100 g.

Mathams and Sutherland (1952), investigating 80 species of pasture plants, found that oxalates were generally highest in immature plants. In the study of oxalates in mangolds, sugar-mangolds, and fodder sugar beets, Baker and Eden (1954) observed high concentrations in the summer months, diminishing as the season advanced.

Fleming (1950) and Cook and Stoddard (1953) found in the annual weed halogeton (*Halogeton glomeratus* L.) that the oxalate content was highest in the early fall and decreased to a minimum by early spring. Morton *et al.* (1959) found that the leaves contained the highest amount of oxalates and the stem fraction relatively small amounts. The oxalates of this species were present mostly in the water-soluble form of sodium oxalate. The above group of researchers have adequately characterized the form, distribution, accumulation, and disappearance of oxalates for this species. It is interesting to note that these characteristics are available for a common weed which is ingested by livestock, but are conspicuously lacking for the spinach plant consumed by humans.

METHODS AND MATERIALS

One of the commercial varieties of spinach, Dark Green Bloomsdale, was employed to determine the distribution of oxalates in the plant. Using a randomized block with four replications the plants were grown in the field as a fall crop in 1962 and a spring crop in 1963.

The fall plots were direct seeded on October 26, 1962. The first samples were collected on December 1, 1962, 32 days after seeding. Leaves most distal to the central axis, the oldest leaves on the plant, were employed for samples. Leaves from ten different plants per replication were collected for one sample. Determinations were made for total oxalates by the method of Moir (1953). Results are expressed as percent anhydrous oxalic acid on a dry-weight basis. Samples were not collected after January 15, 1963, because of plant damage caused by subfreezing temperatures in late January and early February.

The second investigation was on a spring crop of spinach seeded on March 3, 1963. In order to follow the accumulation of oxalates in the entire plant, the plants were divided into three parts for analysis of total and water-soluble oxalates in the roots, petioles, and leaves. Soluble and total oxalates were determined by the method of Moir.

The first samples were harvested 32 days after planting and at 10-day intervals thereafter. Four replications were employed for each harvest date. Ten plants were employed for each sample. Samples were not collected after May 10, 1963, because of damage caused by high temperatures.

RESULTS

The effect of harvest date upon the total oxalate content of leaf tissue in fall-grown spinach is shown in Table 1. The total oxalate content ranged from 8.41 to 10.99% as a result of the development of the plant. The difference of 2.58% anhydrous oxalic acid was significant as indicated by analysis of variance techniques. Leaves harvested on December 1, 1963, 32 days after planting, were higher in total oxalates than those on subsequent harvest dates. There was a decrease in total oxalates on the second and third harvest dates, followed by a small increase on the fourth harvest date. The mean oxalate content on the first harvest date was significantly different from the third harvest date at the 5% probability level.

The plant parts containing the highest amounts of oxalates, both total and soluble, in spring-grown spinach were leaves, followed by the petioles and the roots. The means of all harvest dates for total oxalates in the leaves, petioles, and roots were respectively 12.47, 5.88, and 4.12%, with a difference of 8.35% between the highest and lowest. The means for soluble oxalates in the leaves, petioles, and roots were respectively 10.08, 5.53, and 3.27%, with a difference of 6.81% between the highest and lowest. These differences in both total and soluble oxalate contents of plant parts were highly significant, as indicated in Tables 2 and 3.

Plants harvested on the first harvest date, 32 days after planting, were higher in total oxalates in the leaves, petioles, and roots than plants harvested on subsequent dates (Table 2). The total oxalates were 9.43% as anhydrous oxalic acid. The

Table 1. Effect of date of harvest on the total oxalate content (% anhydrous oxalic acid on dry-weight basis) of Dark Green Bloomsdale spinach leaves grown in the fall.

	Days old and dates			
	32 1 Dec. 62	47 15 Dec. 62	77 15 Jan. 63	62 1 Jan. 63
Means	10.99[a]	9.86[a]	9.46[a]	8.41[b]

[a, b] Indicates 5% level of significance for Duncan's multiple-range test. Values with a common letter are not significantly different.

Table 2. Effect of maturity on the total oxalate content (% anhydrous oxalic acid on dry-wt basis) in the roots, petioles, and leaves of Dark Green Bloomsdale spinach.

	Dates and days old				
	Apr. 5 32	Apr. 15 42	Apr. 25 52	May 5 62	Means
Roots	5.10	4.62	3.04	3.72	4.12[a]
Petioles	9.27	5.77	4.22	4.25	5.88[b]
Leaves	13.91	13.51	11.19	11.28	12.47[c]
Means	9.43[a]	7.97[b]	6.15[c]	6.42[c]	7.49

[a,b,c] Indicates 1% level of significance for Duncan's multiple-range test. Values with a common letter are not significantly different.

Table 3. Effect of maturity on the soluble oxalate content (% anhydrous oxalic acid on dry-wt basis) in the roots, petioles and leaves of Dark Green Bloomsdale spinach.

	Dates and days old				
	Apr. 5 32	Apr. 15 42	Apr. 25 52	May 5 62	Means
Roots	2.94	4.28	2.42	3.45	3.27[a]
Petioles	5.99	5.38	3.82	2.92	4.53[b]
Leaves	11.48	10.43	9.14	9.26	10.08[c]
Means	6.80[a]	6.70[a]	5.13[b]	5.21[b]	5.96

[a,b,c] Indicates 1% level of significance for Duncan's multiple-range test. Values with a common letter are not significantly different.

smallest amount, 6.15%, was observed 52 days after planting, on the third harvest date. The difference was found to be highly significant. No significant differences were found in the total oxalate content on the third and fourth harvest dates.

The soluble oxalate content of the spinach plant followed the same general pattern as the total oxalates in the plant. Soluble oxalates in the leaves, petioles, and roots were 6.80% as anhydrous oxalic acid on the first harvest date. In considering the soluble oxalate content of the entire plant, the greatest quantity was found on the first harvest, 32 days after planting; the smallest amount was found on the third harvest date, 5.13% (Table 3). The difference of 1.67% was highly significant. The means on harvest dates one and two were not significantly different; similarly, the means on harvest dates three and four were not significantly different. The means on harvest dates one and two were highly significantly different from the means on harvest dates three and four.

A highly significant interaction between harvest dates and plant parts was observed for both total and soluble oxalates (Tables 2, 3).

Correlation coefficients determined for all possible combinations of total oxalates, soluble oxalates in the leaves, petioles, and roots for spring-grown spinach are shown in Table 4. Highly significant positive correlations were found for all combinations except combinations involving soluble oxalates in the roots. The relationship between the total oxalates and soluble oxalates in the leaves is presented graphically in Fig. 1.

DISCUSSION

The data for fall-grown spinach agree with Ryder (1930) in that the youngest leaves on the spinach plant contained the highest

Table 4. Correlation coefficients for means of oxalates in spring-grown spinach.

Determinations correlated	Roots total oxalates	Roots soluble oxalates	Petioles total oxalates	Petioles soluble oxalates	Leaves total oxalates	Leaves soluble oxalates	Entire plant total	Entire plant soluble
Total oxalates roots	....	.4810	.8494**	.7488**	.8176**	.8421**	.9252**	.8929**
Soluble oxalates roots		....	.0070	.1862	.4023	.2017	.2337	.4747
Total oxalates petioles			....	.8288**	.7840**	.8770**	.9601**	.8077**
Soluble oxalates petioles				....	.8131**	.7832**	.8606**	.9122**
Total oxalates leaves					....	.8727**	.9154**	.9166**
Soluble oxalates leaves						....	.9253**	.8978**
Total oxalates entire plant							....	.9150**

** 1% level of significance.

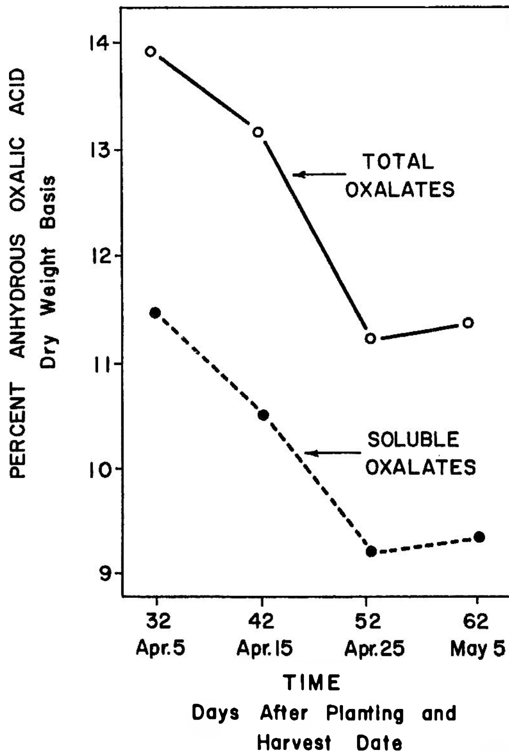

Fig. 1. Effect of maturity on total and soluble oxalates in all leaves on the spinach plant. Spring-grown spinach planted on March 3, 1963.

amount of oxalates. The total oxalate content of the leaves decreased with time. This observation does not agree with published results of DeVilmorin and Bilques (1957) and Doesburg and Sweede (1948) indicating that oxalic acid increased with the development of the plant. The difference may be due to comparative stages of maturity and growing season.

Spinach grown under fall conditions remains in the vegetative state. As the season advances, the growth and development of the plant remains in a more or less stable stage until the spring, when conditions are favorable for rapid growth and maturation. The data presented here represent total oxalates present in the leaves for the vegetative phase of development.

Examination of the data for spring-grown spinach reveals that the plants were initially high in both total and soluble oxalates. As the plant developed vegetatively both the total and soluble oxalates decreased.

The pattern of development of total oxalates in the leaves of spinach was similar for both fall-grown and spring-grown crops. In both cases, early in the development of the plant, the total oxalic acid content was high. As the plant developed vegetatively the total oxalate content decreased.

Preliminary investigations revealed that only traces of oxalates were present in the seed. This investigation revealed that large amounts of oxalates were present in the plants only 32 days old. This would indicate that oxalates are formed rapidly by photosynthetic tissue in the initial stages of plant development, or that oxalates are formed very rapidly from reserve food supplies in the seed.

The concentration of total oxalates was higher in the leaves than in the petioles, which is in agreement with the work of others (Ehrendorfer, 1961; Pierce and Appleman, 1943; Schuphan and Weinmann, 1958). The gradient of both total and soluble oxalates from the leaves to the roots indicates that oxalates may be translocated from the point of production or higher concentration to other parts of the plant. The greater the distance from the leaf tissue, the lower the concentration of both total and soluble oxalates.

The highly significant interaction of plant parts and harvest dates indicated that these two factors did not produce independent effects upon the oxalate content of the spinach plant. This effect could be attributed to variations in the environmental conditions between harvest dates. Doubtless, variations in factors such as light quality and temperature changed the rate of production and produced this effect. Although more theoretical implications of this effect could be postulated, any hypothesis based on the available evidence would be largely speculation.

An interesting aspect of this investigation is the highly significant correlation values for total and soluble oxalates of the various plant parts. The level of total oxalates in the petioles could be predicted from the level of total oxalates present in the leaves. Similarly, the level of total oxalates present in the roots could be predicted from the level of total oxalates present in the leaves or the petioles. The same is true for soluble oxalates except that soluble oxalates in the

roots were not maintained at levels corresponding to those in other plant parts. This difference may be due to difference in translocation of the elaborated oxalic acid or the rate of ionic absorption from the soil.

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S. N. DESHPANDE,[a] W. J. KLINKER,[b] H. N. DRAUDT, and N. W. DESROSIER [c]
Departments of Horticulture and Biochemistry, Purdue University, Lafayette, Indiana 47907

Role of Pectic Constituents and Polyvalent Ions in Firmness of Canned Tomatoes

SUMMARY

Twelve sets of canned tomatoes, each set composed of three fruits harvested from the same plant and subjected to different ripening conditions, were analyzed for pectic constituents. Firmness was highly significantly correlated with total pectic constituents and with the ratio of the carbonyl and pectic content. Correlation between firmness and mineral content was also significant. These results indicate that a high content, large molecular size, and a low methoxyl content of the pectic constituents result in firmer tomatoes. From the results it appears that firmness is related to retention of the original pectic content and controlled demethylation. This induced demethylation increases the extent of ionic bonding and results in firm fruits.

INTRODUCTION

Quality in canned tomatoes is governed in part by wholeness of the fruit. Various canning practices lead to disintegration of the fruits and consequently impair quality. The pectic substances are thought to be responsible for structural integrity in canned tomatoes (Appleman and Conrad, 1927; Kertesz, 1951).

Use of calcium salts is based on this concept (Kertesz, 1949). However, the importance of various factors such as total pectic content, degree of polymerization, methoxyl content, and concentration of polyvalent ions has not been clarified. This investigation was therefore undertaken to correlate firmness with pectic constituents.

The pectic substances are polyuronides composed mostly of any hydrogalacturonic acid residues in the middle lamellar layer of cells in higher plants. They also occur in the primary cell wall of the meristematic and embryonic cells and also in plant juices and extracts of various tissues. The pectic complex is a triad consisting of straight-chain molecules of partially methylated polygalacturonic acid residues to which are attached at undetermined points, side groups of araban and galactan. This structure has an important bearing in regulating the behavior of pectins. The lower the methoxyl content, the greater the number of carboxyl groups; and the greater the number of these latter units the greater is the possibility of ionic bonding (Baker, 1948; Hills and Speiser, 1946; Hills *et al.*, 1945). The term pectin is a group designation or a generic term which embodies protopectin, pectinic acid, and pectic acid loosely. Protopectin is the water-insoluble parent pectic substance occurring in plants, which upon restricted hydrolysis yields pectinic acids. Ripening processes result in disappearance of protopectin and a corresponding increase of pectinic or pectic acids. The term pectinic acids is used for all naturally occurring pectic polyuronides of colloidal nature and of any extent of esterification. Pectic acids are pectic polygalacturonides totally devoid of any degree of esterification (Kertesz, 1951).

This structural variation in the pectic components then governs the properties such as integrity of the texture in fruits and vegetables. These variations can be brought about by pectic enzymes (Baker, 1948; Hills and Speiser, 1946; Hills *et al.*, 1945; Kertesz, 1951).

Mainly, three pectic enzymes influence the alteration in the structure of a natural polygalacturonide. These are: 1) pectin methylesterase or esterase; 2) pectin polygalacturonase; and 3) depolymerase. These jointly bring about the deterioration of the polygalacturonide nucleus. Pectin methyesterase catalyzes the de-esterification of the methoxyl groups.

The second enzyme, pectic polygalacturonase, catalyzes the hydrolysis of the 1,4-glycosidic linkages. The third enzyme, pectic polygalacturonase, is of importance in horticultural processing. Depolymerase is of na-

[a] Present address: School of Agriculture, Tuskegee Institute, Alabama 36088.
[b] Present address: Libby, McNeill and Libby, Chicago, Illinois.
[c] Present address: Beech-Nut Life Savers Inc., 477 Madison Ave., New York, N. Y.

tural occurrence in fruits and vegetables. It is distinguished from the mold polygalacturonase by its specificity for cleavage of the polygalacturonides to large units (Kertesz, 1951).

The edible portions of most fruits and vegetables are composed largely of parenchymatous tissue. The cells of this tissue are generally thin-walled and cemented together by pectic substances of the middle lamella. Similar to changes known to occur during ripening of the fruits, an increase in pectin and pectic acid results in cooking. This hydrolysis of pectin is correlated with an increase in softening. The shape of the parenchymatous cells is such that they do not fit together perfectly. The interstices thus formed are filled with air. During the blanching operations, the air expelled may or may not be replaced by water. This simple factor can affect the firmness; the parenchymatous cells are alive previous to processing, and the method and rate of killing these cells influence the quality of the product (Weir and Stocking, 1949).

MATERIALS AND METHODS

Tomatoes of the variety Urbana, at approximately the canning stage, were subjected to the following tests of maturity and initial firmness: 1) the color intensities of the fruits were measured on the Hunter color-difference meter; and 2) the firmness of the fruits was recorded on the Asco firmness meter. The fruits were scalded for 30 sec in the boiling water, peeled, and packed in 300 × 211 cans. A single fruit was packed per can, with tomato juice as the packing medium. Each fruit was marked with indelible ink and the corresponding numbers were recorded on the containers after peeling to trace the previous history after processing and storage for the cut-out tests. The results of the cut-out tests confirmed the observation that firmness was not goverened entirely by the stage of maturity of the fruit, its initial firmness, processing treatments, or even the length of storage. Evidently the variation in nature of pectic constituents, and probably the variation in total content, determines firmness, and further investigations were concentrated on estimation and characterization of these.

Twelve sets of three fruits of similar maturity of variety Urbana were selected from the same plant. For each replication, one of the fruits was allowed to ripen on the vine. The other two fruits were picked at the turning stage. One of these fruits, in turn, was ripened at room temperature of 63°F. The fruits were canned when they attained equal maturity; a ratio of $a{:}b$ = approximately 1.9 on the Hunter colorimeter was used as the index. In order to avoid contamination from the pectic constituents of tomato juice, ordinarily used to fill cans of tomatoes, all the fruits were packed in phosphate buffer of pH 4.4 and processed for 45 min at 212°F.

Since judging firmness by mere visual observation was prone to subjective errors, an attempt was made to devise a method for measuring firmness objectively. The packs were drained for 2 min on a 0.5-inch-mesh screen, and their weight was recorded. The contents were packed again in fresh cans and agitated over a mechanical shaker for a uniform period of 2 min. They were drained as before, and weighed again.

$$\frac{\text{Weight of strained fruits after shaking, in grams}}{\text{Weight before treatment, in grams}} \times 100$$

Table 1. Correlation of objective evaluation with visual score

Sample no.	x	y	Sample no.	x	y	Sample no.	x	y
1	1	44	13	1	55	25	1	36
2	2	57	14	2	70	26	1	44
3	3	82	15	2	72	27	1	50
4	1	47	16	1	57	28	1	37.5
5	1	42.5	17	3	80	29	2	69
6	3	83	18	2	67	30	1	50
7	2	67	19	2	57	31	1	62.5
8	3	69	20	3	89	32	1	60
9	1	44	21	1	44	33	1	37.5
10	1	36.4	22	2	75	34	1	43
11	1	54	23	2	50	35	2	62.5
12	2	70	24	3	80	36	3	78

x = visual index.
y = objective index.
r = .85482**

Table 2. Vine-ripened fruits; summary of the results of analysis.

Code	*L reading* on Hunter color-imeter	*A/B* ratio on Hunter color-imeter	Visual index of firmness	Objective index of firmness (F.P.I.)	Pectin content expressed as mg% galacturonic acid			Alkoxyl content expressed as mg% of MeOH			Carbonyl content expressed as reducing value of mg% maltose			% Ca g	% Mg g	% K g
					Cold-water fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction			
x-2	30.6	2.10	+1	44.0	15.20	3.40	18.80	16.20	2.44	7.00	38.00	13.50	23.30	0.039	0.023	0.064
x-3	29.3	2.12	+2	57.0	10.40	8.80	14.00	.....	.....	13.12	45.00	27.00	90.00	0.019	0.033	0.057
x-5	32.2	2.03	+3	82.0	37.00	8.60	34.00	12.50	3.50	6.25	26.50	20.00	49.00	0.035	0.035	0.036
x-6	29.6	2.15	+1	47.0	1.70	1.70	9.60	0.55	2.40	4.25	48.00	49.00	49.00	0.016	0.030	0.036
x-7	30.0	2.10	+1	42.5	1.70	0.66	9.65	2.20	2.26	1.18	45.00	38.00	48.00	0.022	0.034	0.041
x-8	30.3	2.00	+3	83.0	38.00	21.00	48.00	2.30	1.13	4.13	48.00	43.00	49.00	0.017	0.028	0.054
x-11	30.9	1.92	+2	67.0	19.00	9.20	36.50	32.50	21.50	5.38	118.00	104.00	164.00	0.015	0.033	0.046
x-12	31.2	2.13	+3	69.0	16.00	41.00	8.00	27.50	37.20	2.50	112.00	35.60	188.00	0.016	0.034	0.053
x-15	30.6	2.14	+1	44.0	9.65	4.10	23.00	4.00	2.20	4.75	81.00	21.00	88.00	0.021	0.029	0.049
x-20	30.3	2.17	+1	36.4	9.50	9.20	33.00	8.75	7.30	4.38	40.00	26.00	100.00	0.019	0.035	0.064
x-24	30.2	2.08	+2	54.0	9.20	51.50	46.50	2.28	2.32	4.50	162.00	172.00	184.00	0.035	0.035	0.061
x-26	30.6	2.15	+2	70.0	75.00	40.00	50.00	6.00	2.22	1.22	240.00	17.80	256.00	70.100	0.035	0.043

Table 3. Fruits harvested in identical ripening stages; ripened at room temperature; summary of the results of analysis.

Code	*L reading* on Hunter color-imeter	*A/B* ratio on Hunter color-imeter	Visual index of firmness	Objective index of firmness	Pectin content expressed as mg% galacturonic acid			Alkoxyl content expressed as mg% of CH_3OH			Carbonyl content expressed as reducing value of mg% maltose			% Ca g	% Mg g	% K g
					Cold-water fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction			
y-2	31.90	2.12	+1	55	3.40	4.80	5.00	4.80	6.00	3.00	11.50	4.80	46.00	0.019	0.028	0.041
y-3	31.60	2.28	+2	70	15.20	15.20	14.00	8.25	9.50	15.00	340.00	17.50	45.00	0.019	0.028	0.032
y-5	32.70	1.90	+2	72	13.00	9.70	44.00	4.75	.....	0.75	6.75	28.00	48.00	0.010	0.025	0.021
y-6	32.60	1.95	+1	57	8.90	2.40	38.00	2.12	1.25	6.50	7.00	35.00	40.00	0.015	0.027	0.028
y-7	30.40	2.27	+3	80	21.00	18.00	31.00	2.07	2.05	3.25	48.00	41.00	48.00	0.016	0.029	0.035
y-8	30.40	2.10	+2	67	23.00	9.80	60.00	3.25	2.00	3.50	49.00	46.00	22.00	0.016	0.029	0.034
y-11	30.80	2.16	+2	57	9.65	7.60	51.50	32.50	19.00	5.73	52.00	31.00	88.00	0.032	0.030	0.030
y-12	31.30	2.17	+3	89	51.50	8.30	8.75	27.50	6.75	4.38	37.00	18.00	14.00	0.020	0.029	0.049
y-15	30.60	2.14	+1	44	9.65	4.10	23.00	4.00	2.20	4.75	81.00	21.00	88.00	0.021	0.029	0.049
y-20	31.50	2.10	+2	75	10.00	8.25	38.00	6.25	6.25	4.625	64.00	28.00	124.00	0.016	0.031	0.038
y-24	30.60	2.22	+1	50	48.00	40.00	75.00	2.18	4.75	6.00	136.00	100.00	192.00	0.023	0.034	0.043
y-26	29.90	2.20	+3	80	60.00	60.00	50.00	4.25	3.50	1.09	148.00	20.00	204.00	......	0.035	0.043

Table 4. Fruits harvested in identical ripening stages; ripened at 63±3°; summary of results of analysis.

Code	*L reading* on Hunter color-imeter	*A/B* ratio on Hunter color-imeter	Visual index of firmness	Objective index of firmness	Pectin content expressed as mg% galacturonic acid: Cold-water soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	Alkoxyl content expressed as mg% of MeOH: Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	Carbonyl content expressed as reducing value of mg% maltose: Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	% Ca g	% Mg g	% K g
z-2	35.1	1.90	+1	36.0	0.72	2.30	10.40	3.00	....	10.00	46.00	46.00	9.00	0.022	0.039	0.047
z-3	34.5	2.10	+1	44.0	16.80	16.80	14.00	9.50	11.80	21.00	12.50	6.75	47.00	0.017	0.031	0.043
z-5	34.4	2.06	+1	50.0	0.46	0.45	7.00	9.50	0.75	3.64	6.75	28.00	48.00	0.010	0.025	0.021
z-6	33.0	1.90	+1	37.5	8.00	4.25	8.25	3.50	2.20	4.88	49.00	7.50	49.00	0.018	0.031	0.035
z-7	32.7	2.07	+2	69.0	43.00	18.50	51.50	1.00	0.68	1.14	48.00	43.00	48.00	0.019	0.033	0.046
z-8	32.3	1.84	+1	50.0	1.70	6.50	7.85	2.20	0.68	2.42	25.00	48.00	49.00	0.015	0.029	0.043
z-11	32.5	1.90	+2	62.5	8.90	8.00	30.00	30.00	28.25	5.25	52.00	46.00	168.00	0.020	0.033	0.041
z-12	34.6	1.98	+1	60.0	8.60	4.10	9.65	2.40	16.20	4.75	50.00	25.00	140.00	0.018	0.033	0.057
z-15	33.5	1.88	+1	37.5	17.00	5.38	9.65	6.00	9.00	3.50	88.00	136.00	98.00	0.040	0.034	0.038
z-20	34.5	2.10	+1	43.0	8.90	6.55	50.00	2.12	2.43	1.38	38.00	26.00	172.00	0.025	0.034	0.045
z-24	33.8	2.00	+1	62.5	19.00	9.40	19.00	4.75	2.13	2.00	102.00	52.00	200.00	0.017	0.033	0.044
z-26	32.9	2.00	+2	78.0	60.00	33.00	67.50	....	2.00	1.19	80.00	14.00	116.00	0.022	0.034	0.039

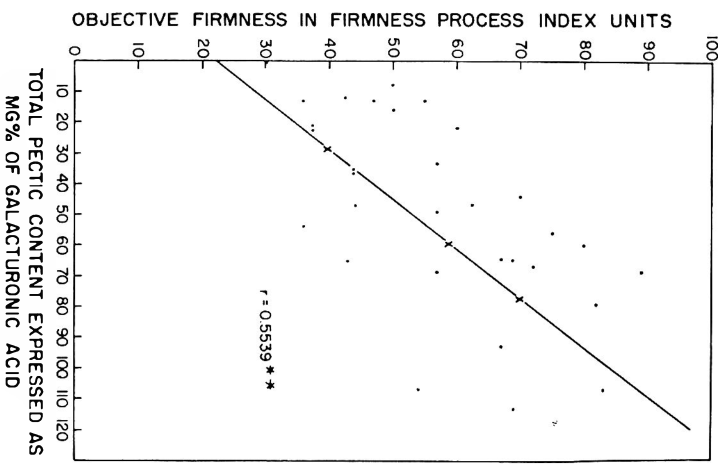

Fig. 1. Correlation of firmness with total pectic content.

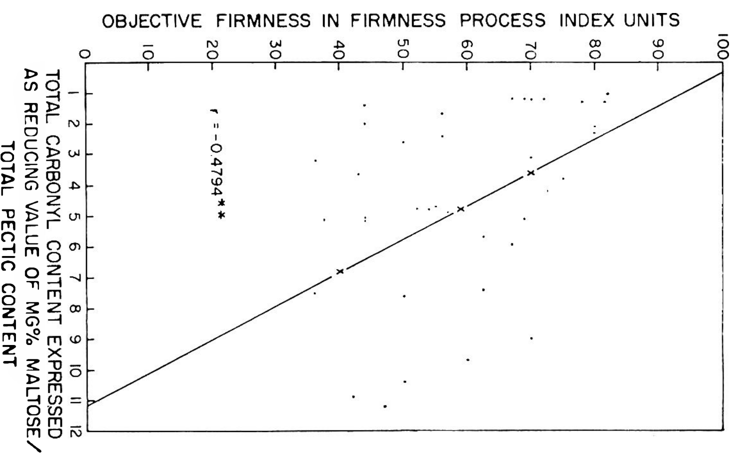

Fig. 2. Correlation of firmness with total carbonyl content.

Table 5. Table of correlations. Correlation of objective firmness with the total pectic content; ratio of total alkoxyl :total pectic content; ratio of total carbonyl :total pectic content; and ratio of total alkoxyl : total mineral content.

	Total pectic content	Total alkoxyl content/total pectic content	Total carbonyl content/total pectic content	Total alkoxyl content/total pectic content	Total mineral content
Objective firmness					
F P I units 1–100					
(*r*) value	.55392	.10592	−.4794	.15973	.3933
Significance	**	NS	**	NS	*
Regression	1.6275	.10921	−.10978	1.73048	−.00019

* Significant (at .05 level).
** Highly significant (at .01 level).
NS = not significant.

gives an objective index as a numerical value. This was called the firmness process index. This value correlated well with the visual score.

A 10-g sample of the canned tomatoes after determination of the objective firmness was wet-ashed for the determination of cations. A second 10-g sample was homogenized in a Waring blender with 95% ethanol and was used for analysis of pectic substances. The pectin was extracted in 3 fractions: 1) cold-water soluble; 2) hot-water soluble; and 3) the residual pectin stripped with 0.5% ethylenediaminetetraacetic acid (EDTA). Each of these fractions had a distinct characteristic.

The McCready-McComb colorimetric method was used for the estimation of pectic content. A 50-ml portion was treated with versene (EDTA) of pH 11.5 for 30 min to sequester cations and to de-esterify pectin. The mixture was acidified with acetic acid to a pH of 5.0–5.5, and then 0.1 g of pectinase (Rohm and Haas) was added. This mixture was agitated for 1 hr over a mechanical shaker, diluted to 100 ml, and filtered, and the filtrate was used for analysis. A 2-ml portion of this sample was added to 12 ml of H_2SO_4 which had been chilled to below 3°C in a 25 × 200-mm tube. This was mixed and cooled to 5°C. The contents of the test tube were heated in boiling water for 10 min and cooled, and 1.0 ml of 0.15% carbozole reagent in ethyl alcohol was added. After allowing the color to develop for about 20 min, the intensity was read at 520 mμ on a Coleman spectrophotometer. The total pectic content was expressed as mg% of anhydrogalacturonic acid (McComb and McCready, 1952; McCready and McComb, 1952). The alkoxyl content in the three pectic fractions was determined. A modification of the method developed by Boos was adapted with chromotropic acid as the reagent (Boos, 1948; Bricker and Johnson, 1945). A 20-ml portion of the corresponding pectic fraction was heated to 80°C, de-esterified with 5 ml of 10% NaOH, and neutralized with (1:4) H_2SO_4 and the CH_3OH condensed was distilled by recovering 70% of the volume, as described by Snell, and then made to 25 ml (Snell and Snell, 1937). A 2-ml portion was used for analysis of the residual pectic fractions. The latter sample was acidified with 4 drops of 5% H_3PO_4 and oxidized with 5 drops of 5% $KMnO_4$ solution for 10 min. The excess $KMnO_4$ was reduced with a few drops of saturated solution of $NaHSO_3$. Then 4 ml of cold H_2SO_4 and 10 drops of the chromotropic acid reagent were added. The samples contained in 10-ml flasks were heated in boiling water for 30 min and cooled, and the color intensity was measured at 580 mμ. The methoxyl content was expressed as μg of CH_3OH obtained from a standard curve previously constructed for pure CH_3OH.

In conjunction with the methoxyl content, the intrinsic content of Ca and Mg was determined by flame photometric method (Beckman spectrophotometer Model B with flame attachment was used). Determination of Na content was not attempted, since phosphate buffer with Na salts was used as a packing medium.

The chain length or the average molecular size of the pectin is assumed to influence the gel structure. It was thought the average molecular size could manifest a trend or relationship with firmness; hence, it was included as one of the determinations. The alkaline ferricyanide method used offers an approximate indication of the chain length by measuring the reducing property of the carbonyl

Table 6. Table of correlations. Correlation of total pectic content with total carbonyl content.

	Total carbonyl content	Total alkoxyl content
Total pectin content		
Correlation coefficient (*r*) value	.54313	.00927
Significance	**	NS
Regression coefficient (*b*) value	1.53342	.000747

** Highly significant.
NS = not significant.

Table 7. Correlation of pectic content of three fractions with the carbonyl content and the alkoxyl content of the corresponding fraction.

	Carbonyl content			Alkoxyl content		
	Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction	Cold-water-soluble fraction	Hot-water-soluble fraction	Residual EDTA-soluble fraction
Pectin content						
Correlation coefficient value	.3746	.27709	.36912	.00493	.00866	—.28021
Significance	*	NS	*	NS	NS	NS
Regression coefficient value	1.3245	.6523	1.2268	—.002662	—.00477	—.00587

* Significant.
NS = not significant.

group. The approximate molecular weight is determined from the relative reducing values, but these results were not translated into the number of monomer units or the molecular weights, since this method is nonstoichiometric (Nussenbaum and Hassid, 1952; Smith and Montgomery, 1956).

A 0.5-ml portion was used for the water-soluble fractions, and a 0.25-ml portion was used for EDTA-soluble fractions. The sample was pipetted into a graduated centrifuge tube and the volume brought to 2 ml with distilled water. One-half ml of carbonate cyanide and ½ ml of 8% K_3FeCN_6 solution were added to the graduated tubes and the tubes were heated 8 min in boiling water. These were further cooled, and 5 ml of ferric sulfate gum ghatti solution was used to develop color. Absorbance was measured at 520 mμ, and the results were expressed as the equivalent reducing value of mg% of maltose.

RESULTS AND DISCUSSION

The results of analysis are reported in the tables. The objective evaluation of firmness was highly significant when correlated with subjective judging, as shown in Table 1. Firmness of the processed tomatoes was found to be directly correlated with total pectic content (Fig. 1).

Total mineral content, including calcium, magnesium, and potassium was correlated with firmness.

Since the carbonyl content in the experiments related to the reducing value of the end group, and hence the average chain length of the pectic fraction, the ratio of carbonyl content, and the pectic content were correlated with the firmness. There was a highly significant negative correlation with firmness and carbonyl content (Fig. 2).

Correlation of the three pectic fractions with firmness is summarized in Tables 5, 6, and 7.

The fruits in each replication were ripened under different conditions in order to determine the effect of the pectic enzymes primarily. However, there were very severe fluctuations in temperature in ripening under refrigeration, and the temperatures could not be regulated in the proposed 60–63°F range. Firmness therefore could not be correlated with the various ripening conditions employed in this study.

In light of the foregoing observations, it appears that regulation of the activity of the two naturally occurring tomato enzymes, pectin methylesterase and depolymerase, would yield a desirable pectic structure. By controlled ripening, the degree of de-esterification of the pectic constituents beyond desirable limits could be avoided. This would protect the polygalacturonides from degradation to small units through the activity of depolymerase. This degradation would otherwise make mushiness of the canned tomatoes inevitable. A certain extent of de-esterification seems to occur during the transient periods of heating when tissue organization has already been disturbed by thermal breakdown, but the enzymes present have not been yet inactivated (Kertesz, 1951). This reaction can also be exploited by controlled blanching and use of inorganic salts to initiate ionic bonding in order to induce wholeness of the canned tomatoes.

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GLENN VAN HULLE,[a] O. FENNEMA, and W. D. POWRIE
Food Science Division, Department of Dairy and Food Industries, University of Wisconsin, Madison, Wisconsin 53706

A Comparison of Methods for the Microscopic Examination of Frozen Tissue

Crystallization of water in tissue has received considerable study (Heard, 1955a,b; Maurer and Murray, 1951; Meryman, 1957; Parkes, 1957; Smith, 1961; Woodroof *et al.*, 1946). In many instances the conclusions concerning tissue disruption and crystal location (intra- or extracellular) are based on observations of specimens from which the ice crystals have been removed. The ice is usually removed by lyophilization, solvent extraction, or a combination of the two (Humason, 1962; Sass, 1961; Heard, 1955 a,b; Rapatz and Luyet, 1959). Specimens prepared in this manner contain numerous voids which, according to Parkes (1957, p. 524) "presumably represent the spaces occupied by ice crystals." To our knowledge, no one has published information demonstrating how accurately the void spaces represent the size and location of ice crystals. The information which follows should be of some value in that respect.

METHODS

Carrots. Specimens measuring approximately 1 cm square × 5 cm long were cut from the centers of small carrots. Slow freezing was accomplished by placing the specimens in covered aluminum pans and allowing them to stand overnight on a block of ice in a chamber maintained at 10°F. Rapid freezing was accomplished by mixing the specimens with finely divided solid carbon dioxide and allowing them to stand for no less than 1 hr. Each specimen was tempered to approximately 22°F and sectioned (Bausch and Lomb rotary microtome) across its major axis. The sections were obtained approximately midway between the ends of the specimens. By this means, it was possible to section slowly frozen carrot specimens to a minimum thickness of 50 μ and rapidly frozen specimens to 40 μ.

Two adjacent sections were selected from each specimen. One section was immediately placed on the cold stage (Thomas-McCrone) of a microscope and allowed to thaw over a period of several minutes. Photomicrographs were taken initially (frozen), after partial thawing, and after complete thawing.

The other section was prepared for viewing by the following procedure:

1) The section was immersed in a fixative of the following composition (volume basis): 70% absolute ethyl alcohol, 15% water, 10% formaldehyde (37–40% by weight), and 5% glacial acetic acid. After 4 hr at 22°F the section and fixative were allowed to warm to room temperature and the section was removed and mounted on an albumenized slide.

2) A parloidin adhesive was applied and precipitated in 95% ethyl alcohol according to conventional procedures.

3) The ethyl alcohol content was gradually reduced by rinsing the specimen in aqueous alcohol solutions of 83, 70, and 30% ethyl alcohol by volume.

4) The specimen was stained with safranin and then rinsed in acidic alcohol, water, aqueous ethyl alcohol solutions of 50, 95, and 100% by volume, carbol-xylene, and xylene (2×).

5) A cover glass was affixed with piccolyte, and the specimen was viewed and photographed at 50×.

Beef muscle. A specimen of prerigor beef muscle (neck) measuring approximately 2 × 2 × 10 cm was thoroughly insulated and frozen slowly over an eight-day period at 22°F. Another specimen of the same prerigor beef muscle measuring approximately 1 × 1 × 4 cm was frozen rapidly by mixing it with finely divided solid carbon dioxide and allowing it to stand for no less than 1 hr. Both specimens were sectioned (Bausch and Lomb rotary microtome) at approximately 22°F. Cuts were made across the fibers near the centers of the specimens. Sections measuring 25 μ thick were obtained from the rapidly frozen specimen, whereas 50 μ was the minimum thickness possible with the slowly frozen specimen. The slowly frozen, fixed and stained section shown in Fig. 3 was 60 μ thick.

Two adjacent sections were selected from each specimen, and, except for the staining procedure, they were treated like the carrot sections. Following fixing and application of the adhesive, the muscle sections were stained and treated as follows:

1) The ethyl alcohol content was gradually reduced by rinsing the specimens in aqueous alcohol

[a] Candidate for M.S. degree.

solutions of 83, 70, 50, 35, and 0% ethyl alcohol by volume.

2) The specimens were stained with Harris' hematoxylin and then rinsed in water (2 ×), acidic water, water (2 ×), alkaline water, water (2×), aqueous alcohol solutions 35, 50, 70, 83, 95, and 100% by volume, xylene-ethyl alcohol (1:1 v/v), and xylene.

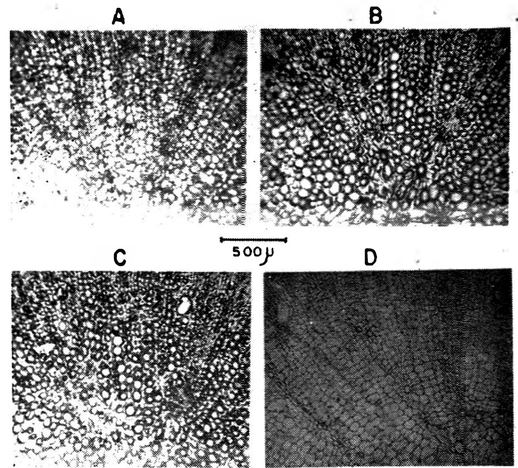
3 Cover glasses were affixed with piccolyte, and the specimens were viewed and photographed at 100 ×.

RESULTS

Photomicrographs of the various sections are shown in Figs. 1–4. In each case, *A* represents a frozen section, *B* the same section and area after partial thawing, *C* the same section and area after complete thawing, and *D* an adjacent section after fixing and staining. An identical area is represented in all four photomicrographs of the slowly frozen carrot (Fig. 1). The same applies to the rapidly frozen carrot (Fig. 2). The small x's appearing at the left center of the photomicrographs in Fig. 1 identify a common point.

A, *B*, and *C* of the slowly frozen muscle section (Fig. 3) represent an identical area of one section, whereas *D* represents a similar area of an adjacent section. The same applies to Fig. 4. With the meat samples, it was not possible to locate the same areas of different sections exactly.

The fixing and staining technique can be evaluated by comparing *D* with the corresponding *A*. In some of the frozen specimens

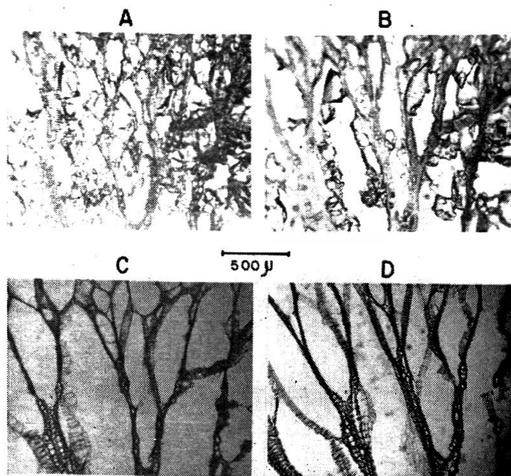


RAPIDLY FROZEN CARROT 50X,40μ

ABC THAWING SEQUENCE

D COMPARABLE SECTION—FIXED WHILE FROZEN

Fig. 2. The breaks in the tissue of photograph *D* are most likely caused by the microtome.

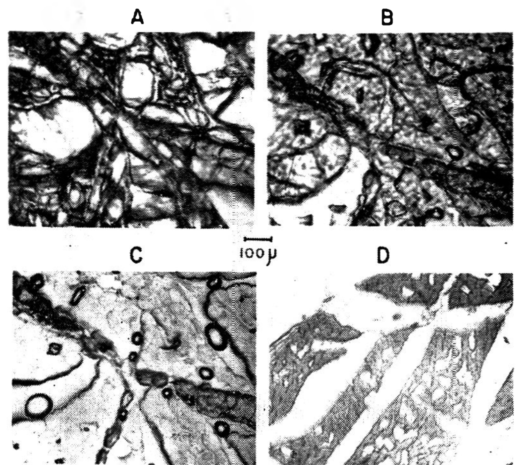


SLOWLY FROZEN CARROT 50X,50μ

ABC THAWING SEQUENCE

D COMPARABLE SECTION—FIXED WHILE FROZEN

Fig. 1. In photograph *D*, the short parallel lines appearing across the center are caused by a grid in the ocular.



SLOWLY FROZEN BEEF MUSCLE 100X,50μ

ABC THAWING SEQUENCE

D COMPARABLE SECTION —FIXED WHILE FROZEN

Fig. 3. In photograph *C*, the circular areas with dark peripheries are air bubbles. In photograph *D*, the short parallel lines appearing across the center are caused by a grid in the ocular.

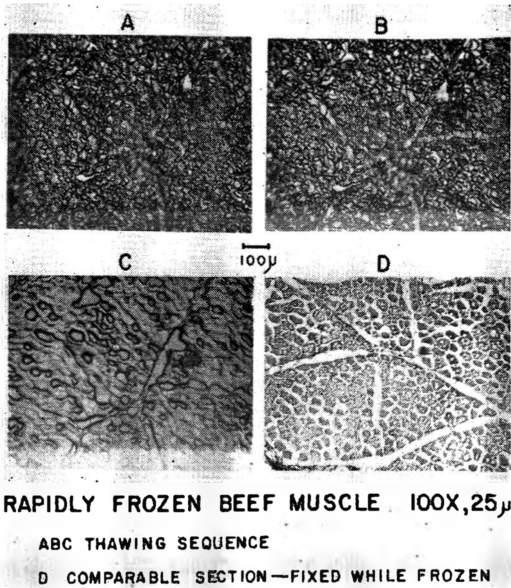


Fig. 4.

it is difficult to distinguish between ice and tissue. In such cases it is helpful to study the thawing sequence to determine which areas melt away.

From these photomicrographs, it is quite evident that the tissue voids were caused by ice formation, and that the fixing and staining procedures used did not result in any gross misrepresentations of the frozen structure. Whether some of the finer structures were altered cannot be ascertained from this work. Nevertheless, these results fail to provide any evidence which would justify criticism of the fixing-staining technique.

Although classical histological techniques are generally accepted as suitable for studies involving frozen tissue, the results of such studies are sometimes interpreted with caution (Parkes, 1957, p. 524; Weier and Stocking, 1949, p. 336). The results presented here should enable investigators who use the fixing-staining procedure for evaluating frozen tissue, to express their results with somewhat greater confidence than was possible previously.

Admittedly, it would be desirable to study the structures in greater detail by using thinner sections and greater magnifications. Such a study would, however, involve a vastly improved means of sectioning the frozen tissue.

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ANN A. HOLCK and M. L. FIELDS

Food Microbiology and Food Processing, Research Laboratories, Department of Horticulture University of Missouri, Columbia, Missouri 65202

The Effects of Storage Conditions Upon Acetylmethylcarbinol, Diacetyl, and Ethyl Alcohol in Apple Juice

SUMMARY

A total of 576 samples of apple juice stored at 0, 37, 72, and 91°F were analyzed during a period of 242 days. Of these, 283 were analyzed for acetylmethylcarbinol (AMC), 195 for diacetyl, and 98 for ethyl alcohol. There was no significant decrease in quantity of AMC or ethyl alcohol in apple juice in tin cans and glass bottles during the storage periods. The level of diacetyl, however, declined at a significant rate when diacetyl had been added to apple juice in bottles and stored at 91 and 72°F for 219 days. Only AMC was tested for its stability during repeated thawing and refreezing periods. No significant changes occurred in the quantities of AMC present in these frozen samples.

INTRODUCTION

Acetylmethylcarbinol (AMC) and diacetyl, metabolic by-products of yeasts, molds, and bacteria, have been used as indicators of the microbial quality of apple juice (Fields, 1962). Fields also proposed standards for AMC content of commercially processed apple juice. Based on data in his study, apple juices with 1.0 ppm AMC or below should be acceptable and are generally indicative of sound raw materials and good processing conditions. Values of 1.1–1.6 ppm AMC would be questionable, and values above 1.6 ppm AMC would indicate poor raw materials and/or poor sanitary conditions.

Although diacetyl is found in very small quantities in fermenting liquids (Brewer *et al.*, 1938), as compared to AMC, both chemicals can be used as indicators of quality. Fields (1964) stated that the main producers of diacetyl in apple juice are yeasts, namely *Saccharomyces* spp. He therefore postulated that diacetyl in apple juice may be considered presumptive evidence of poor sanitary practice in the processing plant. Murdock (1964) stated that diacetyl is destroyed or converted to a non-Voges-Proskauer reactant by yeasts in orange juice, thus possibly limiting the use of diacetyl.

The chemical reactions which bring about changes in canned foods during storage are extremely complicated. It was generally assumed by Murray (1953) that the rate at which chemical reactions occur in canned foods doubles with each 18°F rise in temperature.

Prevailing temperatures reported in a survey of 79 canned-food warehouses throughout the U. S. showed that, even in the hottest areas of the South, the average yearly warehouse temperature did not exceed 80°F (Monroe *et al.*, 1949). In the areas in which the greatest annual volume of canned food is warehoused, the average yearly temperature was about 65°F.

Irvine (1964) stated that the expected shelf life of canned apple juice, based on an average warehouse temperature of 70°F, is approximately 2–2½ years. McConnell (1964) estimated that the shelf life of canned apple juice ranged from 15 months to over 24 months. In any case, a technologist might expect to find apple juice that is over one year old in warehouses. No information could be found on the shelf life of bottled apple juice.

Fields (1964) reported a 23.4% loss of AMC in apple juice after 5 months of storage at 72–74°F. No data have been published on the effects of thawing and refreezing upon the AMC or diacetyl content of apple juice. Such data are important in determining the quality of apple juice.

The ethyl alcohol content has also been suggested as a means of detecting poor raw materials and poor processing conditions in apple juice manufacturing (Hill, 1965). Specific standards have been suggested for ethyl alcohol by Hill. In a 300-ml sample of juice ethyl alcohol values of 0.90 mg per ml in 80 ml of distillate or less are to be considered satisfactory. Values greater than 0.90 mg ethyl alcohol per ml in 80 ml of dis-

tillate are to be considered unsatisfactory. However, no work has been done showing the effects of time and temperature of storage upon ethyl alcohol. If this compound is to be used as an indicator of quality of apple juice, its stability must be determined when juices containing ethyl alcohol are subjected to different storage conditions.

This research was done to determine whether storage conditions influence the quantities of AMC, diacetyl, and ethyl alcohol present in apple juice. Such data are important in establishing a chemical index of quality. If the storage temperature and/or the type of container (glass bottles or tin cans) influences the level of the chemical index, the food technologist should consider these factors in making a final judgment as to whether the product complies with the chemical standard of quality.

EXPERIMENTAL METHODS

Juice with added AMC and diacetyl. Approximately 100 gal. of juice were prepared from Jonathan variety apples with commercial equipment and methods. Sound apples were washed, ground, pressed, and treated with pectinase regular (Takamine) at the rate of 16 oz per 20 gal. of juice. This was 4 times the amount of enzyme recommended by Tressler and Pederson (1938). The increased amount compensated for the added filter in the enzyme preparation.

After the juice was allowed to stand 2 days with the enzyme at refrigerated temperatures, the juice was heated to 150°F to inactivate the enzyme. The filtering process was aided with diatomaceous earth. The juice was pumped through a standard juice filter until clear. The juice was again heated, this time to 160°F, to allow a filling temperature of at least 140°F for both cans and glass bottles.

One-quart bottles with screw-top lids were filled with juice. AMC was added to bottles to yield a concentration of 10 ppm of AMC in 30 ml of distillate. Diacetyl was added to other bottles to yield a concentration of 30 ppm in 30 ml of distillate. The bottles were capped and laid flat to enable sterilization of the caps.

Apple juice was also processed in tin cans in the same manner as the juice in bottles. After the juice was heated the second time it was put into 46-oz cans. Diacetyl was added to specified cans to yield a concentration of 20 ppm in 30 ml of distillate, while AMC was added to other cans of juice to yield a concentration of 10 ppm in 30 ml distillate. The cans were sealed, inverted, and held to sterilize the can.

Juice from apples with added rot. Juice with rots of 0, 1, 3, and 5% were prepared from Golden Delicious apples by weighing exact amounts of rots cut from rot-containing apples and by adding to sound apples at the rate of 0, 1, 3, and 5 lb per hundredweight. These mixtures were ground, pressed, and treated with pectinase regular (Takamine) at the rate of 16 oz per 20 gal. of juice.

The method of processing was as previously described except that the juice was heated to a higher prefilling temperature of 180°F to ensure sterilization because of the inclusion of rot. One-quart bottles were the only containers used in this study.

Juice with added alcohol. Frozen apple juice prepared in 1957 under conditions similar to those described was used for this study. A mixture of 4 parts York, 2 parts Winesap, 4 parts Red Delicious, 1 part Rome Beauty, 1 part Golden Delicious, and 1 part York Imperial was used. To ensure a filling temperature of 140°F, the juice was heated to 160°F. Each 1-quart bottle contained 900 ml of juice to which ethyl alcohol stock solution was added to yield 5.2 mg/ml in 30 ml of distillate using a 300-ml sample of juice.

Influence of thawing and refreezing on AMC. Apple juice used in studying the effects of thawing and refreezing was prepared in 1961 by the procedure given for preparation of apple juice. The juice was stored in 1-gal. glass jugs. The juice was divided into the same experimental categories, Experiment I and Experiment II, as used by Fields (1962), the only difference between the experiments being the varieties of the apples used: in Experiment I, 50% Jonathan, 25% Red Delicious, and 25% Golden Delicious; and in Experiment II, equal amounts of Jonathan and Golden Delicious. At the time of testing, the juice was allowed to thaw at room temperature and was mixed by vigorous shaking. Three hundred ml of juice were removed for each analysis. Immediate refreezing of the apple juice remaining after each testing helped minimize time and temperature effects.

Storage conditions. A walk-in cooler provided storage space for apple juice samples held at 37°F. Samples held at 72°F were stored in an air-conditioned room. A 91°F storage area was achieved in an incubator. These latter samples were likely to show accelerated chemical reactions if any were to occur. Samples were removed at various intervals during storage periods for analyses for AMC, diacetyl, and ethyl alcohol.

Samples of juice used in the thawing and refreezing study were held at 0°F in a cold-storage room locker until sampling time, and were analyzed for AMC.

Determination of AMC and diacetyl. The method used was that of Hill and Wenzel (1957). Three hundred ml of apple juice were distilled in a 1-L Erlenmeyer flask attached to a Liebig condenser with a jacket length of 500 mm. Thirty ml of distillate were collected except when distilling the frozen apple juice, in which case 25 ml were collected.

A standard curve was prepared using Eastman No. P3788 acetylmethylcarbinol and Eastman No. 1591 diacetyl. Ethyl alcohol (95%) was used in preparation of the alpha-naphthol solution (Eastman No. T170); Eastman's No. 951 creatine was used in preparing the creatine-sodium hydroxide solution. Ten minutes after the reagents and the distillate solution were mixed, colorimetric readings were made on a Bausch and Lomb Spectronic 20 colorimeter at 545 mμ.

Determination of ethyl alcohol. Three hundred ml of apple juice which had been adjusted to pH 8 were placed in a 1-L Erlenmeyer flask attached to a Liebig condenser with a jacket length of 500 mm. Eighty ml of the distillate were collected. To 2 ml of the distillate was added 10 ml of potassium dichromate in sulfuric acid solution as recommended by Shupe and Dubowski (1952) and modified by Niss (1953).

Twenty-five ml of 95% ethyl alcohol were added to a volumetric flask and the volume of liquid increased to 1 L with distilled water. Twenty-five ml of ethyl alcohol (95%) at 25°C are equal to 19.99 or 20 mg alcohol/ml in 1 L of water (corrected for temperature and specific gravity. Hodgman. 1932.) Using the stock solution, dilutions were prepared and colorimetric readings were made at 615 mμ. A standard curve was prepared to determine the ethyl alcohol content in stored apple juice.

RESULTS AND DISCUSSION

AMC added. There were 53 samples in glass bottles and 56 samples in tin cans held in storage for periods of 228 days and 242 days, respectively. The samples were analyzed at 5 different times. The levels of AMC were adjusted to give approximately 10 ppm in bottles and tin cans so that the rate of any loss could be followed. The amounts added were well within the limits found in commercial apple juice (Fields. 1964).

There was no significant change in AMC content during storage. The variations found in the amount of AMC present are within the limits of the accuracy of the methods used (Holck, 1964). One can assume that the AMC found in apple juice in these containers is the amount present at processing time. This is contrary to findings by Fields (1964), who stated that an average loss of 24.3% of AMC in apple juice occurred in tin cans during 5 months at 72–74°F. However, in Fields' study the storage data were limited in scope. Other possible reasons for the loss, as reported by Fields, are reactions in the juice and/or reactions with the tin plate due to faulty enamel in the tin cans.

Diacetyl added. Sixty-three samples of apple juice in glass bottles with added diacetyl in the amount of 30 ppm were analyzed at 5 sampling times in a 219-day storage period. As with added AMC, the levels of diacetyl were high enough that the rate of any loss could be followed. It was thought that greater losses might occur with diacetyl than with AMC because of the "extra" ketone group in diacetyl. Of all the samples stored at the 3 different temperatures, only those stored at 91 and 72°F showed a decline in the quantity of diacetyl (Table 1). At present there is no explanation as to why this loss of diacetyl occurred in bottles. Further research needs to be done concerning the effects of storage conditions on diacetyl.

The samples stored at 37°F showed no substantial changes in diacetyl levels; rather, the variations seemed to be due to the greater volatility of diacetyl than of AMC, whether in its purified form or in naturally occurring rots. Before any definite conclusions can be drawn, further study is required with more samples. However, since

Table 1. Storage temperatures and time and their effects upon diacetyl (ppm)[a] added to apple juice in glass bottles.

Storage time (days)	Storage temperature		
	91°F[b]	72°F[c]	37°F
00	30	30	30
22	35	34	28
66	30	30	32
179	23	24	29
219	21	25	27

[a] Mean of four replicate samples.
[b] Any difference between any two means which is greater than the *D* value of 2.9 is significant at the 5% level.
[c] Any difference between any two means which is greater than the *D* value of 4.0 is significant at the 5% level.

the average yearly temperature did not exceed 80°F in the study of Monroe *et al.* (1949), one may conclude that diacetyl would be retained in sufficient quantity during normal warehouse temperatures to be of value as a presumptive indicator of quality.

During a 288-day period, 59 samples of apple juice in tin cans were analyzed for diacetyl at six sampling times. Losses of diacetyl could occur more readily if there were any flaws in the enamel of the tin cans. According to Table 2, however, no substantial changes occurred in any samples, regardless of storage temperature. Variations could, again, be accounted for by the limits of the accuracy of the methods used.

Table 2. Storage temperature and time and their effects upon diacetyl (ppm)[a] added to apple juice in tin cans.

Storage time (days)	No. of samples	Storage temperature 91°F	72°F	37°F
0	02	17	17	17
11	04	21	.....	....
19	12	22	21	21
61	12	22	24	22
174	12	19	20	21
228	17	19	19	21
Mean for storage period		20	20	20

[a] Figures listed as mean.

AMC content with added rot. As shown in Table 3, apple juice samples were tested with 0, 1, 3, and 5% rot. Two samples of each level of rot were analyzed from each of the 3 storage temperatures at each of the 5 testing dates. Zero-time readings were calculated using only 2 samples per rot level.

Since no samples were lost from spoilage, it was possible to report data on all 128 samples prepared. After 219 days of storage, there appeared to be no substantial change in quantities of AMC present in apple juice samples with added rot. This indicated that AMC, as contained in natural rotting apple tissue, was present after storage in concentrations similar to those found during the production of the apple juice. This study emphasized the validity of AMC as an indicator of quality.

Ethyl alcohol with added rot. The same distillates were used for analyses of AMC

Table 3. Influence of storage temperatures and time upon AMC (ppm)[a] in apple juice made from apples with added rot.

No. of days	% rot 0: 91°F	72°F	37°F	1: 91°F	72°F	37°F	3: 91°F	72°F	37°F	5: 91°F	72°F	37°F
00[b]	0.1	0.1	0.1	0.9	0.9	0.9	1.5	1.5	1.5	2.2	2.2	2.2
22	0.1	0.1	0.1	1.5	1.6	1.3	2.1	2.5	2.5	3.0	3.0	2.7
99	0.2	0.2	0.3	0.9	1.0	0.9	1.6	1.8	1.7	2.7	2.7	2.7
187	0.1	0.2	0.1	1.1	1.2	1.3	2.0	2.1	2.1	2.0	2.1	3.0
219	0.1	0.1	0.1	0.9	0.9	1.0	1.5	1.7	2.1	2.1	2.2	2.6
Mean for storage period	0.1	0.1	0.1	1.1	1.1	1.1	1.7	1.9	2.0	2.4	2.4	2.6

[a] Figures listed as mean.
[b] Two samples/temperature/group were tested except at 0 days, when only two samples/group were tested. The total amount of samples tested was 128.

and ethyl alcohol. The total analyses of 128 samples of apple juice for ethyl alcohol are shown in Table 4. Here again, the variations in readings recorded appear to be due to the methods used and not to reactions within the bottled juice during the 219 days of storage at different temperatures.

Ethyl alcohol added. Forty-three samples were analyzed over a 164-day period during which 5 sampling times were selected. Three storage temperatures, as listed previously, were used to show any effects of storage temperature and time upon ethyl alcohol in apple juice. There were no appreciable changes in the ethyl alcohol content of the samples regardless of the temperature at which it was stored. Ethyl alcohol remained stable in apple juice for this period, so it appears that it could be used as a chemical indicator of quality.

Thawing-refreezing. The study was designed to show the influence, if any, of thawing and refreezing upon AMC content in juices made from apples with added rot. As shown in Table 5, there were 4 separate testing times in Experiment I and 3 separate testing times in Experiment II. During these times the samples were thawed and distilled, and readings were recorded using the Voges-Proskauer test. Because of a shortage of the pilot-plant-produced juice, only 1 sample was run per level of rot in each experiment at each sampling time. Forty samples were analyzed in both experiments.

According to results found in these experiments, there appeared to be no loss of AMC contained in the apple juice after 4 successive thawing and refreezing times. This would indicate that the amounts of AMC found in frozen samples would be similar to the amounts found at the time of processing, thus implying that freezing would be an efficient means of handling samples until laboratory work could be carried out.

General conclusions. Both AMC and ethyl alcohol fulfill the criteria for a useful chemical index for determining the microbial quality of apple juice as proposed by Fields (1964). The fact that there was no decline in the quantities of AMC and ethyl alcohol in this study strengthens the use of these compounds as indicators since they reflect the conditions at the time of manufacturing.

Table 4. The influence of storage temperatures and time upon ethyl alcohol (mg/ml)[a] in apple juice made from apples with added rot.

	% rot											
	0			1			3			5		
No. of days	91°F	72°F	37°F	91°F	72°F	37°F	91°F	72°F	37°F	91°F	72°F	37°F
00[b]	0.4	0.4	0.4	0.4	0.4	0.4	0.8	0.8	0.8	0.9	0.9	0.9
22	0.5	0.0	0.5	0.6	0.1	0.4	0.9	0.4	0.7	1.7	1.7	1.0
99	1.0	0.6	0.4	0.7	0.6	0.7	1.2	0.9	0.0	1.4	1.0	1.0
187	0.4	0.6	0.4	0.5	0.7	0.5	0.8	0.8	1.0	1.0	1.3	1.0
219	0.5	0.5	0.4	0.6	0.8	0.7	0.9	1.1	0.9	1.2	1.4	1.2
Mean for storage period	0.6	0.4	0.5	0.6	0.5	0.5	0.9	0.8	0.8	1.2	1.1	1.0

[a] Figures listed as mean.
[b] At 0 day only two samples/rot level were tested. The remaining figures are representative of two samples/temperature/rot level.

Table 5. AMC (ppm)[a] observed by Voges-Proskauer test in frozen pilot-plant-produced apple juice.

Experiment[b]	% rot	Sampling time (days)				Mean for storage period
		00	90	151	185	
I	0	0.2	0.6	0.5	0.6	0.47
	2	1.2	1.5	1.4	1.0	1.27
	4	2.8	4.0	3.5	3.6	3.47
	6	1.6	2.3	2.2	2.3	2.10
	8	5.3	5.2	5.1	4.3	4.97
	10	3.3	3.2	3.7	3.7	3.47
	12	7.5	6.5	6.0	6.7	6.67
II	0	0.5		0.9	0.4	0.60
	4	0.9		1.5	0.9	1.10
	8	1.1		1.9	1.6	1.53
	12	1.8		2.3	2.5	2.20

[a] Figures listed are representative of one sample per testing date.
[b] In Experiment I, a mixture of 50% Jonathan, 25% Red Delicious, and 25% Golden Delicious was used. Juice in Experiment II was 50% Jonathan and 50% Golden Delicious.

Diacetyl, although declining in quantity during storage in this study, would still be effective as an indicator of quality if any were found in a product. Fields (1964) proposed that the presence of diacetyl be used as presumptive evidence of poor sanitary practice in the processing plant because it is produced by yeasts and usually is not found in tissue decomposed by filamentous fungi.

The analyses for AMC and diacetyl and for ethyl alcohol, as described herein, are relatively inexpensive and may be performed rapidly. The validity of the methods used for these indicators are sufficiently accurate for commercial purposes.

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C. K. CROSS and P. ZIEGLER
Research and Development Laboratories, Canada Packers Limited, Toronto 9, Ontario, Canada

A Comparison of the Volatile Fractions from Cured and Uncured Meat

SUMMARY

Gas chromatographic examination of the volatiles of cured and uncured ham showed that hexanal and valeraldehyde were present in appreciable quantities in the uncured product but were barely detectable in the volatiles of the cured meat. The differences were less pronounced in the contents of butyraldehyde, propionaldehyde, and acetaldehyde between cured and uncured ham volatiles, though these aldehydes tended to be more prevalent in the uncured ham. The branched-chain aldehydes (isobutyraldehyde, isovaleraldehyde, 2-methylbutyraldehyde) occurred to the same extent in both meats. Acetone was found to represent a major carbonyl constituent of the volatiles in both cured and uncured ham. The sulfur-containing fractions of the volatiles from both meats were found to comprise hydrogen sulfide and methanethiol.

INTRODUCTION

During the last few years, an ever-increasing number of publications have dealt with the nature of the flavor precursors and flavor components of various types of meat. It is generally agreed (Batzer *et al.*, 1960, 1962; Crocker, 1948; Hornstein *et al.*, 1960; Kramlich and Pearson, 1958) that raw meat has little or no flavor, that the flavor of a particular meat develops on heating, that flavor precursors can be extracted with water from raw meat, and that the characteristic odor of a meat can be produced by heating together the isolated precursors and the fat fraction. It has also been suggested (Hornstein and Crowe, 1960) that the odor derived by heating the water-soluble precursors is the same, regardless of the type of meat from which the precursors are obtained, and that the characteristic flavor differences are due to the contributions of volatiles derived from fat.

The volatile compounds from cooked meat or meat extracts were found (Bender and Ballance, 1961; Hornstein *et al.*, 1960; Yueh and Strong, 1960; Kramlich and Pearson, 1960) to comprise carbonyl compounds, organic acids and alcohols, sulfur compounds, and ammonia. The carbonyl fraction included acetone, methyl ethyl ketone, and diacetyl as well as a variety of normal and branched-chain aldehydes; the acid portion consisted on *n*-alkanoic acids (C_1–C_4) and isobutyric acid; methanol and ethanol represented the only alcohols; the sulfur-containing fraction included hydrogen sulfide, methanethiol, ethanethiol, and dimethyl sulfide. Of all these compounds, the carbonyls and the sulfur-containing substances are believed to be the predominant contributors to meat flavor.

Published reports on the constituents of meat flavor have dealt with various types of meat such as beef, chicken, pork, and lamb, but very little information has become available on the flavor components of cured meats. Ockerman *et al.* (1964) reported very recently on the gas chromatography of the volatiles derived from dry-cured hams; the spectrum of compounds described was very similar to that recorded by other investigators for the volatiles of uncured meat. Our study was undertaken to determine the effects of curing on the volatile constituents of meat flavor.

EXPERIMENTAL

A Perkin-Elmer 154D gas chromatograph with flame ionization detector was employed. The meat volatiles were identified by comparison of their retention times with those of known compounds on two packed columns. A 14-ft copper tube (¼ in. OD), filled with glass microbeads coated with 1% SE-30 silicone gum, was operated at 40°C and at 24 psig, the flow of helium being maintained at 40 cc per min. The second column consisted of a 6-ft stainless-steel tube (¼ in. OD) filled with UCON-LB-550X on diatomaceous earth; this column was operated at 60–80°C and 14 psig, the flow of helium being 35 cc per min. A U-shaped precolumn (Mackay *et al.*, 1959) consisted of a 4-in. copper tube (¼ in. OD) packed with 1% DC-550 silicone oil on glass microbeads. This column was operated in conjunction with the gas-sampling valve. The gas chromatographic examinations were carried out for 40 min. Exploratory experiments for periods up to 1.5 hr or at increased column temperatures (80°C) did not reveal significant amounts of compounds eluted after hexanal.

The cured hams used in the experiments had been artery-injected with pickle to a level of 13%, and curing was carried out for 5 days. The semimembranosus muscle was dissected, canned, and cooked in a water-bath at 75°C until the internal temperature reached 70°C. The cooking time was approximately two hours, after which time the salt content of the ham muscle averaged 2.5%. The samples of uncured ham (semimembranosus muscle) as well as the other meats were canned within one day after slaughter, then cooked immediately in the same manner. All of the cans were stored at 3°C, and were used for examination of meat volatiles within several days after cooking.

Prior to gas chromatography, the volatiles from meat were concentrated by one of two different methods. In the first procedure, samples (40 g) of meat cubes (½ cm) were placed in a test tube fitted with a gas inlet leading to the bottom of the tube. The meat samples, kept at 60°C on a water bath, were purged for 15–20 min with a stream of nitrogen at 30–40 cc per min. The effluent gas then passed into the precolumn, which was cooled by powdered dry ice. The second method employed small cubes of meat (100–300 g) and water (150 ml) in a 500-ml round-bottom flask. While the flask and its contents were kept at 85–90°C, a stream of nitrogen was bubbled through the mixture at 20–25 cc per min for 4–5 hr. The emerging gas stream was then directed through various trapping solutions. Basic compounds were collected in *2N* hydrochloric acid solution, volatile carbonyl compounds were trapped in *2N* hydrochloric acid solution saturated with 2,4-dinitrophenylhydrazine, and the sulfur compounds were precipitated in mercuric chloride or mercuric cyanide solution.

The hydrazones, after extraction with methylene chloride, were passed through a column of neutral alumina (Woelm grade 1) to remove unreacted 2,4-dinitrophenylhydrazine. The eluate was evaporated to dryness, the residue was then hydrolyzed with 20% sulfuric acid, and the liberated carbonyl compounds were introduced directly into the gas chromatograph. A portion of the mixture of hydrazones was examined by thin-layer chromatography according to a modification of a procedure described by Dhont and de Rooy (1961); the derivatives were chromatographed on silica gel G, using methylene chloride-Skelly F (4:1) as developing solvent. The sulfur compounds, precipitated by the mercuric salts, were centrifuged, washed, and dried. The resulting black solid was examined by infrared spectrophotometry, was analyzed for carbon and hydrogen, and was treated with hydriodic acid (Batzer and Doty, 1955) in order to liberate the sulfur compounds, which were subsequently collected in the precolumn.

RESULTS AND DISCUSSION

When the volatiles from samples (100 g) of ham were passed through a *2N* hydrochloric acid solution and this solution was then evaporated to dryness in a desiccator over potassium hydroxide, 0.4 mg of white solid remained as a residue. There was no difference between cured and uncured ham in the amount of residue isolated; all these residues gave strongly positive tests with Nessler's reagent. Because ammonia is known to occur in the volatile fraction of cooked meat and since it does not contribute significantly to meat flavor (Pippen and Eyring, 1957), this basic fraction was not examined further.

Gas chromatographic examination of the volatile carbonyl compounds, obtained by hydrolysis of their 2,4-dinitrophenylhydrazones or by direct concentration from cooked meat, indicated (Fig. 1 and 2) that certain aldehydes were present to a much greater extent in uncured ham than in cured ham. These differences were particularly evident for hexanal and valeraldehyde, which were always major constituents of uncured-ham volatiles but were absent (or present in only minute quantities) in cured-ham volatiles. The differences in content of acetaldehyde, propionaldehyde, and butyraldehyde were not as marked, but these aldehydes do occur in larger amounts in uncured-ham than in cured-ham volatiles. The results were essentially the same when cured and uncured beef or chicken were compared. It seems reasonable to assume that hexanal, valeraldehyde, and, to a lesser extent, butyraldehyde are derived by oxidative cleavage of unsaturated fatty acid residues, probably from linoleate.

Branched-chain aldehydes were found to the same extent in the volatiles from cured and uncured ham. These aldehydes comprise isobutyraldehyde, isovaleraldehyde, and 2-methylbutyraldehyde, which are believed to be respectively derived from valine, leucine, and isoleucine. The branched-chain aldehydes occur in considerably smaller quantities than the straight-chain aldehydes. The above results indicate that curing with nitrite does not affect the conversion of these amino acids to the corresponding aldehydes. Thin-layer chromatography of the 2,4-dinitrophenylhy-

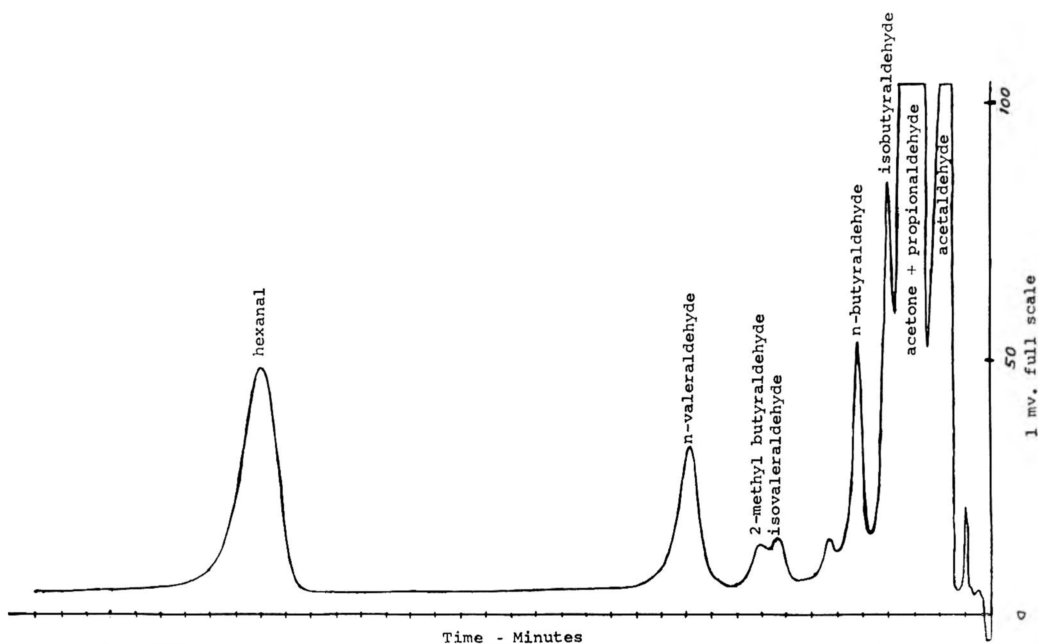

Fig. 1. Gas chromatogram of volatile carbonyl compounds isolated from uncured ham.

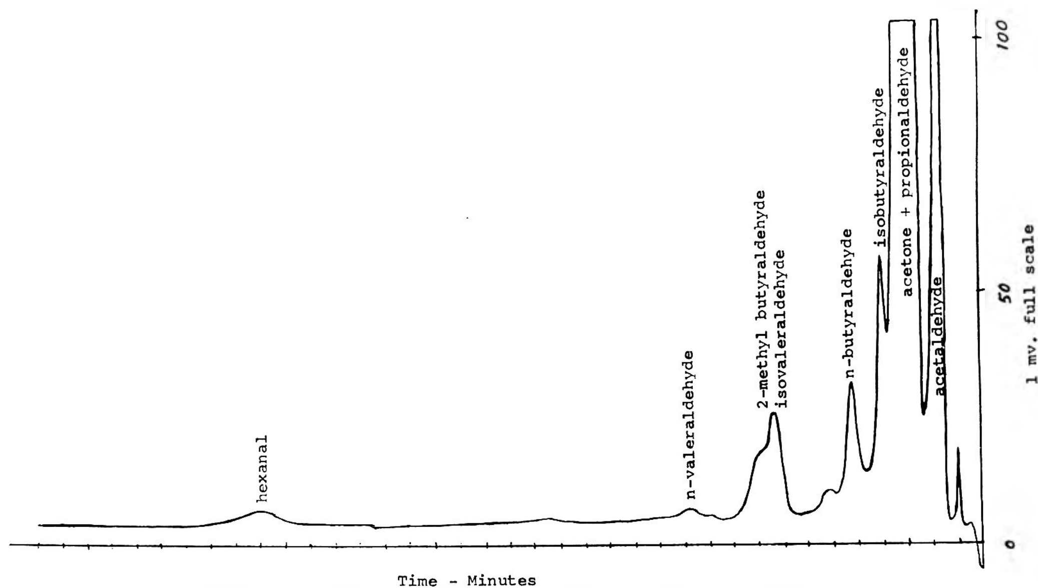

Fig. 2. Gas chromatogram of volatile carbonyl compounds isolated from cured ham.

drazones confirmed the data obtained by gas chromatography; hexanal and valeraldehyde were shown to be present in appreciable amounts in uncured ham but barely detectable in cured ham. Although gas chromatography did not provide a separation between acetone and propionaldehyde, thin-layer chromatography of the hydrazones indicated that acetone represented the larger portion of this mixture. It was concluded that acetone is a major component in the volatiles of cured and uncured ham; although it accounts for approximately 25% and 50% of the volatiles from uncured and cured meat re-

spectively, the total amount of 2,4-dinitrophenylhydrazones obtained from cured ham is only half that isolated from uncured ham. Thus, acetone is present in about the same quantity in both meats. We have not been able to detect methyl ethyl ketone in meat volatiles.

Two different samples (300 g) of uncured ham yielded 9.8 and 7.3 mg of 2,4-dinitrophenylhydrazones, whereas equal amounts of three samples of cured ham provided 3.4, 4.5, and 5.2 mg of carbonyl derivatives. Hydrolysis, and subsequent gas chromatography of the aldehydes and acetone, gave the following respective results, expressed in weight percent:

compounds with hydriodic acid, concentration of the resulting volatiles in the precolumn, and subsequent gas chromatography revealed only dimethyl disulfide. This compound presumably arose from methanethiol by oxidation with iodine during the hydriodic acid treatment.

These experiments show that the main difference between cured-ham and uncured-ham volatiles is the presence of large amounts of valeraldehyde and hexanal in the uncured-ham volatiles. The volatiles from cured and uncured ham contain appreciable quantities of shorter-chain carbonyls (C_1–C_3) but these do not seem to contribute significantly to the characteristic aroma of cured ham. Curing

	Uncured		Cured		
Acetaldehyde	24.8	32.7	33.2	44.1	39.4
Propionaldehyde + acetone	16.5	26.2	58.7	48.3	54.8
n-Butyraldehyde	0.8	0.7	0.5	0.4	0.6
Isovaleraldehyde	1.5	1.6	3.4	3.0	2.7
2-Methylbutyraldehyde	0.7	1.1	2.0	1.8	1.5
n-Valeraldehyde	5.4	3.4	0.7	0.6	0.3
Hexanal	50.4	34.4	1.6	1.7	0.7

The volatiles, after passage through the 2,4-dinitrophenylhydrazine solution, were found to have the characteristic cured-ham aroma, regardless of whether cured or uncured ham was the source of the volatiles. Furthermore, the volatiles of cured or uncured chicken and beef, after having been stripped of carbonyl compounds by passage through 2,4-dinitrophenylhydrazine solutions, possessed an aroma very similar to that of cured ham. The carbonyl-free volatiles were then bubbled through 1% solutions of mercuric chloride or mercuric cyanide, and the effluent gas stream was found to be practically odorless. Thus, all the volatile components, which impart the characteristic odor to cured ham, were trapped by the mercury salts. Samples (250 g) of cured and uncured ham yielded approximately the same amounts (7-14 mg) of black precipitates, which were found to contain 0.4–0.6% carbon and 0.2–0.3% hydrogen. These analyses demonstrated that the precipitates contained only minute amounts of organic matter, and the bulk of the material is considered to be mercuric sulfide. Treatment of the black mercury

with nitrite does not seem to contribute any volatile compounds (other than nitrogen oxides) that are not present in cooked uncured meat. We consider that cured-ham flavor represents the basic meat flavor derived from precursors other than triglycerides, and that the different aromas of the various types of cooked meat depend on the spectra of carbonyl compounds derived by oxidation of fat. It has been reported (Tappel, 1961) that uncured-bacon extracts catalyzed the oxidation of methyl linoleate and that the rate of oxidation was proportional to the amount of extract used; hematin compounds such as hemin, hemoglobin, and cytochrome were found to exert a similar action, which could be inhibited by cyanide ions.

When a sample of uncured ham was injected with sodium cyanide solution, the higher straight-chain aldehydes were completely absent in the volatiles of the cooked product. It is concluded that nitrite as well as cyanide interferes with the oxidation of unsaturated lipids, possibly by deactivating hematin catalysts.

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R. W. PORTER,[a] L. J. BRATZLER, and A. M. PEARSON
Department of Food Science, Michigan State University
East Lansing, Michigan 48823

Fractionation and Study of Compounds in Wood Smoke

SUMMARY

Hard-maple sawdust was sifted on a controlled-temperature hotplate for smoke production. The acids, total phenols, and carbonyl compounds were determined on the steam-volatile and non-steam-volatile portions of both whole smoke and the vapor phase. Most of the compounds in these groups were found to be steam-volatile.

Several steam-volatile monocarboxylic acids and monocarbonyl compounds were isolated and identified from smoke. The C_1—C_{10} acids were found to be present in whole smoke, with the C_1—C_4 acids occurring in the greatest concentrations. Only C_1—C_4 acids were found in the vapor phase of the smoke. The following monocarbonyls were identified in the steam-volatile portion of whole smoke: 2-pentanone, valeraldehyde, 2-butanone, butanal, acetone, propanal, crotonaldehyde, and ethanal. In addition, tentative identification of isovalderaldehyde and methanal was made on the basis of a comparison of chromatographic behavior and absorption spectra of the known and unknown compounds.

INTRODUCTION

Smoke consists of minute particles condensed around nuclei which are suspended in a continuous vapor phase (Foster, 1959). When the particles were removed electrostatically, the appearance, flavor, and keeping qualities of kippers smoked in this manner were not significantly different from those smoked normally, according to Foster and Simpson (1961). They concluded that the deposition of smoke flavor in the cold smoking of fish appeared to be a vapor absorption process. A limited study with bacon by the same workers indicated a similar process to be present in the absorption of smoke flavor in bacon. Using the absorption of phenols as a criterion, Foster *et al.* (1961) found that the particle phase served as a reservoir of volatile constituents when the supply of the vapor was diminished in the smoke. However, this contribution amounted to only about 5% of the absorbed smoke vapors.

Husaini and Cooper (1957) found that most of the phenols, acids, and carbonyl compounds in smoke were steam-volatile. Of the steam-volatile organic acids, acetic, formic, propionic, and butyric acids occurred in decreasing order of concentration, along with 2 unknown acids. Pettet and Lane (1940) reported the presence of formaldehyde, diacetyl, acetone, furfuraldehyde, 5-methylfurfuraldehyde, methyl and ethyl alcohols, acetic and formic acids, and phenols. They also noted that there were no esters or furan hydrocarbons present.

Spanyar *et al.* (1960a) discussed methods for studying the composition of smoke with respect to several variables. In a later report (1960b), those same workers reported the composition of smoke from different woods to vary slightly and that the water content of the sawdust did not influence the composition of the smoke. They also found that the range of generation temperatures from 180 to 440°C yielded increased amounts of CO and CO_2 with constant amounts of incomplete combustion products.

Spanyar and Kevei (1961) reported that the amount of smoke constituents which form "in the smoking chamber" increased with decreasing temperature of this chamber between 80 and 28°C, with a decrease in amount below 28°C. Those workers also found different sausage casings to be unique in the amount of smoke that became deposited on their surface.

The present study was undertaken to determine if any changes occurred in the concentrations of acids, total phenols, and carbonyls in the steam-volatile and non-steam-volatile portions of whole smoke and the vapor phase. Also studied was the qualitative composition of the steam-volatile monocarboxylic acid and monocarbonyl compounds in wood smoke.

EXPERIMENTAL

Hard-maple sawdust from a local sawmill was

[a] Present address: Pillsbury Research and Development Laboratory, 311 Second St., Minneapolis, Minnesota.

screened through hardware cloth with ¼-inch mesh for use throughout the study. Prior to being used it was heated in an open pan for 12 hr at 85°C and allowed to cool in the atmosphere at room temperature.

The smoke was generated by sifting the sawdust from a vibrating pan suspended from a sawdust hopper on a 12 × 12-inch electric hotplate (Fig. 1). Flowrate was adjusted to approximate 60 g of sawdust per hour. The hotplate temperature was determined by placing a thermocouple lead at a point on the hotplate where the sawdust dropped. At temperatures below 300°C (252 and 287), smoke was produced, although the sawdust particles did not glow but merely charred. At temperatures above 300°C the particles first charred, then glowed red, and finally turned to ash. Hotplate temperature was maintained by manually regulating a variable rheostat wired into the power line supplying the plate.

The particle and vapor phases of the smoke were separated by an electrostatic air filter (Trion, 1962) fastened to the chamber surrounding the hotplate. The filter contained thirty-one 23 × 10¾-in. negatively charged aluminum collection plates and thirty-two 21¼ × 9¾-in. positively charged aluminum plates. The plates were spaced 0.3 in. apart. The ionizing section contained ten 0.0065-in.-diameter ionizing wires 2 in. apart midway between nine 2½-in.-wide electrodes. The wires and electrodes were 22 in. long. Voltage across the collection plates and in the ionizing section was 7900 volts DC; the current flow was $<.01$ amperes.

The smoke was collected in a series of four 250-ml traps (ethanol–dry ice) placed at the end of the electrostatic air cleaner. The last (4th) trap was connected to a vacuum line and the vacuum was adjusted until there was <50 ft per min air flow inside the outlet (¼-in I.D.) from the air filter. Smoke was collected in this manner for 12-hr periods. The vapor phase was collected while the electrostatic air filter was on, but the air cleaner was turned off while whole smoke was collected. Both whole smoke and the vapor phase

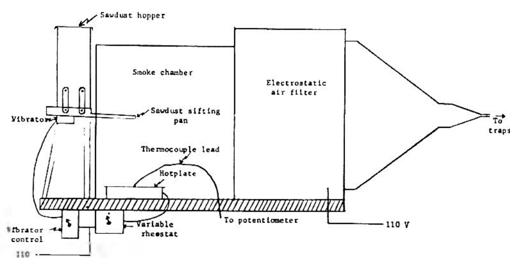

Fig. 1. Schematic diagram of smoke-generating unit.

were collected at hotplate temperatures of 252, 287, 322, 355, and 386°C.

Analysis of smoke condensate. A 5-ml sample of both smoke condensates was steam-distilled at the rate of 550 ml per hour, until 50 ml of each steam distillate were collected. Twenty-five ml were analyzed for total phenols, 10 ml were diluted to 50 ml with distilled water to be titrated for acids, 10 ml were analyzed for total carbonyl compounds, and the remaining 5 ml were discarded. The non-steam-volatile portion of the smoke was diluted to 250 ml with distilled water. One hundred ml were analyzed for total phenols, 100 ml were titrated for acids, 25 ml were analyzed for total carbonyls, and the remaining 25 ml were discarded.

The phenols were first extracted by the method of Braus *et al.* (1952) and the total phenols determined by the procedure of Warshowsky *et al.* (1948), using phenol as a standard. The acids were obtained by titrating the samples to pH 7.0 with 0.1*N* NaOH with an automatic titrator calibrated for that pH. Total carbonyls were determined by the method described by Iddles and Jackson (1934).

Separation and identification of volatile acids. The volatile acids from the 322°C hotplate temperature were prepared for column chromatography as their salts by the procedure of Buyske *et al.* (1957). The samples were applied to columns developed exactly as described by Buyske *et al.* The silicic acid-glycine columns were prepared exactly as prescribed by Corcoran (1956), and columns of pH 2.0, 8.4, and 10.0 were used. One hundred and twenty 5-ml fractions were collected from each column and titrated as described by Corcoran except that .01*N* NaOH was used. The acids were tentatively identified by comparing their eluate volumes with those of known acids run on separate but identically prepared columns. Final identification was accomplished by paper chromatographing the acids as their ammonium salts prepared from the peaks eluted from the column as described by Buyske *et al.* (1957). The ammonium salts of the unknown acids were chromatographed and compared with those of known acids in an ascending direction by the methods of Buyske *et al.* (1957) and Kennedy and Barker (1951).

Separation and identification of volatile monocarbonyls. One hundred ml of condensed whole smoke (386°C hotplate) were steam-distilled in 2 equal portions at atmospheric pressure for 4 hr. The distillates were allowed to react with 2 L of a solution containing 2 g of 2,4-dinitrophenylhydrazine per liter of 2*N* HCl. The monocarbonyl derivatives were then extracted by the scheme described by Day *et al.* (1960). The unknown

mixture of monocarbonyl derivatives was first separated on type B nitromethane-hexane-celite columns (Day *et al.*, 1960) packed in 2.5 × 19.5-cm. chromatographic tubes. Twenty-ml portions were placed on the type B columns, and the forerun fractions obtained from these aliquots were pooled and subsequently placed on type A columns for separation of the longer-chain derivatives.

Known derivatives were prepared and recrystallized from ethanol-water solutions by the procedure of Shriner *et al.* (1956). These derivatives were considered pure when their melting points corresponded to reported values. Melting points were determined in a modified Thiele apparatus, using mineral oil as the bath medium heated with a micro bunsen burner.

The unknown derivatives were rechromatographed on their respective type columns until no new bands appeared. Identification of the various parent carbonyl compounds was based on chain length, class of carbonyl present, and lack of depression in the mixed melting points (uncorrected) of the known and unknown 2,4-dinitrophenylhydrazone derivatives. Chain length was determined by comparing the threshold volumes (Day *et al.*, 1960) of the unknown derivatives with those of known derivatives on identically prepared columns and by the paper chromatographic behavior of the derivatives. The method of Huelin (1952) as extended by Schepartz (1961) was used except that the temperature of the chromatographic chamber was not regulated. In addition, the reverse phase procedure of Seligman and Edmonds (1955), utilizing paper impregnated with a 5.1% solution of olive oil in CCl_4 and a solvent system of methyl acetate–water (10:7) was used with ascending development.

The class of the parent carbonyl compound was determined by comparing the absorption maxima of CCl_4 solutions of the unknown and known 2,4-dinitrophenylhydrazone derivatives (Jones *et al.*, 1956). The unknown derivatives were recrystallized from low-boiling petroleum ether when present in sufficient quantity.

All solvents and column packing were prepared and used as described by Day *et al.* (1960).

RESULTS AND DISCUSSION

The production of smoke from the sawdust at the two lower hotplate temperatures (252, 287°C) differed from that produced at the three higher temperatures (322, 355, 386°C). The exothermic reaction that occurred affected only the rate of smoke production or sawdust burning at the latter temperatures. The total phenol concentration was higher in the whole smoke than in the vapor phase (Table 1). The greatest differences were found between the steam-volatile and non-steam-volatile portions of both whole smoke and the vapor phase.

Table 1. Total phenols in whole smoke and the vapor phase (μg phenol/ml).

Hotplate temp. (°C)	Whole smoke		Vapor phase	
	SV[a]	NSV[b]	SV	NSV
252	88.0	1.7	56.4	1.2
287	90.8	1.6	69.2	1.3
322	153.0	2.9	138.0	1.9
355	196.5	4.7	160.9	1.7
386	165.4	3.5	151.7	1.9

[a] Steam-volatile.
[b] Non-steam-volatile.

Table 2 shows that the difference in acids found in whole smoke and vapor phase was not marked. The amounts shown may be slightly low because titration to pH 7.0 does not fully account for all of the weak acids, but the values should be comparable when the steam-volatile portions are compared with the non-steam-volatile fractions.

The concentration of total carbonyl compounds (steam-volatile and non-steam-volatile) increased as hotplate temperature increased over the range of temperatures studied (Table 3). A comparison of the groups studied indicates that a larger proportion of the carbonyl constituents are non-steam-volatile than the phenols or acids (Tables 1, 2, 3). It was observed that the characteristic smoke odor was most pronounced in the steam-volatile portion, whereas all of the color appeared to be retained in the non-steam-volatile portion of the smoke condensate. This observation has been reported by Husaini and Cooper (1957) and other researchers; thus it appears that the compounds responsible for

Table 2. Acids in whole smoke and the vapor phase (meq acid/ml).

Hotplate temp. (°C)	Whole smoke		Vapor phase	
	SV[a]	NSV[b]	SV	NSV
252	.082	.015	.066	.011
287	.077	.019	.071	.014
322	.121	.023	.115	.019
355	.104	.024	.110	.022
386	.112	.027	.106	.023

[a] Steam-volatile.
[b] Non-steam-volatile.

Table 3. Total carbonyls in whole smoke and the vapor phase (mg acetaldehyde/ml).

Hotplate temp. (°C)	Whole smoke		Vapor phase	
	SV[a]	NSV[b]	SV	NSV
252	19.95	3.10	12.38	1.46
287	20.18	4.75	19.01	3.43
322	27.29	7.71	25.93	5.07
355	31.17	9.64	27.90	7.77
386	37.18	13.34	34.26	10.11

[a] Steam-volatile.
[b] Non-steam-volatile.

characteristic smoke flavor should be sought in the more volatile portion of the smoke.

The identification data for the steam-volatile acids are presented in Table 4. The C_1 to C_{10} monocarboxylic acids were found in whole smoke, whereas only the C_1 to C_4 acids could be detected in the vapor phase with the procedures used. This would indicate that the longer-chain acids were probably condensing in the particles which were removed from the vapor phase. The quantitative recovery of the various acids studied (Table 5) shows acetic, formic, propionic, and butyric acids, in decreasing order of occurrence, to make up most of the steam-volatile acids in each case. The concentration of acids beyond butyric decreased in whole smoke as chain length increased. In both whole smoke and the vapor phase the aliphatic acids did not account for all of the directly titrated acidity in the smoke condensates (Table 5).

The identification data for the steam-volatile monocarboxyl compounds in whole smoke generated at the highest hotplate temperature are presented in Table 6. These results indicate the predominance of short-chained monocarbonyl compounds in the steam-volatile portion of the whole smoke. The unidentified forerun fraction would indicate the presence of monocarbonyl compounds longer than C_5 but in limited amounts, as this fraction was present in much too small a quantity for convenient study.

Table 5. Quantitative analysis of steam-volatile monocarboxylic acids from smoke samples.

Acid	Meq acid/ml smoke condensate	
	Whole smoke	Vapor phase
Formic	.0193	.0235
Acetic	.0699	.0700
Propionic	.0065	.0067
Butyric-isobutyric	.0013	.0011
Above butyric	.0039	....
Total	.1009	.1013
Titrated directly	.1210	.1150

Table 4. Column and paper chromatographic behavior of steam-volatile acids investigated.

Acid	Column pH	Eluate volume (ml)			Solvent I[a] R_f values		Solvent II[b] R_f values	
		Known	Whole smoke	Vapor phase	Unknown	Known	Unknown	Known
Formic	2.0	290–380	275–370	305–410	.31	.32	.31	.31
Acetic	2.0	150–260	160–260	165–285	.36	.34	.35	.36
Propionic	2.0	40–110	60–105	50–130	.39	.39	.49	.48
Butyric-isobutyric	8.4	305–380	325–420	300–430	.50	.48	.54	.54
Valeric-isovaleric	8.4	200–295	195–315	......	.57	.58	.64	.62
Caproic	8.4	55–150	60–185	......	.67	.66	.68	.69
Heptylic	10.0	395–485	375–525	......	.75	.76	.72	.71
Caprylic	10.0	200–310	180–310	......	.79	.80	.76	.76
Nonylic	10.0	80–140	50–140	......	.82	.82	.82	.81
Capric	10.0	10–70	0–40	......	.84	.85	.82	.82

[a] Butanol-H_2O-propylamine.
[b] 95% ethanol-NH_4OH (would not resolve nonylic and capric).

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Table 6. Identification data for the 2,4-dinitrophenylhydrazones of steam-volatile monocarbonyl compounds from whole smoke.

Band no.	2,4-dinitro-phenylhydrazone derivative	TV[a] (ml)		λmax (mμ)		Melting point (°C)		
		Unknown	Known	Unknown	Known	Unknown	Known	Mixture
Forerun	Unidentified	......(A)[b]	......	......	......	......	......	......
1a	2-Pentanone	15.5(A)	15.6	362	363	141–143	142–143.5	140.5–143
b	Isovaleraldehyde	21.8(A)	21.4	358	357	......	......	......
c	Valeraldehyde	22.5(A)	22.5	358	358	104–107	105–106.5	103–106
2a	2-Butanone	23.0(B)	23.4	363	363	112–113	113	110–113.5
b	Butanal	28.7(B)	28.9	358	358	112–113	114–114.5	112–114
3a	Acetone	38.3(B)	38.1	363	363	124–125.5	125.5	123–125
b	Propanal	38.5(B)	38.3	357	358	145–147	146.5	144–146
c	Crotonaldehyde	43.0(B)	43.4	373	373	186–188	188	185–188
4a	Ethanal	51.6(B)	51.2	354	356	164–165.5	166.5	163–166
b	Methanal	90.3(B)	91.5	348	348	......	......	......

[a] TV = threshold volume (Day *et al.*, 1960).
[b] Indicates type of partition column used (Day *et al.*, 1960).

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R. F. ALBACH,[a] A. D. WEBB, and R. E. KEPNER
Departments of Chemistry and of Viticulture and Enology
University of California, Davis 95616

Structures of Acylated Anthocyan Pigments in Vitis vinifera variety Tinta Pinheira. II. Position of Acylation

SUMMARY

Gas chromatographic analysis of the methylated sugar residues obtained from the band-2 pigment of *Vitis vinifera* var. Tinta Pinheira indicated that the acyl function, p-coumaric acid, was most likely attached at the four-position on the glucose portion of the anthocyanins. The possibility of an acid-catalyzed migration of the acyl function during the methylation process cannot, however, be ruled out completely. The acylated pigments of band 2 are thus considered to be the 3-monoglucosides of malvidin, peonidin, petunidin, and delphinidin, each acylated with p-coumaric acid at the four-position on the glucose.

INTRODUCTION

Malic acid (Bockian *et al.*, 1955; Colagrande and Grandi, 1960), tartaric acid (Colagrande and Grandi, 1960), p-coumaric acid (Ribéreau-Gayon, 1959), and chlorogenic acid (Somaatmadja and Powers, 1963) have been reported as the acylating functions in the acylated anthocyan pigments of various *Vitis vinifera* grape varieties. Albach *et al.* (1965) recently presented evidence demonstrating that the pigment from band 2 isolated from the Tinta Pinheira variety is composed of the 3-monoglucosides of malvidin, peonidin, delphinidin, and petunidin, each acylated with p-coumaric acid, while the band-3 pigment consists of the same four anthocyanins each acylated with caffeic acid. Evidence is also presented from partial hydrolysis studies to demonstrate that the anthocyanins are the primary hydrolysis products from the pigments from either band 2 or band 3 from Tinta Pinheira. Harborne (1962), in reviewing current knowledge on acylated anthocyans, concluded that the acyl groups are most likely attached to the anthocyanins through the sugar residues.

This report presents evidence concerning the position of attachment of the acylating functions on the anthocyanins of the Tinta Pinheira skin pigments.

[a] Taken from a Ph.D. thesis by Roger F. Albach, University of California, Davis, 1963.

EXPERIMENTAL PROCEDURES

Acylated anthocyan pigments. Isolation and purification of the various pigment bands from *Vitis vinifera* variety Tinta Pinheira have been described previously (Albach *et al.*, 1965). The same report described investigation of the acylated pigments resulting in identification of the acid, anthocyanidin, and sugar moieties of the pigments in bands 2 and 3.

Boron trifluoride etherate catalyst solution. This catalyst solution was prepared according to the method of Neeman and Hashimoto (1962). One-tenth ml of clear, freshly distilled boron trifluoride etherate was dissolved in 10 ml of a 1:1 mixture of anhydrous diethyl ether and anhydrous methylene chloride.

Synthesis of methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside. This synthesis was an adaptation of a modification of the Kuhn method (1958) as proposed by Bishop and Cooper (1962).

Three ml of methyl iodide and 4 g of silver oxide were added to a solution of 1 g of methyl-α-D-glucopyranoside (Pfanstiehl Chemical Co., Waukegan, Ill.) in 4 ml of anhydrous N,N-dimethylformamide contained in a tightly stoppered 25-ml conical flask. The reaction mixture was stirred 24 hr by a Teflon-coated stirring bar and magnetic stirrer, 15 ml of chloroform were added, resultant precipitate of silver iodide and the unreacted silver oxide were removed by filtration, and the chloroform solution was evaporated to a syrup in a rotary film evaporator. The syrup was dissolved in a few ml of chloroform, and the solution was later used for gas chromatographic analyses.

In the remaining synthetic processes described herein all removals of solvent or evaporations of reaction products to a syrup were conducted in a rotary film evaporator under reduced pressure unless otherwise noted, and the final chloroform solutions of the products were subjected to gas chromatographic analyses.

Synthesis of a mixture of α and β isomers of methyl 2,3,4,6-tetra-O-methylglucopyranoside and methyl 2,3,6-tri-O-methylglucopyranoside. One g of maltose (Paragon Testing Laboratories, Orange, New Jersey) was methylated in the same

manner as described above for methyl-α-D-glucopyranoside. After removal of the N,N-dimethylformamide and chloroform, the residual syrup of octa-O-methylmaltose was dissolved in 15 ml of 5% methanolic hydrochloric acid and the methanolic solution refluxed for 24 hr, cooled, and then neutralized with sodium isopropoxide in isopropanol. The solvent was removed and the product mixture extracted from the sodium chloride with a few ml of chloroform.

Synthesis of a mixture of α and β isomers of methyl 2,3,4,6-tetra-O-methylgalactopyranoside and methyl 2,3,4-tri-O-methylglucopyranoside. Two hundred mg of melibiose, 0.65 ml of methyl iodide, and 625 mg of silver oxide were used to produce methyl hepta-O-methylmelibioside as described above. Methanolysis of the 1-6 glycosidic linkage, and isolation of the products were performed as described in the previous section.

Partial methylation of methyl-α-D-glucopyranoside. The partial methylation was performed in the manner previously described for the synthesis of methyl 2,3,4,6-tetra-O-methylglucopyranoside except that the quantities of methyl iodide and silver oxide were halved. The primary function of this synthesis was the production of the tri- and di-O-methyl derivatives of methyl-α-D-glucopyranoside. The syrupy product was again dissolved in chloroform for later gas chromatographic studies.

Preparation of diazomethane. Methylene chloride or diethyl ether solutions of diazomethane were prepared by the method of DeBoer (1954) from commercially available N-methyl-N-nitroso-p-toluenesulfonamide, "Diazald" (Aldrich Chemmical Company, Inc., Milwaukee, Wisc.). The diazomethane was used within 48 hr after preparation.

The quantity of diazomethane in solution was determined by a modification of the method of Marshall and Acree (1910). The yield of dry diazomethane obtained was 70% in the ether solution, and only 24% in methylene chloride.

Attempted methylation of D-glucose, methyl α-D-glucopyranoside, maltose, methyl p-hydroxybenzoate, p-coumaric acid, ferulic acid, and caffeic acid with diazomethane and boron trifluoride etherate catalyst. One-tenth millimole of the finely powdered compound was added to 5 ml of anhydrous methylene chloride in a 50-ml conical flask protected by a calcium chloride tube, and the mixture, cooled in an ice bath, was stirred vigorously with a magnetic stirrer while cold (0°) 0.2*N* diazomethane in methylene chloride was added from a burette fitted with a Teflon stopcock. After methylation of any carboxyl groups present in a compound, a ten molar excess of diazomethane for each alcoholic hydroxyl group, and a five molar excess for each phenolic hydroxyl present in the molecule was added. Five microliter portions of the boron trifluoride-etherate catalyst solution were then added at 1-hr intervals as long as the yellow color of the solution persisted.

After all of the diazomethane had been consumed, the reaction mixtures were evaporated to dryness in a stream of nitrogen under reduced pressure. The residues remaining from the D-glucose and methyl-α-D-glucopyranoside methylations were each extracted with 1 ml of chloroform, and the chloroform solutions were separated from the insoluble residues and then concentrated to volumes of 0.1 ml by evaporation in a stream of nitrogen under reduced pressure.

The residues remaining from the methyl p-hydroxybenzoate and the p-coumaric, ferulic, and caffeic acid methylations were each dissolved in 1 ml of methanol for gas chromatographic analysis.

The residue remaining from the maltose methylations was dissolved in 10 ml of 5% methanolic hydrochloric acid, refluxed for 6 hr, and then evaporated to dryness. The resulting residue was extracted with 1 ml of chloroform, the insoluble residue separated, and the chloroform extract concentrated to a volume of 0.1 ml by evaporation in a stream of nitrogen under reduced pressure.

Methylation of purified pigment from band 2 with diazomethane and boron trifluoride etherate catalyst. A 50-mg sample of purified pigment from band 2, dissolved in approximately 3 ml of methanol, was evaporated to dryness in a 500-ml round-bottomed flask in a rotary-film evaporator in such a manner that the lower hemispheric surface of the flask was uniformly coated with a film of pigment. Deposition of the film was necessary since the powdered pigment could not be dispersed in methylene chloride because of its tendency to adsorb moisture and to conglomerate. To ensure complete removal of methanol and water from the deposited pigment, 10 ml of dried (distilled from P_2O_5) methylene chloride was added to the flask and then completely removed by distillation at a pressure of 1 mm Hg.

Forty ml of a freshly prepared 0.2*N* solution of diazomethane in methylene chloride was added to the flask containing the pigment, and the flask, protected by a calcium chloride tube, was swirled in an ice bath for 1 hr. The color of the pigment gradually changed from red to blue, and the blue pigment slowly dissolved to give a green solution. After dissolution was essentially complete, 100 ml of dried methylene chloride was added to the flask, which was cooled in an ice bath and stirred with a magnetic stirrer while 0.1-ml portions of the boron trifluoride etherate catalyst solution were added at 10-min intervals until the yellow color

of the diazomethane was dissipated. After another 40 ml of the diazomethane solution was added, the flask was stored overnight at −20° and additional portions of the catalyst solution were then added until the yellow color was again dissipated. Finally, 0.3 ml of the catalyst solution was added to the reaction mixture, and an additional 20 ml of diazomethane solution was added slowly from a burette. After the reaction was completed the solution was a very light yellowish-orange color.

Fifty ml of methanol were added and the reaction mixture was evaporated to dryness. The residue was extracted with methanol, and the methanolic solution was separated from the white polymethylene byproduct and then concentrated to a syrup. This residual light-yellow syrup was dissolved in 30 ml of 5% methanolic hydrochloric acid, producing an orange solution, which was refluxed for 12 hr. The reaction mixture was then evaporated to a dark-red syrup, final removal of solvent being accomplished by heating the flask up to 60°. The residue was dissolved in 2 ml of methylene chloride to give a deep reddish-brown solution which was subjected to gas chromatographic analyses.

Gas chromatographic analyses. The methylated sugar and reference compounds were analyzed on an Aerograph Model 600-B chromatograph (Wilkins Instrument and Research, Inc., Walnut Creek, Calif.) fitted with a hydrogen flame ionization detector and using nitrogen as the carrier gas. The following columns were used:

1) SE-30, 5% on chromosorb, packed in a 6 ft × ⅛-inch stainless-steel tube (a commercial column obtained from Wilkins Instrument and Research, Inc.).
2) Butanediol succinate, 10% on 40-60-mesh firebrick, packed in a 2-ft × ⅛-inch stainless-steel tube.
3) LAC-446, on chromosorb, packed in a 3-ft × ⅛-inch stainless-steel tube (a commercial packing obtained from Wilkins Instrument and Research, Inc.).

Table 1 summarizes the main experimental variables used in the analyses with the above gas chromatographic columns. The concentrations of the various samples being analyzed were empirically adjusted such that a 10-μl sample would have a recorder pen response that stayed on scale at attenuation 64 with an input impedance of 10^{9} ohms.

It was necessary to steam-clean, by means of five consecutive 5-μl water injections, the butanediol succinate column between analytical runs to remove adsorbed compounds which would give false peaks in subsequent runs. This was particularly important when analyzing for the α and β isomers of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside.

Table 1. Gas chromatographic conditions.

Column	Carrier gas		Column temp. (°C)	Injector block voltage
	Inlet press. (psig)	Flow rate (ml/min)		
Butanediol succinate (10%)	8	65	150	60
	10	65	175	60
	8	65	185	70
SE-30 (5%)	40	60	120	60
	14	18	150	70
	14	29	200	70
LAC 446	10	40	150	70
	5	40	200	70
	10	65	200	70

On the SE-30 column, peaks representing the α and β isomers of methyl 2,3,4-tri-O-methyl-D-glucopyranoside and 2,3,6-tri-O-methyl-D-glucopyranoside were found to show large decreases in retention times with increasing sample quantity. Valid comparisons of relative retention times could then be made only when sample sizes were adjusted so as to make the known and unknown peaks approximately equal in area.

RESULTS AND DISCUSSION

The spectral data for the band 2 and 3 pigments from Tinta Pinheira grapes (Albach *et al.*, 1965) indicated the presence of a single acyl function which was most likely attached through the glucose portion of the molecule. Since, of the three acylated bands, the pigments of band 2 were the most stable and were obtainable in largest amounts in the highest state of purity, this material was utilized to study the position of acylation on the glucose moiety. The facile degradation of the pigments from band 3 resulted in large losses of acylated pigments on rechromatography, whereas only minor amounts of hydrolysis of the acyl linkage occurred during rechromatography of the pigments from band 2.

Mueller and Rundel (1958), Mueller *et al.* (1959), and Caserio *et al.* (1958) recently described a mild technique for methylating alcohols utilizing diazomethane with either a boron trifluoride diethyl etherate or fluorboric acid catalyst. Neeman *et al.* (1959) and Neeman and Hashimoto (1962) demonstrated that this method was capable of completely methylating the alcoholic hydroxyl groups in ascorbic acid and in the

methyl ester of estriol monoglucosiduronic acid without cleaving the lactone ring or glycosidic linkage, respectively.

Since the diazomethane reagent requires a nonhydroxylic type of solvent, and anthocyanins are essentially insoluble in nonhydroxylic solvents, any attempt to apply Neeman's method to exhaustive methylation of the acylated anthocyanins would necessarily require that the reaction be run under heterogeneous conditions. A number of model compounds, listed in the experimental section, were subjected to an attempted methylation with diazomethane and boron trifluoride-etherate catalyst in methylene chloride. Except for methyl p-hydroxy benzoate and p-coumaric acid, none of the substrates, even after 8 hr or longer of vigorous stirring, showed any apparent sign of dissolving by virtue of methylation having occurred. Gas chromatographic analyses of the reaction products showed that methyl p-hydroxybenzoate was completely converted to the methyl ether and p-coumaric acid to methyl p-methoxycinnamate. The caffeic and ferulic acids each gave three major and two minor peaks, undoubtedly representing various stages of methylation. The identity of the products produced by the methylations was not established since the major concern was in determining what peaks could be expected to be characteristic of each acid under the conditions of the reaction and to what degree of completeness the reaction would proceed.

Analyses of the possible O-methyl derivatives of glucose obtainable from glucose, methyl α-D-glucopyranoside, or maltose were experimentally limited to detection of the α and β isomers of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside and the α and β isomers of the four possible tri-O-methyl derivatives.

Analyses of the reaction products from the heterogeneous interaction of diazomethane with maltose gave no detectable peaks, glucose gave only two trace peaks corresponding to the α and β isomers of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside, while methyl-α-D-glucopyranoside gave only trace amounts of the five theoretically possible peaks. The almost complete lack of dissolution and methylation of these sugars essentially precluded any meaningful investigations of simple sugar derivatives under the given heterogeneous reaction conditions.

In contrast to the above results with the sugars, the methylation of the band 2 pigments proceeded smoothly under the heterogeneous reaction conditions, with eventual complete solution of the pigment mixture. This was apparently made possible by virtue of the ease with which the phenolic substituents on the aglycone were methylated. The methylation of the phenolic groups on the pigment molecule may have increased the solubility of the glycosides sufficiently to allow the remaining alcoholic hydroxyls on the glucose portion of the molecule to be methylated more easily. Whether the oxonium salt of the anthocyanin persisted in the reaction medium is not known for certain. In the gas chromatographic analysis of the final reaction mixture, attention was focused mainly on determining the nature of the methyl-tri-O-methyl-D-glucopyranosides produced. The fate of the permethylated aglycone was not determined.

The known tri-O-methyl-D-glucopyranosides necessary for reference compounds were obtained by exhaustive methylation of maltose and melibiose by the modified Kuhn method followed by methanolysis, and by partial methylation of methyl α-D-glucopyranoside by the modified Kuhn method. The conversions are summarized in Fig. 1. The gas chromatographic data for each of the above reaction mixtures and for the reaction mixture obtained from the band-2 pigments are summarized in Table 3. The observed retention times were in each case converted to relative retention times with methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside as the reference standard. The peak corresponding to the reference standard was known as a consequence of its preparation by the exhaustive methylation of methyl-α-D-glucopyranoside by the modified Kuhn method. In the maltose, melibiose, and pigment reaction products the α isomers were differentiated from the β isomers by comparison with the mixture containing only α isomers, which was prepared by partial methylation of methyl-α-D-glucopyranoside.

Only three methyl tri-O-methyl-α-D-glucopyranosides were formed in the partial

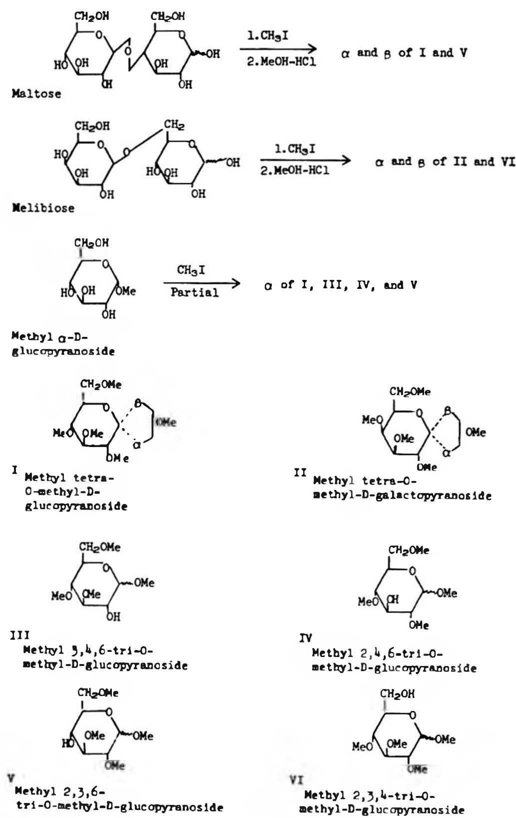

Fig. 1. Structural Formulae of Sugar Methylation Products.

methylation of methyl-α-D-glucopyranoside. From an examination of the relative retention-time data in Table 3 it is evident that the missing component is methyl 2,3,4-tri-O-methyl-α-D-glucopyranoside. This conclusion is theoretically sound since the primary 6-hydroxyl group of a hexose should be more easily methylated than the secondary hydroxyls. The 2,3,6-tri-O-methyl derivative of the α-mixture was identified by virtue of its nearly identical relative retention times with one of the tri-O-methyl component peaks from the maltose methylation. The other two components of the α-mixture were assigned tentative identities based upon relative retention-time data on butanediol succinate columns as reported by Bishop and Cooper (1962), Aspinal (1963), and Klein and Barter (1961). The three tri-O-methyl components in the α-mixture differed sufficiently in amount to permit them to be easily differentiated on different columns by reference to their peak areas, approximately 1:1.5:3 for the 2,4,6:3,4,6:2,3,6 isomers, respectively, even when their order of emergence changed.

Gas chromatographic analyses of the reaction products from the methylation and methanolysis of the pigments from band 2 gave four peaks, approximately equal in size, in the region where the tetra- and tri-O-methyl derivatives would appear. Comparison of the relative retention times of the peaks with the relative retention times of the known reference compounds indicated that the first two peaks represented the β and α isomers of methyl 2,3,4,6-tetra-O-methyl-D-glucopyranoside and the remaining two peaks represented the β and α isomers of methyl 2,3,6-tri-O-methyl-D-glucopyranoside, respectively. The presence of relatively large amounts of the α and β isomers of the tetra-O-methyl derivative indicated that considerable ester hydrolysis occurred under the influence of the boron trifluoride-etherate catalyst. The complete lack of evidence on any of the chromatograms for the presence of the α and β isomers of either the 2,3,4-, 2,4,6-, or 3,4,6-tri-O-methyl derivatives indicated that methylation was essentially complete and that ester hydrolysis led to the formation of the tetra-O-methyl derivatives only (Bourne *et al.*, 1963).

The possibility of migration of acyl groups has been the major problem in investigations of this nature. In the present investigation, to explain the formation of only the 2,3,6-isomers of the possible tri-O-methyl derivatives on the basis of an acyl migration would require complete transfer of the p-coumaryl function from the C-6 to the C-4 position, the most sterically feasible migration, before methylation occurred. Haworth *et al.* (1931), Bouveng *et al.* (1957), and Frohwein and Leibowitz (1963) have reported the facile migration of acetyl groups from the C-4 to the C-6 hydroxyl group in D-glucose under basic conditions. The migration apparently goes via the formation of an intermediate ortho ester, with the primary hydroxyl group the preferred terminus of the migratory acetyl group (Bouveng, 1961). Since the reaction conditions used for the methylation of the band-2-pigments, diazomethane and boron trifluoride-etherate in methylene chloride, are considerably differ-

Table 2. Gas chromatographic data.

	Relative retention times[a]				
	Butanediol succ.		LAC-446		SE-30
Sample and peak components[b] — Col. temp. (°C): N_2 flow (ml/min):	150 65	175 65	200 40	200 65	120 60
Methylated and methanolyzed bd. 2					
a) 2,3,4,6-β-D-glucoP	0.67	0.73	......	0.75	0.78
b) 2,3,4,6-α-D-glucoP	1.00	1.00	......	1.00	1.00
c) 2,3,6-β-D-glucoP	2.48	2.54	......	2.40	1.66[c]
d) 2,3,6-α-D-glucoP	3.32	3.46	......	3.30	1.48[c]
e) Me p-OMe-cinnamate	......	......	......	5.50	......
Methylated and methanolyzed maltose					
a) 2,3,4,6-β-D-glucoP	.68	.73	.73	.75	.78
b) 2,3,4,6-α-D-glucop	1.00	1.00	1.00	1.00	1.00
c) 2,3,6-β-D-glucoP	2.52	2.50	2.43	2.40	1.68[c]
d) 2,3,6-α-D-glucoP	3,76	3.50	3.29	3.30	1.45[c]
Partially methylated Me-α-D-glucopyranoside					
a) 2,3,4,6-α-D-glucoP	1.00	1.00	1.00	1.00	1.00
b) 3,4,6-α-D-glucoP	2.82	2.80	2.69	2.67	1.33[c]
c) 2,3,6-α-D-glucoP	3.77	3.44	3.34	3.30	1.47[c]
d) 2,4,6-α-D-glucoP	7.0	6.24	2.23	2.23	1.82
Methylated and methanolyzed melibiose					
a) 2,3,4,6-β-D-galacP	1.63	1.74	1.93	1.95	.94
b) 2,3,4,6-α-D-galacP	1.04	1.18	3.20	3.10	1.10
c) 2,3,4-β-D-glucoP	2.61	2.59	......	6.64	1.32[c]
d) 2,3,4-α-D-glucoP	3.12	3.15	......	8.30	1.77[c]
Methylated p-coumaric acid					
Mep-OMe-cinnamate	......	......	......	5.50	......

[a] The relative retention times were calculated from uncorrected retention times since the dead volume of the column is insignificant relative to the column performance.
[b] Components abbreviation is based on the following: methyl 2,3,4,6-tetra-O-methyl-α-D-glucopyranoside.
[c] These retention times were recorded at approximately equal peak areas.

ent from the basic conditions in the above studies, one cannot necessarily conclude that the preferred migration would be from C-4 to C-6. However, it is not considered likely that the p-coumaryl function would migrate quantitatively from the primary C-6 position to the less favorable secondary C-4 position. Biological acylation of the C-4 position in the glucose is quite reasonable if one considers the abundance of 1–4 glycosidic linkages in natural polysaccharides as evidence of the biological reactivity of the C-4 position. Although the possibility of an acyl migration during the methylation of the band 2 pigments cannot be completely ruled out, the experimental evidence indicates that the p-coumaryl group is most likely attached at the C-4 position of the glucose portion of the anthocyanin.

On the LAC-446 column a peak at a relative retention time of 5.50 corresponds exactly to the major peak resulting from the methylation of p-coumaric acid with diazomethane.

The diazomethane methylation of the pigment from band 3 was not conducted. The fact that considerable amounts of methyl tetra-O-methyl-D-glucopyranosides were detected in the methylation mixture of the more stable band-2 pigments suggests that perhaps the reaction sequence might not be successful with the more labile band-3 pigments. It is, however, reasonable to generalize that, because of the similar biochemical

nature and origin of bands 2 and 3, the caffeic acid probably esterifies the number-4 hydroxyl of the glucose in the band-3 pigments, just as p-coumaric acid apparently does with the band-2 pigments.

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R. D. PANKEY, H. N. DRAUDT, and N. W. DESROSIER[a]
Departments of Horticulture and Biochemistry
Purdue University, Lafayette, Indiana 47907

Characterization of the Starch of Spirodela polyrrhiza

SUMMARY

Starch was extracted from *Spirodela polyrrhiza* (L.) Schleid. and characterized as to shape, granule size and weight distribution, amylose content, temperature of gelatinization, and viscosity in relation to temperature.

The highest percent of starch for *S. polyrrhiza* was found in the sample collected after 14 hr of illumination in an atmosphere at 5% CO_2. The added CO_2 did not affect the starch content significantly, but did give more luxuriant growth. The starch was found to have simple, centric granules, spherical to slightly ovoid. The granules ranged from 1 to 8 μ, average diameter 3.85 μ. The weight average diameter was 4.86 μ. A dilute suspension of the starch gave a blue color with iodine. The amylose content was found to be 21%. The gelatinization temperature for starch of this size and amylose content was high. The gelatinization range was 94–98°C. The curves obtained from temperature vs. viscosity measurements were typical of those for other starches of similar size and amylose content.

INTRODUCTION

Projection of current knowledge suggests that man may soon be traveling in space. There is speculation also that, in time, the people of the earth may be faced with a food shortage due to the "population explosion." Even today, many parts of the world are faced with malnutrition and starvation. Concern for these problems has prompted workers to explore means of extending the usefulness of familiar foods and to search for new food sources and systems. The design of systems to achieve these aims may be different, but the basic requirements of each are the same: they should be simple, biologically and economically efficient, and nutritionally complete.

Many systems and new food sources have been proposed and studied, but none has yet fulfilled the requirements. The aquatic plants of the family Lemnaceae are now being investigated as a possible food source because of their high carbohydrate content. From this family, selected species of the genera *Wolffia* and *Spirodela* are being studied because of their relatively high starch content and ease of culture.

LITERATURE REVIEW

Nakamura (1961) investigated the family Lemnaceae because of its high carbohydrate content. His work dealt mainly with the genus *Wolffia*. He reports that the taste of *Wolffia* is excellent and sweet, resembling that of cabbage; the decolorized *Wolffia* is as white as flour and, because of its high starch content, has been called the "wheat of the future."

Man's success in extended space exploration may depend upon his ability to provide himself with a livable ecological system. Considering these factors, Wilks (1962) investigated three species of the family Lemnaceae: *Wolffia papulifera, Spirodela polyrrhiza,* and *Lemna minor*. Gas-exchange rates were about equal for *Wolffia* and *Spirodela* and their assimilation quotient approximated the respiration quotient of man. Reproduction of the Lemnaceae is accomplished almost exclusively through vegetable budding, thereby maintaining physiological stability and greatly reducing the probability of genetic change. *S. polyrrhiza* had an average division time of 30 hr.

In a review of the Lemnaceae, Hillman (1961) stated that *S. polyrrhiza,* like many other Lemnaceae, forms turions which constitute a resistant resting stage. These turions are formed continually, but to a greater extent under unfavorable growth conditions, and are heavily loaded with starch granules.

Lautner and Müller (1954) reported that the nutritional value of Lemnaceae compares favorably with that of alfalfa.

Most of the known amino acids were identified in *S. polyrrhiza* (Keser, 1955), suggesting the possible adequacy of *Spirodela* as a forage source for animals.

It appears that *S. polyrrhiza* may be used as a carbohydrate source. It would therefore be of interest to know the microscopic, chemical, and physical characteristics of the starch so that a prediction can be made as to how it might be used in food.

[a] Present address: Beechnut Life Savers, 477 Madison Avenue, New York, New York.

MATERIALS AND METHODS

Material. *Spirodela polyrrhiza* (Greater Duckweed) is an aquatic plant of the family Lemnaceae. Its fronds are ovoid in shape, 2.2–6.5 mm wide, and 2.4–9.5 mm long. Each plant has 4–7 roots that are 7–18 mm long. *S. polyrrhiza* reproduces vegetatively by budding, but also has the capacity to reproduce sexually through flowering, though this is rare. Its natural habitat is lakes, ponds, and swamps. It has a pH tolerance range of 5.8–7.9.

For this study, *S. polyrrhiza* was grown in an enclosed, clear plastic cylinder 45 inches long and 4 inches in diameter. The culture was illuminated with incandescent lamps at a light intensity of 400 ft-c with a photoperiod of 16 hr of light and 8 hr of darkness. The temperature was held at 30°C. An inorganic nutrient solution, Hutner's medium, was used. The cylinders were continually flushed with 5% CO_2.

The starch was extracted from 1500 g of material, fresh weight, by the method of Badenhuizen (1964). The material was homogenized in a Waring blender in a 0.01% $HgCl_2$ solution at 0°C. The suspension was filtered through muslin and centrifuged. Crude impurities were removed with washing. Proteins were denatured and removed by repeated washing with NaCl solution and toluene. The starch was defatted by refluxing with 80% aqueous methanol, then washed with acetone-ether and dried under reduced pressure at 50°C.

Methods. The starch percentage was determined in samples collected after 2, 8, and 14 hr of light in an atmosphere of 5% CO_2, and also after 6 and 12 hr of light, without the addition of CO_2, by the method of Pucher *et al.* (1948). Duplicate samples from two harvest dates were analyzed. A sample of dried *S. polyrrhiza* was extracted with 72% perchloric acid. The starch was precipitated from the extract as an iodine complex. The supernatant was decanted and the iodine-starch complex was decomposed with alcoholic NaOH and then hydrolyzed to glucose with HCl. The glucose was determined by the Somogyi colorimetric methods as described by Nelson (1944).

The microscopic characteristics were evaluated with the aid of photomicrographs enlarged 1000 times. On the photomicrograph, about 1000 randomly selected starch granules were measured to the nearest .1 mm. The starch granules were divided into size ranges, and the percent of each, as well as the average diameter, was calculated (Schoch and Maywald, 1956).

The colorimetric method of MacCready and Hassid (1943) was used to determine the amylose-amylopectin ratio of the starch. One hundred milligrams of the dry starch were dissolved in ethanol and NaOH with heating. Iodine solution was then added and the solution diluted to 500 ml. Percent light transmission, used as the index of color intensity, at a wave length of 610 mμ, was determined with a Bausch and Lomb Spectronic 20 colorimeter. A standard curve was prepared from samples of differing and known amylose-amylopectin content. The experimental conditions were constant for both the standard curve preparation and the experimental sample.

Gelatinization temperature was determined microscopically with an electrically heated hot-stage polarizing microscope (Pfahler *et al.*, 1957). Loss of anisotrophy of the starch granule was used as an index of the point of gelatinization (Alsberg and Rask, 1925). Two or three drops of 2% starch suspension were placed on a microscope slide. The suspension was surrounded with a ring of high-melting vacuum grease and sealed with a coverslip in such a way as to exclude air bubbles. The temperature and rate of heating were controlled with a powerstat to 2°C per min.

The effect of temperature on relative viscosity was determined to obtain information on the pasting characteristic of the starch. Viscosity of 8 ml of the 1% suspension was determined with an Ostwald-Cannon-Fenske No. 100 pipette in an electrically heated ethylene glycol bath. The bath was heated at a rate of 1°C per 5 min to allow for thorough heating. One reading was taken for every degree rise on triplicate samples. Flow time was taken as the measure of viscosity and was plotted against heating time.

RESULTS AND DISCUSSION

Spirodela polyrrhiza is a simple plant with no specialized storage structures. For this reason the entire plant, roots as well as fronds, was analyzed for starch.

Fig. 1 shows the relation of length of illumination on starch content as well as the effect of CO_2 concentration on starch content. The highest percent of starch was found in *S. polyrrhiza* grown under 5% CO_2 and collected after 14 hr of illumination. The data show, however, that the CO_2 content of the culture atmosphere had no significant effect on starch content. It was noted that there was a more rapid rate of growth and a more luxurious growth when 5% CO_2 was added. Increase in starch with increased illumination was to be expected. The fronds of *S. polyrrhiza* are similar in structure and analogous to the leaves of higher plants, whose starch content fluctuates with respira-

Table 1. Microscopic, chemical, and physical properties of *S. polyrrhiza*.

Diam. (μ) granule		Weighted av. diam. (μ)	Amylose content (%)	Color with I	Gelatinization temperatures (°C)	
Range	Av.				Range	Av.
1–8	3.85	4.86	21	blue	94–98	96

tion and translocation of the starch in darkness and synthesis in the light.

The microscopic, chemical, and physical characteristics that were studied are presented in Table 1.

The starch granules of *S. polyrrhiza* are simple, centric granules, spherical to slightly oval. The hilum is readily observable in the

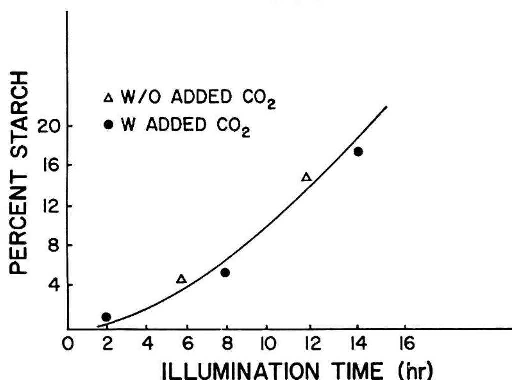

Fig. 1. Starch content of *S. polyrrhiza* grown with or without CO_2 and various periods of illumination.

unstained granules as well as those stained with an iodine solution (Fig. 2). There is a relatively narrow size range of granules between 1 and 8 μ, the average being 3.85 μ Fig. 3). The weighted average diameter is 4.86 μ. This difference means that, although there is a greater number of small starch granules, the larger starch granules contribute more to the mass of the starch. Fig. 4 shows this relation.

The starch granules of *S. polyrrhiza* are smaller than the granules of the more commonly used food starches, but starches of this size are common in other botanical sources.

The amylose content of common food starches ranges from 18% in tapioca to 25% in commercial corn. This is also the general range in most botanical sources, though greater and lesser proportions of amylose are known. The amylose content of *S. polyrrhiza* starch is 21%. A 1% suspension of the starch gives a deep-blue color with a dilute solution of iodine-potassium iodide.

The gelatinization temperature range for

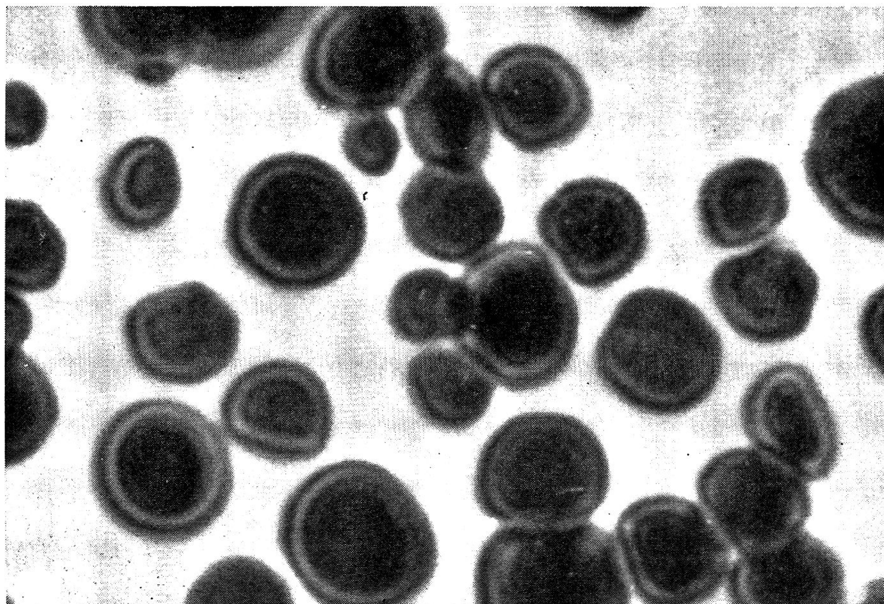

Fig. 2. Starch granules of *S. polyrrhiza* magnified approximately 4600×.

S. polyrrhiza starch is 94–98°C, the average being 96. Most common food starches gelatinize between 55 and 75°C, but higher temperatures are reported for a few non-food starches. Gelatinization temperatures were determined for common corn and wheat starch samples and were found to be 70.9° and 65.2°F respectively, which correspond to the accepted values.

The curves obtained from heating a 1% suspension of *S. polyrrhiza* starch support the results obtained in the gelatinization temperature determinations. The size and shape of the granules were responsible for the initial and steadily decreasing viscosity. The rapid increase in viscosity between 88 and 98°C corresponds closely to the gelatinization range observed by loss of anisotrophy. The curve obtained with the 1% suspension of *S. polyrrhiza* starch (Fig. 5) is typical of the curves obtained from measurements on corn starch (Fig. 6). It also has the same general shape as the viscograms reported by Mullen and Pacsu (1942) for 10% and 15% potato and corn starch.

The increase in viscosity after heating was due to the loss of water from the open ends of the pipette, thus concentrating the starch suspension.

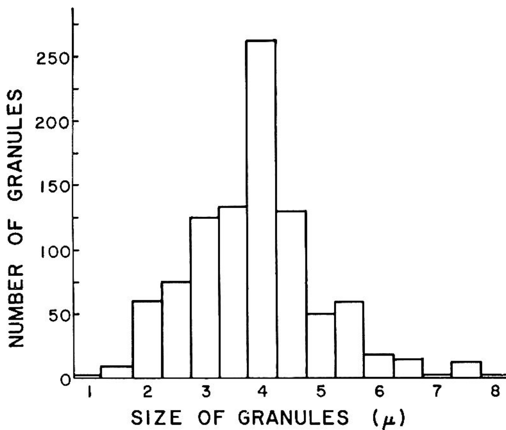

Fig. 3. Size distribution of 956 starch granules on *S. polyrrhiza*.

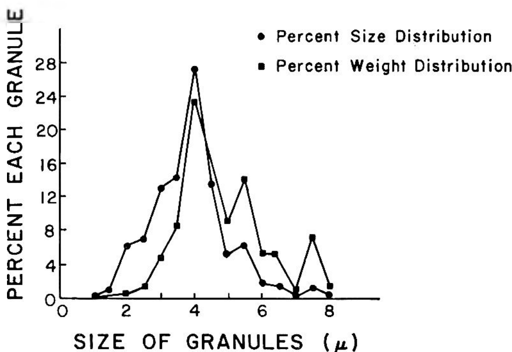

Fig. 4. Percent size and weight distribution of starch of *S. polyrrhiza*.

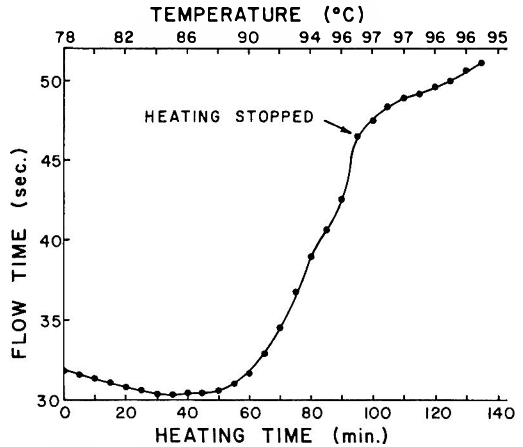

Fig. 5. Temperature vs. viscosity relationship on starch of *S. polyrrhiza*.

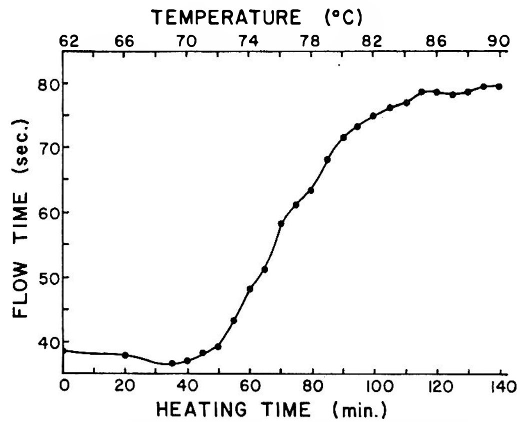

Fig. 6. Temperature vs. viscosity relationship on corn starch.

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R. A. CHUNG,[a] C. L. RAMEY,[b] C. C. LIN,[c] J. A. WALLS,[a] S. H. SETTLER,[d] W. H. FARLEY,[c] and E. T. MILES[e]

School of Agriculture, Tuskegee Institute, Tuskegee Institute, Alabama 36088

Fatty Acid Composition of Tissues of Pigs Fed Whole Peanuts

SUMMARY

The liver of pigs fed a whole-peanut-supplemented diet significantly increased in fatty acid means in 18:1 (carbon chain length:number of double bonds) and 18:2, and significantly decreased in 18:0, compared with the liver of pigs on a control diet. The kidney decreased significantly in 14:0 and 17:0, and the heart decreased significantly in 16:1. These changes were primarily the result of an increased total lipid consumption since the fatty acid compositions of both dietary lipids were very similar. Of all the tissues studied (liver, heart, kidney, ham, shoulder, skin, bacon fat, and chop) the liver contained the largest amount of 18:0, 18:2, and 20:4, and the least amount of 14:0, 16:0, 16:1, 17:1, and 18:1.

INTRODUCTION

Peanuts, an important crop in the southern states, are fed to a considerable extent to pigs. Pigs that eat a large amount of peanuts have a tendency to produce soft pork, which sells at a discount price. Research studies have been reported on the extent to which softening feeds such as peanuts can be used without producing soft pork (Morrison, 1959).

Hilditch and Williams (1964) noted that the softer or more saturated fats of the pig contained quantitative differences in fatty acid composition, especially in palmitic acid content. Several other workers, including Brown (1931) and Hill *et al.* (1961), have reported that different dietary fats change the fatty acid composition of the tissues of the pig.

This investigation was made to determine the fatty acid changes in different organs and tissues of pigs fed a diet supplemented with whole peanuts, and to compare the fatty acid composition of the different parts of the pig.

[a] Department of Food Science and Technology.
[b] Carver Research Fellow.
[c] National Science Foundation Summer Research Participant, Summer, 1964.
[d] Department of Animal Science.
[e] Department of Agronomy.

EXPERIMENTAL PROCEDURES

Eight Hampshire pigs from the Institute herd were conditioned for four weeks on a diet of 20% commercial hog supplement (40% protein; Cosby-Hodges Milling Company, Birmingham, Alabama), 70% ground corn, and 10% alfalfa meal (17% protein; Consolidated Blenders, Inc., Guntersville, Alabama), to an average weight of 175 lb (range 150–202 lb). Four pigs were then placed on a diet in which 10% whole peanuts and 20% whole peanuts were respectively substituted for the same amount of ground corn for the first four weeks and for the last four weeks of experimentation. The other four pigs, continued on the regular diet, served as the control.

The pigs were slaughtered, weighed, and subsequently cut up after eight weeks on the experimental diets. Lipids were extracted from three organs (liver, kidney, and heart) and five body tissues (ham, shoulder, skin, bacon fat, and chop).

The lipids were extracted from the diet and from the different tissues with chloroform-methanol (2:1), and then stored at 8°F under nitrogen, until analysed.

After saponification with 4% potassium hydroxide in ethanol and acidification with dilute hydrochloric acid the fatty acids were esterified with methanol, with sulfuric acid used as the catalyst.

The fatty acid methyl esters were separated and quantitatively determined by gas-liquid chromatography. The gas chromatograph used was an Aerograph model A-350-B instrument with dual column and a hot wire thermal conductivity detector system. The polar liquid phase was diethylene glycol succinate (20%, w/w) on 30–60-mesh firebrick. Ten-foot copper columns were used. Column temperature was 215°C, and helium gas flow 120 ml/min.

Fatty acid mixtures *A, C, D,* and *F*, obtained from the National Institutes of Health, were used to calibrate the instrument and to provide information on retention times for identification of sample components. Peak areas were calculated by a disc integrator. Peak areas of the standard mixtures were proportional to the weight percentages of the fatty acid methyl esters.

Lipids were analyzed for each animal and the results were reported as averages for four pigs. Experimental data were analyzed by analysis of variance (Snedecor, 1959). Differences between

means were located with Newman-Keuls test (Duncan, 1955).

RESULTS AND DISCUSSION

Analysis of variance (Table 1) showed significant differences between means of 16:1, 17:1, 18:1, 18:2, and 20:1 acids due to treatment effects. These differences were primarily between the means of the fatty acids found in the different organs (Table 2). The liver of the pigs fed the whole peanuts showed a significant increase in 18:1 and 18:2, and a significant decrease in 18:0, compared with the control diet. The kidney decreased significantly in 14:0 and 17:0, and the heart decreased significantly in 17:0 and 17:1. These changes occurred despite the very slight differences in the fatty acid composition of either diet (Table 4).

The 18:2 acid had a tendency to increase, and the 18:1 and 18:0 contents to decrease, in the kidney and heart with the whole peanut diet. However, these differences were not significant. The apparent increase in 18:2 in all the organs of pigs on the whole-peanut-supplemented diet could be explained by the increase in fat content of this diet (Table 4) and the consequent increase in consumption of 18:2, since there was no difference in the concentration of this acid in either diet.

Despite the higher lipid content of the whole-peanut-supplemented diet, no significant differences were evident between the means of any fatty acid of the body tissues studied except in the bacon fat, where 16:1 decreased when whole peanut was consumed (Table 3). All the fatty acids shown in Table 1, except 20:2, exhibited a significant difference among the parts of the pig. This difference was between the organs and the body tissues. There was no significant difference between the means of the different body tissues.

The organs appeared to contain large amounts of 20:4, with the greatest concentration in the liver. No trace was detected of several of the lower fatty acids (10:0, 13:0, 14:0, 15:0, and 15:1) in the liver.

The predominant fatty acid in the liver was 18:0 when the control diet was consumed, and 18:1 when the whole peanut was consumed. Apparently the liver fat differed from the body fat, kidney, and heart fat in that the liver had 18:0 as the principal saturated acid. In general the organs exhibited a more active fatty acid metabolism than the body tissues.

Table 1. Summary of the analyses of variance of the methyl esters of the component fatty acids.

	Source of variation							
	Treatment		Part		Treatment × part		Error	
Fatty acid[a]	d.f.	Mean square	d.f.	Mean square	d.f.	Mean square	d.f.	Mean square
12:0	1	0.0042	7	0.6633**	7	0.0254	48	0.0093
14:0	1	0.0361	7	1.0804**	7	0.0259	48	0.0261
16:0	1	1.3865	7	39.4602**	7	3.0343	48	2.0267
16:1	1	1.3370*	7	10.1476**	7	0.4247	48	0.2325
17:0	1	0.5353**	7	0.5679**	7	0.1387	48	0.0725
17:1	1	0.5264**	7	0.2406**	7	0.0496	48	0.0345
18:0	1	0.0086	7	253.6499**	7	6.7595	48	4.1910
18:1	1	18.2009*	7	878.1257**	7	7.4131	48	4.2102
18:2	1	26.8065**	7	74.3209**	7	1.1733	48	1.6403
18:3	1	0.0395	7	0.2317**	7	0.0283	48	0.0508
20:1	1	0.9385**	7	1.0410**	7	0.1380	48	0.1259
20:2[b]	1	0.0900	2	0.1645	2	0.6686	18	0.4556
20.3[b]	1	0.0988	2	2.2347*	2	0.7365	18	0.5474
20:4	1	2.1134	7	236.4832**	7	2.5627*	48	0.9738

[a] Carbon chain length: number of double bonds.
[b] Only data from organ tissues (liver, heart, and kidney) were used.
* $P<.05$.
** $P<.01$.

Table 2. Fatty acid composition of organ tissues of pigs on a control and a peanut-supplemented diet.

Fatty acid[a]	Liver[b]		Kidney[b]		Heart[b]	
	Peanut	Control	Peanut	Control	Peanut	Control
10:0	......	......	......	......	0.04	0.06
12:0	0.12	0.07	0.39	0.30	0.10	0.09
13:0	......		0.10	0.12	0.26	0.35
14:0	0.51	0.39	0.98	1.34*	1.51	1.47
14:1	......	......	0.02	0.08	0.21	0.13
15:0	......	......	0.15	0.22	0.11	0.16
15:1	......	......	0.33	0.45	0.36	0.27
16:0	18.99	18.32	23.38	25.68	19.94	21.52
16:1	1.24	1.06	1.52	1.99	2.35	1.97
17.0	0.66	0.71	0.35	0.94**	0.52	1.15**
17:1	0.02	0.21	0.32	0.69	0.35	0.82**
18.0	22.74	26.04*	16.54	18.49	14.93	14.66
18:1	23.15	20.13	37.06	38.26	38.49	40.20
18:2	15.25*	13.05	8.53	6.24	12.99	12.80
18:3	0.27	0.15	0.46	0.34	0.68	0.66
20:0	0.31	0.25	......	......	......	......
20:1	0.29	0.10	0.83	0.67	0.75	0.73
20:2	0.15	0.13	0.58	0.42	0.31	0.36
20:3	0.92	1.20	0.77	0.34	0.28	0.32
20:4	15.57	16.92	5.40*	3.33	4.03	3.60

[a] Carbon chain length: number of double bonds.
[b] Means for 4 pigs per treatment.
* P<.05.
** P<.01.

Table 3. Fatty acid composition of body tissues of pigs on a control and a peanut-supplemented diet.

Fatty acid[a]	Ham[b]		Shoulder[b]		Skin[b]		Bacon fat[b]		Chop[b]	
	Peanut	Control	Peanut	Control	Peanut	Control	Peanut	Control	Peanut	Control
10:0	0.09	0.10	0.08	0.10	0.05	0.08	0.08	0.05	0.08	0.08
12:0	0.07	0.05	0.08	0.09	0.07	0.10	0.10	0.10	0.07	0.07
13:0	0.10	0.07	0.02	0.03	......	......	......	......	......	......
14:0	1.57	1.58	1.36	1.45	1.51	1.51	1.41	1.41	1.41	1.50
15:0	0.10	0.90	0.11	0.06	0.06	0.10	0.10	0.12	0.03	0.04
16:0	24.37	24.58	24.02	25.15	22.93	23.91	22.86	22.72	24.61	25.49
16:1	3.65	4.11	3.60	3.85	3.54	4.28	2.39	3.34*	4.03	4.03
17:0	0.16	0.20	0.10	0.24	0.27	0.27	0.47	0.48	0.18	0.19
17:1	0.20	0.31	0.26	0.36	0.24	0.41	0.52	0.60	0.23	0.22
18:0	8.99	8.91	9.44	8.85	7.67	7.77	11.85	9.92	12.23	9.82
18:1	48.74	49.71	49.85	51.36	52.47	53.05	46.50	50.30	49.25	51.24
18:2	8.48	7.61	8.03	7.49	7.53	6.22	9.87	8.39	6.13	4.59
18:3	0.47	0.64	0.63	0.45	0.54	0.61	0.75	0.72	0.49	0.35
20:1	0.98	0.95	1.15	0.60	1.58	0.82	1.58	1.27	0.81	0.81
20:2	0.60	0.17	0.32	0.15	0.67	0.12	0.76	0.16	0.19	0.38
20:4	0.93	0.80	0.79	0.55	0.77	0.56	0.66	0.33	0.36	0.67

[a, b] Same as footnotes in Table 2.
* P<.05.

Table 4. Fatty composition of lipids extracted from the diets.

Fatty acid[a]	Control[b]	Peanut[b]
10:0	0.13	0.07
12:0	0.56	0.39
14:0	0.43	0.35
16:0	16.86	13.65
16:1	0.87	0.80
18:0	3.51	2.02
18:1	30.79	36.08
18:2	41.66	41.01
18:3	5.15	2.34
20:1	......	0.94
22:0	......	2.20

[a] Carbon chain length: number of double bonds.
[b] Lipid content of the control diet was 3.6% on wet-weight basis and of the whole-peanut-supplemented diet (20%) was 7.8%.

In contrast to the liver and the kidney, the shoulder, bacon fat, and chop tended to increase in 18:0 with the whole-peanut diet. These means were not significant.

Only in the liver was 20:0 detected and 20:3 was detected only in the liver, kidney, and heart.

The presence of odd-carbon fatty acids in the tissues confirmed the report of Sink *et al.* (1964), who found these acids in the fat depot of pigs.

Of all the tissues studied, the liver contained the largest amount of 18:0, 18:2, and 20:4, and the least amount of 14:0, 16:0, 16:1, 17:1, and 18:1. The heart and kidney generally followed a similar pattern in comparison with the body tissues.

It was observed that the average weight of pigs was greater on the whole-peanut-supplemented diet than on the control diet. This increase was accounted for primarily by an increase in the bacon and fat trimming weights.

CONCLUSIONS

Significant changes in fatty acid composition due to dietary whole peanuts were found primarily in the organ tissues as compared to the body tissues. Of the three organs studied, the heart was the least prone to fatty acid changes. Each organ exhibited characteristic changes in response to dietary whole peanuts.

The 18:2 acid tended to increase in all organs and body tissues with the whole-peanut-supplemented diet, but only in liver were the differences significant. The liver increased in 18:1, while all other tissues decreased in this acid, when whole peanut was consumed.

Despite the higher lipid content of the whole-peanut-supplemented diet, the total polyunsaturated fatty acid content of all tissues studied changed very slightly.

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J. H. RAMPTON,[a] A. F. ANGLEMIER, and M. W. MONTGOMERY
Department of Food Science and Technology
Oregon State University, Corvallis, Oregon 97331

Fractionation of Bovine Sarcoplasmic Proteins by DEAE-Cellulose Chromatography

SUMMARY

A Tris buffer system (starting buffer, 0.04*M* Tris phosphate, pH 9.0; limiting buffer, 0.5*M* Tris H_2PO_4, pH 3.6) and a concave gradient elution procedure were developed for the fractionation of beef sarcoplasmic proteins by diethylaminoethyl cellulose ion-exchange chromatography. Sarcoplasmic proteins extracted 2 hr post-mortem were separated chromatographically into 16–18 distinct fractions. Duplication of the results was excellent. Some alterations occurring in these proteins during 10 days' post-mortem aging were detected by this technique. Such changes observed were the disappearance of one fraction and appearance of new components, while other fractions diminished.

INTRODUCTION

Research on the proteolytic changes occurring in beef skeletal muscle during post-mortem aging has been hindered by a lack of appropriate procedures for the quantitative separation of muscle proteins. Thus, a procedure capable of detecting and following such changes systematically could be of immense value in future meat investigations.

Cellulose ion-exchange chromatography has been applied very advantageously for the separation and fractionation of protein constituents of several biological materials of rather diverse complexity. This technique has been used successfully for the separation of milk proteins (Yaguchi *et al.*, 1961), analysis of blood sera proteins (Huisman *et al.*, 1958; Sober *et al.*, 1956), purification of certain hormones and enzymes (Keller *et al.*, 1958; Sober and Peterson, 1960), and characterization of egg white proteins (Mandeles, 1960; Rhodes *et al.*, 1958).

Some success in fractionating the water-soluble proteins of chicken muscle by DEAE-cellulose (diethylaminoethyl-cellulose) anion-exchange chromatography has been reported by Fischer (1963) and Mandeles (1960). Hartshorne and Perry (1962), using stepwise elution, and Mohasseb (1963), employing nonlinear gradient elution, showed that DEAE-cellulose chromatography offered some possibility of separating mammalian muscle proteins quantitatively. Fujimaki and Deatherage (1964) recently chromatographed beef sarcoplasmic proteins on cellulose phosphate (cationic) columns, and by stepwise elution obtained excellent fractionation of these proteins. Although those researchers were optimistic about using this procedure in future studies on muscle proteins, they did emphasize the need for additional research to clarify some of the problems that became evident.

The study reported herein pertains to fractionation of the sarcoplasmic proteins of beef skeletal muscle by DEAE-cellulose ion-exchange chromatography. This report differs from the above muscle studies in that a concave gradient elution procedure and a Tris phosphate buffer system were developed to facilitate the fractionation technique.

EXPERIMENTAL

Muscle extract. About 45 min after slaughter, an intact longissimus dorsi muscle was removed from a beef carcass and taken to the laboratory, where it was held at 3°C until used. Fifty grams of muscle from which the visible external fat and membranes had been removed were diced and homogenized for 2 min with an equal weight of distilled water in a Waring blender. The homogenate was centrifuged for 20 min at 10,000 × G, and the supernatant was filtered through Whatman No. 12 to remove fat particles. A clear filtrate was obtained which indicated the absence of visual particulate material. The sarcoplasmic extract was then dialyzed for 24 hr against two 100-fold portions of the starting buffer (Peterson and Sober, 1962) before being chromatographed.

Chromatography. DEAE-cellulose (Type 20, 1.0 meq/g, obtained from Brown Company, Berlin, New Hampshire) was prewashed and packed (2 × 40-cm columns) under pressure by the method of Peterson and Sober (1962). All columns were packed from the same lot of washed DEAE-cellulose. The columns were rotated slowly about the

[a] Present address: Department of Food Science, Michigan State University, East Lansing, Michigan.

long axis during the packing procedure to provide the necessary agitation to obtain a level packing deposition of the cellulose. In certain instances, the cellulose columns were regenerated for reuse by passing 750 ml of 0.1*M* Na_3PO_4–0.2% Triton X-100 (nonionic detergent) solution through the columns under gravity followed by prolonged rinsing with distilled water (Peterson and Chiazze, 1962). Twenty-five ml of 0.1*M* Tris [tris(hydroxymethyl) amino methane] phosphate, pH 9.0, were then passed into the columns followed by re-equilibration with the starting buffer, 0.04*M* Tris adjusted with H_3PO_4 to pH 9.0.

Twelve ml of dialyzed sarcoplasmic extract (360 mg protein) were carefully washed onto a column by several small portions of starting buffer. After 120 ml of the starting buffer had passed through the column, the gradient elution procedure was started. A nine-chambered variable-gradient mixer, the Varigrad (Peterson and Sober, 1959), produced the desired combination pH-ionic strength gradient, beginning with the starting buffer and increasing to a final concentration of 0.5*M* Tris H_2PO_4, pH 3.6. Total volume of the gradient was 2250 ml, which was prepared by mixing the following percentages of the final buffer with the starting buffer in the order of 0, 0.4, 0, 1.6, 0, 3, 0, 5, and 100% for the nine chambers. The gradient was followed with several 100-ml additions of final buffer to the ninth chamber of the Varigrad until the column was completely titrated to the pH of the final buffer before elution was halted. Flow rate was kept constant at 80 ml/hr by inclusion of a micropump between the Varigrad and the chromatographic column. All fractionation work was completed at 3°C.

The effluent was monitored continuously at 280 mμ by a Beckman DB recording spectrophotometer and then collected by a fraction collector equipped with a 10-ml volumetric siphon. A Beckman DK-1 recording spectrophotometer was used to obtain the absorption spectrum, 700–230 mμ, of the peak tube of each fraction. The pH gradient was determined by measuring every tenth tube of effluent with a Beckman pH meter, Model G. Total nitrogen of the sarcoplasmic extracts was determined by the micro-Kjeldahl technique, while the amount of protein recovered from the columns was derived from the absorbancy measurements (Dixon and Webb, 1964).

The results were evaluated largely on the number of distinct fractions separated and on the similarities or differences between chromatograms in relation to both the effluent volume and pH at which the various fractions were eluted. The fractions were arbitrarily labeled in numerical order according to their sequence of elution from the cellulose columns.

RESULTS AND DISCUSSION

Several trial runs involving different buffer and gradient elution procedures were completed initially to determine optimal conditions for separation of the sarcoplasmic proteins by DEAE-cellulose ion-exchange chromatography. A buffer system (starting buffer of 0.005*M* Tris phosphate, pH 8.6; limiting buffer of 0.5*M* Tris H_2PO_4, pH 3.6) that proved excellent for the fractionation of blood sera proteins (Peterson and Chiazze, 1962; Sober and Peterson, 1958) was used originally. Although the sarcoplasmic proteins were separated into 11 fractions, compared to only six reported in previous work by different buffer and elution procedures (Hartshorne and Perry, 1962; Mohasseb, 1963), two large doublet peaks were eluted during the early stages of chromatography, which necessitated changes in the initial conditions of the starting buffer. After several alterations were tested, the starting buffer mentioned in the preceding section was selected since most of the protein was initially retained on the cellulose.

Fig. 1 shows the chromatograms of duplicate runs in which 12-ml samples (360 mg protein) of sarcoplasmic extract (2 hr post-mortem) were fractionated on each of two

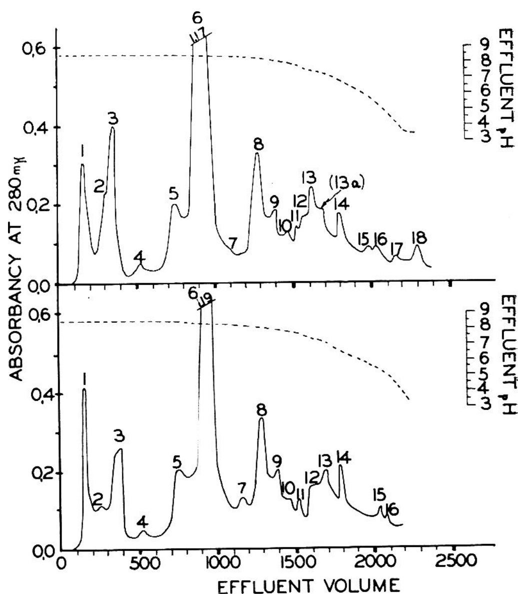

Fig. 1. Chromatography of beef sarcoplasmic proteins extracted 2 hr post-mortem. Lower and upper curves are respectively the first and second separations.

freshly packed chromatographic columns. Since only one monitoring and collection assembly was available during the study, the runs were not completed simultaneously. The second run was started 30 hr after the beginning of the first. At least 16–18 fractions were noted, which agrees well with findings of Fujimaki and Deatherage (1964), who observed the separation of at least 14 fractions by cellulose phosphate chromatography. An equipment failure occurred in the first run after 2200 ml of effluent had passed from the column. Thus the lower curve in Fig. 1 does not show the two fractions eluted at the end of the second run. In comparing these two chromatograms, many similarities are evident, especially in regard to the positions of the eluted fractions on the profile and the pH at which they were eluted. There are, however, differences between the heights of some of the corresponding peaks, indicating differences in the protein content of the fractions. Moreover, an additional peak (13a) was eluted between Fractions 13 and 14 on the second run. Some of these differences might be the result of slight alterations occurring during storage (3°C) of the extract used for the second run since there was a 30-hr time differential between runs.

The large component (Fraction 6) emerging at about 900 ml of effluent, pH 8.5, in both chromatographs of Fig. 1 contained about 90% of the red pigments in the sarcoplasmic extract. This fraction showed absorption maximum (λ_{max} 542 and 580 mμ) corresponding to that of oxymyoglobin (Fujimaki and Deatherage, 1964). Absorption spectra also showed that the remainder of the pigment was in those fractions (8, 9, 10, and 11) eluted between 1280 and 1530 ml at a pH range of 8.2–7.7. The last components (Fractions 15 through 18) to be released from the columns between 1980 and 2280 ml, pH range 5.8–3.5, possessed almost as much absorbancy at 254 mμ as at 280 mμ. Fujimaki and Deatherage (1964) eluted a fraction from cellulose phosphate columns at pH 5.0 that showed a high optical density at 260 mμ. They speculated that this fraction was a nucleoprotein.

The chromatograms shown in Fig. 2 are the results of another test of reproducibility of the technique, although the column used in the second run was the same as that used for the first separation after the DEAE-cellulose had been cleaned and re-equilibrated in the column as described earlier. The sarcoplasmic proteins used for both of these separations were extracted 3 hr post-mortem and stored for three weeks at −20°C, and therefore cannot be directly compared with the results in Fig. 1. These chromatograms show a high degree of similarity in regard

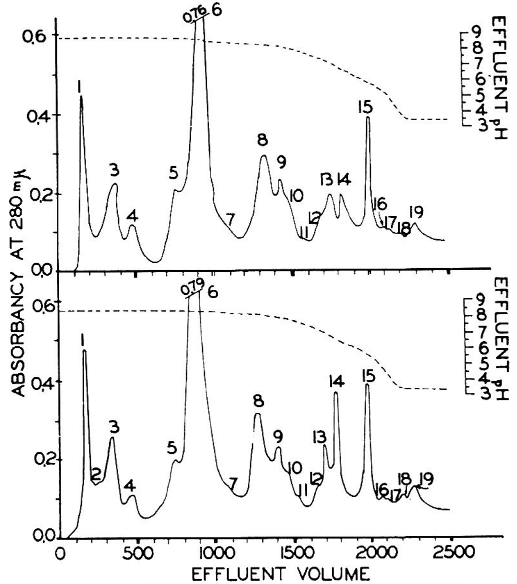

Fig. 2. Reproducibility of chromatography of beef sarcoplasmic proteins extracted 3 hr post-mortem and stored for 3 weeks at −20°C. Lower curve, new cellulose column; upper curve, used, cleaned, regenerated column.

to number of fractions separated, the effluent volumes required for their elution, and the pH at which they were eluted. The only appreciable differences between these profiles are the appearance of the minute peak for Fraction 2 and a higher peak for Fraction 14 when separation was completed on freshly packed cellulose than occurred on cellulose which had been used, cleaned, and regenerated in the column. Other differences between these chromatograms appear to be of a minor nature.

Although Fujimaki and Deatherage (1964) obtained reproducible results with their fractionation system, various enzymes tested for appeared in several of the peaks.

Thus it must be emphasized that an individual eluted peak is not necessarily a particular homogeneous protein nor does all of any one protein reside in one specific fraction.

Fig. 3 shows the chromatography of the sarcoplasmic proteins extracted 10 days post-mortem from the same muscle that served as sample source for Fig. 1. In order to maintain continuity in numbering the fractions, this curve was transposed over the lower diagram in Fig. 1 and the numbering of fractions was designated accordingly. It can readily be noted that after 10 days of aging, one fraction disappeared (7), others diminished (1, 3, 12, 13, 14, 15 and 16), certain new fractions appeared (1a, 1b and 1c), and the emergence of several fractions was delayed (1–3 and 8–16). The upper diagram of Fig. 1 can also be compared with Fig. 3 to denote aging differences. These results indicate that some of the changes occurring in the sarcoplasmic proteins during aging can be detected by DEAE-cellulose ion-exchange chromatography. Fujimaki and Deatherage (1964) also reported chromatographic differences in these proteins after 12 days post-mortem aging.

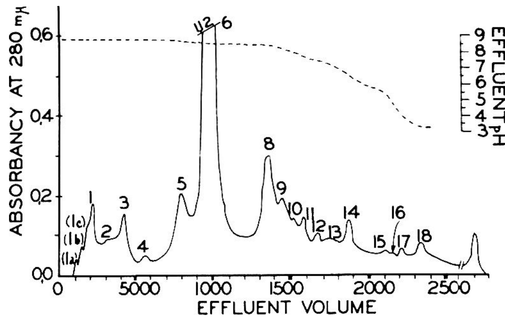

Fig. 3. Chromatography of beef sarcoplasmic proteins extracted 10 days post-mortem. The last fraction (not numbered) was removed from the column with 0.1*N* NaOH.

When 360 mg of protein were applied to the columns, 325–330 mg were recovered in the effluent, indicating a recovery of more than 90%. Subsequent washing of the columns with 0.1*N* NaOH removed an additional 1–2% of the protein originally applied. An example of this residual protein is the peak shown to be eluted at 2700 ml in the chromatogram in Fig. 3. These results compare quite well with recovery data recorded by others (Mohasseb, 1963; Sober *et al.*, 1956).

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D. E. HEINZ, R. K. CREVELING, and W. G. JENNINGS
Department of Food Science and Technology
University of California, Davis 95616

Direct Determination of Aroma Compounds as an Index of Pear Maturity

SUMMARY

The production of esters of trans:2-cis:4-decadienoic acid, which have been identified as contributory flavor compounds of Bartlett pear, are shown to increase rapidly at the climacteric, and continue to increase as ethylene and CO_2 production decline. The production of the decadienoates can be readily followed by ultraviolet spectrophotometry, and their measurement offers a simple and convenient criterion of fruit maturity that is a direct function of fruit flavor. Gamma irradiation of pears, which produces a flavorless and subnormal fruit, also severely inhibits the production of the decadienoates.

Characteristic changes that occur in fruit during the ripening process include softening of the flesh, pigment transformation, quantitative and qualitative changes in the amount of soluble solids, the total titratable acids, and the volatile constituents (Audigie, 1958; Hulme, 1958; Leonard *et al.*, 1954; Luh *et al.*, 1955; Tindale *et al.*, 1938). Most of these have been suggested as indices of maturity, and some have found acceptance for that purpose. Aroma is probably the quality which most influences consumer acceptance, and the various maturity indices have been used because they correlated, to a greater or lesser degree, with fruit flavor and aroma. A direct measurement of these aroma compounds would appear to be more meaningful than the indirect approach of measuring a property which correlates statistically with aroma.

Antoniani and Serini (1955), Antoniani *et al.* (1954), and Serini (1956) observed that the amounts and ratios of 2,3-butylene glycol and acetoin in pears and apples varied with the maturity of the fruit, and suggested that these variations might serve as an index of ripeness. However, because of difficulties in determining the concentrations of these compounds, the method has not found acceptance. Gerhardt (1954) studied the rates of emanation of volatiles from pears and apples by bubbling the evolved gases through sulfuric acid and oxidizing with ceric sulfate. His study would appear to be directed toward low-boiling volatiles capable of oxidation under these conditions, e.g. acetaldehyde. Lim (1963) followed the production of individual low-boiling volatile compounds (ethylene, acetaldehyde, and the normal acetates up to hexyl acetate) by gas chromatographic analysis of the headspace vapors of fruit ripened in closed containers. These methods, which have been applied to pears, apples, nectarines, and peaches, should be applicable to many foods.

Higher-boiling compounds (esters of trans:2-cis:4-decadiencoic acid) have been found to be important to Bartlett pear aroma (Jennings *et al.*, 1964). Gas chromatographic analysis of ether extracts of fresh pear indicate that the methyl and ethyl esters of 2,4-decadienoic acid are the major high-boiling compounds contained in the volatile essence of pears. These triconjugated esters can be conveniently measured by following their ultraviolet absorption, which correlates well with the concentration of pear flavor as determined by sensory evaluations (Heinz *et al.*, 1964). The work reported here indicates that the biochemical production of these esters in the fruit (Table 1) correlates with the production of total volatiles as measured by Gerhardt (1954) and Lim (1963). This study also showed that production of the 2,4-decadienoate moiety is decreased in irradiated pear and that the ripening process is also markedly inhibited.

METHODS

Trans: 2-cis: 4-decadienoate ester production. Bartlett pears were obtained green (18–20 lb penetrometer pressure) from commercial orchards near Placerville, California. The pears were stored at 0°C for 30 days prior to study. Twenty-four of these pears were selected and placed in individual glass containers at 20°C. Air, saturated with water vapor, was continuously passed over the pears at flow rates of 40–60 ml/min. The diconjugated ester production was monitored by

Table 1. Production of 2,4-decadienoates (as methyl ester), CO_2, and C_2H_4 in ripening Bartlett pears.

Days' storage at 20°C	1	2	3	4	5	6	7	8	9	10	11	12	13
μmoles decadienoates/kg	...	0.45	1.6	2.7	5.7	38	52	118	177	246	237	258	270
μl C_2H_4/Kg hr	17.8	15.8	20.3	24.7	32.9	36.3	37.8	36.5	35.2	32.8	28.0	29.7	25.9
mg CO_2/Kg hr	......	3.8	11.9	28.3	64.8	96.3	93.6	83.6	87.0	51.8	29.3	13.9	7.5

ultraviolet measurements (Heinz *et al.*, 1964). On each day after the second day, two of the pears were blended separately with an equal weight of 0.01*M* potassium phosphate buffer at pH 7.3. Equal volumes of this blended material, water, and iso-octane (spectro-quality reagent, Matheson, Coleman, and Bell) were then shaken vigorously together. The organic phase was separated by centrifugation and scanned from 320 to 210 mμ on a Beckman DB spectrophotometer, and absorbance at 260 mμ was measured from an interpolated baseline (Heinz *et al.*, 1964). Production of the 2,4-decadienoic moiety is expressed as micromoles (μ*M*) of ester per kg of pear. The μ*M* of the decadienoic moiety were calculated using the methyl ester extinction coefficient of 22,000 (Crombie, 1955).

Gas Chromatography. A selected ripe pear (36 days at 0°C, 12 days at 20°C) was blended with an equal weight of distilled water and immediately extracted by shaking with an equal volume of freshly distilled reagent-grade ether. This mixture was centrifuged, and the ether solvent was removed from the aqueous pear residue. The ether was separated from the higher-boiling lipid residue by fractional distillation. The volatiles of interest were separated from the yellow oily residue in a one-plate molecular still at a pressure of 0.2 mm Hg. The colorless distillate was collected on a cold finger cooled with a slurry of dry ice and acetone. A small amount of ether was then used to remove this high-boiling fraction of pear essence, which possessed a desirable pear aroma. Two-tenths μl of this product was injected onto a 200-ft × 0.01-inch Carbo-wax 20 M column using N_2 carrier gas (4 ml/min), temperature programming (60–200°C), and hydrogen flame detection (20 ml/min H_2). A similar injection was made on a 300-ft × 0.01-inch Apiezon L capillary column under the same conditions. Based on duplicate runs with internal standards, the two major high-boiling compounds in both cases were the methyl and ethyl 2,4-decadienoate esters. These two esters had been found in pear previously (Jennings *et al.*, 1964).

CO_2 and ethylene production. CO_2 evolution was measured by the method of Claypool and Keefer (1942). The effluent air passing over pears stored at 20°C in glass containers was bubbled through a sodium bicarbonate buffer containing bromthymol blue indicator. The equilibrated solutions were then read in an Evelyn colorimeter at 320 mμ, and these readings were compared with a standard curve. The results are expressed as mg CO_2 per kg of pear per hour.

Ethylene evolution was determined gas chromatographically by injecting 2-ml gas samples of effluent gas on a ⅛-inch × 5-ft column containing 60–80-mesh alumina at 62°C, and utilizing flame ionization detection. Peak heights were compared with a 6.27-ppm standard ethylene mixture and expressed as μl ethylene formed per hour, per kg of pear.

Effect of radiation. After a twelve-day ripening period, Bartlett pears irradiated with 300 Krad gamma radiation from a cobalt-60 source possessed only 1/30 the 2,4-decadienoate esters of control pears that had not been irradiated. It was evident that irradiation influenced the ripening process, for the irradiated pears were noticeably greener, firmer, and less desirable than the control pears. This is consistent with the results obtained by others (Maxie and Sommer, 1963).

RESULTS AND DISCUSSION

Table 1 shows the averaged values of the CO_2 and ethylene emanation compared with production of the esters of 2,4-decadienoic acid in the ripening Bartlett pears. The plotting of the data on a physiological basis was quite similar to the chronological plotting, since the fruit ripened quite uniformly. The rapid increase of the 2,4-decadienoate moiety occurs at the climacteric peak and increases as CO_2 and ethylene production decline. Fig. 1 shows the same data as Table 1, except the first derivative of the 2,4-decadienoate concentration is plotted with CO_2 and ethylene production. This plot shows not the total concentrations, but rates of production for each of the three compounds. The incremental increase of decadienoate was cal-

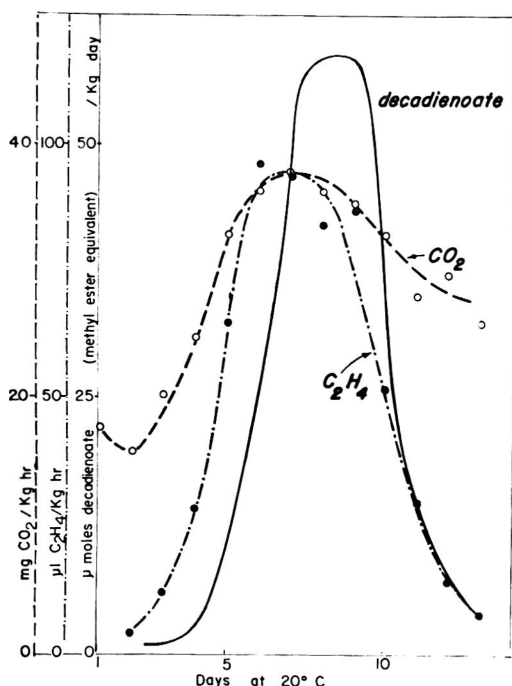

Fig. 1. Rates of production of carbon dioxide, ethylene, and decadienoates by the ripening Bartlett pear.

culated from a smooth curve drawn through the data from Table 1. It can be seen that maximum production of the high-boiling esters occurs approximately 1–2 days after the maximum production of both ethylene and CO_2; this coincided with optimum eating quality of the fruit (8–9 days' storage).

Because methyl and ethyl 2,4-decadienoate are quite important to pear flavor, and because their concentrations can be easily measured, it would seem that measurement of this moiety in pear maturity studies or pear processing operations could serve to evaluate the effect of variables on pear flavor.

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HORACE D. GRAHAM
Biology Department and Nuclear Center
University of Puerto Rico, Mayaguez, Puerto Rico

Quantitative Determination of Piperine. I. The Komarowsky Reaction

SUMMARY

A highly stable reagent consisting of para-dimethylaminobenzaldehyde (I) or para-hydroxybenzaldehyde (II) or other cylic aldehydes, plus thioureau in concentrated sulfuric acid, has been developed for use in determining piperine. The slightly yellow reagent, if stored under refrigeration in a brown bottle, maintains a constant chromogenic capacity for 4–6 weeks. Piperine when heated with this reagent for 15 min at 100°C develops a red color (I max. 490 mμ) or purple color (II max. 570 mμ) whose intensity is proportional to the amount of piperine present. Water inhibits color development, but a small amount of methyl alcohol is necessary. Ethyl alcohol cannot be used, since it develops color with the reagent. Critical factors for reproducible, quantitative assay are the concentration of acid and thiourea in the reagent and the time and temperature of heating the reaction mixture. For I, the most sensitive of the aldehydes used, good precision, with a standard deviation of ± 1.34%, was attained. Between the levels of 0.01 and 0.06μM of piperine per ml of reagent, there is a linear relation which may be described by the least-squares equation.

$$X = 0.088 \quad Y + 0.00175$$

where X **= concentration (**μM**) of piperine per ml of reagent**

Y **= absorbance at 490 mμ**

Percent piperine in sample =

$$\frac{X\ (0.28533)\ (\text{dilution factor})}{\text{Sample weight (mg)}} \times 100$$

INTRODUCTION

Black pepper, one of the most important of spices, is used for both aroma and flavor. Piperine is generally thought to be the predominant bite principle in pepper (black and white). The quality of the oleoresin of pepper is generally based on its piperine content, and some interest has been shown in its metabolism (Acheson and Alkins, 1961). Methods available for the detection and/or quantitative assay of piperine include Kjeldahl nitrogen determination (AOAC., 1950), ultraviolet spectrophotometry (Fagen *et al.*, 1955), adaptation of the chromotropic acid test for formaldehyde (Lee, 1956), infrared analysis (Pleat *et al.*, 1951), gas chromatography (Parker *et al.*, 1963) or determination as the reineckate (Kum-Tatt, 1961). The Kjeldahl method also measures other nitrogenous compounds and always gives high results (Lee, 1956; Genest *et al.*, 1963; Rao *et al.*, 1960; Tausig *et al.*, 1956). Lee (1956) pointed out that the spice trade and other segments of the food industry need simple rapid, sensitive tests for the determination of piperine, but, as far as can be ascertained, efforts to exploit some of the known color reactions of piperine have not been fruitful. Piperine in concentrated sulfuric acid forms a red color which changes to brown and finally to gray-brown (Beilstein, 1935; Winton and Winton, 1939). Such instability has negated attempts to quantitatively determine piperine by this simple method. There is little information on the factors affecting the stability of this initial red color except the notation that water abolishes the color (Beilstein, 1935). If para-dimethylaminobenzaldehyde or one of several other cyclic aldehydes in concentrated sulfuric acid is employed, the color persists but increases drastically with time. Heating the mixture for 5–15 min at 100°C stabilizes the color. The addition of thiourea to the sulfuric acid–aldehyde mixture provided a reagent which, if stored in a brown bottle and refrigerated, maintained a constant chromogenic capacity for at least four weeks. This communication reports on the factors governing reproducible quantitative assay of piperine with the above reagent. Also given are data on the reaction with concentrated sulfuric acid plus other aromatic aldehydes, known as the Komarowsky reaction (Coles and Tournay, 1942; Nogare and Mitchell, 1953).

EXPERIMENTAL

Materials. The various aldehydes tested, all CP grade, are listed in Table 1. Reagent-grade *p*-dimethylaminobenzaldehyde (m.p 75–76°C), was

used. *p*-Hydroxybenzaldehyde (m.p. 116–117°C) and thiourea (m.p. 174–176°C) were recrystallized twice from water. Piperine (Inland Alkaloid, Tipton, Indiana and K & K Laboratories, Long Island, N. Y.) was recrystallized three times from ethyl alcohol to a constant melting point of 129–130°C. Samples were also prepared from ground black pepper (Marion, 1950) and recrystallized from alcohol to a constant melting point of 129–130°C, and the purity was checked by the ultraviolet method (Fagen *et al.*, 1955). Sulfuric acid was reagent grade, 95–98%, sp. gravity 1.8407–1.8437. Methanol was CP grade and gave reagent blanks of absorbance similar to those given by carbonyl-free methanol.

The color reagent was prepared by dissolving 5 g of thiourea in 60 ml of concentrated sulfuric acid in a 100-ml volumetric flask. Two hundred mg of para-dimethylaminobenzaldehyde was then added to the flask, which was then placed 10 min in a water bath at 80°C. After cooling, the contents were made up to volume with concentrated sulfuric acid, poured into a brown, glass-stoppered bottle, and placed in a refrigerator. The other aldehyde reagents were similarly prepared except that 240 mg of the aldehyde was used for para-hydroxybenzaldehyde and no thiourea was used with vanillin. The reagents were allowed to age for at least 12 hr before use.

Equipment. The equipment used was a Beckman DU spectrophotometer, volumetric pipettes, and Pyrex glass-stoppered test tubes.

General procedure. One ml containing 0.125–1.5 micromole of piperine dissolved in methanol was placed in a series of Pyrex glass-stoppered test tubes. The tubes were cooled well in an ice bath, and 5 ml of the cold color reagent was added slowly from a burette, with the tubes immersed in the ice bath and care being taken to let the reagent run down the side of the tubes. The tubes were shaken, while immersed in the ice bath, to mix the contents well and then placed 15 min in a boiling-water bath (100°C). Then they were cooled for 5 min in an ice bath, the volume was made up to 10 ml with concentrated H_2SO_4 and allowed to stand at room temperature for 30 min, and the intensity of the color developed was measured at the wave length of maximum absorption, vs. the appropriate reagent blank. These details refer to the *p*-dimethylaminobenzaldehyde, and, except for heating time, are the same for all other aldehydes.

To establish the wave length of maximum absorption for the color(s) developed, mixture(s) of piperine and the color reagent(s) were treated according to the general procedure. The colors developed were measured over the wave length range of 380–700mμ, with distilled water as the blank. Reagent blanks were examined in a similar manner. A plot of the absorbance vs. wave length established the wave length of maximum absorption, as shown in Figs. 1 and 2 and Table 1.

Influence of variables on the reaction. Preliminary experiments indicated that several factors affected color production. Therefore, these were studied. Except for the particular variable under consideration, the conditions of the general procedure were followed. Absorbance readings were made against reagent blanks at the wave length of maximum absorption. The results are summarized in Table 2.

Vanillin was used at a level of 50 mg per 100 ml of concentrated sulfuric acid, and no thioureau was added. Liquid aldehydes were weighed out in glass containers. Conditions for the other alde-

Table 1. Characteristics of colors developed by piperine and its degradation products in the Komarowsky reaction.

Reagent	Color of rgt.	Piperine		Piperic acid		Piperonal		Piperidine	
		Color	λ_{max}	Color	λ_{max}	Color	λ_{max}	Color	λ_{max}
H_2SO_4 only	N.C.	R	490	R	490	Y-B	400	N.C.	
Reagent I	Y	R	490	R	490	Y	400	Y	400
Reagent II	Y	P	570	P	570	Y	380	Y	400
Reagent III	Y	Y-G	520	Y-G	580	Y	420	Y	420
Reagent IV	Y	P	540	P	540	Y	380	Y	380
Reagent V	Y	R-P	540	R-P	540	Y	400	Y	380
Reagent VI	Y	P	540	P	540	Y	400	Y	380
Reagent VII	Y	B-Y	540	B-Y	540	Y	380	Y	380

Reagents: I, conc. H_2SO_4 + *p*-dimethylaminobenzaldehyde + thiourea
II + *p*-hydroxybenzaldehyde + thiourea
III + vanillin
IV + salicyaldehyde + thiourea
V + benzaldehyde + thiourea
VI + *p*-chlorobenzaldehyde + thiourea
VII + cinnamaldehyde + thiourea
R, red; B, brown; Y, yellow; O, orange; P, purple; G, green; NC, no color.

Table 2. Influence of variables on color development in the determination of piperine by the Komarowsky reaction.

Variable	Range investigated	Limits for reproducibility				Value or condition selected for color measurement	
		Minimum		Maximum			
		A	B	A	B	A[a]	B[b]
Temperature of heating (°C)	60–100	95	95	100	100	100	100
Time of heating at 100°C (min)	2–60	5	20	20	40	15	30
Concentration of sulfuric acid in the reagent (*M*)	16.2–18	16.2	16.2	18	18	18	18
Concentration of aldehyde in the medium (mg/100 ml of medium)	25–500	50	50	500	500	200	240
Concentration of thiourea in the medium (% w/v)	1–10	3.5	2.0	5.0+	3.0	5.0	2.5
Presence of water in the medium (% v/v)	0.0–10	0.0	0.0	0.05	0.05	0.0	0.0
Stability of color developed (hr)	1–96	0.25	0.25	4+	4+	0.5	0.5
Color stability of reagent (wk)	1–10	4	4	6+	6+	6	4
Solvent for piperine	H_2O inhibits color formation; Ethanol develops color with reagent						Methanol selected

[a] Reagent A, concentrated sulfuric acid + *p*-dimethylaminobenzaldehyde + thiourea.
[b] Reagent B, concentrated sulfuric acid + *p*-hydroxybenzaldehyde + thiourea.

hydes were the same as for para-dimethylaminobenzaldehyde.

Quantitative determination of piperine. To test the quantitative nature of the method developed, aliquots of piperine were treated according to the general procedure, and standard curves were established as shown in Fig. 2. Piperine was then extracted from ground black pepper with ethyl alcohol according to the procedure of Lee (1956). Aliquots of the extract after evaporation of the ethyl alcohol and the addition of 1 ml of methanol were treated according to the general procedure and the piperine content determined from the standard curve. The results are shown in Table 4. Recovery tests were also done, and the results are shown in Table 3.

Comparison with other methods. To compare the proposed method with two of those now in use, aliquots of the extracts from pepper were assayed by the ultraviolet spectrophotometric method (Fagen *et al.*, 1955) and the colorimetric method of Lee (1956). The results are shown in Table 4.

RESULTS AND DISCUSSION

Several variables are rather critical for reproducible results. With all the aldehydes used, slight variations in acid concentration will lead to drastic changes in color intensity. The time and temperature of heating must also be closely controlled. Below the minimum temperature, the color produced will continue to increase on aging, whereas, above the maximum temperature shown in Table 2, a diminution of color intensity may occur. Heating much beyond the selected times (Table 2) produced little change in the absorbance of the reagent blanks except in the case of *p*-hydroxybenzaldehyde and *p*-chlorobenzaldehyde. Another critical variable is the concentration of aldehyde in the reagent. As the concentration of the particular aldehyde increased, the absorbance of the color also increased. The blank absorbance increased correspondingly, hence aldehyde concentrations were selected which would be adequate for the range of piperine investigated and, at the same time, give reasonably low blank readings at the wave length of maximum absorption.

In all cases, water in the medium impedes or reduces color formation. This has been noted by other workers in the determination of aliphatic alcohols (Nogare and Mitchell,

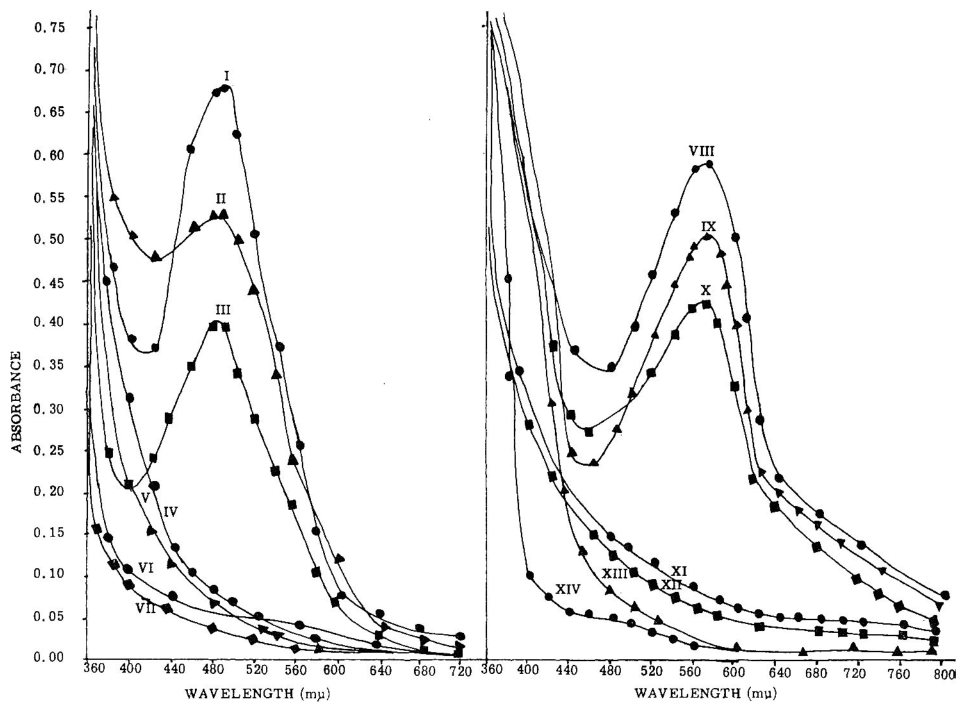

Fig. 1. Absorption spectra of the colors developed by piperine in the Komarowsky reaction.
I Piperine + PDMABA + thiourea + H_2SO_4
II Piperine + PDMABA + H_2SO_4
III Piperic acid + PDMABA + thiourea + H_2SO_4
IV Piperonal + PDMABA + thiourea + H_2SO_4
V Piperidine + PDMABA + thiourea + H_2SO_4
VI Reagent blank (PDMABA + thiourea + H_2SO_4)
VII Reagent blank (PDMABA + H_2SO_4)
VIII Piperine + PHBA + thiourea + H_2SO_4
IX Piperine + PHBA + H_2SO_4
X Piperic acid + PHBA + thiourea + H_2SO_4
XI Piperonal + PHBA + thiourea + H_2SO_4
XII Piperidine + PHBA + thiourea + H_2SO_4
XIII Reagent blank (PHBA + thiourea + H_2SO_4)
XIV Reagent blank (PHBA + H_2SO_4)
Each point on the curves represents an average of two different determinations.

1953) and in the reaction of piperine with sulfuric acid (Beilstein, 1935).

The addition of thiourea to the aldehyde-acid mixtures gave highly stable reagents for as long as 4–6 weeks provided the mixtures were stored in amber bottles under refrigeration. In this respect, the method is more advantageous than the chromotropic acid procedure, where the reagent must be made up daily. The yellow tinge resulting from the addition of thioureau to the aldehyde-acid mixture is due, apparently, to a reaction between the thioureau and the aldehyde, since thiourea in the acid gives no color. The yellow coloration, being given by similar types of compounds such as urea (Nadai, 1958), hydrazine semicarbazide (Watt and Chrisp, 1952, 1954), and isonicotinic hydrazide (Machek, 1956), has been used for their quantitative determination. This preliminary interaction does not prevent the reaction of the aldehyde with piperine in the presence of concentrated sulfuric acid. The thiourea serves as an antioxidant to prevent or retard color development in the aldehyde-H_2SO_4 mixture. Instability of *p*-dimethylaminobenzaldehyde solutions has been noted (Bowman *et al.*, 1959), and solutions of the aldehydes used

Table 3. Recovery of piperine added to pepper samples.

Piperine added (μM)	Piperine recovered [a] (μM)	Percent recovery
10	9.92	99.2
20	20.1	100.5
30	30.5	101.7

[a] Average of 5 determinations at each level.

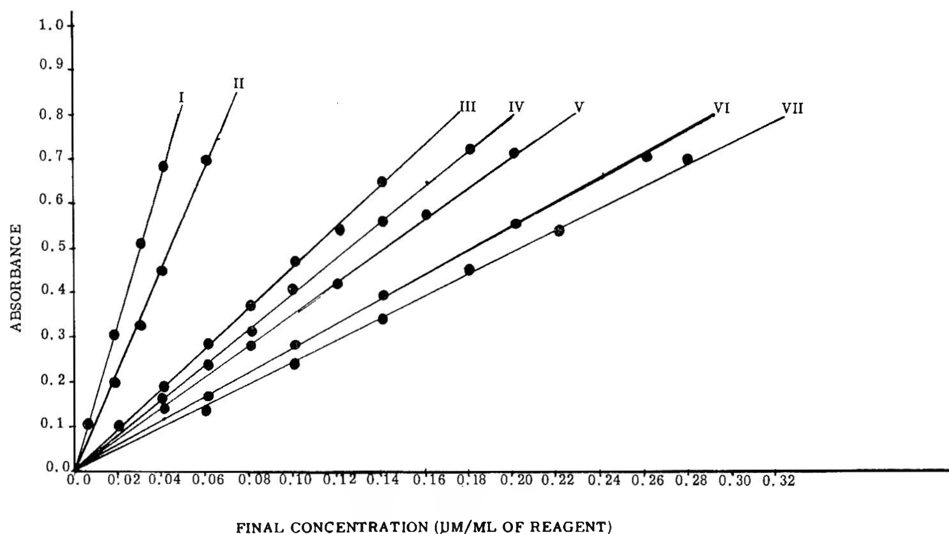

Fig. 2. Quantitative response of piperine in the Komarowsky reaction and comparison with the chromotropic acid method.
I Chromotropic acid
II Para-dimethylaminobenzaldehyde
III Para-hydroxybenzaldehyde (furfural gave similar results)
IV Para-chlorobenzaldehyde
V Benzaldehyde
VI Salicylaldehyde
VII Vanillin
Each point on the curves represents an average of five different determinations.

in the Komarowsky reaction are usually prepared fresh daily. Therefore, the recommended procedure eliminates this necessity and results in the stabilization of the piperine-H_2SO_4 color. Perhaps, thioureau also serves to retard decomposition of the chromogen formed by piperine in the presence of the aldehyde and concentrated sulfuric acid. This may be largely an antioxidant effect, but some direct participation cannot be ignored.

Of various aldehydes tried, *p*-dimethylaminobenzaldehyde, *p*-hydroxybenzaldehyde, *p*-chlorobenzaldehyde, vanillin, cinnamaldehyde, furfural, salicylaldehyde, and benzaldehyde were selected for detailed study. Para-dimethylaminobenzaldehyde seems to be the most sensitive aldehyde, but control of the reaction conditions must be very rigid. Para-hydroxybenzaldehyde also gave consistent results which were comparable to the chromotropic acid method, but it is not as sensitive as para-dimethylaminobenzaldehyde. Although the yellow H_2SO_4-vanillin color does not need stabilization, the chromogen formed with piperine is a nondistinctive yellowish-green with a rather nonincisive absorption maximum at 520–540 mμ. Para-chlorobenzaldehyde is comparable to *p*-hydroxybenzaldehyde. The other aldehydes, though useful, are liquids and cannot be as readily weighed out or purified (by recrystallization) as the solid ones.

In the Komarowsky reaction it is thought that dehydration of the compound by the strong sulfuric acid occurs to produce olefinic

Table 4. Comparison of determination of piperine content of pepper samples by different methods.

Method used	Piperine content of pepper samples[a] (% by weight of material analyzed)		
	P_1	P_2	P_3[b]
UV spectrophotometry	6.70	5.70	5.00
Chromotropic acid	7.80	7.40	6.35
Komarowsky reaction			
PDMABA[c]	7.74	7.41	6.40
PHBA	7.82	7.34	6.50
PCBA	7.60	7.50	6.30
Vanillin	7.76	7.32	6.30
Benzaldehyde	7.65	7.28	6.28
Salicylaldehyde	7.70	7.34	6.34
Furfural	7.74	7.36	6.32

[a] Average of 5 determinations.
[b] White pepper.
[c] PDMABA, para-dimethylaminobenzaldehyde; PHBA, para-hydroxybenzaldehyde; PCBA, para-chlorobenzaldehyde.

type intermediates which then condense with the aldehyde to give the characteristic colors (Nogare and Mitchell, 1953). With alcohols, a condensation mechanism involving formation of carbonium ions, followed by coupling to form colored reaction product(s), has also been proposed (Duke, 1947). The inhibition or elimination of color formation by added water indicates that dehydration is involved. Since piperine is already highly unsaturated, its ready production of a red color in the presence of concentrated sulfuric acid is expected. The rapid change of the red color to other hues may be due to oxidation and other factors. However, oxidation may not be the sole cause, since the incorporation of thioureau, a good antioxidant, into the acid did not prevent the color change. However, when one of the aldehydes was added, the red color persisted. Apparently, the compound(s) formed by the action of concentrated sulfuric acid on piperine condense with the aldehyde to form a more stable red chromogen than the red piperine-H_2SO_4 reaction product(s).

The Komarowsky reaction is exhibited by unsaturated hydrocarbons and their derivatives and substances, which on treatment with concentrated sulfuric acid are converted into unsaturated compounds, including aldehydes and ketones. In this respect, the method is not as specific as the chromotropic acid or ultraviolet spectrophotometric method. When the method is applied to the analysis of pepper, the degradation products of piperine must be considered. As seen from Figs. 1 and 2 and Table 1, piperic acid gives a positive reaction in all cases whereas piperidine and piperonal do not. Therefore, the unsaturated portion of the molecule is necessary for color production. As a result, piperettine (Genest *et al.,* 1963; Spring and Stark, 1950) and chavicine (Winton and Winton, 1939; Marion, 1950) would be expected to react. Unsaturated analogs of piperine would probably also react, but since these have not been found in pepper (Genest *et al.*, 1963) interference from this source can, at the moment be disregarded. Of the other substances found in pepper, starch (sugars), proteins, and pentosans must be considered as possible interferences. All these compounds, in the presence of concentrated sulfuric acid, will yield the typical colors. If pepper oil constituents such as α-pinene, β-pinene, myrcene, limonene, and others (Guenther, 1952; Hasselstrom *et al.,* 1957, Jennings and Wrolstad, 1961; Ikeda *et al.,* 1962; and Sharma *et al.,* 1963), are present, then these may be expected to interfere also. Thiourea in the medium inhibits but does not eliminate color production by the proteins, as demonstrated in tests with vitamin-free casein and other proteins. Since ethanol is used to extract the piperine, the amount of these substances in the extract is minimized.

The heat of reaction occurring on the addition of the acid reagent to the methanolic piperine mixture must be closely controlled or results may be erratic. Precooling of the piperine sample and use of cold reagent and an ice bath during manipulations will aid in obviating these difficulties.

As seen from Table 4, the values obtained for the samples of pepper analyzed are higher than those obtained by the ultraviolet spectrophotometric method. Genest *et al.* (1963), in a critical study of piperine analysis, pointed out this trend in the (chromotropic acid) colorimetric method. As with the chromotropic acid method, piperettine, which is likely to be always present in samples of black and white pepper (Genest *et al.,* 1963; Spring and Stark, 1950; Newman, 1953), would be measured by the present method as apparent piperine. Therefore, the method measures the total "bite principle" of pepper, whereas the ultraviolet method, as usually applied measures piperine only.

With adequate control of variables the proposed method can serve for the determination of piperine in samples of pepper. The main advantage is the availability of a highly stable reagent, thus avoiding the necessity of daily preparation, as in the chromotropic acid method. Moreover, it can serve as a routine method in laboratories which lack equipment for ultraviolet spectrophotometry. Any one of several aldehydes can be used with no great difference in analytical results. The procedure is highly reproducible, with a standard deviation for paradimethylamino-

benzaldehyde of 1.38% when applied to commercial piperine, and 1.34% of the total piperine present as 100% when applied to the assay of piperine in black pepper. Recoveries of added piperine were high in all cases (99.2–101.7%).

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HORACE D. GRAHAM
Department of Biology and Nuclear Center, University of Puerto Rico
Mayaguez, Puerto Rico

Quantitative Determination of Piperine. II. Direct Determination with Phosphoric Acid

SUMMARY

Piperine in 85% phosphoric acid forms an unstable yellow color which, when heated for 8 min at 100°C, becomes a stable bluish-green with absorption maximum at 635 mμ. This reaction was exploited for the direct determination of the piperine content of pepper samples. Pepper, ground to pass a standard U. S. 80-mesh sieve, was treated 30 min at room temperature (29 ± 1°C) with 85% phosphoric acid. The resulting yellow color was heated 8 min at 100°C, and cooled, and the absorbance was measured against a reagent blank at 635 mμ. Results were comparable to those obtained with the chromotropic acid and H_2SO_4–para-dimethylaminobenzaldehyde methods. Beers' law was obeyed between the levels of 0.04–0.28 μM of piperine per ml of 85% H_3PO_4. The method requires only simple, readily available chemicals and equipment, and is rapid and reproducible with a standard deviation of ± 1.01% of the total amount of piperine as 100%.

INTRODUCTION

Piperine is considered to be the major bite principle of pepper (*Piper nigrum* L.) (Tausig *et al.*, 1956; Rao *et al.*, 1960; Newman, 1953), and its concentration in a particular batch of pepper is of interest to the spice trade in particular and to the food industry in general. For this reason, a simple, rapid, sensitive method of assay is highly desirable. Methods proposed or now used for its estimation include Kjeldahl nitrogen determination (AOAC, 1955), ultraviolet spectrophotometry (Fagen *et al.*, 1955), adaptation of the chromotropic acid test for formaldehyde (Lee, 1956), infrared analysis (Pleat *et al.*, 1951), gas chromatography (Parker *et al.*, 1963), or determination as the reineckate (Kum-Tatt, 1961).

It has long been known that piperine reacts with certain acids to produce colors (Beilstein, 1935), but these reactions have not been exploited for quantitative assay because of the instability of the colors or other reasons (Lee, 1956). While reviewing these color reactions, it was observed that, under appropriate conditions, piperine when heated with 85% phosphoric acid forms a highly stable bluish-green color with absorption maximum at 635 mμ. The intensity of this color is proportional to the concentration of piperine present. Pepper can be assayed directly for its piperine content by treating a weighed amount in 85% H_3PO_4, filtering the yellow color, heating 8 min at 100°C, and measuring absorbance at 635 mμ. This article summarizes the details for quantitative assay and the various factors which govern color formation.

EXPERIMENTAL

Reagents. The reagents used were piperine, m.p. 129–130°C; piperic acid; piperonal (K and K Laboratories, New York); and phosphoric acid, 85% C. P. Piperine was recrystallized three times from absolute ethanol, and its purity was checked by the ultraviolet spectrophotometric method (Fagen *et al.*, 1955).

Equipment. The equipment involved is a Beckman model DU spectrophotometer or any other suitable spectrophotometer, 25-ml glass-stoppered volumetric flasks, 200 × 16-mm Kimax test tubes permanently etched at the 25-ml mark, and glass wool washed thoroughly with distilled water and then with 85% H_3PO_4. Filtration columns were made of glass tubings 150 mm long × 5–20 mm in diameter. (Broken burettes or 10-ml pipettes will suffice.) The columns were packed to a height of 5 cm so as to allow the passage of 85% H_3PO_4 at the rate of 1 ml per min. Also used were medium fritted-glass filter funnels.

General procedure. Twenty-five mg of pepper ground to pass a U.S. standard 80-mesh sieve was placed in duplicate dry 25-ml volumetric flasks, and 85% H_3PO_4 was added to the mark. The flasks were allowed to stand 30 min at room temperature (29±1°C), during which time they were shaken every 5 min. The contents were then filtered through a medium fritted-glass funnel or over glass wool. Five to 10 ml of the filtrate was collected, heated 8 min at 100°C, cooled, and allowed to stand 30 min at room temperature, and absorbance was then read at 635 mμ against a reagent blank treated similarly. The piperine content was read from a standard curve for piperine

or calculated from an equation describing the curve.

Wave length of maximum absorption. Convenient concentrations of piperine and its degradation products were treated according to the general procedure except that filtration was not done. Since alcohol interferes, this solvent for the piperine was first removed by heating the flasks for about 5 min over a steam bath. The colors developed were measured against distilled water over the wave length range of 380–800 mμ. A plot of the absorbance as a function of the wave length established the wave length of maximum absorption. The spectrum of a reagent blank was also scanned. The results are shown in Fig. 1.

Construction of standard curve for piperine. For this, 0.0–7.0 μM of piperine was placed in consecutive 25-ml volumetric flasks, and 85% H_3PO_4 was added to the mark. The color was developed and measured as described in the general procedure. The results are shown in Fig. 2.

Influence of variables on the reaction. Several variables influencing color development were investigated, using pure piperine and black pepper samples. Constant quantities of piperine (5 μM) and black pepper (25 mg) were treated, separately, according to the general procedure except that the conditions were varied, one at a time, between the limits shown in Table 1. Absorbances were measured at 635 mμ against reagent blanks treated similarly.

Application of proposed method and comparison with other methods. After all the influencing factors were delineated, 25 mg of 3 different batches of pepper was treated and assayed according to the general procedure. The piperine content of the pepper samples was also determined by the ultraviolet spectrophotometric method (Fagen *et al.*, 1955), chromotropic acid method (Lee, 1956), and the Komarowsky reaction (Graham, 1965). The results are shown in Table 2.

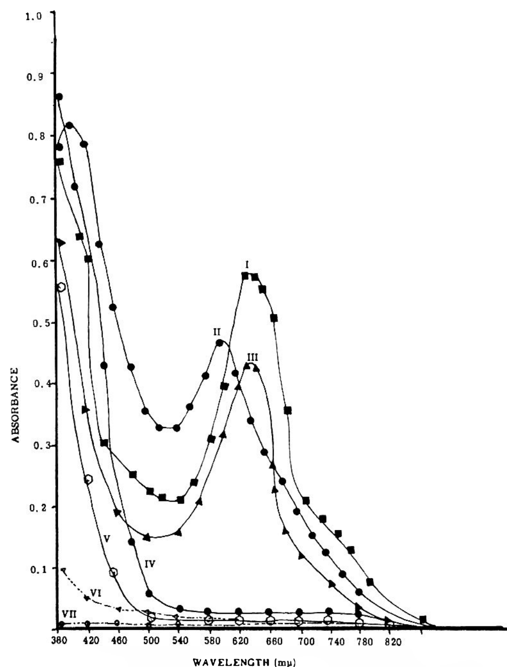

Fig. 1. Absorption spectra of colors produced by piperine and its degradation products after heating 8 min at 100°C in 85% phosphoric acid.

I Piperine
II Piperic acid
III Chromogen from pepper obtained according to the general procedure.
IV Piperonal
V Piperine-H_3PO_4 yellow color (before heating)
VI Piperidine
VII Reagent blank (85% H_3PO_4)

Interferences. To assess possible interferences, several compounds of various classes were treated according to the general procedure, and any color

Table 1. Influence of variables on color development in the reaction of piperine with 85% H_3PO_4.

Variable investigated	Range investigated	Conditions for reproducibility Min.	Max.	Value selected
Concentration of acid (*M*)	7.60–18.1	17.74	18.10	18.1
Temperature of heating (°C)	50–100	95	100	100
Time of heating at 100°C (min)	2–30	6.0	8.0	8.0
Reaction time for H_3PO_4- piperine at 29±1°C (hr)	0–24	0.25	0.66	0.5 hr
Stability of blue color (hr)	0.1–96	0.3	24	0.5 after color development
Diluent for blue color				85% H_3PO_4 selected
H_3PO_4-pepper reaction time (hr)	0–4	0.30	0.75	0.5
Mesh size of ground pepper	10–200	60	140	80

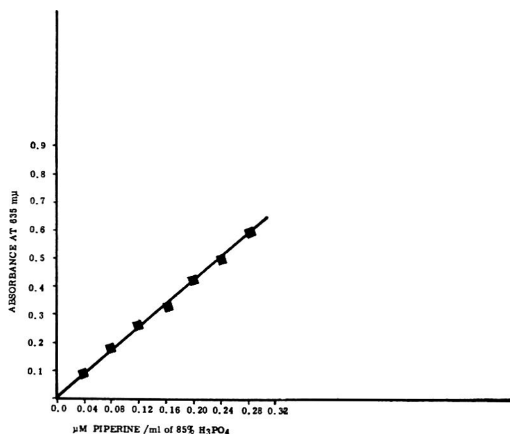

Fig. 2. Quantitative response of piperine when heated 8 min at 100°C in 85% phosphoric acid. Each point represents the average of five different determinations.

developed was noted. Table 3 summarizes the results.

Precision of the method. To establish the accuracy and precision of the method, 10 determinations were made at each of three levels of piperine and of black pepper, and the standard deviation of the values obtained was calculated.

RESULTS AND DISCUSSION

Although the color reactions of piperine with acids have been known for a long time (Beilstein, 1935; Winton and Winton, 1939; Lee 1956), its reaction with phosphoric acid, as far as can be ascertained, has never been strongly emphasized. Jones *et al* (1952) determined piperonyl butoxide (3,4-methylenedioxy-6-propylbenzyl) (butyl diethylene glycol) ether with tannic acid in a mixture of acetic acid and phosphoric acid. They claimed a high degree of specificity of the reagent for the compound. Since the purification of tannic acid is rather tedious (Jones *et al.*, 1952), gallic acid was used instead. However, piperine, piperic acid, and piperonal all gave a purple color. Moreover, color development was independent of the presence of acetic acid in the medium. The proposed method, in contrast, exhibits a high specificity for piperine. Piperic acid responds, but the absorption maximum of the color developed occurs at 600 mμ instead of 635 mμ. No other acid reacted in this way, and the color is obtained if phosphorus pentoxide is mixed with piperine, and triple-distilled water is added.

In further attempts to check the specificity of the method and to elucidate the mechanism of reaction, other compounds were tested. Of over 100 compounds of several classes tested, only quinine and quinidine, at relatively high concentrations, gave faint blue colors. However these alkaloids do not occur in pepper and have never been reported as adulterants. Therefore, any possible interference from them can be readily dismissed. Hydrastine and berberine, two methylenedioxyphenyl compounds, cinnamic acid, sorbic acid, and oleic acid all failed to give the characteristic bluish-green color (Table 3). On the basis of these observations and since piperonal does not respond at all whereas piperic acid gives a closely related color, it seems as if the minimum requirement for color formation is the piperic acid "core structure." Although piperidine itself does not give a color, its attachment to the piperic acid moiety seems to enhance color formation and to shift the wave length of maximum absorption from 600 to 635 mμ. Further work to characterize the compound(s) formed is in progress.

Several variables are rather critical for

Table 2. Comparison of determination of piperine content of pepper samples by different methods.

Method used	Piperine content of pepper samples (% by weight of material analyzed)		
	P_1	P_2	P_3[a]
Ultraviolet spectrophotometry	6.70	5.70	5.00
Chromotropic acid	7.80	7.40	6.35
H_2SO_4-paradimethylamino-benzaldehyde	7.74	7.41	6.40
H_3PO_4			
a) After extraction of piperine with ethanol	7.76	7.35	6.25
b) Direct determination (80 mesh)	7.70	7.44	6.38

[a] White pepper.

Table 3. Response of compounds of various classes when heated 8 min at 100°C in 85% H_3PO_4.

Compound or class of compound tested	No. tested	Response
Piperine	1	+
Piperic acid	1	±[a]
Piperonal	1	—
Piperidine	1	—
Cinnamic acid	1	—
Sorbic acid	1	—
Sugars and carbohydrates	30	—
Sugar alcohols	8	—
Proteins, amino acids, and polypeptides	35	—
Alkaloids	32	—[b]
Fats and fatty acids	10	—
Steroids	16	—

[a] Max = 600 mμ.
[b] High concentrations of quinine and quinidine give slight blue color.

the quantitative determination of piperine by the proposed method. Acid concentration is extremely important, and, below 17.74*M*, color intensity decreases rapidly. Thus, the amount of water in the medium is a limiting factor, and 85% H_3PO_4 was used throughout the studies.

Several solvents for piperine, including methanol, ethanol, and chloroform, were tried, but all inhibited color development. Therefore, prior to addition of the acid, all solvent must be removed by evaporation over a steam bath or by heating in a water bath.

Although color formation will occur at 70°C, maximum intensity results only by heating at or above 95°C. Heating time is also critical. Diminution of color intensity is rapid if heating continues for more than 10 min, while development is maximum after 5 min. A heating time of 8 min at 100°C was chosen.

The bluish-green color is very stable, and measurements made as long as 12 hr after development were similar to those obtained after 30 min. The color is destroyed by alkalis, water, alcohol, and other solvents, and its formation is inhibited by inorganic ions such as Na^+, Ca^{++}, Mg^{++}, etc.

The piperine-H_3PO_4 reaction time greatly influences the final intensity of the bluish-green color. Color formation is maximum after a reaction time of 15–40 min. After 40 min the yellow color begins to fade, resulting in a corresponding decrease in intensity of the bluish-green color.

When applied to the direct determination of piperine in pepper, the pepper-H_3PO_4 reaction time has essentially the same importance as when commercial piperine was employed (Table 1). However, the mesh size (fineness of grind) is extremely important (Table 1 and Fig. 3). With mesh sizes of 10–40, results were much lower than those obtained with the chromotropic acid method (Lee, 1956) or the H_2SO_4–para-dimethylaminobenzaldehyde method (Graham, 1964).

Mesh sizes of 60–100 consistently gave good reproducible results. Therefore, a mesh size of 80 was chosen. Above 100 mesh there was always a tendency of the pepper to "cake" upon addition of the acid. Although the "cake" can be broken up with a "policeman" if test tubes are used, when volumetric flasks are employed this becomes less easy and results will be erratic.

Filtration through a medium fritted-glass funnel or over a column of H_3PO_4-washed glass wool must be done before heating of the yellow pigment; otherwise, the solution will darken, with consequent erroneous results.

Table 2 shows that the method, when applied directly or indirectly, gives results which are much higher than those with the ultraviolet method, but close to those given by the other colorimetric methods. Genest *et al.* (1963) gave a lucid account of reasons for differences between the ultraviolet spectrophotometric and the chromotropic acid methods. These reasons, to a great extent, hold true for the H_3PO_4 method. Piperettine and chavicine (Genest *et al.*, 1963; Spring and Stark, 1950; Marion, 1950) would most likely also react with H_3PO_4 to give colors similar to that obtained with piperine. Piperic acid, although producing a color with a lower wave length of maximum absorption, would also contribute to any final readings. It is unlikely that other analogs of piperine (Genest *et al.*, 1963) would react, and, since these do not occur in pepper, their interference is a remote possibility. Sugars

and proteins, which make direct determination by other colorimetric procedures impossible, do not respond here.

The proposed method involves no unstable or expensive reagents, and can be executed easily and rapidly. The high stability of the color produced is a strong attribute, and the method can be scaled down or up without any serious loss in accuracy or precision. As proposed, precision (10 different determinations) was good, with a standard deviation of ± 0.92% when applied to piperine, and ± 1.01% of the total amount of piperine as 100% when applied to pepper samples. Between the levels of 0.04–0.28 μM of piperine per ml of 85% phosphoric acid, a linear relationship was observed between the concentration of piperine and the absorbance of the color developed. The relationship can be described by the least squares equation:

$$X = 0.4950\ Y - 0.0047$$

$X = \mu M$ of piperine per ml of 85% H_3PO_4

Y = absorbance of color developed at 635 mμ

Percent piperine in sample =

$$\frac{X(0.28533)\ (\text{dilution factor})}{\text{Sample weight (mg)}} \times 100$$

The color developed has a molar extinction coefficient of 1.783×10^3.

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HERMAN BECKMAN
Agricultural Toxicology and Residue Research Laboratory
University of California, Davis, California 95616
and WAYNE THORNBURG
Emeryville Research Laboratories, California Packing Corporation
Emeryville, California

Effect of Frozen Storage on Parathion Residues

SUMMARY

A field experiment for the control of aphids on spinach with parathion was conducted to develop data for a complete decline curve following treatment. Data show that a tolerance of 1 ppm cannot be achieved within the seven-day withdrawal period. Data were then developed over a six-month period of frozen storage for residue levels. The program allowed a comparative study of methods of analysis, a study of extraction methods, and a study of the residue level of parathion on spinach over an extended period of frozen storage. The effect of storage on the parathion residues is discussed.

INTRODUCTION

Studies to show the effect of extraction and clean-up procedures on the recovery of residues from crops were reported by Van Middelem *et al.* (1963). They compared tumbling with blending, and an alcohol-benzene mixture with benzene alone, for the recovery of parathion from leafy vegetables. The blending operation was shown to be essential to high recovery, without regard to the solvent system used. All samples were held in a frozen condition prior to analysis.

Thornburg (1963) determined that fresh leafy materials, such as spinach, need not be macerated in a blender for a high recovery of parathion. If great care is taken to avoid leaf damage, a stripping action with benzene will achieve residue recovery equivalent to a complete blending operation. If, however, the samples are not strictly fresh, or have been frozen for any period, a blending procedure is essential.

Hardin and Sarten (1962) investigated several methods for the extraction of DDT from field-treated collards. A complete blending operation of the frozen samples was found necessary for highest residue levels. No attempt was made to compare the extraction processes with freshly collected field samples.

A study by Laws (1959) made use of both total phosphorus and anticholinesterase measurements to determine the stability of phosdrin during frozen storage. A study of his data reveals an erratic but distinct loss of insecticide at six weeks of frozen storage. At six months of frozen storage, the data reveal an even more erratic picture.

OBJECTIVES

This collaborative study regarding the analysis of spinach for parathion residues was undertaken to evaluate: 1) the effect of the type of extraction on the recovery of parathion residues from fresh and frozen field-treated spinach; 2) the effect of frozen storage on the stability of parathion on spinach; 3) three analytical procedures for parathion; and 4) comparison of the results of parathion analysis at two laboratories.

An extensive field experiment for the control of aphids on spinach with parathion was conducted to develop data for a complete decline curve following treatment. The treatment schedules included 3 rates of application. Six replicate samples from each treatment were taken for the California Packing Corporation at each of the time periods following application. Triplicate samples of the same treatments were taken for the Agricultural Toxicology and Residue Research Laboratory.

Application of parathion. Parathion (4 lb per gal. EC) was applied at 0.5, 0.75, and 1.0 lb actual per acre to spinach at Yolo, California. Application was made with a hand sprayer (3-gal. capacity) to 0.01-acre plots on March 5, 1963. Each plot was replicated 3 times.

Sampling and extraction procedures. At the appropriate sampling times, samples from each plot were taken for the California Packing Corporation Laboratory to a cold-storage plant, and frozen. Three similar 1-lb samples were taken to the University of California Laboratory, and a portion was extracted immediately. The remainder of each sample was frozen for subsequent analysis.

Two types of extraction were used for these samples: 1) A portion of the fresh leaves was weighed into an extraction can, a volume of benzene three times the weight of the samples was added, and the can was tumbled 30 min. The

benzene extract was decanted through a filter and held at room temperature. 2) A portion of the frozen sample was chopped and weighed into a blender, and a volume of benzene equal to 3 times the weight of the sample was added. The sample was blended 3 min, transferred to an extraction can, and tumbled 30 min. The can was allowed to remain upright for a few minutes, and the extract was decanted through some sodium sulfate in the filter and held for analysis.

Frozen storage of samples. Samples were held at −20°C at the University in a standard chest-type freezer. Samples for the California Packing Corporation Laboratory were frozen as previously noted, and after ten days were transferred without thawing to a chest-type freezer at −20°C. Samples were removed from the freezer, and subsample was taken for analysis while still frozen. At no time were the samples thawed and refrozen.

Purification and clean-up of extracts prior to analysis. The benzene extracts of fresh samples were not purified prior to analysis. Benzene extracts of frozen spinach contained a large amount of pigment and were purified by the following procedure. A suitable aliquot of extract was evaporated to 5 ml in a rotary vacuum evaporator. The sample was transferred to a basic alumina column containing 5% Darco G-60. The parathion was eluted with 200 ml of 5% isopropyl alcohol in benzene, and the eluate was evaporated to near dryness in a rotary vacuum evaporator. The residue was dissolved in isopropyl alcohol, and the color was developed as described by Averell and Norris (1948).

The benzene extracts for electron-capture gas chromatography were cleaned up with acid-washed Nuchar-Attaclay.

Benzene extracts for Dohrmann gas chromatography were concentrated and transferred to unactivated Florisil using a small amount of benzene. The parathion residue was eluted using 5% ethyl ether in benzene. The solvent was removed and the waxes precipitated from acetonitrile at 0°C. The precipitated waxes were removed by filtration into a sedimentation tube. The filtrate was concentrated with a stream of air. The final volume was noted, and a suitable aliquot was injected into the Dohrmann instrument.

Methods of parathion analysis. The California Packing Corporation Laboratory analyzed samples by the Averell-Norris colorimetric procedure and by electron-capture gas chromatography. The University Laboratory analyzed samples by the Averell-Norris colorimetric procedure and by the Dohrmann gas chromatographic procedure.

Results indicated by *a* in Tables 1 and 2 were

Table 1. Analysis of spinach treated with parathion.

Rate and time appl. to sampling (days)	Sample no.	Parathion residue (ppm)				
		Extracted fresh U.C.D.	Held frozen 10 days C.P.C.	Six months' frozen storage		
				C.P.C.		U.C.D.
				a	b	c
0.5 lb per acre						
0	1	16.1	20.0			
	2		11.6			
	3	18.5	25.4			
	4		17.6			
	5	19.4	33.4			
	6		18.4			
1	1	12.7	16.0	8.6	5.5	
	2		16.0	9.5	4.2	
	3	12.4	19.0	7.5	2.6	
	4		19.0	8.3	5.2	
	5	11.4	14.0	8.5	6.8	
	6		20.6	8.0	4.0	
3	1	6.4	6.8	3.8	2.4	3.4
	2		8.0	2.6	2.1	
	3	7.8	11.6	3.5	1.8	3.9
	4		7.2	3.0	1.5	
	5	7.8	7.2	3.7	2.8	
	6		5.6	5.0	3.0	
5	1	2.3	2.6			
	2		1.6			

(continued)

Table 1. Continued

Rate and time appl. to sampling (days)	Sample no.	Parathion residue (ppm) Extracted fresh U.C.D.	Held frozen 10 days C.P.C.	Six months' frozen storage C.P.C. a	b	U.C.D. c
	3	3.1	3.6			
	4		2.6			
	5	3.1	1.6			
	6		2.0			
7	1	7.2				
	2					0.52
	3	2.5				
	4					
	5	2.5				
	6					
10	1	0.9	0.4	0.4	0.3	
	2		0.3	0.4	0.4	0.8
	3	1.2	0.6	0.8	0.7	
	4		0.5	0.5	0.5	
	5	1.1	0.5	0.6	0.5	
	6		0.8	0.7	0.7	
14	1	0.7	0.3			
	2		0.3			
	3	0.4	0.3			0.3
	4		0.6			
	5	0.9	1.0			
	6		0.5			
21	1	0.3	0.5			0.1
	2		0.6			
	3	0.4	0.6			0.1
	4		0.5			
	5	0.4	0.7			
	6		0.3			
28	1	0.03	0.3			
	2		0.2			
	3	0.03	0.3			
	4		0.3			
	5	0.03	0.3			
	6		0.3			
0.75 lb per acre						
0	1	28.8	23.2			8.3
	2		25.8			
	3	21.1	27.4			8.8
	4		30.0			
	5	34.4	30.6			8.6
	6		25.4			
1	1	23.4	22.6			
	2		25.2			
	3	19.6	23.0			
	4		27.8			
	5	24.5	20.6			
	6		24.6			
3	1	10.6	16.4			
	2		13.8			

(continued)

Table 1. Continued

Rate and time appl. to sampling (days)	Sample no.	Parathion residue (ppm) Extracted fresh U.C.D.	Held frozen 10 days C.P.C.	Six months' frozen storage C.P.C. a	C.P.C. b	U.C.D. c
	3	12.5	11.8			
	4		13.8			
	5	11.0	8.4			
	6		10.8			
5	1	13.4	5.8			1.9
	2		4.2			
	3	5.2	4.8			
	4		5.8			
	5	6.8	5.4			
	6		4.2			
7	1	3.7				
	2					
	3	4.6				
	4					
	5	3.4				
	6					
10	1	3.4	1.6	0.9	1.0	1.3
	2		1.1	1.5	1.4	
	3	3.1	1.4	1.0	1.2	
	4		1.0	1.0	1.1	
	5	2.6	1.1	1.1	0.9	1.5
	6		1.2	1.0	1.2	
14	1	1.6	1.1			0.9
	2		0.9			
	3	1.0	0.6			
	4		0.6			
	5	1.1	0.8			
	6		1.2			
21	1	0.5	0.2			0.2
	2		0.5			
	3	0.4	0.5			
	4		0.4			
	5	0.4	0.4			
	6		0.3			
28	1	0.04	0.3			
	2		0.3			
	3	0.03	0.3			
	4		0.3			
	5	0.06	0.3			
1.0 lb per acre						
0	1	37.4	30.4			
	2		25.0			
	3	40.0	30.4			
	4		37.4			
	5	38.8	36.6			
1	1	19.6	29.8	17.3	5.0	
	2		25.8	14.6	11.5	
	3	22.4	32.6	13.8	10.7	

(continued)

Table 1. Concluded

Rate and time appl. to sampling (days)	Sample no.	Parathion residue (ppm)				
				Six months' frozen storage		
				C.P.C.		U.C.D.
		Extracted fresh U.C.D.	Held frozen 10 days C.P.C.	[a]	[b]	[c]
	4		29.8	24.8	16.6	
	5	25.1	28.0	22.0	13.6	
	6		31.1	19.0	8.4	
3	1	11.4	14.0			
	2		12.2			
	3	11.3	12.6			6.1
	4		10.4			
	5	13.8	10.6			9.6
	6		11.6			
5	1	13.5	6.8			10.5
	2		6.8			
	3	7.0	7.0			
	4		8.8			
	5	7.5	10.2			
	6		7.6			
7	1					
	2	7.5				
	3					
	4	5.7				
	5					
	6	5.4				
10	1	2.5	0.8	0.6	0.8	1.4
	2		1.0	0.8	1.2	
	3	2.6	1.2	0.8	0.7	
	4		0.9	1.0	0.8	
	5	3.2	2.1	0.9	0.9	1.4
	6		1.2	1.3	1.0	
14	1	0.9	0.5			
	2		1.2			
	3	1.1	0.9			
	4		1.0			
	5	1.0	1.4			
	6		0.7			
21	1	0.3	0.6			
	2		0.3			
	3	0.3	0.2			
	4		0.3			
	5	0.5	0.2			
	6		0.3			
28	1	0.03	0.3			
	2		0.3			
	3	0.06	0.3			
	4		0.3			
	5	0.08	0.2			
	6		0.3			

[a] Averell-Norris colorimetric procedure.
[b] Electron capture gas chromatography.
[c] Dohrmann gas chromatography.

Table 2. Summary of average parathion found at storage times indicated.

Time appl. to sampling (days)	Parathion lb/acre	Fresh[a]	10 days[a]	2 mo.[a]	4 mo[a]	6 mo[b]
0	0.5	18.0	20.9			
	0.75	28.2	27.0			
	1.0	38.7	30.7			
1	0.5	12.1	17.4			
	0.75	22.5	23.9			
	1.0	22.3	29.5			
3	0.5	7.3	7.7			3.6
	0.75	11.3	12.5			8.6
	1.0	12.1	11.9			7.8
5	0.5	2.8	2.3	1.2	2.3	1.9
	0.75	8.5	5.0	2.9	2.2	
	1.0	9.3	7.9	5.1		
7	0.5	2.4		1.0	1.3	0.5
	0.75	4.6		2.5	3.0	
	1.0	6.2		4.6	2.6	
10	0.5	1.1	0.5		0.8	0.8
	0.75	3.0	1.2		1.4	1.4
	1.0	2.8	1.2			1.4
14	0.5	0.6	0.5	0.2	0.5	0.3
	0.75	1.2	0.9	0.9	0.5	0.9
	1.0	1.0	1.0	0.8	0.8	
21	0.5	0.4	0.5	0.1		0.1
	0.75	0.4	0.4			0.2
	1.0	0.4	0.3	0.1		
28	0.5	0.03	0.3			
	0.75	0.04	0.3			
	1.0	0.05	0.3			

[a] Averell-Norris colorimetric procedure.
[b] Analyzed by Dohrmann gas chromatography.

obtained by the Averell-Norris colorimetric procedure. Results indicated by *b* were obtained by an electron-capture detector using an Aerograph Hy-Fi equipped with a 4-ft × ⅛-in. Pyrex column packed with 2% Dow 200 silicone oil on 60–80-mesh Anakrom ABS. Column temperature was 195°C, and retention time was 2½ min. Results indicated by *c* were obtained by Dohrmann gas chromatograph equipped with a sulfur-sensitive coulometric cell. A 6-ft × ¼-in. quartz column packed with 20% purified Dow 11 silicone oil on 40–60 Chromosorb P was used. Column temperature was 200°C, block temperature was 260°C, and retention time was 4 min.

RESULTS AND DISCUSSIONS

The Averell-Norris procedure detects both parathion and its possible breakdown products para-amino-parathion and paraoxon while either gas chromatographic procedure detects only parathion. This probably accounts for the somewhat uneven results given by gas chromatography. Table 2 lists averages of residues found on fresh and frozen samples at 10 days and 2, 4, and 6 months.

Table 1 shows the level of the residue after 6 months of frozen storage. The values indicate a general decline and, in some instances, a significant degradation on the basis of gas chromatographic analysis. The 2 gas chromatographic procedures give generally lower values since the response is due to the parathion *per se*. The decline appears somewhat erratic, which is not unlike previously observed data. If such data were obtained and not interpreted in the light of the fresh-sample data shown for comparison, it would

appear that the samples were well within tolerance in the acceptable time limitation. An additional observation is that the greatest apparent decline in residue levels is associated with the samples of highest initial residue, that is, that the greatest decline was associated with samples taken 5–7 days from treatment. Further evidence of this is shown in Table 2. The greatest amount of decline took place within the first two months of storage, with smaller and less spectacular changes during succeeding periods. This table shows averages. The same samples were not necessarily analyzed at each time period, which accounts for the occasional apparent increase in level after two months. This was necessary in order to have material left for analysis at the later intervals.

We feel that the data found in this collaborative study indicate the following: 1) spinach samples held frozen for parathion analysis should be analyzed within two weeks of sampling; 2) reasonably good agreement was found between the two laboratories and between different procedures; 3) at the lowest rate (0.5 lb per acre) the allowed level of 1 ppm was not achieved until 14 days after application.

CONCLUSIONS

Future work associated with this problem will include residue stability during storage of extracts under various conditions. It is suspected that if this information was available for other pesticide crop combinations similar responses would be observed.

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K. YASUMATSU,[a] T. O. M. NAKAYAMA, and C. O. CHICHESTER
Department of Food Science and Technology
University of California, Davis, California 95616

Flavonoids of Sorghum

SUMMARY

Three anthocyanogen-type flavonoids—chromogens I, II, III—were found by chromatography of a methanol extract of sorghum. Hydrolysis by boiling with acid yielded the same two flavonoids for each of the three chromogens. One was a flavanone, probably eriodictyol; the other was an anthocyanidin—pelargonidin.

INTRODUCTION

Although sorghum is an important grain crop in the world for human food, in the United States it is primarily a feed for animals (Kramer, 1959).

An objectionable feature of sorghum as human food appears to be connected with the seed coats of most sorghums, which show a reddish or brownish color (Elder, 1963). Furthermore, it is reported that there are pigment precursors which become visible upon the addition of acid or alkali (Blessin *et al.*, 1963). These pigments leach out during the steeping operation for wet milling and give a slightly brown or pinkish off-color to the product.

The chemical nature of these materials is ill-defined, and only a few references on it are available. Nakayama (1963), from investigation of the use of these cereals for adjunct materials in brewing, reported R_f values, absorption spectra, locations of the pigments in the grains, and color differences among sorghum varieties. Additionally, Blessin *et al.* (1963) purified the main pigment by a procedure based on adsorption on a strongly basic ion-exchange resin. They tentatively identified fisetinidin as one of the reaction products that resulted from treatment of the anthocyanogens with 12*N* hydrochloric acid.

This is a report of further investigation of the chemical structures of the pigments in sorghums.

[a] Present address: Takeda Chemical Industries, Ltd., Osaka, Japan.

METHODS AND MATERIALS

Material. Seed coats of commercial sorghum were used because they are rich in pigments. They were furnished by Grain Products, Inc., of Dodge City, Kansas.

Extraction and concentration of chromogens. Four hundred grams of seed coat were suspended in 3.5 L of methanol and left overnight at room temperature. The mixture was filtered and the residue was re-extracted with 2 L of methanol. The combined methanol extract was concentrated to dryness under vacuum on a rotary evaporator. The reddish-brown solids were dissolved in 60 ml of *n*-butanol, and the resulting clear reddish-brown butanol solution was washed with an equal amount of water.

Column chromatography. Commercial microcrystalline cellulose powder was used in separation and purification. Thirty ml of butanol solution of the pigments was placed on a column of dry cellulose powder (85 g, 4 × 20 cm), followed by petroleum ether saturated with water. A clear yellow effluent, fraction *A*, was obtained which did not turn pink upon the addition of concentrated hydrochloric acid. Two additional bands were eluted with 99% aqueous acetone. The first band was designated fraction *B*, and the latter fraction *C*. A further fraction *D*, was eluted with 90% acetone, and a fraction *E* with 70% methanol. Fractions *B–E* turned clear pink upon the addition of hydrochloric acid at room temperature. Each fraction still contained a small amount of a fluorescent substance, so they were concentrated under reduced pressure and repurified by preparative paper chromatography with water used as solvent. Finally, a light yellow paste was obtained. This, when dissolved in *n*-butanol and rechromatographed, moved as one spot on chromatograms developed with three solvents (butanol–acetic acid–water, 4:1:5; 2% acetic acid; and water saturated with 2-butanol).

Acid hydrolysis. Each fraction (*B-E*) was boiled individually with 3*N* hydrochloric acid for 30 min and extracted with butanol. The extract was chromatographed with a 1:1 mixture of formic acid and 3*N* hydrochloric acid used as solvent. Each fraction yielded the same two flavonoids upon hydrolysis. One was light yellow and the other pale orange. The dark-brown spots remaining at the origin probably represented polymerized substances.

Alkaline hydrolysis. Degradation on a micro-

scale of the acid-hydrolyzed products (light-yellow and pale-orange fraction) was carried out by a modification of Lindstedt and Misiarny's method (1951). About 0.2 mg of sample in a small test tube was heated for 10 min with 0.5 ml of 50% potassium hydroxide in boiling water, diluted with water, acidified with dilute acetic acid, and extracted with ether. The extract was run on paper chromatograms in a solvent of a mixture of butanol–acetic acid–water (4:1:5) and benzene–acetic acid–water (2:2:1). The phenolic compounds obtained were detected under UV light or visualized after spraying with diazotized benzidine (Lindstedt and Misiarny, 1950). Samples of known phenols were run on the same chromatogram.

Spectrophotometric measurements. The light yellow and orange bands (acid-hydrolyzed products of fraction *B–E*) were eluted from the paper chromatograms with 95% methanol, concentrated, and spotted on one-half Whatman filter paper No. 1 (45 × 55 cm). After development, the band and a blank (the other half of the paper) were cut out and eluted separately with 95% ethanol.

The shift in the absorption maxima was determined by adding three drops of either a 5% ethanolic solution of aluminum chloride or of 1*N* ethanolic sodium ethoxide to 3 ml of solution (Geissman *et al.*, 1956). The shifts caused by sodium acetate were measured as follows: an excess of anhydrous fused sodium acetate was added to both sample and blank. The spectra were measured after 5–10 min (Jurd and Horowitz, 1957).

The spectra of the eluted pigments were determined after sodium borohydride reductions as follows: 500 mg of sodium borohydride were added to 2 ml of concentrated eluate. The solutions were kept 20 min at room temperature, neutralized with 2 ml of 50% acetic acid in methanol, diluted with water, and extracted with butanol. The butanol extracts were dried on the flash evaporator, and residues were dissolved in 95% methanol for measurements of the absorption spectra. All measurements were made with a Beckman DK-2.

Authentic eriodictyol and pelargonidin for comparison measurements were respectively supplied by Drs. R. M. Horowitz and S. Ito.

RESULTS AND DISCUSSION

Fig. 1 is a two-dimensional chromatogram of the methanol extract of sorghum seed coats.

Spot *A* had an orange color and turned purple on exposure of the paper to the vapors of ammonia, which was suggestive of anthocyanins. Spot *B* had a blue fluorescence under ultraviolet light. Application of 2% phosphomolybdic spray followed by exposure to ammonia fumes revealed spot *B* together with spots I, II, III (referred to hereinafter as chromogens I, II, III). Spot *B* is presumably a dihydroxyphenol (Riley, 1950).

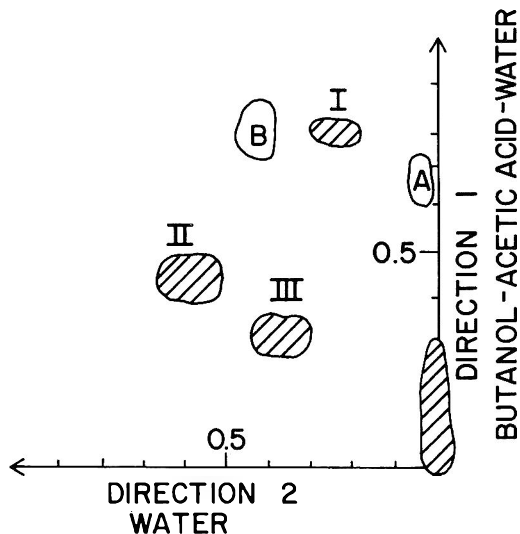

Fig. 1. Two-dimensional chromatogram of methanol extracts of sorghum. The solution was chromatographed with an *n*-butanol–acetic acid–water mixture (4:1:5) in direction 1, and with water in direction 2.

Chromogens I, II, III turned pink when sprayed with acid or 3% *p*-toluene sulfonic acid in ethanol, which suggests that they are anthocyanogens or "leuco" compounds (Roux, 1957).

Several kinds of adsorbents were tried in preliminary investigations in an attempt to separate these compounds. Both crystalline cellulose powder and Celite 545 were used successfully for separation of chromogens I, II, III, but the former adsorbent was used in the main portion of the experiment since it appeared to give slightly better separations. Fractions *B, C, D,* and *E* were eluted from the cellulose column using 99% acetone, 90% acetone, and 70% methanol, sequentially. Each fraction was further purified by preparative chromatography, concentrated, and chromatographed with a mixture of butanol–acetic acid–water (4:1:5). The results are shown in Fig. 2.

The R_f values show that fraction *B* is chromogen I; fraction *C*, chromogen II;

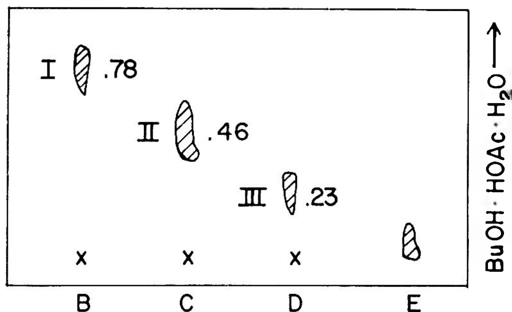

Fig. 2. Chromatogram of eluate from cellulase column. The number shown in the chromatogram is R_f value.

Table 1. R_f values of chromogens I, II, III in different kinds of solvents.

Chromogen-developing solvent	I	II	III
Butanol–acetic acid–water (4:1:5)	0.22	0.44	0.78
2% acetic acid	0.41	0.44	0.30
Water-saturated 2-butanol	0	0.01	0.57
Water	0.39	0.57	0.24

and fraction *D*, chromogen III. The R_f values in other developing agents are shown in Table 1. These chromogens turn distinctly pink 1 or 2 min after concentrated hydrochloric acid at room temperature is added to the eluates.

These were then boiled with hydrochloric acid and chromatographed with a 1:1 mixture of formic acid and 3*N* hydrochloric acid used as solvent. The chromatograms are shown in Fig. 3.

The figure shows that these chromogens yield the same two flavonoids upon acid hydrolysis. One is light yellow, the other is pale orange. Color reactions were carried out on the ethanolic eluates of the light-

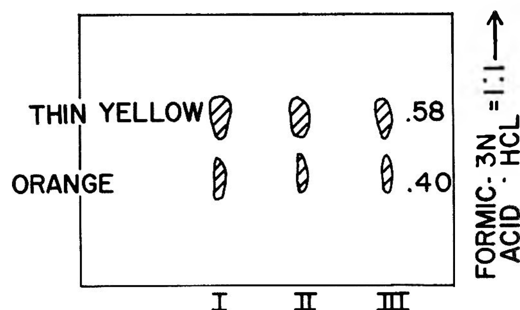

Fig. 3. Chromatogram of acid hydrolysate of chromogen I, II, and III.

Table 2. Color reaction of light-yellow component in acid-hydrolyzed product of chromogens.

Reagent	Color
Conc. H_2SO_4	Orange
1% Na_2CO_3	Pink
NaOH	Pink → brown
$NaBH_4$ + conc. HCl	Pink
Mg + conc. HCl	Pink
Zn + conc. HCl	Colorless

yellow spot. The results are presented in Table 2.

These reactions show that the light-yellow band is a flavanone (Venkataraman, 1962). The magenta color produced by reduction with sodium borohydride followed by addition of hydrochloric acid is very indicative in detecting the presence of flavanones (Horowitz, 1957). Moreover, negative results in a zinc reduction test showed that this is not a 3-flavanol (Pew, 1948).

Table 3 lists the R_f values of the yellow band in different developing agents. These values clearly coincide with those of authentic eriodictyol, but the color reactions of our sample to alkali differed slightly from those of authentic eriodictyol. The authentic sample turned purple in sodium hydroxide, but our sample turned pink. Upon chromatography and spraying with Tollens' reagent, however, the color reactions of the unknown were identical to eriodictyol.

The absorption spectra in ethanol are shown in Fig. 4. These revealed no difference between the sample and authentic eriodictyol. Furthermore, aluminum chloride, fused sodium acetate, and sodium borohydride reduction all caused identical shifts in the maxima, as shown in Fig. 5.

Horowitz and Jurd (1961) reported that bathochromic shifts of flavanones caused by

Table 3. R_f values of eriodictyol and light-yellow component in acid hydrolysate of chromogens I, II and III.

Developing-solvent	Light-yellow part	Eriodictyol
Forestal solvent	0.81	0.81
Formic acid–3*N* HCl (1:1)	0.51	0.51
n-Butanol–acetic acid–water (4:1:5)	0.76	0.77
30% acetic acid	0.62	0.61

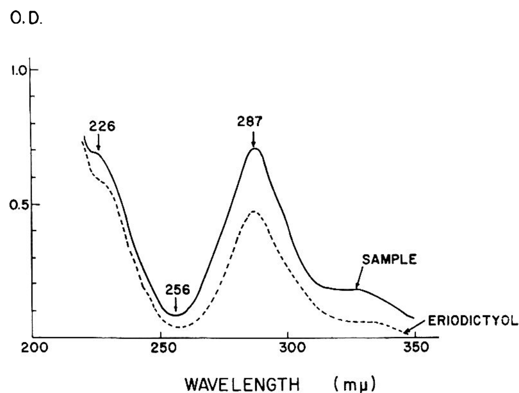

Fig. 4. Absorption spectra of one component of acid-hydrolyzed chromogens in 95% ethanol.

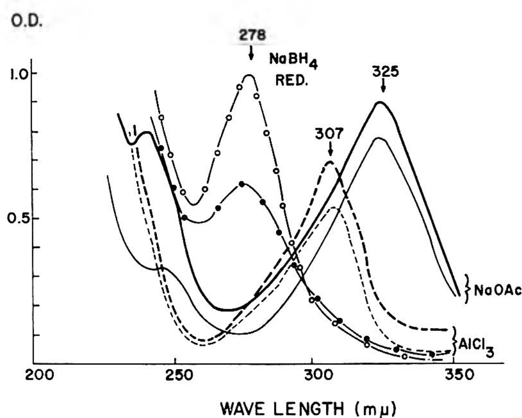

Fig. 5. Shift of absorption maxima with the addition of various reagents. Heavy lines are sample, and light lines are authentic eriodictyol.

aluminum chloride are due to a free 5-hydroxyl group, and shifts by sodium acetate are due to a free 7-hydroxyl group. The shift by sodium borohydride and mineral acid is ascribed to the reduction of a flavanone to a flavylium salt. Thus, these results all support the argument that the compound was eriodictyol.

Alkaline hydrolysis of this fraction was carried out after Lindstedt's method. Results with this fraction were not satisfactory; although phloroglucinol was clearly detected, neither caffeic acid nor protocatechuic acid was found. It is possible that these phenols were destroyed during the alkaline degradation (Dunlap and Wender, 1960).

The other acid-hydrolyzed product—the orange compound—appears to be an anthocyanidin. Color tests (Table 4) show that

Table 4. Color reaction of pink component.

Reagent	Color
Conc. H_2SO_4	Pink
1% Na_2CO_3	Purple
NaOH	Purple → yellow
Cyanidin reagent	Mostly extracted
Amyl alcohol–sodium acetate	Violet red
Amyl alcohol–sodium acetate + $FeCl_3$	No change

Table 5. R_f values of pelargonidin and pink component.

Developing-solvent	Pink part	Pelargonidin
Forestal solvent	0.67	0.67
Formic acid–3N HCl (1:1)	0.37	0.37
30% acetic acid	0.44	0.44

this anthocyanidin is of the pelargonidin type (Hayashi, 1962). The R_f values coincide with those of authentic pelargonidin (Table 5). The absorption spectrum in ethanol-HCl was identical with pelargonidin. Several kinds of chromogens have been reported which turn pink by acid; although most of them are basically flavan-3,4-diols, other compounds (called proanthocyanidine) have been suggested (Robinson, 1963). These were considered to be dimers or polymers, which upon heating with acid form anthocyanidin, catechin, and other products (Forsyth and Roberts, 1960).

CONCLUSION

The present study revealed that chromogens I, II, and III isolated from sorghum yielded two flavonoids (a flavanone and an anthocyanidin) upon hydrolysis. The present compounds are apparently polymerized substances similar to that reported in baggasse lignin, which was suggested by Robinson (1963). The flavonoids were identified as eriodictyol and pelargonidin.

Further work is needed to ascertain how these flavonoids are combined and what structural differences exist among chromogens I, II, and III.

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ROBERT M. SILVERSTEIN, J. O. RODIN, C. M. HIMEL
Stanford Research Institute, Menlo Park, California 94025
and ROBERT W. LEEPER
Pineapple Research Institute of Hawaii, Honolulu, Hawaii

Volatile Flavor and Aroma Components of Pineapple II. Isolation and Identification of Chavicol and γ-Caprolactone

SUMMARY

Chavicol (p-allylphenol) and γ-caprolactone were isolated from a fresh pineapple concentrate and identified by mass, infrared, and NMR spectrometry.

INTRODUCTION

There are many approaches to the chemistry of flavor, and there are levels of sophistication within each approach. It is not appropriate that any of these approaches be derogated; each accomplishes an objective commensurate with the effort, talent, and instrumentation available. For example, in the early days of gas chromatography a fair amount of information was gleaned with the nose used as a combined detector and analyzer.

The widespread use of gas chromatography as a tool for both isolation and "identification" has the inherent limitation that the matching of retention times does not really constitute identification in the true sense. The unique, the unexpected, will not be discovered; the trivial will be sought and found. In addition, a retention time is a single parameter unless several substrates are used. An additional parameter can be added by carrying out chemical group tests either on the mixture or on trapped fractions (Connell, 1964; Hoff and Feit, 1964).

To avoid the problems involved in trapping fractions, several investigators have connected a gas chromatographic instrument (with packed or capillary columns) directly to a mass spectrometer. This represents a quantum leap in sophistication (and expense). For leading references, see Day and Libbey (1964) and Anderson and von Sydow (1964). It is sometimes possible to identify simple molecules with no information other than a good mass spectrum when the parent peak is clearly recognizable and the fragmentation pattern straightforward. Unfortunately the parent peak can be weak, absent, or obscured by impurities, and the fragmentation pattern complicated by rearrangements that are more readily rationalized than recognized. Recourse is usually had to matching spectra of unknowns with those of authentic samples. Preconceived ideas and preliminary studies are frequently prerequisites for such investigations, though this aspect may be overshadowed by the technical virtuosity involved.

Recent developments in fast-scan infrared spectrometers should make it feasible in the near future to obtain a satisfactory infrared spectrum on a sample en route from a gas chromatography column to the mass spectrometer. Such an arrangement will extend the possibility of identification manyfold. The next generation of chemists will probably obtain mass spectral data accurate to 1 millimass unit, and query a center for data storage and retrieval. Both the required high-resolution mass spectrometers and computers are currently available. A massive data-gathering program is under study. Yet, all these procedures that depend on matching data against stored information utilize basically a clerical rather than a chemical approach. Again, as with the more primitive matching of retention times, the methodology is limited by the number of reference compounds available. A new compound—i.e., one not previously reported in the chemical literature—will frustrate the search.

The methodology used in these laboratories involves isolation (trapping) of pure components and identification by a combination of whatever techniques are required. Usually a combination of two, three, or four spectra suffices for most molecules that can be put through a gas chromatographic column. For compounds of greater complexity, such information will permit intelligent selection of chemical procedures, and the resulting products can be identified spectrometrically. A fraction of a milligram of a compound of moderate molecular weight can

give usable mass, infrared, and ultraviolet spectra; it is difficult to get a satisfactory (integrated) nuclear magnetic resonance spectrum on less than 1–2 mg. Practical limitations are encountered mainly in techniques of handling minute amounts of compounds: trapping, transferring, solvent removal, prevention of contamination, and decomposition of unstable components. On the other hand, this approach requires no *a priori* assumptions as to possible components. Reference spectra are used for confirmation only after a tentative identification has been made.

Application of this methodology to pineapple flavor resulted in tentative identification of a compound that not only had not previously been found in pineapple, but was a new compound in itself (Rodin *et al.*, 1965). We now report the isolation and identification of two additional major components that have not been previously found in pineapple.

ISOLATION

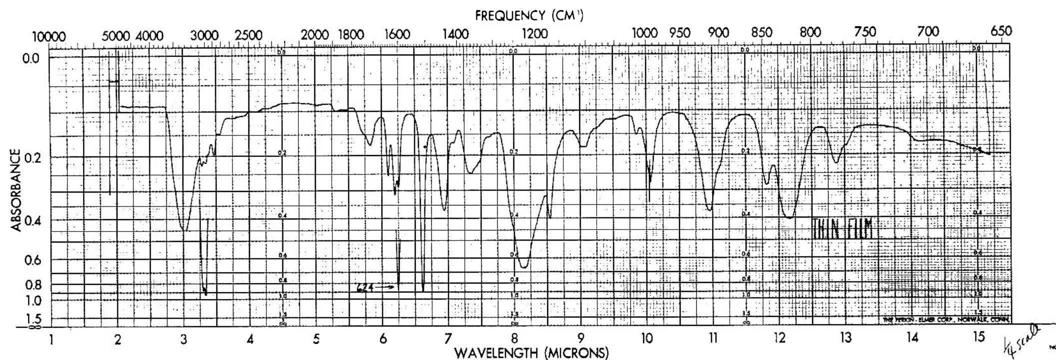
This study was carried out on a flavor concentrate prepared from 250 freshly-picked pineapples. Preparation of the concentrate was described in the previous paper (Rodin *et al.*, 1965). The concentrate (18 g) was subjected to short-path distillation at bath temperatures ranging from room temperature to 120°C at one atmosphere pressure to give distillation cuts 1 and 2. Two receivers were then attached in series to the distillation flask containing the residue. The first receiver was held at room temperature, and the second cooled in a dry-ice (acetone) bath. The pressure was reduced to 0.1 mm Hg, and the bath temperature was increased from room temperature to 120°C. The distillate in the second (cooled) receiver consisted of 1.4 g of a two-phase mixture. This mixture was separated, and the aqueous phase was extracted with ether; the ether extract and the organic phase were combined and dried over sodium sulfate; the ether was removed, and the residue (*ca.* 0.5 g) was labeled distillation cut 3. The distillate in the first receiver (0.94 g) was labeled distillation cut 4. Cuts 3 and 4 are of immediate interest.

Distillation cut 4 was fractionated by gas chromatography into 5 fractions (A through E) (G.E.-SF 96 silicone, 20% on firebrick, 6 ft × ¼ in., 140°C, 40 ml helium/min, each injection 50 μl of a 50% methanol solution, injection block 155°C, on-column injection, Aerograph A350B). The largest of these fractions was 4B (300 mg); its workup and tentative identification as 2,5-dimethyl-4-hydroxy-3(2H)-furanone were described in the previous paper (Rodin *et al.*, 1965). The next-largest fraction, 4D, (retention time 17–22 min, 70 mg) was refractionated into two fractions on a Carbowax column (Carbowax 20M, 15% on Chromosorb W, 3 ft × ¼ in., 157°C, 70 ml helium/min, each injection 20 μl of a 50% methanol solution, injector block 200°C, on-column injection, Aerograph A90C). Fraction 4D2 (23 mg) was collected from 25 to 30 min. Its infrared, mass, and NMR spectra are presented in Fig. 1.

Distillation cut 3 was fractionated by gas chromatography into 8 fractions, A through H (Silicone QF1, 15% on Chromosorb W, 12 ft × ⅜ in., 155–175°C, 46–70 ml helium/min, each injection 40 μl of sample, injector block 200°C, on-column injection, Aerograph A90C). The temperature and flow-rate were programmed as follows: 155°C, 46 ml/min for 23 min; 155–175°C, 46 ml/min for 8 min; 175°C, 46 ml/min for 11 min; 175°C, 70 ml/min. Fraction 3H was collected from 54 to 62 min. Fraction 3H (17 mg) was investigated first because it appeared to be a relatively simple mixture. It was refractionated on a Carbowax 20M column (15% on Chromosorb W, 6 ft × ¼ in.). The major peak was collected as fraction 3H1 (9 mg) from 30 to 39 min at 115°C and a flowrate of 48 ml/min. The infrared, mass, and NMR spectra of fraction 3H1 are presented in Fig. 2.

Identification of Fraction 4D2

The parent peak of the mass spectrum is mass 134. The parent + 2 peak (P + 2) is too small to allow for the presence of sulfur, chlorine, or bromine. From the calculated contributions of isotopes to the P + 1 and P + 2 peaks, we can select $C_9H_{10}O$ as the best tentative fit to our P + 1 peak of 9.89% and P + 2 peak of 0.65% (Silverstein and Bassler, 1963). The fact that the parent peak is the strongest peak in the spectrum is an indication of a structure stable to electron bombardment. This indication of aromaticity is quickly confirmed by the tentative molecular formula, by the general appearance of the infrared spectrum, by the low-field peaks in the NMR spectrum, and by the "benzene" peaks (77, 78, and 79) in the mass spectrum. Under these circumstances, the bands in the infrared spectrum at 3.03 and 8.15 μ could respectively be accounted for as the O—H stretching and the C—O stretching absorption of a phenolic hydroxyl group. This could have been confirmed by a bathochromic shift in an ultraviolet spectrum on adding base. The very nice AB pattern centered at τ 3.20 indicates p-disubstitution on a benzene ring. This possibility is reinforced by the strong band at 12.15 μ in the infrared spectrum, though little reliance should be placed on such assignments when polar groups are involved.



MASS SPECTRAL DATA (RELATIVE INTENSITIES)

m/e	% OF BASE PEAK	m/e	% OF BASE PEAK	m/e	% OF BASE PEAK
27	11.	66	5.0	108	4.6
38	3.3	77	20.	115	11.
39	15.	78	7.1	116	5.8
50	5.6	79	7.8	117	9.6
51	12.	91	10.	119	9.4
52	4.7	94	3.1	131	5.7
53	6.9	103	6.5	132	5.9
55	7.0	105	21.	133	71.
63	5.6	106	3.3	134(P)	100.
65	5.1	107	55.	135(P + 1)	9.89
				136(P + 2)	0.65

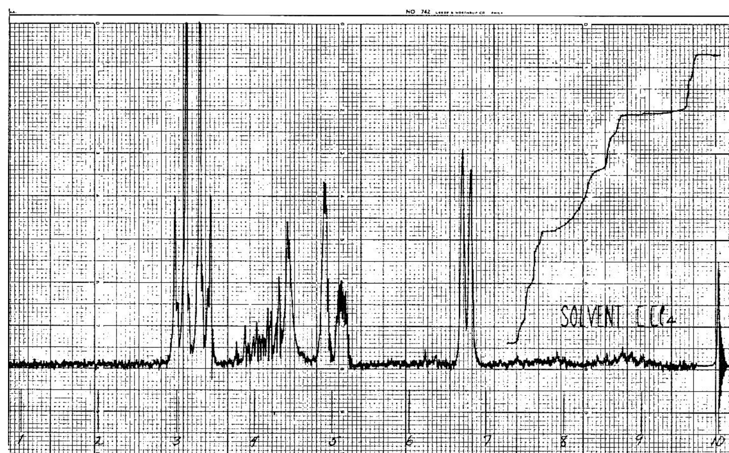
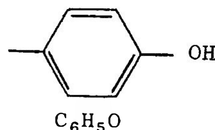
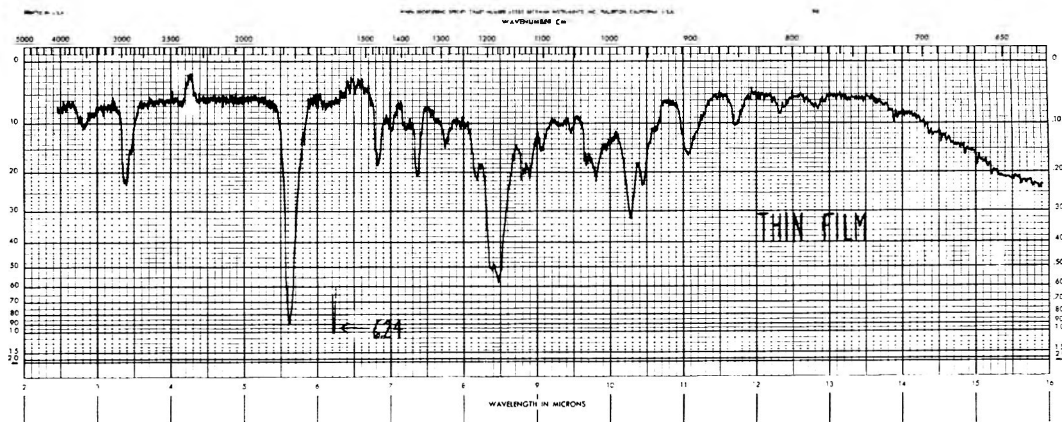


Fig. 1. IR, mass, and NMR spectra of 4D2.

Our data, thus far, give us the following partial structure.



Subtracting this moiety from the tentative molecular formula, $C_9H_{10}O$, leaves C_3H_5, which can only be an allyl, a propenyl, or a cyclopropyl group. Peaks in the olefinic region (τ 4–5) of the NMR spectrum, and a C = C stretching band at 6.10 μ in the infrared spectrum narrow the choice to allyl or propenyl. We note the bands at 10.07 and 10.95 μ in the infrared, and we are inclined toward the allyl structure. Confirmation follows from the 2-proton (compared with the 4-proton aromatic peaks) doublet at τ 6.77; this is the proper shift position for a methylene group situated between a vinyl group and a benzene ring. The characteristic disposition of the vinyl protons (2 protons centered at about τ 5 and a single proton multiplet centered near τ 4.1) lends further credence to the proposed structure. The vinyl absorption pattern



MASS SPECTRAL DATA (RELATIVE INTENSITIES)

m/e	% OF BASE PEAK	m/e	% OF BASE PEAK
14	2.8	42	19.
15	8.3	43	16.
18	5.8	55	12.
26	7.0	56	16.
27	27.	57	15.
28	22.	66	3.8
29	49.	70	10.
39	13.	85	100.
40	4.0	86	9.3
41	10.	113	1.6
		114(P)	1.4
		(128)	(0.4)

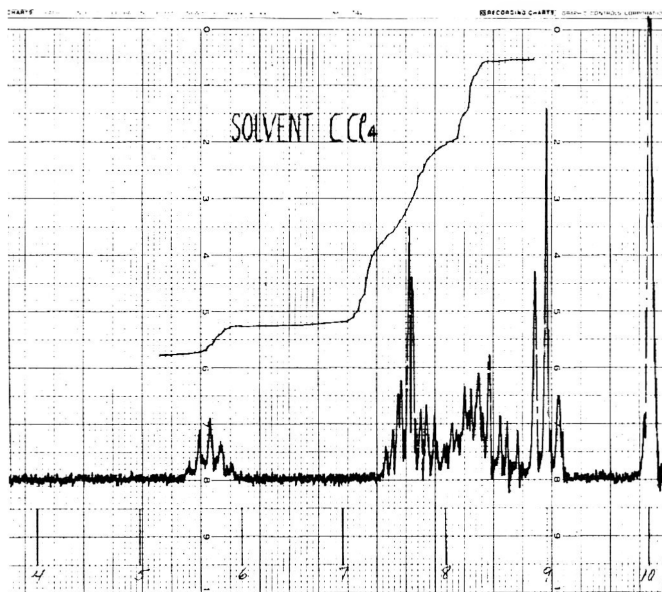
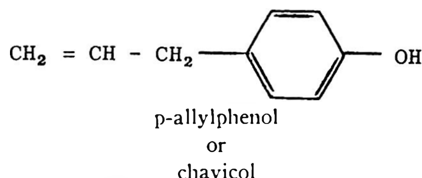


Fig. 2. IR, mass, and NMR spectra of 3H1.

is distorted by the hydroxylic proton peak at τ 4.42. This peak could have been caused to shift, relative to the other peaks, by changing temperature, concentration, or solvent; it could have been eliminated by shaking the solution with D_2O. The fragmentation pattern of the mass spectrum is not especially informative; the major peak at mass 107 probably represents cleavage of the $CH_2 = CH$-group.

We have now constructed the molecule

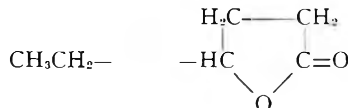


p-allylphenol
or
chavicol

Confirmation was obtained by peak-by-peak matching of the infrared spectrum with that of an authentic sample.

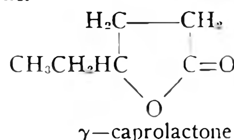
Identification of Fraction 3H1

Initial efforts failed to locate the parent peak of the mass spectrum. There are no large peaks beyond the base peak of mass 85, and some contaminant was evident. The carbonyl band at 5.63 μ in the infrared spectrum leads us to consider a 5-membered lactone ring; the broad strong band at about 8.45 μ is the C—O stretching absorption. Neither the infrared nor the NMR spectrum show any sign of aromatic or olefinic structure. The base peak of mass 85 is confirmation for a 5-membered ring lactone substituted at C_4; this represents facile cleavage of the bond which is at a branched carbon atom, and is also β to an oxygen atom. The NMR spectrum clearly shows a triplet at τ 9.00. This must represent a methyl group adjacent to (split by) a methylene group. We can write the following fragments.



The methine proton adjacent to the oxygen atom can be seen in the NMR spectrum as a multiplet centered at about τ 5.7. The integration ratios point to a C_6, or possibly a C_7, lactone.

With the foregoing information, we can look again at the mass spectrum. The molecular weight of γ-caprolactone is 114, and that of γ-hydroxyheptanoic acid lactone is 128. Unfortunately, both peaks are present and no more can be learned from the data at hand. Comparison of the mass spectrum and the gas chromatographic retention time of the unknown compound with those of authentic samples of both likely reference compounds confirmed the identity of the unknown compound as γ-caprolactone.



γ—caprolactone

EXPERIMENTAL

Infrared spectra were run on a Perkin-Elmer 221 and a Beckman IR5, NMR spectra on a Varian HR60, and mass spectra on a CEC 21-103C. Mass peaks of less than 3% relative to the base peak (except for parent and isotope peaks) were not reported. Gas chromatography instruments used were Wilkens Instruments Aerographs A90C and A600B with aluminum columns.

The reference sample of γ-caprolactone was purchased from K and K Laboratories, New York. p-Allylphenol was synthesized according to Grignard and Ritz, 1936.

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JOHN GORTON DAVIS and LEO KLINE
Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture Albany, California 94710

Vapor Pressure of Water and Carbon Dioxide Over Whole Egg Solids Containing Added Carbohydrates

SUMMARY

Water and carbon dioxide vapor pressures were determined at 25°C for freeze-dried whole egg powders containing various levels of sucrose and of corn syrup solids added before drying. The manometric method used permitted determinations of vapor pressures over a range of moisture levels with a single sample.

At moisture levels commonly encountered in commercial whole egg solids (2-6%), addition of both types of carbohydrate consistently increased equilibrium water vapor pressures over the dried products. Equilibrium values for carbon dioxide partial pressures were not obtained but the amounts found made it necessary to apply appropriate corrections in the determinations of water vapor pressure.

The results are discussed in relation to Maillard-type browning reactions and oxidative flavor deterioration in dried whole egg.

INTRODUCTION

Brooks and Hawthorne (1943) showed that addition of substantial levels of carbohydrate to dried whole egg (10% or more on a liquid basis) retarded manifestations of Maillard or browning-type reactions, and Bate-Smith and Hawthorne (1945) examined the reactions in greater detail. More recently, Kline *et al.* (1964) showed that levels of carbohydrate below 10% retard oxidative flavor deterioration under conditions where browning reactions are not a factor. The role of water in these two different types of deterioration has not been elucidated, although it has been shown that the rate of browning-type reactions in whole egg powders increases with increasing water content (Bate-Smith *et al.*, 1943; Hawthorne, 1943; Kline *et al.*, 1951).

The equilibrium moisture content of whole-egg, white and yolk solids at various relative humidities has been reported by Gane (1943). Makower (1945) confirmed Gane's results for whole egg solids by direct measurement of equilibrium water-vapor pressures as a function of moisture content and extended the data to cover a wider range of temperatures and moisture contents. Both investigators found no significant difference between spray-dried and freeze-dried egg preparations, but Makower was able to show a small but definite effect of denaturation. Makower also found that the data conformed to theory for the adsorption of gases on solids.

The present report is concerned primarily with the effect of carbohydrate addition to whole egg solids before drying on the relations between water content and vapor pressure. Studies were limited to the determination of equilibrium vapor pressures at 25°C of freeze-dried whole egg solids containing graded levels of sucrose and of regular conversion corn syrup solids (42% dextrose equivalent acid-hydrolyzed corn starch. The Corn Industries Research Foundation, Inc., gives the following typical analysis: 18.5% dextrose, 13.9% maltose, 42.2% tri- to hepta-saccharides, and 25.4% higher saccharides). Since such spray-dried formulations are substantial items of commerce, the relationships found should be useful in determining limiting drying conditions.

The technique used in the present work permitted the determination of carbon dioxide pressures as well as water vapor pressures. This was important because carbon dioxide was found to contribute significantly to the total pressure at all except the lowest moisture levels. In contrast, Makower (1945) found carbon dioxide to be significant only at temperatures above 70°C. Data on carbon dioxide content and vapor pressure are also included.

MATERIALS AND METHODS

Samples of whole egg solids were prepared from fresh eggs broken out and blended, divided into portions, and blended again to dissolve varying amounts of carbohydrate. After freeze-drying, the samples were rubbed through a sieve and stored at 2°C until used.

Vapor pressure was measured by the method of Makower and Myers (1943) with modifications suggested by the work of Taylor (1961). These modifications permit the determination of equilibrium pressures at various moisture levels from a single sample rather than requiring a separate sample for each level. The equilibrium pressure is first measured at the highest moisture level and then the moisture is progressively reduced by vacuum distillation into weighing traps without removing the sample from the system. The initial samples were prepared by allowing water vapor to diffuse into the freeze-dried materials in an evacuated desiccator.

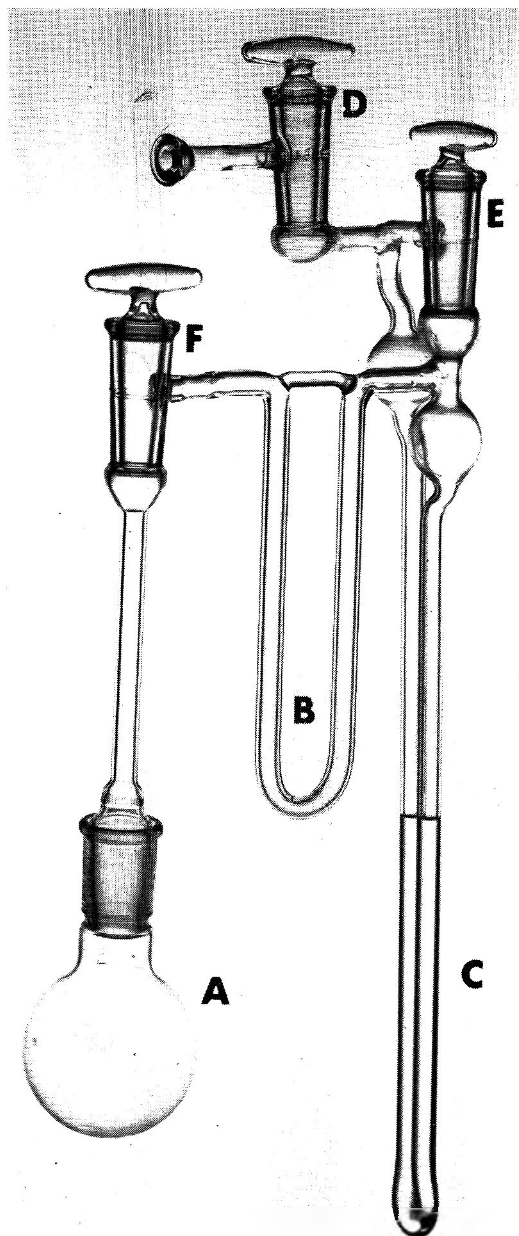

Fig. 1. Vapor pressure manometer. A. sample flask; B, cold trap; C. oil manometer; D, evacuation stopcock; E, manometer bypass; F, vapor stopcock.

The apparatus used is shown in Figs. 1 and 2. The ground joints were individually matched and lapped, and the stopcocks used (DEF) were designed for high vacuum (Corning Nos. 7546 and 7548). An O-ring joint was used for the exhaust connection. Oblique-bore stopcocks with lapped plugs were used on the concentric-tube collection traps (Fig. 2). A silicone diffusion pump oil (Dow 704) was used as the manometer fluid, and the levels were read to the nearest 0.1 mm with a cathetometer. The readings were converted to mm Hg, but the sensitivity was about 10 times that obtainable with mercury.

The sample (10 g) was weighed into the 100-ml flask (A) and the flask attached to the manometer. The trap (*B*) was then immersed in liquid nitrogen and the system evacuated to less than 0.01 mm Hg with all stopcocks open. During this evacuation, the manometer fluid was boiled with an open flame to remove dissolved gases. Stopcocks *E* and *D* were then closed and the apparatus disconnected from the vacuum and placed in a constant-temperature water bath. This bath had plate-glass sides so that readings could be made of the immersed manometers without optical distortion. The temperature was controlled at 25±.05°C. The bath accommodated four manom-

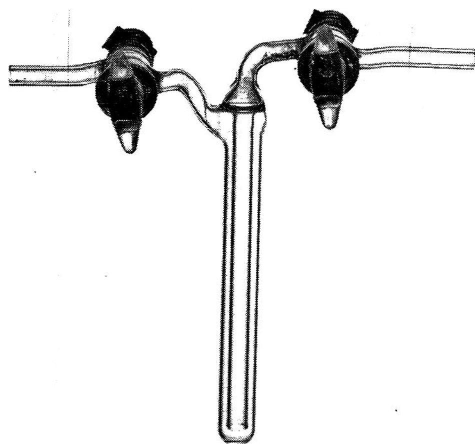

Fig. 2. Concentric-tube collection trap.

eters so that a control and three levels of carbohydrate-containing samples could be run simultaneously.

After equilibration for 24–48 hr, the total vapor pressure was determined. The stopcock *F* was then closed, isolating the sample flask, and the assembly was removed from the thermostat to a ring stand. The trap *B* was immersed in a solid carbon dioxide-acetone bath to a mark one inch from the top and the pressure again determined. This pressure, when corrected (see below) was deducted from the total pressure to give the water vapor pressure. A third pressure determination was made with the trap *B* immersed to the same level in liquid nitrogen. The difference between the second and third corrected pressures was the carbon dioxide pressure, and the third corrected pressure was that of the absorbed gases not condensable at liquid nitrogen temperature. The carbon dioxide was identified by mass spectrometry, and the noncondensable gases were similarly identified as nitrogen and oxygen.

The corrections applied to the second and third pressure measurements were gas-law compensations for the reduced temperature in the trap and immediate vicinity. Because the volume relationships of the various manometers used were not uniform, a calibration curve was required for each. These curves were constructed by determining the pressure loss over a range of total pressures with the trap *B* immersed to a fixed mark in solid carbon dioxide-acetone or liquid nitrogen when the manometers contained dry air. No differences in correction curves were found with dry air, dry nitrogen, or carbon dioxide.

After the initial pressure measurements were complete, the manometer assembly was returned to the thermostat bath, and three concentric tube traps (Fig. 2), previously evacuated and tared, were connected in series between the manometer exhaust connection and the vacuum pump. This assembly extended outside the thermostat so that the two traps nearest the manometer could be immersed in solid carbon dioxide-acetone baths and the third in liquid nitrogen. The collection traps and connections were evacuated and then immersed in the cold baths. After about 10 min of further pumping, stopcocks *D, E,* and *F* were opened, and pumping was continued to distill off water and carbon dioxide from the sample. Distillation required from about 1 hr, at the highest moisture level, to 8 hr, at the lowest. After distillation was complete, stopcocks *D* and *E* and the collection trap stopcocks were closed and the manometer allowed to equilibrate at the lower moisture level. The collection traps were allowed to come to room temperature, dried by wiping, and weighed to constant weight to be sure no condensed water remained on the outside surfaces.

The water distilled off collected in the two traps nearest the manometer which were immersed in solid carbon dioxide-acetone baths. All but traces were found in the first trap, but the second trap was retained in the system to prevent contamination of the carbon dioxide. The carbon dioxide collected in the third trap immersed in liquid nitrogen. Nothing detectable collected when a fourth trap, immersed in liquid nitrogen, was included in the collection train.

When the moisture level had been reduced to 0.1–0.2%, the evacuation time required to distill off an appreciable quantity of water had increased to about 8 hr. At this point, the residual moisture was determined by transfering duplicate portions of the samples to tared weighing bottles and drying to constant weight over P_2O_5 in an evacuated desiccator. The transfer was made in a plastic bag flushed with dry nitrogen, and constant weight was attained in 2–4 days. The water content of the sample at each point was then calculated from the original weight, the residual water, and the amounts withdrawn at each equilibration point.

RESULTS AND DISCUSSION

Procedure. The procedure described here was adopted because it allowed a number of points on the vapor equilibrium curve to be determined from a single sample and permitted determination of the water content without exposure to excessive heat. An added advantage was determination of the contribution of carbon dioxide to the total vapor pressure and weight loss. Samples of whole dried egg with and without added carbohydrate showed marked browning when moisture was determined in a vacuum oven (5 hr, 100°C), and the results were much more variable than drying to constant weight over P_2O_5 in an evacuated desiccator. Karl Fischer titration showed even greater variability and was not usable at low water levels because of the size of sample required. The principal disadvantage of the present procedure is the extreme care required in all manipulations because an error at any point will affect all preceding values and may invalidate all of the data for a particular sample.

Effect of added carbohydrate on water vapor pressure. Fig. 3 shows the relationship between water contents and water vapor pressures at 25°C for freeze-dried whole egg powders to which 0, 5, 10, or 15 g of sucrose

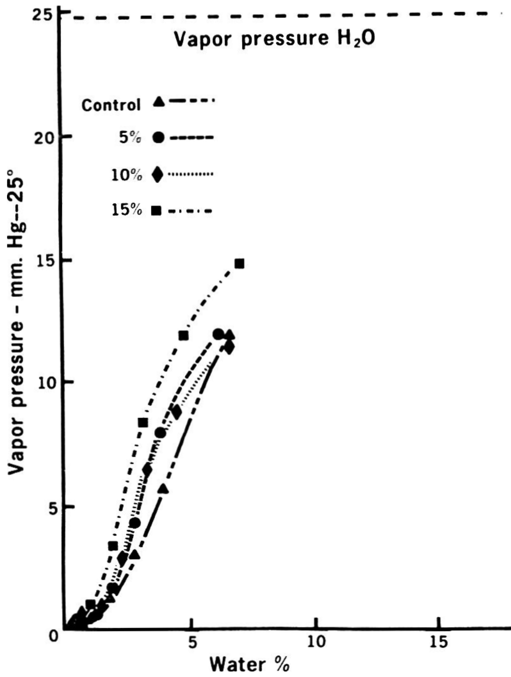

Fig. 3. Vapor pressure of water over whole egg solids with sucrose added before drying.

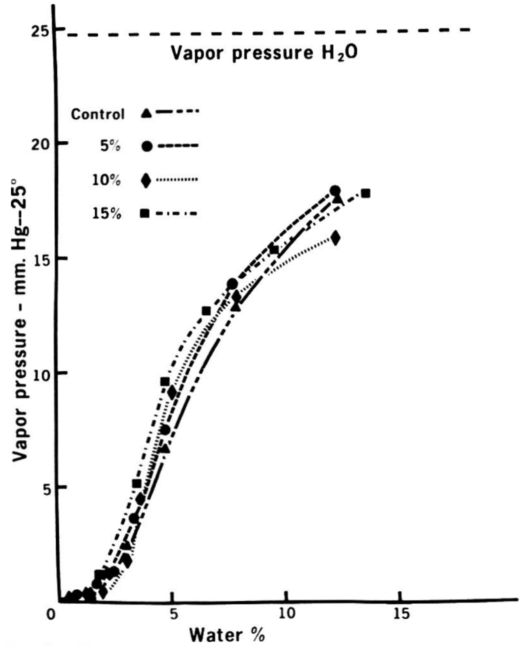

Fig. 4. Vapor pressure of water over whole egg solids with corn syrup solids added before drying.

per 100 g of liquid whole egg had been added before drying. These are referred to herein as 0, 5, 10, or 15% powders. On a dry-solids basis, these proportions are respectively equivalent to 0, 16, 28, and 36½%. Fig. 4 gives similar data for whole egg powders prepared from different raw material with like levels of corn syrup solids of 42 dextrose equivalent (42 DE css).

Water vapor pressures for the whole egg powders without added carbohydrates agreed well with those reported by Makower (1945) over the moisture range 1.0–5.5%. In the 2–6% moisture range added carbohydrate increased water vapor pressure above that of the unsugared controls of equivalent moisture content. Thus, the maximum difference in water vapor pressures at equivalent moisture contents is at 15% sucrose. For example, at 4% moisture the water vapor pressures of the two samples are 10.1 and 5.5 mm Hg, respectively. A moisture content of 5.7% in the control sample is required to reach a water vapor pressure (10.1 mm Hg) equivalent to that in the sugared sample.

Two principal types of deterioration occur in whole egg powders (Kline *et al.*, 1964). First are the Maillard-type reactions, which result in the browning and insolubility that occur during storage at moderate to elevated temperatures. Table 1, taken from the data of Kline *et al.* (1964), shows that these reactions are inhibited progressively as the level of added carbohydrate increases. Second are oxidative flavor deteriorations, which occur at low temperatures where browning reactions are not a factor. These reactions are first progressively inhibited by increasing amounts of added carbohydrate to a point of maximum stability that is followed, however, by an abrupt transition to marked flavor instability as the carbohydrate is further increased. A similar abrupt change occurs in the degree of dispersion of the egg lipids. At the lower levels of added carbohydrate, where oxidative flavor deterioration is inhibited, the lipids exist in a coalesced or large globule state as a consequence of emulsion breakdown during drying. In this state the lipids are easily extractable at room temperature with a petroleum solvent. At the higher levels of carbohydrate, where oxidative flavor deterioration is very rapid, the lipids are finely dispersed as in the original whole egg liquid. These finely dispersed lipids are extractable

Table 1. Effect of added carbohydrates on browning reaction in whole egg powders.[a]

Added solids, liquid basis (%)	Viscosity increase on reconstitution [b] (Δ cps)	Protein solubility decrease (%)	Browning of powders: Average % reflectance loss (530–640 mμ)	Lipid browning of powders: Increase in absorbancy of $CHCl_3$ extract (370 mμ)
Sucrose				
0	690	16	10	0.12
2	395	8	11	0.15
5	13	1	5	0.09
10	<1	0	3	0.04
15	<1	0	2	0.02
42 DE css[c]				
0	3500	19	....	0.08
4	20	0	....	0.03
7½	6	0	....	0.01
10	5	0	....	0.00
15	7	0	.	0.00

[a] Moisture content of powders: Sucrose series, 3.0 ± 0.5%; 42 DE css series, 3.4 ± 0.2%. Storage 3½ mo @ 86°F in N_2, Kline *et al.* (1964).

[b] Over control powders held at 34°F in N_2.

[c] 42 dextrose equivalent corn syrup solids.

with difficulty or nonextractable under the above conditions. Table 2, also from Kline *et al.* (1964), shows these changes for the carbohydrates used in the present investigation.

The increasing water vapor pressure resulting from carbohydrate addition suggests that the carbohydrate may compete with water for the same adsorption sites. Makower's (1945) calculations indicate that the water adsorption sites of whole egg powders are principally on the proteins. There is no *a priori* reason for assuming that these same sites are not involved in glucose-protein browning reactions. If this is so, then added carbohydrate may inhibit browning-type reactions by a mass-action effect which excludes water from the secondary phases of reactions at these sites. This hypothesis accords with the increasing protective effect of added carbohydrate with increasing concentration.

It has been established that there is some relationship between water content and oxidative flavor deteriorations in dehydrated foods. Holm *et al.* (1927) found that oxi-

Table 2. Effect of added carbohydrates on flavor stability of whole egg powders.[a]

Added solids liquid bases (%)	Mean flavor rank[b] (custards)	No. of off-flavor judgments per category: None–slight	No. of off-flavor judgments per category: Moderate–pronounced	Skelly B extractable fat (% of theoretical)
Sucrose				
0	2.7	9	9	74
2	2.8	9	9	74
5	1.3	18	0	72
10	5.0	0	18	34
15	3.2	6	12	2
42 DE css[c]				
0	3.3	2	6	74
4	1.4	8	0	67
7½	1.4	8	0	71
10	3.9	1	7	44
15	4.9	0	8	1

[a] Storage 5 mo @ 55°F in air (Kline *et al.* 1964).

[b] 1 = *least* off-flavor.

[c] 42 dextrose equivalent corn syrup solids.

dative deteriorations were accelerated in whole milk powders at very low moisture levels and concluded that "free moisture has a decidedly retarding effect upon susceptibility to oxidation." Degener's (1960) findings are similar. Salwin (1963) reported similar conclusions for several other dehydrated foods and suggested that maximum stability toward oxidative flavor deteriorations occurs when the water content is the amount required to form a monomolecular layer. This implies that water acts as a physical barrier to the penetration of oxygen. If this is true, then the effects of low levels of carbohydrate in preventing oxidative flavor deterioration in whole egg powders could again be accounted for in terms of competition for adsorption sites and the formation of a more effective barrier to oxygen penetration. It is also possible that water liberated by added carbohydrate is a reactant inhibiting initial autoxidation.

Carbon dioxide vapor pressure. The amount of carbon dioxide found and the carbon dioxide partial pressures corresponding to the water vapor pressure data are given in Table 3. It should be emphasized that these are values obtained after approximately 48 hr of equilibration for use as corrections in the determination of water and its vapor pressure. After 20 days, the carbon dioxide vapor pressures are approximately 75% of the total pressure but equilibrium was not attained even after this time. This proportion applies only to the products discussed in this report. Other types of egg products have different rates.

Table 3. Amount and vapor pressures of CO_2 in whole egg powders.

% Carbohydrate added before drying	Sucrose added				Corn syrup solids added			
	% H_2O in powder	% CO_2 in powder	CO_2 vapor pressure mm Hg	CO_2 vapor pressure % of total	% H_2O in powder	% CO_2 in powder	CO_2 vapor pressure mm Hg	CO_2 vapor pressure % of total
0					12.15		0.57	3.1
	6.65	0.086	1.61	12.0	7.85	0.042	0.44	3.3
	4.00	0.058	0.61	10.0	4.72	0.024	0.18	2.7
	2.90	0.044	0.21	6.8	3.11	0.018	0.05	2.0
	1.90	0.024	0.12	8.8	2.11	0.005	0.03	2.7
	1.19	0.004	0.08	17.0	1.07		0.01	
	0.12				0.75		0.00	
5					12.18		0.40	2.2
	6.21	0.090	2.09	15.4	7.77	0.044	0.15	1.1
	3.90	0.023	1.01	11.4	4.80	0.038	0.05	0.7
	2.76	0.011	0.44	9.6	3.36	0.019	0.12	3.2
	1.88		0.26	15.5	2.35	0.015	0.02	1.6
	1.22		0.12	19.1	1.49	0.006	0.00	0.0
	0.21		0.04	50.0	0.92		0.01	
10					12.33	0.027	1.67	9.6
	6.60	0.054	2.60	18.6	7.99	0.014	0.07	0.5
	4.64	0.046	1.64	15.6	5.06	0.011	0.00	0.0
	3.39	0.040	0.98	13.5	3.72	0.007	0.00	0.0
	2.30	0.026	0.39	12.1	2.57	0.003	0.01	0.6
	1.50	0.017	0.20	19.0	1.55		0.02	
	0.50		0.10	48.0	0.95		0.01	
15					13.60	0.034	0.44	2.4
	7.08	0.046	3.22	18.4	9.56	0.024	0.17	1.1
	4.82	0.029	1.92	14.0	6.63	0.016	0.04	0.3
	3.21	0.018	0.99	10.8	4.83	0.010	0.04	0.4
	1.92	0.007	0.41	11.3	3.50	0.005	0.00	0.0
	1.04		0.16	16.8	1.95	0.001	0.02	
	0.23		0.10	90.0	1.22		0.01	

The present technique, which includes measurement of the carbon dioxide pressure as well as a gas law correction, is preferred by the writers because carbon dioxide is frequently encountered in egg powders and may be a large factor in the total vapor pressure. For example, examination of a series of spray-dried egg white powders to which 0, 5, and 10% sucrose (liquid basis) had been added before drying gave carbon dioxide partial pressures 75, 45, and 30%, respectively, of the total pressure after 2 months at 75°F.

The source of the carbon dioxide found was not determined. While freeze-drying should remove most of the dissolved carbon dioxide, no particular precautions were taken to ensure complete removal or to prevent subsequent adsorption of atmospheric carbon dioxide.

The amounts of carbon dioxide found were similar for the two series of powders, but the partial pressures were markedly different in two respects. First, the carbon dioxide pressures were all considerably higher in the sucrose series than in the corn syrup solids series, even in controls. Second, the partial pressure of carbon dioxide as percent of the total pressure is roughly constant in the sucrose series. However, in the corn syrup solids series, it is constant only in the control and decreases with decreasing water content in the sugared samples.

Gas-law correction. The gas-law corrections become important only when carbon dioxide or noncondensable gases contribute significantly to the total pressure. With manometers of the proportions used in this investigation, the corrections amounted to 7–9% of the observed values for carbon dioxide and 35–40% of the observed values for noncondensable gas. In the method proposed by Taylor (1961), where the "water vapor" is condensed in the sample with liquid oxygen, carbon dioxide will also condense and thus be included in the "water" vapor pressure.

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BERNARD J. BRANDWEIN
Chemistry Department, South Dakota State University
Brookings, South Dakota 57007

The Pigments in Three Cultivars of the Common Onion (Allium cepa)

SUMMARY

The flavonoid and anthocyanin pigments were isolated and identified in three onion cultivars: Southport Yellow Globe, Southport White Globe, and Southport Red Globe. All three varieties contain quercetin-4′-glucoside, quercetin-3,4′-diglucoside, quercetin-4′,7-diglucoside, and quercetin-3-glucoside. In addition, Southport Yellow Globe contains quercetin, and Southport Red Globe contains quercetin and peonidin-3-arabinoside.

INTRODUCTION

In a lengthy survey of the anthocyanin content of representative plants, G. M. Robinson and R. Robinson (1932) list Allium cepa as containing a cyanidin pentose glycoside. Their results were based upon simple color and solubility tests, and no attempt was made to isolate and identify individual constituents. Herrmann (1956) reported the isolation of three quercetin glycosides from onions, no variety mentioned. The compounds were separated by paper chromatography. One of them was identified as spiraeoside (quercetin-4′-glucoside); the other two were unidentified although he stated that one of them behaved as though the carbohydrate, glucose, was on the C-3 position. This same worker later reported on the phenols and flavonols of the onion; again, no varieties given (Herrmann, 1958). Spiraeoside and quercetin were isolated and identified, and two others were isolated and partially identified. Methylation and hydrolysis of one of the latter gave rise to 3′,4′,5,7-tetra-o-methylquercetin and 2,3,4,6-tetra-o-methylglucose. The other was found to give quercetin and glucose on hydrolysis. These compounds, as well as some simpler phenols, were isolated by countercurrent distribution. The yields were very small. Two South African workers, Koeppen and Van der Spuy (1961), isolated spiraeoside and quercetin, as well as some uncharacterized quercetin glycosides, from an unidentified variety of onion. This paper presents identification of the flavonols, flavonol glycosides, and anthocyanin glycosides from three cultivars of the onion.

EXPERIMENTAL MATERIALS

Onions. The three varieties studied were taken from commercially available onions sold for home consumption. The Southport Yellow Globe was taken from the 1962, 1963, and 1964 crops and the Southport White Globe and Red Globe from the 1963 and 1964 crops. Only the bulbs were used.

Chromatographic equipment. Whatman Nos. 3MM and 1 papers were used in sheets 57 by 46 cm for preparative separations and R_f determinations respectively. Chromatogram development was carried out in a Reco Chromatocab, model A.

Solvent systems for paper chromatography were:

BAW	n-butyl alcohol–acetic acid–water, 6:1:2 by vol. (Nordström and Swain, 1953).
BuHCl	n-butyl alcohol–2N HCl, 1:1 by vol. (upper phase) (Harborne, 1959).
AW1	acetic acid–water, 15:85 by vol. (Hänsel, 1955).
AW2	acetic acid–water, 3:2 by vol. (Hänsel, 1955).
pH	phenol-water, 73:27 by vol. (Jurd, 1962).
EtFW	ethyl acetate–formic acid–water, 10:2:3 by vol. (upper phase) (Hörhammer *et al.*, 1959).
HCl	concentrated HCl–water, 1:99 by vol. (Harborne, 1959).
AHW1	acetic acid–concentrated HCl–water, 15:3:82 by vol. (Harborne, 1959).
AHW2	acetic acid–concentrated HCl–water, 30:3:10 by vol. (Harborne, 1959).
FHW	formic acid–concentrated HCl–water, 5:2:3 by vol. (Harborne, 1959).

Chromatographic spray reagents for location of phenols and carbohydrates on paper chromatograms were:

Partridge reagent (1949)—0.91 ml aniline and 1.66 g phthalic acid dissolved in 100 ml of water-saturated n-butyl alcohol. Papers sprayed with this reagent were air-dried and then heated for 10 min in an oven at 95°C. Carbohydrates give red to brown colors on a light tan background under these conditions.

$AlCl_3$ reagent (Harborne, 1960)—3.5 g of $AlCl_3$ dissolved in 100 ml 95% ethyl alcohol. Papers sprayed with this reagent were air-dried and examined under ultraviolet light (UV).

Ninhydrin reagent (Consden *et al.*, 1944)—0.1 g of ninhydrin dissolved in 100 ml of water-saturated n-butyl alcohol. Papers sprayed with this reagent were air-dried, and heated 5 min in an oven at 90°C.

EXPERIMENTAL PROCEDURES

Pigment extraction. Dried, discolored outer scales were removed. Pigment was then extracted by blending 200–300-g samples of each onion variety for 2 min in a Waring blender with sufficient hot ethyl alcohol to give a final concentration of 80% alcohol. A trace of $CACO_3$ was added to neutralize plant acids. The resultant extract was filtered with suction while hot to give yellow, yellow, and red alcoholic solutions respectively from the Southport Yellow, White, and Red Globe varieties.

Preliminary purification. Each extract was concentrated under reduced pressure at 40°C to remove the alcohol, and the residue taken up in 200 ml of water. To separate the flavonoids from the nonflavonoid constituents, the aqueous solutions were passed over a column of Amberlite IRC-50 (H-form) 40 by 5 cm, and after extensive washing with distilled water the flavonoids were eluted with 1 L of 95% ethyl alcohol. The alcoholic eluates were concentrated to 50 ml under reduced pressure at 40°C. These concentrates, respectively labeled Y, W, and P for Yellow, White and Red Globe varieties, were used for the isolations. The concentrates contained no free amino acids or carbohydrates as shown by paper chromatography in BAW.

Pigment isolation. Twenty-ml portions of concentrates Y, W, and P were each streaked on 24 pieces of Whatman 3MM filter paper 57 by 46 cm. The chromatograms were developed by descending chromatography in a Reco Chromatocab, model A, with solvent AW1. The developed chromatograms were air-dried, and a thin vertical strip was cut from each, sprayed with $AlCl_3$ spray reagent, air-dried, and visualized under UV to locate the pigments present. Using the sprayed strip as a marker the positions of the pigment bands were located on the unsprayed portion of the paper. The unsprayed yellow bands were cut from the paper, and eluted with 80% ethyl alcohol. A reddish band appearing on the chromatogram from P concentrate was eluted with 1% methanolic HCl. The alcoholic eluates were concentrated to a small volume (20–30 ml, depending upon the amount of pigment present), by evaporation under reduced pressure at a temperature of 35–40°C. The yellow flavonoid bands were numbered from the origin to the solvent front. Y concentrate revealed 5 yellow bands labeled Y-1, Y-2, Y-3, Y-4, and Y-5. W concentrate revealed only 4 bands, labeled W-2, W-3, W-4, and W-5, at the same R_f values as the corresponding bands from Y concentrate. P concentrate revealed 5 yellow bands, again at the same R_f values as the corresponding band from Y concentrate. These latter bands were labeled P-1, P-2, P-3, P-4, P-5. There was also present a reddish band visible under daylight conditions which was labeled P-A (Table 1).

Purification of isolated bands. Bands Y-1 and P-1, by repeated chromatography on Whatman 1 paper sheets with four solvents, were found to contain no detectable impurities. The bands were evaporated to dryness under reduced pressure at 35–40°C, and taken up in 15 ml of absolute ethyl alcohol.

Bands Y-2, W-2, and P-2 were contaminated by a yellow phenolic impurity. They were separated from this impurity by streaking each concentrate on 4 pieces of Whatman 3MM paper and developing by descending chromatography with solvent AW2. With this solvent, the impurity moved ahead of the flavonoid band and could be removed by cutting the paper between the impurity and the flavonoid band. The positions of the flavonoid and the impurity were located by viewing the chromatogram under UV. The flavonoid bands were cut from the remainder of the paper and eluted with 80% ethyl alcohol. The eluates were evaporated to dryness under reduced pressure as before, and taken up in 15 ml of absolute ethyl alcohol. Repeated chromatography on Whatman 1 paper with 5 solvents revealed no other impurities present.

Bands Y-3, W-3, and P-3 were purified in the same manner as bands 2. The solvent used for purification was BAW.

Bands Y-4, W-4, and P-4 were purified in the same manner as bands 2. The solvent used for purification was water. R_f values for Y-4, W-4, and P-4 were 0.09, 0.09, and 0.10.

Bands Y-5, W-5, and P-5 were purified in the same manner as bands 2. The solvent used for purification was water. R_f values for Y-5, W-5, and P-5 were 0.26, 0.25, and 0.26.

Band P-A was found to have no impurities present by chromatography in 6 solvents, and therefore was subjected to no further purification treatment.

Characterization of isolated bands. Each isolated band was characterized by calculation of R_f values in selected solvents, hydrolysis and chromatography of aglycone and carbohydrate in selected solvents, calculation of carbohydrate and aglycone ratios, and spectral data under specific conditions.

Table 1. R_f values of isolated products and reference compounds.

Compound	AW1	BAW	Ph	EtFW	BuHCl	HCl	AHW1
P-A	0.33	0.41		0.20	0.28	0.08	0.28
Y-1	.03	.67		.80	.79		
P-1	.03	.67		.80	.78		
Y-2	.11	.44	.40	.53	.66		
W-2	.14	.43	.42	.52	.65		
P-2	.13	.44	.41	.54	.65		
Y-3	.20	.18	.43	.10	.18		
W-3	.21	.18	.43	.10	.17		
P-3	.21	.17	.45	.10	.18		
Y-4	.37	.52	.53	.56			
W-4	.38	.51	.54	.57			
P-4	.38	.52	.54	.57			
Y-5	.58	.38	.48	.23	.52	.28	
W-5	.60	.39	.48	.23	.51	.29	
P-5	.58	.38	.48	.24	.50	.31	
Quercetin	.03	.67	.33	.80	.79		
Isoquercitrin[a]	.38	.55[d]	.53	.55			
Spiraeoside[b]	.13	.46[d]	.40	.56			
Peonidin 3-glucoside[c]		.41[d]			.30	.09	.33

[a] Literature values (Harborne, 1960; Hänsel, 1955).

[b] Literature values (Koeppen and Van der Spuy, 1961; Herrmann, 1956).

[c] Literature values (Harborne, 1959).

[d] The solvent actually used was BAW (4:1:5), upper phase. The values are roughly comparable.

Determination of R_f values. The purified bands were chromatographed on Whatman 1 paper by descending chromatography, along with quercetin for reference purposes. Solvents used were AW1, BAW, Ph, EtFW, BuHCl, HCl, and AHW1. Spot positions were noted by examining the dried chromatogram under UV light before and after spraying with the $AlCl_3$ reagent. Results are given in Table 1. P-A was visible in daylight as a rose-colored band.

Hydrolysis and chromatography of aglycone and carbohydrate. The yellow flavonoid materials were hydrolyzed by refluxing 2 ml of sample and 2 ml of 2*N* H_2SO_4 for 2 hr (Hänsel, 1955). The hydrolysates were cooled and extracted 3 times with 5-ml portions of ethyl acetate. The combined ethyl acetate portions, containing the aglycones, were evaporated to dryness under reduced pressure at 20°C, and the residues taken up in 1 ml of 95% ethyl alcohol. Chromatography on Whatman 1 paper, with quercetin for comparative purposes, was carried out with BAW, AW2, and AHW2 solvents (Table 2). The aqueous portions, containing the carbohydrates, were neutralized by passage over a column of Amberlite IR-4B (bicarbonate form) (20 by 3 cm). The neutralized solutions were evaporated to dryness under reduced pressure at 40°C, and the residues taken up in 10 drops of distilled water. The resultant solutions were chromatographed on Whatman 1 paper with BAW by descending chromatography for 72 hr. Standard carbohydrates were included for comparative purposes and the results are calculated relative to glucose (Table 2).

A 5-ml portion of the P-A band was evaporated to dryness under reduced pressure at 20°C and the residue taken up in 5 ml of distilled water. An equal volume of concentrated H_2SO_4 was added and the solution was heated for 3 min on a steam bath. After cooling, 5 ml of isoamyl alcohol was shaken with the hydrolysate to remove the colored aglycone. The aqueous portion, containing the carbohydrate material, was treated as above. The isoamyl alcohol portion was used directly for chromatographic identification of the aglycone (Table 2).

Carbohydrate-aglycone ratios. Hydrolysis was carried out as above. The hydrolysate was cooled and diluted to 25 or 50 ml in a volumetric flask. The concentration of aglycone was determined by measuring the absorbance at 375 mμ in a Spectronic 20 colorimeter and calculating the concentration in micromoles by reference to a standard curve of quercetin treated in the same way (Pratt, 1962). The carbohydrate concentration in micromoles was measured using the phenol-H_2SO_4 method on a 1-ml portion of the solution (Whistler and Wolfrom, 1962). The absorbance was again read in a Spectronic 20 colorimeter against a standard

Table 2. R_f and $R_{glucose}$ values of hydrolysis products of isolated bands.

Compound	Aglycone-R_f				Carbohydrate-$R_{glucose}$
	BAW	AW2	AHW2	FHW	BAW
P-A	0.70		0.66	0.29	1.74
Y-2	.65	0.36	.43		0.98
W-2	.67	.34	.42		0.97
P-2	.67	.35	.43		0.98
Y-3	.67	.34	.43		1.05
W-3	.67	.33	.43		1.04
P-3	.67	.33	.43		1.00
Y-4	.67	.37	.43		0.98
W-4	.67	.37	.43		0.98
P-4	.67	.37	.43		0.96
Y-5	.65	.33	.45		1.02
W-5	.67	.33	.45		1.01
P-5	.67	.35	.45		1.02
Quercetin	.67	.36	.43		
Peonidin[a]	.70		.63	.30	
Glucose					1.00
Arabinose					1.78
Xylose					2.02
Galactose					0.85
Fructose					1.33
Rhamnose					3.88

[a]Literature values (Harborne, 1959).

curve prepared by treating glucose in the same fashion. The results are listed in Table 3.

Spectral data (Jurd, 1962). The spectral curves for the flavonoids were measured in alcoholic solution, using a Beckman DB spectrophotometer, in the range from 220 to 500 mμ. The P-A band was measured in 1% methanolic HCl solution from 450 to 600 mμ. The flavonoids showed two peaks—the first in the 300–400-mμ range (peak I) and the second in the 250–300-mμ range (peak II). The P-A band showed a peak in the 500–550-mμ range. Spectral shifts were measured 5 min after: a) addition of 5 drops of 3.5% alcoholic $AlCl_3$ solution; b) addition of fused $NaC_2H_3O_2$ to saturation; c) addition of H_3BO_3 to 1/5 saturation, and fused $NaC_2H_3O_2$ to saturation; d) addition of 5 drops of 2*N* NaOH solution (Table 4).

Table 3. Carbohydrate-aglycone ratio of hydrolysates of isolated bands.

Hydrolysate	Carbohydrate	Aglycone
Y-2	1.1	1.0
W-2	0.8	1.0
P-2	0.8	1.0
Y-3	2.0	1.0
W-3	1.9	1.0
P-3	2.2	1.0
Y-4	1.3	1.0
W-4	0.8	1.0
P-4	1.2	1.0
Y-5	2.0	1.0
W-5	1.8	1.0
P-5	2.1	1.0

RESULTS AND DISCUSSION

Bands Y-1 and P-1. R_f values coincide with those of quercetin, and hydrolysis did not change these values. The spectral data coincide with those of quercetin. Y-1 and P-1 are therefore identified as quercetin. These compounds were present in only trace amounts, and therefore could be artifacts resulting from hydrolysis of glycosides during the separation and purification procedures.

Bands Y-2, W-2 and P-2. These are the major flavonoid constituents present. Their R_f values agree very well with the recorded values of spiraeoside. The aglycone is quercetin, the carbohydrate is glucose, and the carbohydrate-aglycone ratio is essentially 1:1. The stability of peak I in NaOH solution shows that position 3 or 4′ does not contain a free hydroxyl group. The high bathochromic spectral shift after the addition of $AlCl_3$, as well as the lack of a bathochromic shift after the addition of $NaC_2H_3O_2$ and H_3BO_3, indicates that the 3-position contains a free hydroxyl group, while the 4′-

Table 4. Spectral data for isolated bands and reference compounds.

Band	λ_{max} (mμ)									
			$AlCl_3$		$NaC_2H_3O_2$		$NaC_2H_3O_2$ H_3BO_3		NaOH	
	I	II	I	II	I	II	I	II	I	II
Y-1	370	257	427	265	385	267	389	265	dec.	
P-1	370	256	426	266	383	268	384	264	dec.	
Y-2	366	253	421	262	390	269	368	253	435	282
W-2	363	254	423	263	382	274	372	255	438	278
P-2	365	254	423	263	380	270	370	255	432	281
Y-3	360	255	415	263	358	257	358	254	420	260
W-3	358	252	415	262						
P-3	357	256	415	261	355	259	355	257	421	259
Y-4	365	255	405	275	371	280	375	278	405	278
W-4	360	252	405	273						
P-4	363	255	402	272	373	273	378	283	404	278
Y-5	354	263	388	270	363	270	356	270	380	280
W-5	354	258	388	265	360	273	357	269	378	278
P-5	353	263	390	274	362	270	358	272	380	280
P-A	523		523							
P-A aglycone	533		533							
Quercetin	372	257	423	265	387	269	386	264	dec.	
Isoquercitrin[a]	363	259	420	275	390	275			413	275
Spiraeoside[a]	365	255	423	265	386	276	367	253		
Peonidin[a]	532		532							
Cyanidin[a]	535		555							
Peonidin 3-glucoside[a]	523		523							

[a] Literature values (Jurd, 1962).

position is blocked. These bands are therefore spiraeoside (quercetin-4′-glucoside).

Bands Y-3, W-3 and P-3. The hydrolysis data indicate that the aglycone is quercetin and the carbohydrate is glucose. There is a carbohydrate-aglycone ratio of 2:1. The positions of the carbohydrate on the flavonoid nucleus were determined from the spectral data. The large bathochromic spectral shift after the addition of $AlCl_3$ is indicative of free 3- and 5-hydroxyl groups, as is the color of unsprayed chromatogram under UV illumination. The lack of a bathochromic shift after the addition of $NaC_2H_3O_2$ and H_3BO_3, as well as the stability of peak I in NaOH solution, means that the 4′-position does not contain a free hydroxyl group. The small hypsochromic shift of peak I and the lack of a bathochromic shift of peak II after addition of fused $NaC_2H_3O_2$ are typical of a blocked 7-hydroxyl group. These compounds, then, are in all probability quercetin-4′,7-diglucoside. Herrmann (1956) reported the presence of a quercetin glycoside in onions, and his R_f values in solvent AW1 are the same, but slightly different in BAW. He used, however, the upper phase of a solvent ratio of 4:1:5 by volume, while the values in this paper are the result of a solvent ratio of 6:1:2. This 6:1:2 ratio is only an approximation of the organic phase of the 4:1:5 ratio and could account for the difference in R_f values.

Bands Y-4, W-4 and P-4. The R_f values agree very well with recorded values for isoquercitrin, as do the hydrolysis chromatographic data and carbohydrate-aglycone ratio. Unsprayed chromatograms under UV illumination give a dark spot for these compounds, which is indicative of a blocked 3-hydroxyl group. Spectral stability in NaOH solution is again indicative of a blocked 3-hydroxyl group or a blocked 4′-hydroxyl group. The compound is not spiraeoside, and therefore must be isoquercitrin (quercetin-3-glucoside). These materials are present in only trace amounts and, again, could possibly be artifacts resulting from the isolation and purification procedures. It is interesting that no quercimeritrin (quercetin-

7-glucoside) was found, which would be expected from random partial hydrolysis of the quercetin diglycosides present.

Bands Y-5, W-5 and P-5. The carbohydrate-aglycone ratio and the chromatographic data of the hydrolysis products indicate a quercetin diglucoside. The color of unsprayed chromatograms containing these compounds is characteristic of a blocked 3-hydroxyl group. The spectral data of 34–37 bathochromic shifts after addition of $AlCl_3$ support this contention. The other glucose residue is probably on the 3′-position or 4′-position as shown by the lack of a bathochromic shift after addition of $NaC_2H_3O_2$ and H_3BO_3. A partial hydrolysis of bands Y-5 and P-5 gave spots with R_f values identical to quercetin-3-glucoside, quercetin-4′-glucoside, and quercetin as well as unchanged Y-5 and P-5. The R_f values are those reported by Herrmann (1956) for one of his unknown quercetin glucosides. Very likely, then, bands 5 are quercetin-3,4′-diglucoside. Bands 5 are present in an amount only a little less than bands 2, the major flavonoid pigment.

Band P-A. When the dried chromatogram was fused with NH_3 or on adding base to the methanolic solution, band P-A turned blue, a behavior characteristic of anthocyanins. R_f values of aglycone and carbohydrate correspond to those expected of a cyanidin or peonidin arabinoside. Spectral data identify it as a peonidin arabinoside, there being no bathochromic shift of the aglycone peak after the addition of $AlCl_3$. The R_f values of P-A are very close to those reported in Harborne (1959) for peonidin-3-glucoside, but differ greatly from those for peonidin-3-diglucoside or -3,5-diglucoside. P-A is therefore considered to be peonidin-3-arabinoside.

The absolute amounts of flavonoids are greatest in the Yellow Globe variety, followed closely by the Red Globe. The White Globe has much smaller quantities present. Within each species the major flavonoid is quercetin-4′-glucoside, closely followed by quercetin-3,4′-diglucoside. Quercetin-4′,7-diglucoside is present in relatively small amounts, and only traces of quercetin-3-glucoside and quercetin are found. Peonidin-3-arabinoside is a major constituent in the Red Globe variety.

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L. J. MINOR, A. M. PEARSON, L. E. DAWSON, and B. S. SCHWEIGERT
Department of Food Science, Michigan State University
East Lansing, Michigan 48823

Chicken Flavor: the Identification of Some Chemical Components and the Importance of Sulfur Compounds in the Cooked Volatile Fraction

SUMMARY

Meat and water slurries of both leg and breast muscle from heavy hens were cooked in a nitrogen atmosphere. Some of the chemical components in the volatile fraction were identified by solubility classification, derivative preparation, and/or functional group analysis in combination with gas chromatography and/or qualitative chemical analyses and odor evaluation. Twenty-nine compounds in the volatiles from leg muscle and 25 compounds from breast muscle were identified by the functional-group trapping technique followed by gas chromatography of the effluent fractions. Qualitative chemical tests revealed 19 major classes of compounds and a few specific compounds.

Removal of sulfur compounds resulted in an almost complete loss of "meaty odor" in both dark and light meat. Removal of the carbonyls from the volatile fraction resulted in a loss of "chickeny-flavor" and intensification of the "meaty or beef-like odor."

INTRODUCTION

Bouthilet (1950, 1951a,b) made the first attempts to isolate and identify the components responsible for chicken flavor by using a high-vacuum distillation technique followed by either solvent extraction or an ion-exchange treatment. Pippen *et al.* (1958) and Pippen and Nonaka (1960) made extensive identifications of the carbonyl compounds in the volatiles from cooked chicken by identifying their 2,4-DNPHS (2,4-dinitrophenylhydrazine derivatives). Although hydrogen sulfide has been identified in chicken skin, muscle, and broth (Sadikov, 1934; Bouthilet, 1951b; Pippen and Eyring, 1957; Kazeniac, 1961), it was not until recently that Mecchi *et al.* (1964) demonstrated that cystine and/or cysteine in the glutathione and protein fraction of muscle served as the precursor(s) for hydrogen sulfide, which was released on heating muscle. Pippen and Nonaka (1963) recently demonstrated that gas chromatography can be used advantageously for separation and identification of the volatile fraction from cooked chicken.

The present study was made to identify the chemical constituents in the volatile fraction of both leg and breast meat from cooked chicken using a solubility classification method, a trap-reaction technique for functional group analysis, and chemical tests in combination with gas chromatography and odor evaluation.

MATERIALS AND METHODS

Chickens. Twenty-five Cornish cross hens were obtained immediately after slaughter from a known commercial source. The average eviscerated weight of each bird was 6 lb. After the giblets, kidney fat, and skin were removed, the bones, tendons, cartilage, and veins were cut away to give a careful separation of light and dark muscle. The two kinds of muscle were ground, weighed, and vacuum-packaged separately in 100-g portions in 4 × 4-in. Cryovac bags. Then the packages were quick-frozen at −34°C in a blast freezer and stored at −23°C until used.

Cooking and distillation. A slurry consisting of 2 kg of chicken meat and 3 L of hot deionized-distilled H_2O was heated in a 12-L flask under reflux as 40 ml/min of pure nitrogen gas was passed through the system to give the "oxidation-inhibiting conditions" as outlined by Pippen *et al.* (1958). A perspective view of the cooking and distillation apparatus is shown in Fig. 1.

Fractionating and trapping apparatus. An original radial-manifold stream-splitter (Fig. 2) was designed for simultaneously splitting the total volatile stream into 8 or fewer separate fractions. Large absorption traps containing 25 ml of reagent were used for the solubility classification of the volatiles and also for the preparations of sulfur and carbonyl derivatives (Fig. 2). The small traps (Fig. 2) were used for functional group analysis and as secondary traps for solubility classification or derivative-preparation reactions. A 1-L round-bottom flask containing 300 ml of deionized-distilled H_2O, serving as a water trap, was connected between the reflux condenser and the radial-manifold splitting device as shown in Fig. 3.

Solubility classification of compounds. The

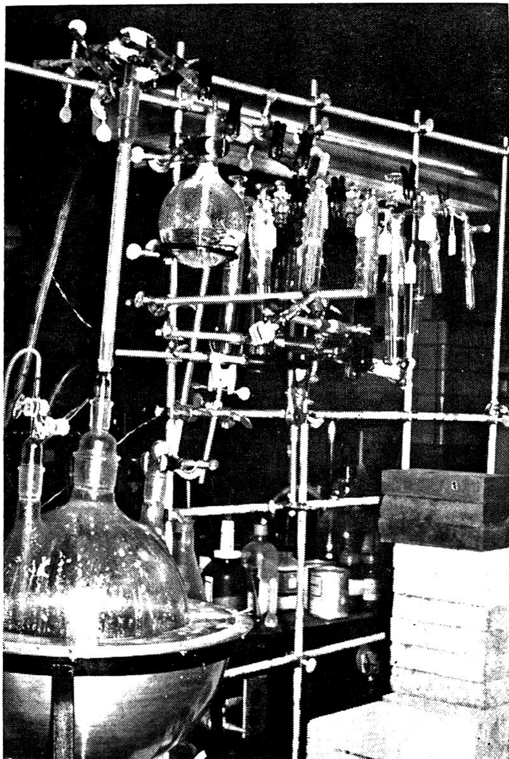

Fig. 1. Perspective view of cooking-distillation apparatus.

solubility classification system of Cheronis and Entrikin (1961) was used in all runs for both light and dark muscle, except that the ether solvent trap that they describe was not used. Table 1 summarizes the solubility classification method used.

Preparation of carbonyl and sulfur derivatives. The carbonyls were trapped in 2,4-DNP (dinitrophenylhydrazine solution) as outlined by Pippen *et al.* (1958). Derivatives of sulfides, disulfides, and mercaptans were prepared by the methods of Challenger and Rawlings (1937), Hasselstrom (1957), and Folkard and Joyce (1963). Three series of three traps each were used for trapping the sulfur compounds in the volatile fraction. In each series, one large and two small traps were used (Fig. 2). The first series of traps was for mercaptans. The large trap contained 25 ml of mercuric cyanide (4% w/v), and each small trap contained 3 ml mercuric chloride (3% w/v). In the second series, which was for sulfides and disulfides, the large trap contained 25 ml of mercuric chloride (3% w/v), and each small trap contained 3 ml of mercuric cyanide (4% w/v). In the third series, which was for hydrogen sulfide and methyl mercaptan, the large trap contained 25 ml of bismuth nitrate reagent (Koren and Gierlinger, 1953), and each of two small traps contained 3 ml of mercuric acetate (4% w/v) as described by Martin *et al.* (1962). Each series of traps was coupled to one of the eight 12/5 ball outlets leading from the radial-manifold stream-splitter.

Qualitative chemical tests. A total of 8 reagent traps surrounding the radial manifold stream-splitter were used (Fig. 2). Of these traps, four plus the water trap were used for solubility classification of the volatiles, while the remaining traps were used for preparation of carbonyl or sulfur compound derivatives from the cooked volatiles. To facilitate qualitative chemical testing, eight auxiliary traps, each containing 10 ml of distilled H_2O, were connected to the trap outlets, and the emerging water-soluble volatiles were collected and tested by procedures described by Cheronis and Entrikin (1961). Tests for mono- and poly-carbonyl derivatives were made on the 2,4-DNPH precipitate by the methods of Pippen *et al.* (1958) and Pippen and Nonaka (1960).

Sulfur compounds were recovered as their mercuric chloride and mercuric cyanide derivatives

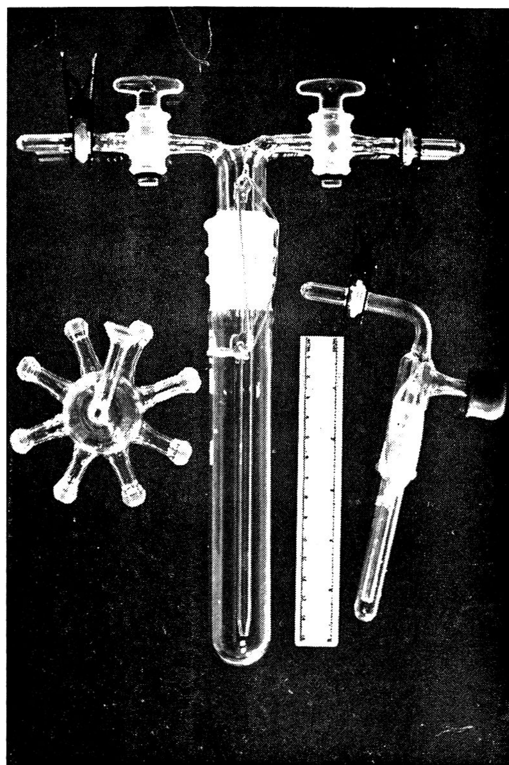

Fig. 2. (L to R) Radial-manifold stream-splitter. Large absorption-trap sealed with 12/5 ball and socket plugs. A 12-cm rule. Small absorption trap with surgical seal and 12/5 plug in place to retain volatiles for gas chromatographic analysis.

Table 1. Solubility classification method.

Division	Reagent(s)	Reagent(s) volume (ml) Primary trap	Secondary trap(s)	Classifying function of reagent
S_2[a]	H_2O	300	....	Removed H_2O soluble compounds
B	1.2N HCl	25	....	Removes amines
A_2	2.5N $NaHCO_3$:: 2.5N NaOH	25	(2) 3 ml ea.	Removed compounds insoluble in H_2O and 2.5N $NaHO_3$ but soluble in 2.5N NaOH
A_1	1.5N $NaHCO_3$:: 2.5N NaOH	25	(2) 3 ml ea.	Removed compounds insoluble in H_2O and 1.5N $NaHCO_3$ but soluble in 2.5N NaOH
N	Conc. H_2SO_4	25	....	Removed compounds insoluble in H_2O but soluble in conc. H_2SO_4

[a] According to Cheronis and Entrikin (1961).

(Hasselstrom, 1957). The mercuric cyanide and mercuric chloride derivatives were collected during 6 separate runs for both dark (leg) and light (breast) meat. The black precipitate from the cyanide trap and the white precipitate from the chloride trap were recovered by filtering, washed, and dried. Attempts to purify these derivatives by recrystallization from hot water, ethanol or ethyl acetate failed to yield material with a sufficiently sharp melting point for identification, which Challenger (1959) had previously reported to be difficult. Thus, identification was achieved by releasing the sulfur-containing material as volatiles by using the acid or alkaline decomposition methods of Self *et al.* (1963) and Folkard and Joyce (1963). This was accomplished by adding 2 ml of either 8N HCl or 10% NaOH to 0.2-g portions of the dry derivatives in a 1 × 4-cm reaction trap. Tests for disulfides and mercaptans were made using absorption trains containing the reagents described by Folkard and Joyce (1963) or the sodium borohydride method of Stahl and Siggia (1957). Hydrogen sulfide and methyl mercaptan were identified by the method of Marbach and Doty (1956).

Additional evidence for the presence of mercaptans, sulfides, and disulfides was obtained by subjecting the decomposition products of the mercuric derivatives to gas chromatography. This was accomplished by decomposing 50 mg of derivatives (25 mg of mercuric chloride and 25 mg of mercuric cyanide derivatives) in a small trap (Folkard and Joyce, 1963). The washed volatiles were collected in a 1 × 4-cm trap (Fig. 2) at −196°C. Samples were then subjected to gas chromatography, and tentative identifications achieved by comparing retention times with those for authentic samples of sulfides, disulfides, and mercaptans.

Functional-group analysis. The syringe reaction technique of Hoff and Feit (1963), the head-space gas analysis method of Bassette *et al.* (1962), and the vial technique of Walsh and Merritt (1960) were modified to give a trap reaction technique for functional group analysis. The functional group reagents that were used included basic and acidic hydroxylamine (Bassette *et al.*, 1962); mercuric chloride and mercuric cyanide (Folkard and Joyce, 1963); saturated potassium permanganate, sodium borohydride solution, sodium nitrite solution, acetic anhydride solution with additional treatment in a secondary trap containing sodium bicarbonate solution, hydrogen chloride solution, and concentrated sulfuric acid solution (Hoff and Feit, 1963); and a deionized-distilled H_2O control.

In the present study, the volatiles collected during 36 hr of cooking distillation were passed through each of the different traps, which contained 2–3 ml of an appropriate functional group reagent. Two cold traps (1 × 6-cm ID) were then connected to the functional group-reagent trap in series (Fig. 4). The first trap was immersed in ethanol-dry ice (−80°C) and the second in liquid nitrogen (−196°C). Thus, the cooked volatiles were passed through the functional-group reagent, the ethanol-dry ice, and the liquid nitrogen traps in that order at a gas flow rate of 10 ml/min for 30 min (Fig. 4). About 1 ml of liquid nitrogen accumulated in the liquid nitrogen trap together with the condensed volatiles. Then, after 30 min,

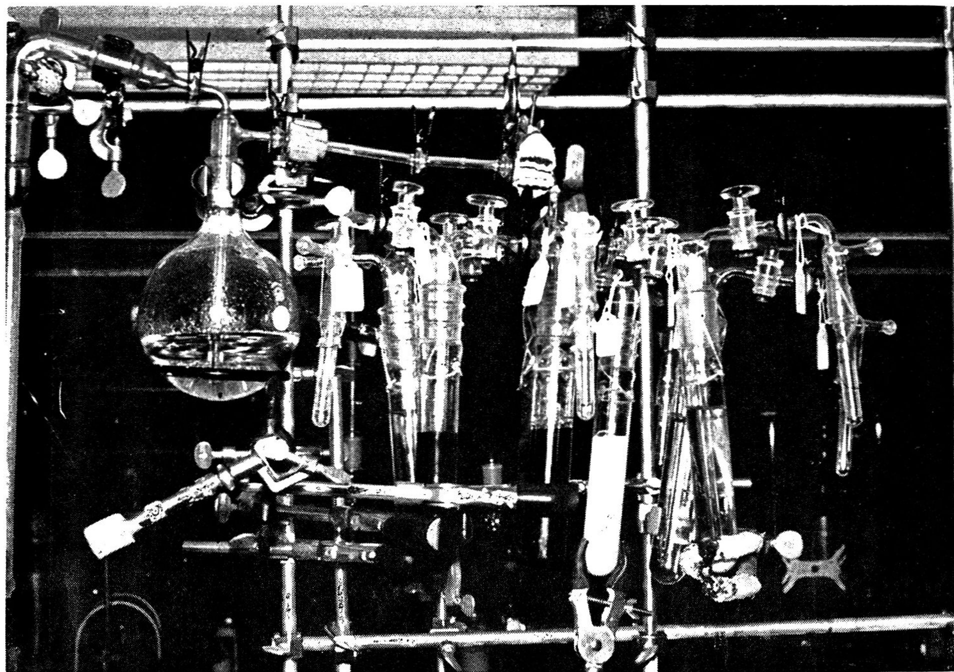

Fig. 3. Closeup view of water trap and a cluster of 8 reagent traps surrounding the radial-manifold stream-splitter. Small auxiliary traps are also shown.

the accumulated liquid nitrogen and other low-boiling constituents, such as hydrogen sulfide, and possibly some other low-molecular-weight compounds which had condensed in the liquid nitrogen trap, were eliminated by prewarming the trap slightly from −196 to −140°C and immersing it in a 2,2,4-trimethylpentane-ethanol-dry ice bath. Release of these gases relieved the pressure so that the trap could then be warmed to room temperature prior to sampling for gas chromatography without disturbing either the trap joints or seals shown in Fig. 2. A 2-ml sample of the gases was then removed from the trap by inserting a gas-tight syringe through the rubber seal (Fig. 2). The sample was injected into the gas chromatograph. All chromatograms were made under the same conditions, using temperature programming from 100 to 250°C at 15°C/min rise in temperature and a chart speed of 80 sec/in.

Control samples of the total cooked volatile fractions from thigh and breast muscle were taken in like manner. However, two different methods were tested in taking the control samples. In the first method, the volatile fraction was passed through the water trap and then trapped in liquid nitrogen, and the effluent was tested. In the second method, the water trap was replaced by an identical flask containing 100 g of anhydrous sodium sulfate in place of the 300 ml of water. The system was then purged with nitrogen gas at a flow rate of 100 ml/min for 4 hr, while leaving open 1 of the 8 outlets leading from the radial-manifold. This was the same 12/5 outlet to which each of the functional-group analysis reagent traps was attached in turn. Since the water-trap technique resulted in better separation on the chromatograms, it was adopted for all subsequent studies.

Gas chromatography. An F & M gas chromatograph (F and M Scientific Corp., Avondale, Penn.), model 500, was used. Although the chromatograph was equipped with both thermal-conductivity and flame-ionization (Model 1609) detectors, only the hydrogen flame detector was used. A ⅛-in.-OD × 8-ft coiled copper column was packed with 15% Apiezon L on 60-80-mesh Chromosorb W. Before use, the column was preconditioned at 250°C for 72 hr. Prior to each run the column was again conditioned at 150°C for 16 hr or until the signal response from the column gave a steady baseline.

The block heater was set at 200°C, and the injection port at 100°C. Air pressure to the hydrogen flame detector was held at 6 lb, hydrogen at 2 lb, and helium at 50 lb to maintain a constant flow rate of 55 ml/min. A sight gauge indicator showed any fluctuations in the flow of helium, hydrogen, and air. Limits of flow rate were established on the 3 scales and verified by tests with known compounds—redistilled acetone and methanol. Vapor samples of 0.5 ml were taken above

the liquid level of these solvents with a gas-tight syringe.

The gas chromatograph was operated in its most sensitive range. The attenuator was set on 8 at the start of each run. The chart speed was 80 sec/in. The temperature programmer was set to provide a 15°C/min increase in column temperature. A starting temperature of 100°C and a final temperature of 250°C were used.

Control chromatograms of the total cooked volatile fractions of either thigh or breast muscle were compared with the corresponding chromatogram which resulted when the volatiles were passed through any particular functional-group reagent. Thus, changes in chromatograms as shown by the elimination or partial elimination of peaks or the production of new peaks were studied. Since temperature programming was uniformly maintained at 100–250°C at an increase of 15°C/min for all control and test samples, peak changes were noted by superimposing the control on the unknown chromatogram. Retention times were then measured and compared with those of authentic known compounds from commercial sources.

Organoleptic evaluation. Organoleptic evaluations were made on the volatiles both before and after passing them through the various reagent traps. This permitted the judges to note any changes in the characteristic odor when any particular class of compound was removed from the volatile fraction. The odor evaluations were made on the untreated cooked volatiles from both leg and breast muscle as they emerged from the reflux condenser. Evaluation of the odors after chemical treatment was accomplished on the volatiles in the auxiliary traps, each of which contained 10 ml of deionized-distilled H_2O and was attached to one of the 8 traps, which surrounded the radial manifold stream splitter (Fig. 2) and removed various classes of compounds by chemical treatment. All results were based on six separate runs using dark meat and an equal number on light meat. A panel of 7 judges was used throughout and utilized an open-session discussion of the flavor-profile procedure as described by Nelson (1960). Since all judgments were subjective, the data were unsuitable for statistical analysis.

RESULTS AND DISCUSSION

Derivative yields. The yield of 2,4-DNPHS was small, and appreciable quantities were not evident until about 4 hr after cooking-distillation began. In contrast, yields of mercuric cyanide and mercuric chloride derivatives were good. Formation of the sul-

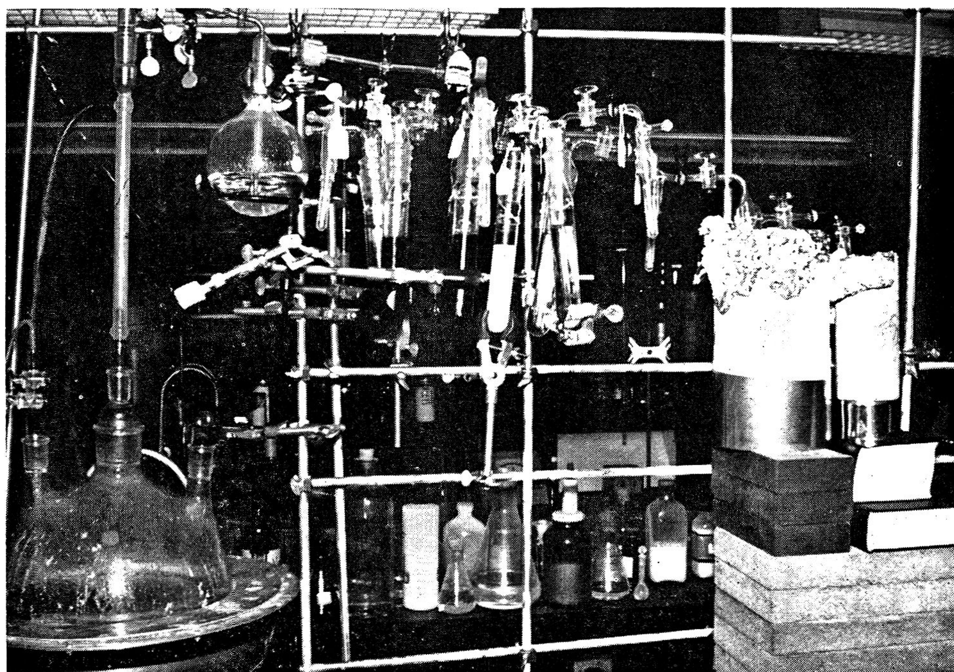

Fig. 4. Closeup of cooking-distillation apparatus for trapping cooked chicken volatiles in ethanol-dry ice (−80°C) and liquid nitrogen (−196°C) with the traps connected in series to a small trap containing a functional group reagent, which is in turn connected to a 12/5 outlet from the radial-manifold stream-splitter.

fur derivatives in the mercuric cyanide and mercuric chloride traps was noted within ½-hr after cooking-distillation began. Results were similar in the bismuth nitrate trap. Sulfur derivatives were formed in large quantities and continued to be evolved as long as cooking-distillation was continued. Results confirm those of Mecchi *et al.* (1964), who found that large quantities of hydrogen sulfide were released when chicken was cooked, and of Yueh and Strong (1960), who observed that sulfur components were released continuously when beef was cooked.

Qualitative chemical tests. The qualitative chemical tests are summarized in Table 2. A total of 19 classes of compounds and a few individual compounds were found to be present in cooked chicken volatiles. These included nitro-sulfonic acid, ethanol, ethanal, methyl ketones, aldehydes, mono-, di- and polybasic acids, enols, phenols, sulfinic acid(s), ammonia, amines, mercaptans, alcohols, esters, hydrogen sulfide, organic sulfides, and disulfides.

Disulfides and mercaptans were identified as their respective 2,4-dinitrophenyl ethers by the method of Folkard and Joyce (1963). The identification of disulfides was confirmed by treatment with sodium borohydride according to the method described by Stahl and Siggia (1957). The method of Marbach and Doty (1956) gave a positive test for hydrogen sulfide, which was confirmed by exposing lead acetate paper to the stream of volatiles with the resulting black precipitate. Thus, the presence of hydrogen sulfide confirmed results of several earlier workers as recently summarized by Mecchi *et al.* (1964).

Table 2. Results of qualitative tests based on the solubility classification method[a] and sulfur and carbonyl component tests by indicated methods.

Division or compound tested	Reagent or method	Result	Major class(es) detected
S_2	ferrous hydroxide test	+ (red-brown ppt)	Nitro-sulfinic acid
	sodium hyporodite	+ (yellow ppt)	Ethanol, ethanal, methyl ketones
	cupric ions	+ (yellow-red)	Aldehydes
	iodate-iodine starch	+ (blue)	Acids (dibasic, polybasic)
	bromine in H_2O etc.	+ (tan to blue)	Enols and phenols
	ferric chloride test	+ (red)	Sulfonic acid
B	ferricyanide	+ (green-blue)	Amines
A_1	iodate-iodide starch	+ (blue)	Acids
	ferric chloride test	+ (red)	Sulfonic acids
A_2	iodate-iodide starch	+ (blue)	Acids
	1-Cl-2,4-DNB	+	Mercaptans
N	iodic acid	+ (brown)	Alcohols
	nitrochromic acid	+ (blue)	Alcohols
	ferric chloride hydroxylamine	+ (blue-red)	Esters
Sulfur	Bismuth nitrate reagent, p-phenylanediamine (Marbach and Doty, 1956)	+ (blue)	Hydrogen sulfide Sulfides
	Folkard and Joyce (1963)	+ (black ppt)	Mercaptans
	Stahl and Siggia (1957)	+ (ppt)	Disulfides
	Folkard and Joyce (1963)	+ (white ppt)	Sulfides
Carbonyls	2,4-DNP reagent (Pippen *et al.*, 1958)	+ (ppt)	Mono- and poly-carbonyls

[a] According to Cheronis and Entrikin (1961).

Nine peaks were obtained by gas chromatography of the washed volatiles from acid or alkaline decomposition of mercuric chloride and mercuric cyanide derivatives. The principal compounds tentatively identified in the mixed mercuric sulfur derivative complex included dimethyl sulfide, ethyl mercaptan, methyl-ethyl sulfide, n-propyl mercaptan, diethyl sulfide, methyl disulfide, ethyl-n-propyl sulfide, di-n-propyl sulfide and n-hexyl mercaptan (Table 3 and Fig. 5). The absence of methyl mercaptan may mean that its high vapor pressure could cause instantaneous escape when the reaction trap was being sealed following addition of acid to the mercuric sulfur derivatives (a mercaptan odor was evident at that time). It is possible that altering the technique might have resulted in recovery of this compound. A small preliminary peak (Fig. 5) may have been due to methyl mercaptan, since the observed retention time of about 30 sec corresponded closely to that of an authentic sample. However, the response noted for the preliminary peak was not adequate to be used for identification.

It is realized that identification by gas chromatography with only a single column packing is only tentative. However, the identification in this case is more positive,

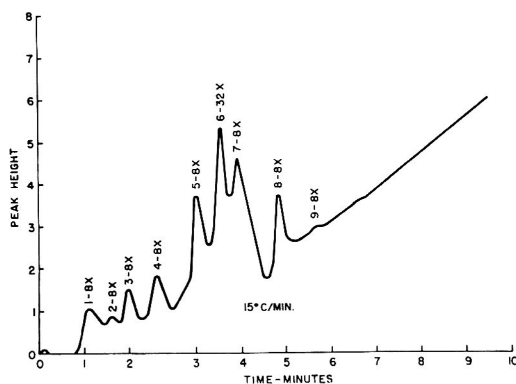

Fig. 5. Monolinear temperature-programmed separation of 5 ml of volatiles from acid decomposition of mercuric cyanide and mercuric chloride derivatives of cooked chicken volatiles. (Detector, flame ionization; column, ⅛-in.-OD × 8-ft coiled copper; packing, 15% Apiezon L on 85% of 60 to 80 mesh Chromosorb W; temperature range, 100 to 250°C; helium pressure, 50 psi; attenuator at 8, range 1; chart speed, 80 sec/min; temperature programmer set @ 15°C/min; F and M, model 500).

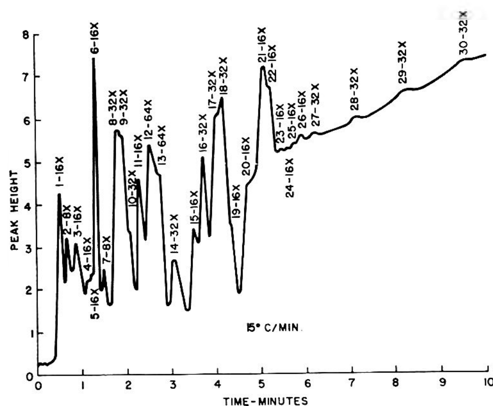

Fig. 6. Nonlinear temperature-programmed separation of 2 ml of total cooked volatiles from heavy hen leg muscle. (Detector, flame ionization; column ⅛-in.-OD × 8-ft coiled copper; packing, 15% Apiezon L on 85% of 60 to 80-mesh Chromosorb W; temperature range, 100 to 250°C; helium pressure, 50 psi; attenuator at 8, range 1; chart speed, 80 sec/in.; temperature programmer set @ 15°C/min; F and M, model 500).

since all compounds present in the mercuric cyanide and chloride derivative complex would be sulfur compounds and thus separation more complete.

Functional group analysis. A gas chromatogram of the total cooked volatiles from heavy hen leg muscle is shown in Fig. 6. Chromatograms obtained after passing the total cooked volatile fraction through the traps (Fig. 4) containing specific functional group reagents were compared with control chromatograms and also with chromatograms of known compounds. Results of these comparisons are summarized in Fig. 7.

Fig. 6 shows that 30 peaks were obtained in the control chromatogram of the total cooked volatile fraction from heavy hen leg muscle. Fig. 7 shows the tentative identification made by functional group analysis followed by gas chromatography. Six sulfides, 3 mercaptans, and 2 disulfides were tentatively identified, totaling 11 sulfur compounds. In addition, 3 aldehydes and 4 ketones were identified, totaling 7 carbonyl compounds. There were 2 alkanes, 1 amine, and 1 alcohol-amine, totaling 2 amines. There were 6 alcohols and acetylmethylcarbinol, totaling 7 alcohols. There were no esters, and 3 unidentified compounds. Thus, the principal components of the cooked volatile fraction from dark muscle were tenta-

PEAK NO.	PEAK HEIGHTS HEAVY HEN LEG MUSCLE	FUNCTIONAL-GROUP REAGENT'S EFFECT										FUNCTIONAL GROUP	TENTATIVE IDENTITY
		ACIDIC NH_2OH	BASIC NH_2OH	$KMnO_4$	$HgCl_2$	$Hg(CN)_2$	$NaNO_2$	$NaBH_4$	$(AcO)_2C$	HCl	H_2SO_4		
1	16×*-4.3											R-H	ETHANE PROPANE
2	8×-3.2	□	□	□			□		□			R-SH	METHYL MERCAPTAN
3	16×-3.1			■				□	╱			RR'-CO	ACETONE
4	16×-2.2	╱	╱	╱□			□	╱	□			R-OH	METHANOL
5	16×-2.3	╱	╱	□					□			R-S-R	DIMETHYL SULFIDE
6	16×-7.4	╱	╱	□					□	╱		R-S-R'	METHYL ETHYL SULFIDE
7	8×-2.5						□		□			$R-NH_2$	METHYLAMINE
8	32×-5.7	╱	╱	□				╱	□	╱		R-S-R' R-OH	DIETHYL SULFIDE ETHANOL
9	32×-5.6							□	╱□			R-CHO	ACETALDEHYDE
10	32×-3.4	╱	╱	□					□	╱		R-S-R'	METHYL ISOPROPYL SULFIDE
11	16×-4.6		╱	╱□			□	□	□			R-CO-CO-R'	2,3-BUTANEDIONE
12	64×-5.4	╱□	╱					□	□	╱		R-S-S-R'	METHYL DISULFIDE
13	64×-4.7			╱				□	╱			R-CO-CHOH-R'	ACETOIN
14	32×-2.7	╱	╱	□					□	╱		R-S-R'	ETHYL-N-PROPYL SULFIDE
15	16×-3.4	╱□	╱	╱				□	□	╱		R-S-S-R'	ETHYL DISULFIDE
16	32×-5.1	╱	╱	╱								×	×
17	32×-6.1	□	□	□			□		□			R-SH	ETHYL MERCAPTAN SYM. TRITHIANE
18	32×-6.5	╱	╱	□						╱		R-S-R'	DIPROPYL SULFIDE
19	16×-3.4	□	□	□			□		□			R-SH	N-PROPYL MERCAPTAN
20	16×-4.4							□	╱□			R-CHO	N-HEXANAL
21	16× 7.2			■				□	╱			RR'-CO	2,4-PENTANEDIONE
22	16×-6.7	╱	╱	╱□			□	╱	□			R-OH	ISO-AMYL ALCOHOL
23	16×-5.3	╱	╱	╱□			□	╱	□			R-OH	N-AMYL ALCOHOL
24	16×-5.3							□	╱□			R-CHO	N-HEPTANAL
25	16×-5.4	╱	╱	╱□			□		□			R-C<OH, NH_2	ETHANOLAMINE
26	16×-5.6	╱	╱	╱□			□	╱	□			R-OH	N-HEXANOL
27	32×-5.6			■				□	╱			RR'-CO	2-HEPTANONE
28	32×-6.0	╱	╱	╱□			□	╱	□			R-OH	N-HEPTANOL
29	32×-6.6						□					×	×
30	32×-7.3						□		□			×	×

* ATTENUATION OF PEAK HEIGHT

Symbol	Meaning
(blank, shaded)	ELIMINATION
□	ELIMINATION NEW PRODUCTS
╱	SEVERE DECREASE
╱□	SEVERE DECREASE NEW PRODUCTS
■	SLIGHT DECREASE
(blank)	NO EFFECT

Fig. 7. Composite of gas chromatographic analysis of cooked volatiles from heavy hen leg muscle by functional-group analysis method.

tively identified by total retention volumes as being sulfur compounds, carbonyls, and alcohols, in that order. Two alkanes constituted one peak. Ethanol and dimethyl sulfide were found together in another. Hence, 32 compounds were present in 30 peaks, of which 29 were tentatively identified.

Fig. 8 shows that 25 peaks were obtained from gas chromatography of the cooked volatile fraction from heavy hen breast muscle. Fig. 9 shows the compounds identified, which include: 4 sulfides, 2 disulfides, and 1 mercaptan, totaling 7 sulfur compounds: 4 aldehydes and 4 ketones, totaling 8 carbonyls; 6 alcohols, 2 alkanes, 1 ester, 1 amine, and 2 unidentified compounds. Two alkanes constituted one of the peaks. Ethanol and diethyl sulfide were contained in another. Thus, 27 compounds were present in 25 peaks, of which 25 were tentatively identified. The principal components of the total cooked volatile fraction from breast muscle were tentatively identified by total retention volumes as being carbonyls, alcohols, and sulfur compounds, in that order. The complexity in identification of the constituents of chicken flavor has been well

Table 3. Gas chromatographic analysis of volatiles obtained by acid decomposition of the mercuric cyanide and mercuric chloride derivatives of cooked chicken volatiles.

Peak No.	Retention times (min) Unknown	Known	Tentative identity of peaks
1	1.2	1.4	dimethyl sulfide
2	1.7	1.8	ethyl mercaptan
3	2.0	2.5	methylethyl sulfide
4	2.6	2.8	n-propyl mercaptan
5	3.0	3.2	diethyl sulfide
6	3.7	3.8	methyl disulfide
7	3.8	4.1	ethyl n-propyl sulfide
8	5.0	5.2	Di-n-propyl sulfide
9	5.8	6.0	n-hexyl mercaptan

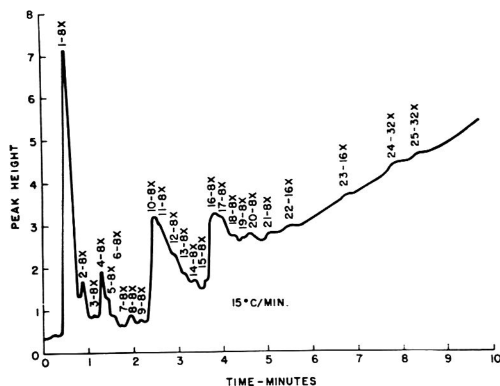

Fig. 8. Nonlinear temperature-programmed separation of 2 ml of total cooked volatiles from heavy hen breast muscle. (Detector, flame ionization; column, ⅛-in.-OD × 8-ft coiled copper; packing, 15% Apiezon L on 85% of 60 to 80 mesh Chromosorb W; temperature range, 100 to 250°C; helium pressure, 50 psi; attenuator at 8, range 1; chart speed, 80 sec/in.; temperature programmer set @ 15°C/min; F and M, model 500).

stated by Kazeniac (1961), and the present study confirms his observations.

Organoleptic evaluation. Results of the organoleptic evaluation are summarized in Table 4. Odor evaluation of chicken breast muscle by the panel indicated it to have a typical "chickeny" aroma, whereas leg meat was described as having a "chicken-like odor" but with a definite "red-meat note" reminiscent of beef. Removal of the sulfur compounds from the cooked volatile stream by passing through either mercuric chloride or mercuric cyanide solutions resulted in an almost complete elimination of the "meaty" aroma. On the other hand, removal of the carbonyl compounds by passing the volatiles from breast meat through 2,4-DNP (Pippen *et al.*, 1958) appeared to remove the characteristic "chickeny" aroma, but enhanced the "meaty" note. In fact, the panel described the resulting aroma as being "beefy." Results confirm the earlier observation of Crocker (1948) to the effect that chicken is composed of two principal flavoring components—a meaty flavor and a flavor peculiar to poultry meat.

Although both sulfides (Sadikov *et al.*, 1934; Bouthilet, 1951a; Pippen and Eyring, 1957; Kazeniac, 1961; Mecchi *et al.*, 1964) and carbonyl compounds (Pippen *et al.*, 1958; Pippen and Nonaka, 1960, 1963;

Table 4. Organoleptic evaluation of the volatile fraction from various reagent traps and untreated controls.

Reagent trap[a]	Odor characteristics of effluent stream	pH of effluent trapped in H_2O after 40 hr[b]
Mercuric chloride reagent	Sweet, chemical odor, meaty aroma absent	6.6
Mercuric cyanide reagent	Chemical odor, meaty aroma absent	6.9
Bismuth nitrate reagent	Strong sauerkraut odor, acidic note, sulfide odor, slightly beefy note, chicken aroma absent	4.3
2,4-DNP	Strong sulfide odor, pronounced roast beef odor, chicken aroma absent	....
Water	Roasted peanuts, burnt popcorn, pungent-strong sulfide and rubber odor	9.4
Leg muscle control (untreated)	Beefy or red meat aroma predominant, chicken note evident	....
Breast muscle control (untreated)	Strong, typical chicken odor, no beefy note evident	....

[a] The odor evaluations were made on the effluent volatiles collected in water after passing through the reagent, except for the controls which were evaluated directly from the reflux condenser.
[b] Evaluations were made after cooking-distillation for 40 hr.

PEAK NO.	PEAK HEIGHTS HEAVY HEN BREAST MUSCLE	FUNCTIONAL-GROUP REAGENT'S EFFECT										FUNCTIONAL GROUP	TENTATIVE IDENTITY
		ACIDIC NH_2OH	BASIC NH_2OH	$KMnO_4$	$HgCl_2$	$Hg(CN)_2$	$NaNO_2$	$NaBH_4$	$(AcO)_2O$	1.2N HCl	H_2SO_4		
1	8x*-7.0											R-H	ETHANE PROPANE
2	8x-1.7	E	E	■				□	/		E	RR'-CO	ACETONE
3	8x-0.9	/	/	/△			□	/	□		E	R-OH	METHANOL
4	8x-1.9	/	/	□	E				□	E	E	R-S-R'	DIMETHYL SULFIDE
5	8x-1.3						□		□	E	E	R-NH$_2$	METHYLAMINE
6	8x-0.9		E						■		E	R-COO-R'	METHYL FORMATE
7	8x-0.7	/	/	□	E			/	□	/	E	R-S-R', R-OH	DIETHYL-SULFIDE ETHANOL
8	8x-0.9	E	E	E				□	/△		E	R-CHO	ACETALDEHYDE
9	8x-0.8		/	/△			□	□	□		E	R-CO-CO-R	2,3-BUTANEDIONE
10	8x-3.2	/△	/	E	E			□	□	/	E	R-S-S-R'	METHYL DISULFIDE
11	8x-3.0	E	E	/				□	/		E	R-CO-CHOH-R'	ACETOIN
12	8x-2.3	E	E	E				□	/△		E	R-CHO	N-PENTANAL
13	8x-1.8	/	/	□					□	/	E	R-S-R'	ETHYL-N-PROPYL SULFIDE
14	8x-1.7										E	R-OH	ISO-BUTANOL
15	8x-1.7	/△	/	/	E			□	□	/	E	R-S-S-R'	ETHYL DISULFIDE
16	8x-3.3	E	E	■				□	/		E	R-CO-R'	×
17	8x-3.2	/	/	/△			□	/	□		E	ROH	N-BUTANOL
18	8x-2.7	/	/	□	E					/	E	R-S-R'	DIPROPYL SULFIDE
19	8x-2.7	□	□	□			□		□		E	R-SH	N-PROPYL MERCAPTAN
20	8x-2.8	E	E					□	/△		E	R-CHO	N-HEXANAL
21	8x-2.8	E	E	■				□	/			R-CO-R'	2,4-PENTANEDIONE
22	16x-3.0	E	E					□	/△			R-CHO	N-HEPTANAL
23	16x-3.7	E		■				□	/			R-COR'	2-HEPTANONE
24	32x-4.4	/	/	/△			□	/	□		E	R-OH	N-HEPTANOL
25	32x-4.7											×	×

* ATTENUATION OF PEAK HEIGHT

Symbol	Meaning
E (shaded)	ELIMINATION
□	ELIMINATION NEW PRODUCTS
/	SEVERE DECREASE
/△	SEVERE DECREASE NEW PRODUCTS
■	SLIGHT DECREASE
(blank)	NO EFFECT

Fig. 9. Composite of gas chromatographic analysis of cooked volatiles from heavy hen breast muscle by a functional group method.

Kazeniac, 1961) have been identified in the volatiles from cooked chicken, their contribution, if any, to chicken flavor has not been established. Thus, the panel studies reported herein were used in an attempt to relate chicken aroma to the volatile chemical constituents released upon cooking, as was so succinctly stated by Stewart (1963) and quoted by Burr (1964).

The present study indicates that the sulfur compounds are responsible for the "meaty" aroma of cooked chicken muscle, while the "chickeny" aroma is due to the presence of carbonyls. Thus, it is postulated that both sulfur compounds and carbonyls play major roles in the volatile flavor fraction of cooked chicken.

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Ms. rec'd 11/27/64.

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H. A. HAMID and R. L. SAFFLE
*Food Technology Department, University of Georgia
Athens, Georgia 30601*

Isolation and Identification of the Volatile Fatty Acids Present in Hickory Sawdust Smoke

SUMMARY

Acetic, propionic, butyric, *iso*-valeric, *n*-valeric, *iso*-caproic, and *n*-caproic acids were identified in hickory sawdust smoke by gas chromatography, and the relative amounts of each were determined. Formic acid could not be identified, because the flame ionization detector was not sensitive to this acid. A total of eight columns were evaluated, and three of these which gave best separation were used for identification by retention times as well as infrared spectrophotometry.

INTRODUCTION

Wood smoke contains substances that inhibit the growth of spoilage organisms and gives an agreeable taste and appearance to cured meats and meat products. A few studies have been made to determine the chemical differences in smoke in order to explain the organoleptic differences between sawdust-generated smoke and friction-generated smoke (Husaini and Cooper, 1957.). It has been reported that approximately 200 compounds have been found in the destructive distillation of woods (Wilson, 1961), but relatively few of these compounds have been identified in smoke used for meat. Since smoke is a very complex mixture of many compounds, it would appear that the most logical approach would be to determine its composition in units of chemically similar compounds. The objective of this study was to isolate and identify only the volatile fatty acids present in smoke from hickory-wood sawdust.

MATERIALS AND METHODS

Materials. A small laboratory smoke generator was constructed from an iron pipe of 24 × 3 in. reduced at one end to ¼ in. Pure hickory sawdust was loosely packed in the smoke-generating pipe, which was held vertically and connected at the bottom by ¼-in.-diameter glass tubing to a train of four specially designed glass chemical traps, each trap containing 100 ml of 1.0*N* NaOH solution. The traps were connected to a vacuum line (Fig. 1).

An F & M flame ionization gas chromatographic unit, Model 609, was used throughout this investigation. The gas chromatographic columns used consisted of ¼-in. × 5-ft copper columns packed with the following materials coated on 60–80-mesh firebrick (10% by weight): LAC-446 (polyethylene glycol adipate polyester), Craig polyester succinate, diethylene glycol succinate, carbowax 20M, UCON polar, UCON nonpolar, Apiezon L, and ethylene glycol succinate. Preliminary trials indicated that the first three stationary phases gave the best separation. Therefore, they were the only ones used for the remainder of the study.

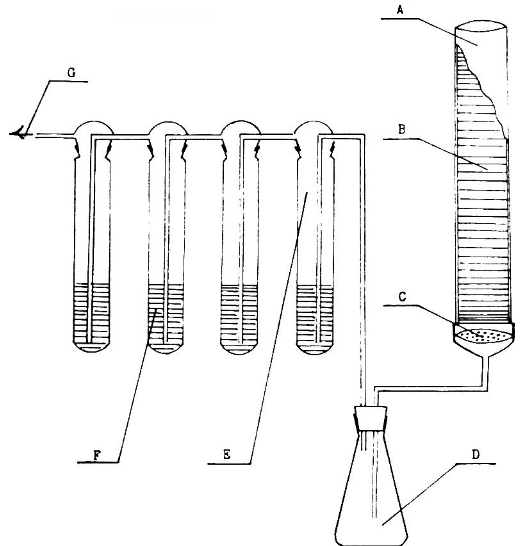

Fig. 1. Smoke generator: (A) iron pipe, 3 × 24 in.; (B) hickory-wood sawdust; (C) iron plate; (D) water trap; (E) glass trap, 2 × 12 in.; (F) 1.0*N* sodium hydroxide; (G) to vacuum line.

Methods. Moisture content of the hickory-wood sawdust was 10.2% as determined by drying 12 hr in an oven at 105°C. Moisture content of the sawdust was brought to 50% (Husaini and Cooper, 1957) by adding a calculated amount of distilled water. The sawdust was allowed to burn from the top, and the flowrate of the vacuum line was set at approximately 60 bubbles per min issuing from the ¼-in.-diameter glass tubing.

The volatile fatty acids present in the generated smoke were reacted with 1.0*N* sodium hydroxide in the glass traps. When the pH of the solution in the traps reached 8.0–8.5, the traps were disconnected and the solutions transferred to a 500-

ml beaker. The solution was then evaporated to dryness on a steam bath to obtain the sodium salts of the volatile fatty acids.

The sodium salts of the volatile fatty acids were acidified with a slight excess of 7N dichloroacetic acid in acetone to liberate the free fatty acids. The solution was centrifuged at 2°C, and the precipitated sodium dichloroacetate was discarded. An aliquot of acetone solution containing the fatty acids was then injected into the gas chromatographic column for identification (Hunter *et al.*, 1960). An internal standard was also used in which 1×10^{-5} moles of each of the known fatty acids were added to the dichloroacetic acid which contained the unknown acids. An aliquot was injected into the gas chromatographic column.

The gas chromatographic column temperature was 135°C, controlled to ±0.5°C. The flow rate of the carrier gas (nitrogen) was 60 ml per min, and the chart speed was 15 in. per hr. The detector sensitivity was Range 10 and Attenuation 32, with a sample size of 10 µl per injection.

RESULTS AND DISCUSSION

Seven volatile fatty acids were found in the hickory-wood-sawdust smoke. They were identified as acetic, propionic, butyric, iso-valeric, *n*-valeric, iso-caproic, and *n*-caproic acids. Fig. 2 shows the chromatogram with the LAC-446 column; Fig. 3, with the Craig polyester succinate; and Fig. 4, with the diethylene glycol succinate.

The retention times of the fatty acids from hickory sawdust smoke and authentic commercial volatile fatty acids are given in Table 1. The retention times varied only to a small degree. This small variation was probably due to normal experimental error such as speed of injection, injection port temperature, and measuring the center of the peak height. Chromatograms of the internal standards showed no new peaking but showed the expected increase in area of the peak. Fig. 5 shows a graph of the carbon number of the volatile fatty acids versus log of retention times on LAC-446, Fig. 6 on Craig polyester succinate, and Fig. 7 on diethylene glycol succinate. The retention times of the acids increased with increasing carbon number, and the *iso*-forms of the acids had a shorter retention time than their *n*-forms. Although it was quite easy to dis-

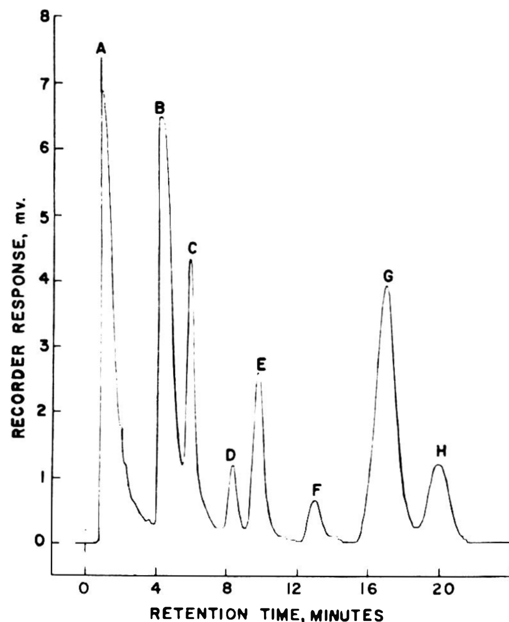

Fig. 2. LAC-446 chromatogram of the volatile fatty acids isolated from hickory wood sawdust smoke—chromatogram peaks: (A) acetone and decomposition products of dichloroacetic acid, (B) acetic acid, (C) propionic acid, (D) butyric acid, (E) *iso*-valeric acid, (F) *n*-valeric acid, (G) *iso*-caproic acid, (H) *n*-caproic acid.

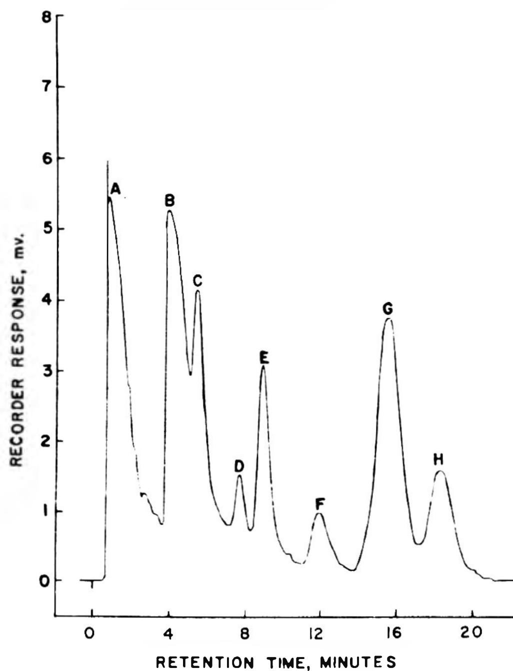

Fig. 3. Craig polyester succinate chromatogram of the volatile fatty acids isolated from hickory wood sawdust smoke—chromatogram peaks: (A) acetone and decomposition products of dichloroacetic acid, (B) acetic acid, (C) propionic acid, (D) butyric acid, (E) *iso*-valeric acid, (F) *n*-valeric acid, (G) *iso*-caproic acid, (H) *n*-caproic acid.

tinguish between the *n*- and *iso*-forms of the acids, it was not possible to distinguish between the various *iso*-forms of the same acid. As expected, a straight line was obtained between the log of retention time and carbon number for *n*-fatty acids, which is in good agreement with results of Sugisawa *et al.* (1962).

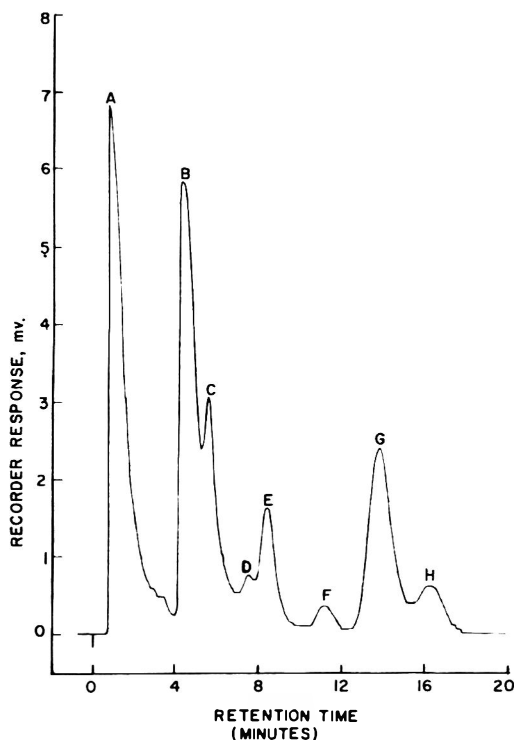

Fig. 4. Diethylene glycol succinate chromatogram of the volatile fatty acids isolated from hickory wood sawdust smoke—chromatogram peaks: (A) acetone and decomposition products of dichloroacetic acid, (B) acetic acid, (C) propionic acid, (D) butyric acid, (E) *iso*-valeric acid, (F) *n*-valeric acid, (G) *iso*-caproic acid, (H) *n*-caproic acid.

Previous workers, using column chromatographic separation of the volatile fatty acids present in sawdust smoke, identified formic, acetic, propionic, and butyric acids (Husaini and Cooper, 1957). In this investigation more volatile fatty acids were identified because of the more sensitive method of isolation and identification—the chemical traps and gas chromatography.

Formic acid was not identified in this study. If a different type of detector (thermal conductivity, B-ionization) had been used, formic acid would probably have been detected. The flame ionization detector, however, did not respond to formic acid (Lovelock, 1961; Kabot and Ettre, 1963).

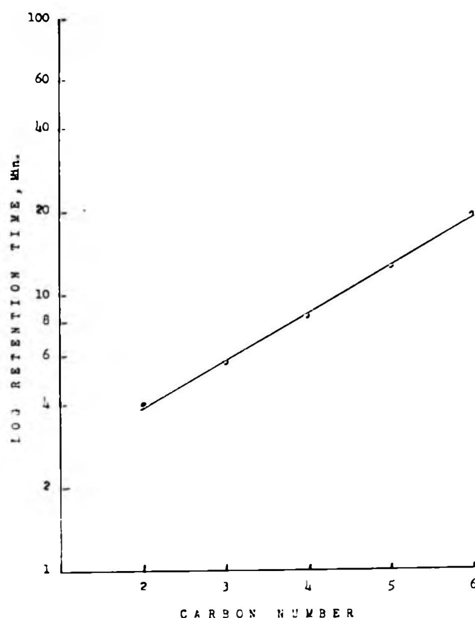

Fig. 5. Log retention time versus carbon number of *n*-fatty acids for LAC-446 column.

Table 1. Retention times of authentic and volatile fatty acid from hickory wood smoke.

	Retention time (min)					
	LAC-446 column		Craig polyester succinate column		Diethylene glycol column	
Acid	Authentic	Smoke	Authentic	Smoke	Authentic	Smoke
Acetic	4.2	4.0	4.0	3.8	4.2	4.0
Propionic	5.8	5.6	5.2	5.2	5.4	5.2
Butyric	8.4	8.2	7.4	7.6	7.2	7.2
Iso-Valeric	9.6	9.6	8.8	8.8	8.2	8.2
n-Valeric	12.8	12.8	11.8	11.8	10.6	10.8
iso-Caproic	16.8	16.8	15.6	15.4	13.6	13.4
n-Caproic	19.6	19.6	18.2	18.2	15.6	15.8

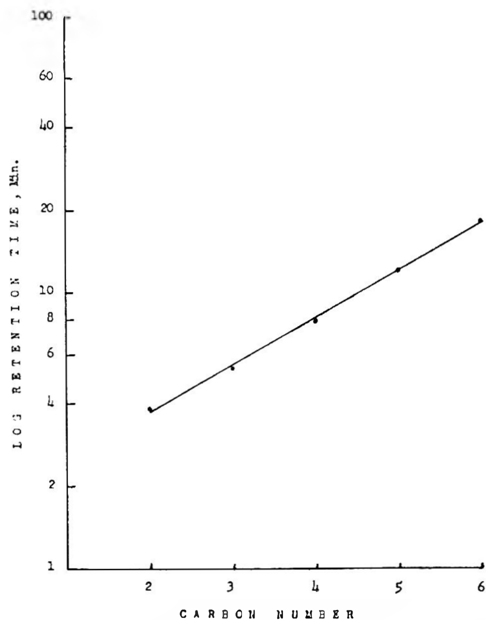

Fig. 6. Log retention time versus carbon number of n-fatty acids for Craig polyester succinate column.

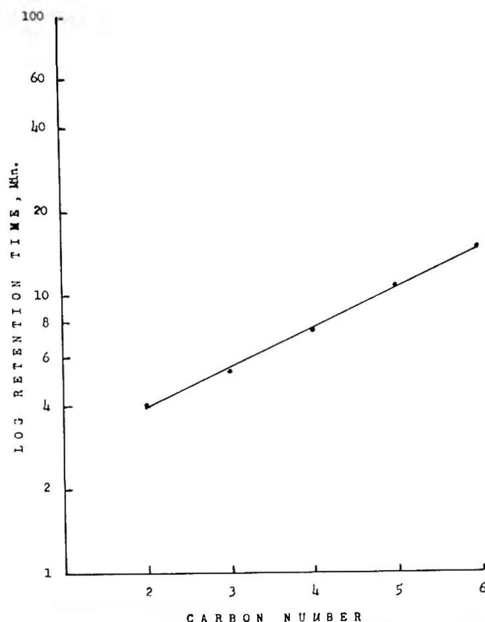

Fig. 7. Log retention time versus carbon number of n-fatty acids for diethylene glycol succinate column.

For the quantitative analysis the detector response values per 1×10^{-5} moles were determined for each acid. Table 2 summarizes these data, and Fig. 8. shows the plot of the detector response values for 1×10^{-5} moles versus carbon number. It may be seen that the area of peak increased linearly with the carbon number of equal molar quantities of the acids. Thus, total area of the peaks for different acids cannot be compared directly as to the relative amounts of each. Kabot and Ettre (1963) also obtained a straight-line plot for carbon number versus molar response. Gehrke and Lamkin (1961) have also shown that the peak area was a function of concentration for the volatile fatty acids. Peak areas were determined by multiplying peak height by peak width at half height, as suggested by Pecsok (1961).

The use of three different columns permitted identification of the volatile fatty acids. To confirm the gas chromatographic identification, the peaks were collected in acetone and their infrared spectra obtained. The extremely small amounts of samples collected did not permit positive identification of each compound; however, the characteristic absorptions of volatile fatty acids at 2500–300 cm^{-1} for the OH group, and at

Table 2. Calculation of moles of acids in smoke from detector response values and peak areas for known amount of the acids.

Acid	Molecular weight	Mole injected	Known peak area	Detector response value for 1×10^{-5} mole	Smoke peak area	Moles of acid in smoke [a]
Acetic	60.05	1.74×10^{-5}	1.94	1.12	1.24	1.107×10^{-5}
Propionic	74.08	1.34×10^{-5}	1.71	1.28	0.494	0.386×10^{-5}
Butyric	88.10	1.08×10^{-5}	1.52	1.42	0.12	0.085×10^{-5}
Iso-Valeric	102.13	0.917×10^{-5}	1.36	1.48	0.44	0.297×10^{-5}
n-Valeric	102.13	0.992×10^{-5}	1.44	1.56	0.12	0.077×10^{-5}
iso-Caproic	116.16	0.796×10^{5}	1.30	1.63	1.17	0.718×10^{-5}
n-Caproic	116.16	0.813×10^{-5}	1.40	1.72	0.36	0.209×10^{-5}

[a] This is the moles of C_2–C_6 in 10 μl of the acetone and acid solution injected.

1700 cm^{-1}, which is the carbonyl frequency for simple saturated fatty acids, were present for each sample.

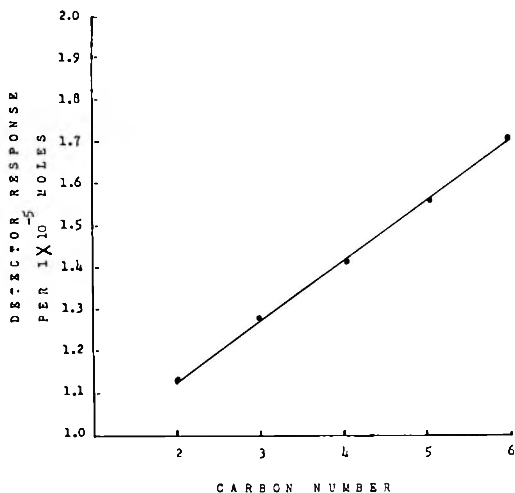

Fig. 8. Detector response values for 1 × 10^{-5} moles versus carbon number.

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Data from a Ph.D. thesis by the senior author.

HENRY KLOSTERGAARD

Division of Science and Mathematics, San Fernando Valley State College Northridge, California

Simple Mathematical Solutions of Problems Associated with Heat Sterilization of Milk

SUMMARY

Three simple equations have been developed along with tabulated values that allow calculation of sterilizing time, sterilizing value, or heat penetration factor for a process when only two of the three are known. A short trial-and-error calculation can yield the retort temperature if other data are known. A mathematical derivation associated with the problem is included as an appendix.

INTRODUCTION

In the manufacture of evaporated milk it is well known that the heat stability and viscosity properties of the milk often necessitate changes in the sterilizing schedule. Time and temperature, however, cannot be freely changed; rather, the operator must consult charts or he makes "a safe guess." In dealing with modified products, modified processes, or modified containers the process must be calculated with rather complicated procedures involving a graphical or empirical approach or using tables that are rarely on hand. It is probably safe to say that the average operator will shy away from the calculations, and, as a result, sterilizing research is impeded.

This paper presents a simple mathematical solution to the problems of calculating some of the fundamental parameters occurring in the sterilizing process. It happens that these parameters enter the final equations in a very simple functional form that allows a direct inspection of their importance. It turns out that the whole treatment can be made using only moderately advanced mathematical developments, and it is considered worthwhile to include some of these in an appendix, which may, however, be skipped over by readers interested primarily in the final results.

Symbols used in this paper:

- e base of natural logarithm (= 2.71828...)
- ln natural logarithm
- log logarithm base 10
- N ln 10 or ln/log
- γ Euler's constant ($\lim_{n \to \infty} \sum_{1}^{n} 1/n - ln\ n$; = 0.5772...)
- C e^{γ} (= 1.781...)
- RT temperature of retort (°F)
- IT temperature of material (can) as it enters retort (°F)
- TD $RT - IT$ (°F)
- CT temperature of material (can) during process (°F). It is a function of t.
- T temperature in general (°F)
- z increase in temperature (°F) required for a tenfold increase in rate of destruction of the microorganism to be treated, a characteristic of the microorganism. It also depends on the medium.
- t time (min) the material has been in the retort.
- f time (min) required in retort to obtain a tenfold decrease in difference $RT - CT$
- l time lag (min) before ideal convection heating is obtained.
- j lag factor ($j = 10^{l/f}$)
- B_B total time material is in retort (min)
- F_o lethality (min) in equivalent min at 250°F
- A log z/CN
- Φ $10^{\frac{RT - 250}{z}}$
- u $\frac{N}{z}(RT - CT)$
- u_o $\frac{N}{z}(TD)$
- u_B value of u for $t = B_B$

DERIVATION OF THE FUNDAMENTAL HEAT PENETRATION EQUATION

In this derivation we shall assume that the temperature of material inside the can is uniform so that any temperature rise is due to convection. Let K be the number of calories required to heat the can and content 1°F. The amount of heat going through the can wall per second will be $c\ (RT - CT)$, where we shall consider c constant although it depends on burn-on, speed of rotation of can, and other factors. In a short time, dt, the temperature of the can will increase by dT, and since the amount of heat going through the wall equals that taken up by the can and content we have

$$c\ (RT - CT)\ dt = KdT \qquad [1]$$

Since the temperature increase is equal to the decrease in difference between retort and can temperatures we have

$$dT = -d(RT - CT)$$

so Eq. 1 can then be written as

$$dt = -\frac{K}{c}\frac{d\ (RT - CT)}{RT - CT} \qquad [2]$$

This equation relates the change in can temperature during a short time interval. By integration of Eq. 2 we can obtain the can temperature at any time during the sterilizing. Eq. 2 gives

$$t + a = -\frac{K}{c}\int\frac{d\ (RT - CT)}{(RT - CT)} \qquad [3]$$

where a is an integration constant. Since $\int\frac{dx}{x} = lnx$ Eq. 3 gives

$$t + a = -\frac{K}{c}\ ln\ (RT - CT) \qquad [4]$$

When $t = 0$ we have $CT = IT$, so Eq. 4 gives

$$a = -\frac{K}{c}\ ln\ (RT - IT) \qquad [5]$$

Subtract Eq. 5 from Eq. 4, and rearrange:

$$t = \frac{K}{c}\ ln\ \frac{TD}{RT - CT} = \frac{KN}{c}\ \log\ \frac{TD}{RT - CT} \qquad [6]$$

After f min in the retort, we have $\frac{TD}{RT - CT} = 10$, so, using Eq. 6, we get:

$$f = \frac{KN}{c} \qquad [7]$$

Combine Eqs. 6 and 7, and get:

$$t = f\ \log\ \frac{TD}{RT - CT} \qquad [8]$$

Eq. 8, the fundamental heat penetration equation, can be used to calculate the product temperature at any time during the sterilization process.

Experiments with a variety of products show that during "retorting" the temperature-time relation is indeed often of the form given in Eq. 8. This is especially true for evaporated milk (1). In many cases, however, the logarithmic relation in Eq. 8, arises only after an initial lag period. The relation between CT and t can then be expressed (see symbols)

$$t - l = f\ \log\ \frac{TD}{RT - CT}$$

or

$$t = f\ \log\ \frac{j\ (TD)}{RT - CT} \qquad [9]$$

which holds *after* l min of processing. If l is small compared to f (and j therefore not much greater than 1) CT will not at time $t = l$ min have obtained any significant lethality rate and no significant error is committed by letting Eq. 9 apply throughout the entire "retorting." [For evaporated milk a j value of 1.2 has been used commercially in process calculations (2)]. In the subsequent development $j(TD)$ may be used instead of TD; the final equations will also be given in a form that includes the lag factor j.

LETHALITY OF STERILIZING PROCESS

The lethality, F_o, of a process is usually expressed by the number of minutes at 250°F that produces the same destruction of microorganisms as the process. Experiments have shown that the lethality of a process consisting solely of t min at T°F is

$$F_o = t \cdot 10^{\frac{T - 250}{z}}$$

A process consisting of dt min at CT°F gives, then,

$$dF_o = dt\ 10^{\frac{CT - 250}{z}} \qquad [10]$$

Now $CT - 250 = (RT - 250) - (RT - CT)$, and therefore

$$10^{\frac{CT-250}{z}} = 10^{\frac{RT-250}{z}} \times 10^{-\frac{RT-CT}{z}} = \Phi \times 10^{-\frac{RT-CT}{z}}$$

which is used in Eq. 10. On integration we get the lethality of the treatment in the retort (from $t = 0$ to $t = B_B$)

$$F_o = \Phi \int_0^{B_B} 10^{-\frac{RT-CT}{z}} dt \qquad [11]$$

Now, $10 = e^N$, and, using symbols, we have

$$F_o = \Phi \int_0^{B_B} e^{-u} dt \qquad [12]$$

From Eq. 7 we have $\frac{K}{c} = \frac{f}{N}$, which in Eq. 2 gives, as $u = \frac{N}{z}(RT - CT)$:

$$dt = -\frac{f}{N}\frac{du}{u} \qquad [13]$$

Eqs. 12 and 13 give:

$$F_o = -\frac{\Phi f}{N}\int_{t=0}^{t=B_B} \frac{e^{-u}}{u} du \quad \text{or}$$

$$F_o = \frac{\Phi f}{N}\int_{u_B}^{u_o} \frac{e^{-u}}{u} du \qquad [14]$$

which may be rearranged

$$\frac{F_o N}{f\Phi} = \int_{u_B}^{\infty} \frac{e^{-u}}{u} du - \int_{u_o}^{\infty} \frac{e^{-u}}{u} du = H(u_B) - H(u_o) \qquad [15]$$

$H(u)$ is listed in Jahnke and Emde's "Tables of Functions" as $-Ei(-x)$.

From Eq. 8 we get $\log \frac{TD}{RT - CT} = \frac{t}{f}$ or

$$ln \frac{RT - CT}{TD} = -\frac{Nt}{f}$$

so $RT - CT = e^{-\frac{Nt}{f}} \times TD$

which when multiplied by $\frac{N}{z}$ gives

$$u = u_o \times e^{-\frac{Nt}{f}} \qquad [16]$$

For $t = B_B$ we have

$$u_B = u_o e^{-\frac{NB_B}{f}} = u_o 10^{-\frac{B_B}{f}} \qquad [17]$$

Along with $u_o = \frac{N}{z} \times TD$, Eqs. 17 and 15 allow a precise calculation of F_o. The values for $H(u)$ in Eq. 15 would generally be obtained from tables in "Handbook of Chemistry and Physics" (late editions), Chemical Rubber Publishing Co., Cleveland, Ohio, or Jahnke and Emde: Tables of Functions, Dover Publications, New York, or similar books. The values of the integral, however, are not available with adequate accuracy for the values of u_B normally encountered in milk sterilizing. We shall therefore calculate F_o without using such tables.

It can be shown (see appendix) that

$$\int_a^{\infty} \frac{e^{-u}}{u} du = -\gamma - ln\, a + a - \frac{a^2}{2!2} + \frac{a^3}{3!3} - \ldots\ldots \qquad [18]$$

Using Eq. 18 with Eq. 15, we get, after rearranging:

$$\frac{F_o N}{\Phi f} = ln \frac{u_o}{u_B} - (u_o - u_B) + \frac{u_o^2 - u_B^2}{2!2} - \frac{u_o^3 - u_B^3}{3!3} + \ldots$$

but from Eq. 17 we have $ln \frac{u_o}{u_B} = \frac{NB_B}{f}$

which we insert and get

$$F_o = \Phi B_B - \frac{f\Phi}{N} \times \left[u_o - u_B - \frac{u_o^2 - u_B^2}{2!2} + \frac{u_o^3 - u_B^3}{3!3} - \ldots\right] \qquad [19]$$

Eq. 19 allows an exact calculation of F_o, using Eq. 17 and a table for Φ. Under conditions normally present in sterilization of milk, a much simpler formula can be developed that gives results of excellent accuracy. In order to develop this formula we start with Eq. 15 and investigate the integrand

$\frac{e^{-u}}{u}$. We find

u	$\frac{e^{-u}}{u}$	u	$\frac{e^{-u}}{u}$	u	$\frac{e^{-u}}{u}$
0		0.40	1.68	4.00	0.0046
0.01	99.0	0.50	1.21	5.00	0.0015
0.02	49.0	0.60	0.91	6.00	0.00041
0.05	19.0	0.80	0.56	7.00	0.00013
0.10	9.0	1.00	0.36	8.00	0.000042
0.20	4.09	2.00	0.068	9.00	0.000014
0.30	2.47	3.00	0.017	10.00	0.0000045

It is readily seen that the integrand decreases very rapidly as u increases, and therefore only low u values contribute to the integral

$$\int_a^\infty \frac{e^{-u}}{u}\, du.$$

We shall therefore disregard $H(u_o)$ in Eq. 15, since u_O is normally much larger than u_B. Eq. 15 is then changed to:

$$\frac{F_oN}{f\Phi} = H(u_B) \quad [20]$$

This involves an error, which we shall assess, in conjunction with another error introduced, by subsequently taking into account only the first two terms of the expression in Eq. 18, so $H(u_B) = -\gamma - ln\ u_B$ [21]

Inspection shows that the two errors counteract each other, and therefore impose less severe restrictions on the conditions than either error would impose by itself. The restrictions will be stated later. Eqs. 20 and 21 give

$$\frac{F_oN}{\Phi f} = -\gamma - ln\ u_B \quad [22]$$

From Eq. 17 we get

$$ln\ u_B = ln\ u_o - \frac{NB_B}{f} \quad \text{or}$$

$$ln\ u_B = ln\ N - ln\ z + ln\ TD - \frac{NB_B}{f}$$

which we insert in Eq. 22 and use $\gamma = ln\ C$

$$\frac{F_oN}{\Phi f} = -\ ln\ C - ln\ N + ln\ z - ln\ TD + \frac{NB_B}{f} \quad [23]$$

But $\quad -\ ln\ C - ln\ N + ln\ z =$

$$ln\ \frac{z}{NC} = N\log\frac{z}{NC} = N \times A,$$

so Eq. 23 can be written

$$\frac{F_oN}{\Phi f} = N \times A - N\log TD + \frac{NB_B}{f} \quad \text{or}$$

$$F_o = \Phi\left[B_B - f(\log TD - A)\right] \quad \text{or} \quad [24]$$

$$B_B = \frac{F_o}{\Phi} + f(\log TD - A) \quad \text{or} \quad [25]$$

$$f = \frac{B_B - \frac{F_o}{\Phi}}{\log TD - A} \quad [26]$$

These final simple equations are valid within 1% error for these conditions

$\frac{B_C}{f}$	z	<$RT - IT$<	
2.0	16	13	27
	18	15	30
	20	17	33
2.5	16	14	64
	18	16	72
	20	18	80
3.0	16	13	(205)
	18	15	(230)
	20	17	(255)
4.0	16	12	—
	18	13	—
	20	15	—
5.0	16	11	—
	18	12	—
	20	13	—

It is clear that these conditions are satisfied in the normal sterilization process of mil.k

Only when $\frac{B_B}{f}$ is 2 are the restrictions of importance.

To facilitate the use of these equations a table is given below of values of Φ and A at frequently used retort temperatures and a range of z values.

If l is not 0 min, but constant and still small compared to f, j can be incorporated in the three equations as follows:

$$F_o = \Phi\left[B_B - f(\log j + \log TD - A)\right] \quad [24a]$$

$$B_B = \frac{F_o}{\Phi} + f(\log j + \log TD - A) \quad [25a]$$

$$f = \frac{B_B - \frac{F_o}{\Phi}}{\log j + \log TD - A} \quad [26a]$$

T°F	$z = 16$	$z = 17$	$z = 18$	$z = 19$	$z = 20$	$z = 21$
240	0.2371	0.2580	0.2783	0.2976	0.3162	0.3340
241	0.2738	0.2955	0.3162	0.3360	0.3548	0.3728
242	0.3162	0.3384	0.3594	0.3793	0.3981	0.4160
243	0.3652	0.3875	0.4084	0.4281	0.4467	0.4642
244	0.4217	0.4437	0.4642	0.4833	0.5012	0.5179
245	0.4870	0.5080	0.5275	0.5456	0.5623	0.5780
246	0.5623	0.5817	0.5995	0.6158	0.6310	0.6449
247	0.6494	0.6661	0.6813	0.6952	0.7080	0.7197
248	0.7499	0.7627	0.7743	0.7848	0.7943	0.8031
249	0.8860	0.8733	0.8799	0.8859	0.8912	0.8982
250	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
251	1.1548	1.1450	1.1364	1.1288	1.1220	1.1159
252	1.3335	1.3111	1.2915	1.2743	1.2589	1.2452
253	1.5399	1.5013	1.4678	1.4384	1.4125	1.3895
254	1.7783	1.7190	1.6681	1.6239	1.5849	1.5505
255	2.0535	1.9684	1.8957	1.8330	1.7783	1.7302
256	2.3714	2.2539	2.1544	2.0691	1.9953	1.9307
257	2.7384	2.5808	2.4484	2.3357	2.2387	2.1544
258	3.1623	2.9552	2.7825	2.6366	2.5119	2.4041
259	3.6518	3.3839	3.1623	2.9763	2.8184	2.6828
260	4.2170	3.8747	3.5938	3.3598	3.1623	2.9936
A	0.5912	0.6176	0.6424	0.6659	0.6881	0.7093

EXAMPLES

1) Calculate F_o

$RT = 248°F \quad IT = 205°F \quad f = 2.7$ min
$z = 18°F \quad B_B = 9$ min

From the table we get $\Phi = 0.7743$ and $A = 0.6424$

Eq. 24 gives

$$F_o = 0.7743 \times \left[9 - 2.7\,(1.6335 - 0.6424)\right] \cong 4.9 \text{ min}$$

Note that $\frac{B_B}{f} \sim 3$, so $RT - IT$ is not restricted.

If $j = 1.2$ F_o would be reduced by 0.17 min.

2) Calculate B_B

$RT = 248°F \quad IT = 208°F \quad f = 2.8$ min
$z = 18°F \quad F_o = 5.2$ min

Eq. 25 gives

$$B_B = \frac{5.2}{0.7743} + 2.8\,(1.6021 - 0.6424) \cong 9.4 \text{ min}$$

If $j = 1.2$ B_B would be increased by 0.22 min.

3) Calculate f

$RT = 245°F \quad IT = 205°F \quad z = 18°F$
$B_B = 10$ min

Inoculated pack test indicated an unsafe F_o of 2.8 min

Eq. 26 gives

$$f = \frac{10^{-\frac{2.8}{0.5275}}}{\log 40 - 0.6424} \cong 4.9 \text{ min}$$

If this f value is unusually high, burn-on might be responsible for the unsafe process.

4) Calculate RT

$IT = 205°F \quad z = 18°F \quad F_o = 5.0$ min
$f = 2.6$ min

Can speed requires $B_B = 8.5$ min

Eq. 24 gives

$$5.0 = \Phi\left[8.5 - 2.7\,(\log TD - 0.6424)\right]$$

The term involving TD is less sensitive to variation in RT than is Φ.

Its value is of the order 1, so we have approximately $5.0 \cong \Phi\,(8.5 - 2.7)$ or $\Phi \cong 0.862$ from which we get (table) $RT = 249°F$

Reusing Eq. 24 with $RT = 249°F$, we get $F_o = 5.1$ min, very close to the required $F_o = 5.0$ min.

If $j = 1.2$, the F_o value at 249°F would be reduced to 4.9 min and RT would have to be increased to 249.3°F to balance the j effect.

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APPENDIX

From the well-known series

$$e^u = 1 + \frac{u}{1!} + \frac{u}{2!} + \ldots\ldots\ldots\ldots + \frac{u^n}{n!} + \ldots$$

one easily obtains

$$\int \frac{e^{-u}}{u}\,du = ln\,u - u + \frac{u^2}{2!2} - \frac{u^3}{3!3} + \ldots + \text{const.} \quad [I]$$

$$\text{Let } L(u) = ln\,u - u + \frac{u^2}{2!2} - \frac{u^3}{3!3} + \ldots\ldots$$

In order to find

$$H(u) = \int_u^\infty \frac{e^{-u}}{u}\,du \quad \text{we must find } L(\infty).$$

We have

$$H(a) = L(\infty) - \left[ln\,a - a + \frac{a^2}{2!2} - \frac{a^3}{3!3} + \ldots\right] \quad [II]$$

and also

$$H(a) = \int_a^\infty \frac{e^{-u}}{u}\,du = \int_{u=a}^\infty e^{-u}\,d(ln\,u) = \text{(integration by parts)}$$

$$= e^{-u}\,ln\,u\Big|_a^\infty - \int_{u=a}^\infty ln\,u\,d(e^{-u}) =$$

$$= e^{-u}\,ln\,u\Big|_a^\infty + \int_a^\infty e^{-u}\,ln\,u\,du \quad [III]$$

As $u \to \infty$ $e^{-u} \ln u \to 0$ (as u increases by 1, $\ln u$ changes little whereas e^{-u} becomes approx 2.7 times smaller), so

$$H(a) = -e^{-a} \ln a + \int_a^\infty e^{-u} \ln u \, du \qquad [IV]$$

Eqs. II and IV can now be combined, and we obtain:

$$L(\infty) = \left[1 - e^{-a}\right] \ln a - a + \frac{a^2}{2!2} - \frac{a^3}{3!3} + \ldots + \int_a^\infty e^{-u} \ln u \, du$$

$L(\infty)$ is independent of a, so we may choose any value of a in calculating $L(\infty)$ and we shall choose $a = 0$.

As $a \to 0$ $(1 - e^{-a}) \ln a \to 0$

since $(1 - e^{-a}) = 1 - 1 + \frac{a}{1!} - \frac{a^2}{2!} + \ldots =$

$$a\left(1 - \frac{a}{2!} + \frac{a^2}{3!} - \ldots\right)$$

so $(1 - e^{-a}) \ln a = a \ln a \left(1 - \frac{a}{2!} + \frac{a^2}{3!} - \ldots\ldots\right)$

For $a = 0$ the value of the bracket is 1. If we let $a \to 0$ through the series $e^{-1}, e^{-2}, e^{-3}, \ldots$ etc., we see that for each step, a decreases by the factor approx. 2.7, whereas $|\ln a|$ increases only by 1. Therefore:

$$L(\infty) = \int_0^\infty e^{-u} \ln u \, du \qquad [V]$$

This integral can now be evaluated using the gamma function which is defined as

$$\Gamma(x) = \int_0^\infty e^{-u} u^x \, du$$

Writing $e^{-u} u^x$ as $-u^x d(e^{-u})$ and integrating by parts, we obtain:

$$\Gamma(x) = -u^x e^{-u} \Big|_{u=0}^{u=\infty} + \int_0^\infty e^{-u} d(u^x).$$

As $u \to \infty$, $-u^x e^{-u} \to 0$ (if u is doubled, the former factor becomes 2^x times greater, the latter e^u times smaller) so:

$$\Gamma(x) = \int_0^\infty e^{-u} d(u^x) = x\int_0^\infty e^{-u} u^{x-1} du = x\Gamma(x-1)$$

For integral values of x we now have by induction that:

$$\Gamma(x) = x(x-1)(x-2) \ldots\ldots \Gamma(0)$$

$\Gamma(0) = \int_0^\infty e^{-u} du$ (since $u^0 = 1$) and this is equal to

$$-e^{-u} \Big|_0^\infty = -0 - (-1) = 1$$

and therefore for positive integral values of x

$$\Gamma(x) = x! \qquad [VI]$$

We now differentiate $\Gamma(x)$ using that $u^x = e^{x \ln u}$

so that $\frac{d(u^x)}{dx} = e^{x \ln u} \ln u = u^x \ln u$

and obtain $\Gamma'(x) = \int_0^\infty u^x e^{-u} \ln u \, du$

$$\text{and therefore } \Gamma'(0) = \int_0^\infty e^{-u} \ln u \, du \qquad [VII]$$

and in combining Eqs. V and VII, we have:

$$L(\infty) = \Gamma'(0)$$

For integral n and x, we have:

$$\Gamma(n + x) = \Gamma(n)(n + 1)(n + 2) \ldots\ldots (n + x)$$

But also:

$\Gamma(n + x) = \Gamma(x)(x + 1)(x + 2) \ldots\ldots (n + x)$, so:

$$\Gamma(x) = \Gamma(n)n^x \frac{\left(1 + \frac{1}{n}\right)\left(1 + \frac{2}{n}\right) \ldots\ldots \left(1 + \frac{x}{n}\right)}{(x + 1)(x + 2) \ldots\ldots (x + n)} \qquad [VIII]$$

This identity holds for all n and therefore also for $n \to \infty$, so we may write:

$$\Gamma(x) = \lim_{n \to \infty} \frac{\Gamma(n) n^x}{(x + 1)(x + 2) \ldots (x + n)} \qquad [IX]$$

(The x factors of form $1 + \frac{x}{n} \to 1$ as $n \to \infty$, and therefore disappear.) From Eq. IX we obtain:

$$\ln \Gamma(x) = \lim_{n \to \infty} \Big[\ln \Gamma(n) + x \ln n - \ln(x+1) - \ln(x+2) \ldots - \ln(x+n)\Big]$$

We differentiate with respect to x and obtain

$$\frac{\Gamma'(x)}{\Gamma(x)} = \lim_{n \to \infty} \left[ln\ n - \frac{1}{x+1} - \frac{1}{x+2} - \ldots - \frac{1}{x+n} \right]$$

Since $\Gamma(0) = 1$, we can obtain finally

$$\Gamma'(0) = \lim_{n \to \infty} \left[ln\ n - \frac{1}{1} - \frac{1}{2} - \frac{1}{3} - \ldots - \frac{1}{n} \right] \quad [X]$$

The right side of Eq. X tends to a limit $-\gamma$, where γ is Euler's constant. Now $L(\infty) = \Gamma'(0) = -\gamma$, and Eq. II now gives:

$$H(a) = -\gamma - ln\ a + a - \frac{a^2}{2!2} + \ldots\ldots\ldots$$

which is Eq. 18.

γ can be estimated from the following:

n	sum of n first reciprocals	$ln\ n$	Δ
10	2.928968254	2.30259	0.62638
20	3.597739661	2.99573	0.60201
30	3.994987149	3.40120	0.59379
40	4.278543054	3.68888	0.58966
50	4.499205342	3.91202	0.58719
60	4.679870411	4.09434	0.58553
70	4.83281675	4.24850	0.58432
100	5.18735752	4.60517	0.58219

$\Delta \to \gamma = 0.577215665 \ldots$

JOSEPH A. LIUZZO, ARTHUR F. NOVAK, and JESSE R. ORTEGO
Department of Food Science and Technology
Louisiana State University, Baton Rouge, Louisiana 70803

Physiological Changes Induced by Gamma Irradiation of Bacteria From Shrimp

SUMMARY

The morphology and metabolism of seven radiation-resistant bacteria isolated from fresh Gulf shrimp were studied to ascertain if radiation altered their characteristics. Radiation-induced differences were observed in optimum incubation temperature, chromogenesis, carbohydrate and vitamin utilization, and action on litmus milk.

INTRODUCTION

The success of preserving many perishable foods by radiation pasteurization or sterilization is primarily attributed to the destruction of microorganisms which enhance food spoilage. The storage life of many foods cannot be extended by radiation doses which result in sterilization because artifacts produced by these high levels are detrimental to the flavor and odor of the foods. Consequently, the lower pasteurization doses employed often result in the survival of certain microorganisms which resist radiation or which may recover from radiation effects upon storage in a suitable environment. Kelner and co-workers (Kelner *et al.*, 1955) reported that the physiological state of the organisms at irradiation determines to some extent their sensitivity to radiation, and that under certain conditions a fraction of the bacterial population can recover from the damaging effects of ionizing radiation. Fields and his co-workers (Fields *et al.*, 1961) working at the radiation doses generally applied in food preservation, observed that the physical form of a medium subjected to radiation can determine the status of organism resistance.

Radiation-resistant organisms in seafoods have been reported primarily for codfish (Mac Lean and Welander, 1960); flounder, rockfish, and salmon (Lerke *et al.*, 1961); haddock and clams (Nickerson *et al.*, 1962); and canned shrimp and crabmeat (Scholz *et al.*, 1962).

This study was undertaken to isolate and identify bacteria from Gulf shrimp which are resistant to radiation-pasteurization doses, and to ascertain if physiological changes are induced by the gamma radiation treatment. A knowledge of these organisms and of any changes produced could aid future workers in the development of new chemical and physical processes for reducing their numbers and in conjunction with low-dose irradiation render the food in question virtually free of spoilage bacteria.

EXPERIMENTAL

The fresh shrimp (*Penaeus aztecus*) used in this investigation were caught near Barataria Bay by personnel from the Louisiana Wildlife and Fisheries Commission, on Grand Terre Island, Louisiana. The operations were supervised by members of the laboratory staff to ensure that the shrimp procured were of the best quality present in this locale of the Gulf of Mexico.

The shrimp were packed in layers of ice immediately after being caught, and transported to the Marine Laboratory on the Island, where they were washed in cold tap water, deheaded, packed in ice, and transported to the campus laboratories. Upon arrival, they were washed again to ensure against contamination from the ice. Several 50-g samples were independently homogenized with sterile distilled water in a Waring blender and diluted to produce 30 to 300 bacterial colonies when seeded in a petri dish containing Difco Bacto nutrient agar supplemented with 0.2% NaCl. After the seeded dishes were incubated at 25°C until moderate growth appeared (24–72 hr), the colonies were picked from the plates and streaked on fresh agar plates, which were also incubated at the same temperature and time. Two agar slants of each culture were made from the latter series of plates. After incubation, one slant of each culture was stored under refrigeration while its mate was irradiated at 100,000 rads with cobalt-60 in the 10,000-curie irradiator at the L.S.U. Nuclear Science Center.

The radiation dose employed has been reported as being within the optimum range that can be used for preserving fresh shrimp without destroying their quality (Novak and Liuzzo, 1962). After exposure, the cultures were transferred immediately to fresh slants since reports indicate that irradiated medium ingredients can destroy certain microorganisms (Fields *et al.*, 1961).

Morphological and metabolic tests for the purpose of culture identification were conducted according to methods suggested in *Bergey's Manual* (1962). The unirradiated cultures were used for the identification tests. After identification, comparable tests were performed on both cultures, the irradiated and unirradiated, to ascertain if the treatment induced differences in their physiology. The major tests included: Gram stain, characteristics of agar stroke, inoculation into lactose, glucose, nitrate, tryptone (indole test) broths, litmus milk, methyl red-Voges Proskauer (MR-VP) medium, and Knox gelatin. All metabolic tests were conducted at different temperatures (10, 25, 37°C) and periods (1–10 days) to ensure that metabolic reactions would occur under optimum conditions for the organism.

Vitamin utilization assays were also conducted to ascertain if irradiation produced changes in specific enzyme reactions of the bacteria, since these nutrients are generally active moieties of enzyme-coenzyme systems. The vitamins investigated were niacin, pyridoxine, pantothenic acid, riboflavin, thiamine, and folic acid. The assays were performed by employing the Difco Bacto assay medium for the vitamin under investigation and according to the techniques cited in the *Difco Manual* (1953). The degree of bacterial growth in the vitamin-supplemented medium was measured turbidimetrically using a Bausch and Lomb Spectronic 20 spectrophotometer at 640 mμ.

RESULTS AND DISCUSSION

Seven radiation-resistant bacteria were isolated and identified from fresh Gulf shrimp. These bacteria were classed into the following genera and species: *Brevibacterium minutiferula*, *Gaffkya homari*, *Sarcina flava*, *Bacillus megaterium*, *Achromobacter eurydice*, *Bacillus firmus*, and *Bacillus pumilus*.

Irradiation of the cultures affected the optimum incubation temperature of 3 organisms (Table 1). In each case the temperature required for best growth was lowered 12–15°C. Since shellfish are usually preserved under refrigeration, the ability of the organisms to grow more rapidly at lower temperatures could become a problem in extension of the storage life of irradiated shrimp.

One of the most important changes induced by radiation was in culture chromogenesis in the agar stroke technique for *S. flava* and *B. firmus* (Table 1). This determination was repeated several times to confirm this alteration in visual cultural coloration. In each case the distinct color change was observed. Chromogenesis, in general, is greatly influenced by composition and pH of the medium, incubation temperature, intensity of light, and oxygen tension. It is also generally accepted that pigments produced during chromogenesis are a result of metabolism. The irradiated organisms that altered pigmentation were treated similarly to their unirradiated counterparts with respect to the above influencing factors. Therefore, it is suggestive that the changes in chromogenesis were due to alterations in metabolism as a result of radiation.

Table 1. Radiation effect upon optimum incubation temperature, chromogenesis, and utilization of glucose of resistant organisms.

Bacteria		Temp (°C)	Chromogenesis	Utilization of glucose	
				Acid	Gas
S. flava	UI[a]	37	yellow		
	R	25	white		
A. eurydice	UI	25			
	R	10			
B. firmus	UI	25	white	+	—
	R	10	pink	—	—
B. pumilus	UI			+	—
	R			—	—

[a] *R* designates irradiated culture; *UI*, the unirradiated.

The only changes in carbohydrate utilization resulting from radiation were experienced by *B. firmus* and *B. pumilus* (Table 1). In each case the irradiated culture failed to ferment glucose as the unirradiated counterpart. Changes were also observed in the action on litmus milk by *B. pumilus* and *B. megaterium* (Table 2). The unirradiated culture of the former bacterium showed a neutral reaction and no reduction after 7 days while the irradiated culture displayed an acid reaction after 4 days and reduction at 7 days. The major difference observed in the latter bacterium was observed in curd formation. The unirradiated culture formed a curd at 4 days whereas the irradiated culture failed to display curd formation at 7 days.

Three bacteria were affected in the utilization of B-vitamins by irradiation (Table 3). *Bacillus minutiferula* lost its ability to utilize niacin and pantothenic acid by irradi-

Table 2. Differences induced by radiation on culture action on litmus milk.[a]

Bacteria		Reaction[c]	Curd	Peptonization	Reduction
B. pumilus	UI[b]	n (7)	+ (2)	+ (4)	− (7)
	R	a (4)	+ (7)	+ (7)	+ (7)
B. megaterium	UI	n (7)	+ (4)	+ (7)	− (7)
	R	n (7)	− (7)	+ (7)	− (7)

[a] Number of days required for action is shown in parentheses.
[b] *R* designates irradiated culture; *UI*, the unirradiated.
[c] Reactions classed as neutral (n) or acid (a).

Table 3. Differences induced by radiation in culture utilization of certain B-vitamins.[a]

Vitamin	Amount per assay tube	Culture growth as % transmission					
		B. minutifervula		*G. homari*		*B. megaterium*	
		UI	R	UI	R	UI	R
Niacin	0.05 μg	69	100				
	0.1	73	100				
	0.2	68	100				
	0.3	56	100				
Pantothenic acid	0.01 μg	49	100				
	0.03	52	100				
	0.05	45	100				
	0.1	42	100				
	0.2	40	100				
	0.5	42	100				
Thiamine	10 mμg			64	100	100	79
	20			53	100	100	80
	30			52	100	100	85
	50			50	100	100	90

[a] Values are averages of two assays determined in duplicate. *R* designates irradiated culture; *UI*, the unirradiated.

ation. *Gaffkya homari* did not use thiamine after treatment while *B. megaterium's* use of the vitamin was enhanced by the low-dose radiation. These results suggest that the metabolic roles of NAD, coenzyme A, and thiamine pyrrophosphate have been changed for the respective bacteria.

Irradiation did not alter the organisms' metabolism of nitrate, hydrolysis of gelatin, motility, production of hydrogen sulfide, reaction to the MR-VP tests, their growth characteristics in nutrient broth, and reaction in tryptone medium for the production of indole. Since the production of indole has been employed as an index of shrimp decomposition (Duggan and Strasburger, 1946), it was interesting to note that none of the radiation-resistant organisms possessed the ability to produce this compound. These results emphasized the role of irradiation in eliminating most of the microorganisms responsible for more rapid decomposition of shrimp.

A summary of differences in physiological responses induced by low-dose gamma radiation shows that all radiation-resistant bacteria isolated from shrimp were affected in at least one attribute (Table 4). These results suggest that the pattern of metabolism in radiation-resistant bacteria can be altered by low-dose radiation.

It is possible that changes produced in the physiology of bacteria are only temporary and most noticeable immediately after irradiation. In some instances, the resistant bacteria revert back to their original metabolic patterns after several subcultures. However, this is not the case in changes reported here. These resistant bacteria have been subcultured monthly over a period of two-and-one half years. During this interval the metabolic determinations were repeated several

Table 4. Summary of differences in metabolism induced by radiation.[a]

Bacteria	Incubation temperature	Chromo-genesis	Carbohydrate utilization	Litmus milk	Vitamin utilization
B. minutiferula	—	—	—	—	X
G. homari	—	—	—	—	X
S. flava	X	X	—	—	—
B. megaterium	—	—	—	X	X
A. eurydice	X	—	—	—	—
B. firmus	X	X	X	—	—
B. pumilus	—	—	X	X	—

[a] *X* designates a difference between the unirradiated and irradiated cultures; "—" designates no difference.

times, emphasizing the permanency of the change. This is suggestive of mutational changes induced in the bacteria by the low level irradiation employed.

A knowledge of these altered pathways can be helpful in the development of chemical and physical techniques to render foods sterile in the extension of their storage life. This is especially true when foods cannot be subjected to high levels of radiation because of changes induced in them by the treatment.

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M. L. FIELDS and LAWRENCE W. SCOTT

Food Microbiology Research Laboratory, Department of Horticulture, University of Missouri, Columbia, Missouri 65202
and
Department of Chemistry, Wisconsin State University, River Falls, Wisconsin

An Investigation of Substrate Effect on AMC Production by Rhizopus nigricans

SUMMARY

In production of acetylmethylcarbinol (AMC), *Rhizopus nigricans* was more efficient with monosaccharides than with disaccharides as carbon sources. Starch permitted intermediate growth, but only a low AMC production. Greatest growth resulted when galactose was the substrate. AMC levels were higher when glucose was the substrate, thus giving lower (better) efficiency values. AMC was produced from pyruvate and acetaldehyde substrates but absent in the presence of sulfite, indicating that acetaldehyde is an essential intermediate for the production of AMC by *R. nigricans*. Diacetyl substrate was changed the most, AMC substrate next, and butanediol substrate the least by *R. nigricans* mycelial mats.

INTRODUCTION

Studies by Beisel *et al.,* (1954), Byer (1954), Fields (1962a, 1964b), Hill and Wenzel (1957), and O'Neil (1957) have established the usefulness of Voges-Proskauer (V-P) reactants as chemical indices of food quality. Although the relationship of the V-P reactants to the degree of contamination has been established and the influence of some factors on the production of acetylmethylcarbinol (AMC) studied (Fields, 1964a), no data have been presented relating AMC production to variation in the carbohydrate source. If one is to employ the V-P reaction as an index of microbial quality to foods of different kinds and composition, a knowledge of the quantity of AMC produced in the presence of various carbohydrates would be helpful. *R. nigricans* was selected for the current study because previous work in this laboratory (Fields, 1962b) indicated that it did not produce diacetyl, thus permitting determination of AMC without interference from diacetyl.

MATERIALS AND METHODS

Test organism. *Rhizopus nigricans*, strain NRRL1478 was grown on slants at room temperature, then suspensions prepared from these slants were used to inoculate media for mat growth. For the studies with preformed mycelial mats, 300 ml of the growth media was inoculated and incubated at room temperature until the mats were strong enough to withstand decanting of the growth media and washing three times with sterile distilled water. Then 300 ml of the substrate being tested was added to the washed mat in the 1-L Erlenmeyer flask. After 12- or 24-hr contact, the substrate medium was separated from the mat and the AMC determined. For the study in which spores were used as the inoculum, 110 ml of the substrate, in 300-ml Erlenmeyer flasks, was inoculated with spore suspension and the fungi allowed to grow 5 days. The substrate was separated from the mat by filtration through a Büchner funnel into 250-ml Erlenmeyer flasks, and then the Büchner funnel was moved to a filtration flask to permit washing of the mat.

Growth media. The media for growth of the mycelial mats contained (g/L) glucose 10.0, tryptone 2.0, yeast extract 2.5, KCl 0.5, $MgSO_4$ 0.5, KH_2PO_4 1.0, and $FeSO_4$ 0.001. The basal medium for the "growth from spores study" was the same except for omission of the glucose and yeast extract.

Carbohydrate substrates. The carbohydrate substrates were made up in 1/15*M* phosphate buffer, pH 5.6, at equivalent carbon concentrations, glucose and galactose at 0.2*M*, sucrose and maltose at 0.1*M* and a starch suspension at 32.4 g/L (based on mean molecular weight of 162 g per 6 carbons; Hodgman, 1932). For the study in which spores were used, each carbohydrate was added to the basal medium in sufficient quantity to give one-half of the above concentration. A solution of glucose in buffer was also prepared at a concentration of 20 g/L (0.111*M*).

Other substrates. Pyruvate solution was prepared by the addition of 5 g pyruvic acid (Matheson-Coleman and Bell P5300) to 800 ml of distilled water and adjusting the pH to 7.2 before sterilization. After dilution to 1 L, the pH was adjusted to 7.0 with sterile HCl and NaOH. Acetaldehyde (Matheson-Coleman and Bell P2724) solutions (pH 6.6) were prepared by aseptic addition of acetaldehyde to sterile distilled water (0.375 mg/ml). The AMC and 2,3-butanediol solutions were

prepared in sterile buffer by aseptic addition of the quantity to yield 10 μg/ml, while diacetyl was prepared at 5 μg/ml.

Sulfite. Sodium sulfite, as a binder for acetaldehyde, was added (1.75 mg/ml) to some of the flasks.

AMC determinations. AMC was determined by the method of Hill and Wenzel (1957), which is a modification of the V-P test. To obtain the AMC free of sugars, 25 ml were distilled from 200 ml of the substrate sample and 15 ml from 120 ml for the experiments in which spores were used as the inoculum. For the comparison of diacetyl substrates, both first and fourth 25-ml fractions of the distillate were collected. Since acetaldehyde and pyruvate, at the levels used, do not interfere with the AMC color reaction, filtration was used to remove mold mycelium and spores, and the color reactions were run directly on the substrate without distillation. For easier comparison of the AMC produced in the 20 g/L glucose substrate with that of the pyruvate and acetaldehyde substrates, the distilled AMC value of the former was divided by 1.39, which has been found to be the concentration factor obtained by distillation of 25 ml from 200 ml. For all determinations, 10 ml of the sample (or diluted sample) was shaken with 5 ml of alpha-naphthol solution (5 g in 100 ml of 95% ethyl alcohol) and 2 ml of creatine-sodium hydroxide (0.3 g creatine in 100 ml of 40% NaOH). Standards between 0 and 5 μg/ml were run with each group of samples. The absorbancy of the solutions at 545 mμ was determined with the Bausch and Lomb Spectronic 20 Colorimeter, 10 min after the addition of creatine-sodium hydroxide solution.

Mat weight. The mats collected on weighed filter paper were washed with distilled water to remove substrates, then dried overnight at 55°C before weighing.

Reducing sugar determinations. To determine the amount of glucose which had been utilized in the experiment, the Lane-Eynon method (AOAC, 1960) was used.

Acetaldehyde determination. The Underkoffler *et al.* (1951) modification of the iodimetric method of Tomoda (1929) which measures bound sulfite was employed.

RESULTS AND DISCUSSION

The amounts of AMC found in the 5 carbohydrate substrates after 12 hr of contact with the preformed mats are given in Table 1. The amount of AMC found, using buffer alone as the substrate, served as a control, and its average (0.6 μg/ml) was subtracted from the values to give those

Table 1. Effect of carbohydrates on AMC production by *R. nigricans*.[a]

Substrate	Replicate flasks	Mean AMC μg/ml	Efficiency ratio mg mat wt./μg/ml
Glucose, 0.2*M*	3	4.2	107[c]
Galactose, 0.2*M*	3	1.9	192
Sucrose, 0.1*M*	3	0.6[b]	317
Maltose, 0.1*M*	3	0.5	425
Starch, 32.4 g/L	3	0.0	847

[a] Preformed mats, 12-hr contact time at room temperature. AMC values corrected for media and buffer blanks. Media buffered at pH 5.6.

[b] Any two means not spanned by the same line are significantly different at the 1% level by Duncan's test (1955).

[c] Any two means not spanned by the same line are significantly different at the 5% level by Duncan's test (1955).

shown in Table 1. *R. nigricans* produced significantly more (1% level; Duncan, 1955) AMC on glucose than on galactose, and both of these averaged significantly higher than the production by sucrose, maltose, and starch. The latter were not significantly different from each other.

Another measure previously used by Fields (1964a) is the efficiency ratio—the mat weight in mg divided by the μg/ml AMC found. These values, calculated prior to subtraction of the buffer-AMC value, are also presented in Table 1. The lower the efficiency ratio, the more effective the mat was in producing AMC. There was no significant difference between the efficiency of production of AMC by *R. nigricans* on glucose and galactose substrates. The efficiency ratios of the galactose and sucrose substrates were also not significantly different, but the efficiency ratio of glucose was significantly different from that of sucrose, maltose, and starch. The efficiency ratio of sucrose was not significantly different from that of maltose, but it was from the efficiency ratio of starch.

The size of the carbohydrate had an influence on the efficiency ratio; for example, *R. nigricans* was 3.9× as efficient on glucose as on maltose, while *R. nigricans* was 7.9× as efficient on glucose as on starch. In another experiment similar results were obtained. The data suggest that, for foods containing primarily starch or the disaccharides such as sucrose and maltose, a low AMC content would not be indicative of

contamination even though considerable growth might have occurred. However, most plant tissues have free glucose, which would tend to make AMC a valid indicator of quality.

In the experiment in which growth was obtained from spores, 100 ml of substrate was diluted to 120 ml to obtain a sufficient quantity for distillation. The AMC values were corrected for this dilution. Glucose and galactose were found to be different in AMC production at the 5% level (Duncan, 1955) in this experiment, as contrasted to difference at the 1% level reported in the preformed mat experiment. The addition of sucrose or maltose to the basic medium significantly increased the 5-day growth (Table 2). The addition of starch, glucose, or galactose to the basic medium produced significantly more growth than the dissaccharides. The AMC content and efficiency ratios are also given in Table 2. The efficiency ratio was not calculated for either the basic medium or the maltose-containing medium because the individual AMC values were zero.

The results of our experiment in which mycelial mats were produced from spores differ from those of Margolin (1942), who, with 8 g/L of carbon from carbohydrate, found *R. nigricans* to grow equally well on D-glucose, D-galactose, and maltose, and no significant growth on sucrose, in 4 days at 25°C. In our study, however, *R. nigricans* grew equally well on both maltose and sucrose. This may be accounted for by strain difference.

Acetaldehyde is known to be an intermediate in AMC synthesis by some organisms and tissues (Gross and Werkman, 1947; Juni 1952a; Neuberg and Simon, 1925; Tomiyasu, 1937), while acetolactic is the intermediate in other cases (Dolin and Gunsalus, 1951; Juni, 1952b; and Watt and Krampitz, 1947). The pathway, however, is not known for the filamentous fungi. Since sulfite complexes acetaldehyde, no AMC would be formed with sulfite present, if acetaldehyde is an essential intermediate for *R. nigricans*. Table 3 shows the effect of addition of sulfite to glucose and to pyruvate substrates. In both cases, the sulfite significantly reduced AMC production. The sugar used by the mycelial mat was about 0.1 of the amount furnished in the absence of sulfite, with utilization only about ¾ as great in the presence of sulfite. Bound acetaldehyde averaged 69 μg/ml with the glucose sulfite medium, showing that production of acetaldehyde was not prevented. This could not be determined in the pyruvate substrate, however, because the pyruvate itself, at the concentration used, reacted slowly with iodine. Also indicated in this table is the ability of *R. nigricans* to utilize pyruvate and acetaldehyde for AMC synthesis. This utilization, when considered with the blocking effect of sulfite, indicates that both pyruvate and acetaldehyde are intermediates in the formation of AMC from glucose, and that acetaldehyde is essential for this synthesis by *R. nigricans*.

Table 2. Effect of carbohydrates on mycelial growth from spores and AMC production by *R. nigricans*.[a]

Substrate	Replicate flasks	Mean AMC μg/ml	Weight mat (mg)	Efficiency ratio
Basic	6	0.0[b]	32.2	[d]
Maltose 0.05M	6	0.1	52.7[c]	[d]
Sucrose 0.05M	6	0.2	52.5	281
Starch 16.2 g/L	5	0.9	154	187
Galactose 0.1M	6	3.4	204	72[b]
Glucose 0.1M	6	4.3	181	43

[a] Incubated at room temperature for 5 days. Each sugar added to basal media.
[b] Any two means not spanned by the same line are significantly different at the 5% level by Duncan's test (1955).
[c] Any two means not spanned by the same line are significantly different at the 1% level by Duncan's test (1955).
[d] Efficiency not calculated because several individual AMC values were zero.

Table 3. Effect of metabolic intermediates on AMC production by *R. nigricans*.[a]

Substrate	Replicate flasks	Contact time (hr)	Mean AMC μg/ml[c]
Glucose, 20 mg/ml	5	12	1.1
Glucose plus sulfite[b]	5	12	0.0
Pyruvate, 5 mg/ml	4	12	0.5
Pyruvate, 5 mg/ml	3	36	2.3
Pyruvate plus sulfite[b]	4	12	0.0
Pyruvate plus sulfite[b]	4	36	0.0
Acetaldehyde 0.375 mg/ml	5	12	6.3

[a] Preformed mats. Mats and substrates incubated at room temperature.
[b] Sulfite was present at 1.75 mg Na_2SO_3/ml of the solution.
[c] All AMC values on undistilled basis corrected for media blanks.

To determine whether AMC, diacetyl, and 2,3-butanediol would be metabolized by *R. nigricans*, solutions were prepared of each and incubated overnight at room temperature, with and without mycelial mats. The samples were distilled, and the first and fourth 25-ml fractions were collected. The first fraction contains both AMC and diacetyl, while the fourth fraction contains only AMC if the diacetyl is not present in large amounts. The original amount of AMC and diacetyl can be obtained by dividing the V-P value in the first 25-ml fraction by 9.46, and the original AMC can be obtained by dividing the fourth 25-ml fraction by 1.39. As shown in Table 4, there was a difference of 1.7 ppm between the AMC substrate with and without a mat. This difference may be due to differences in distillation of the samples.

With diacetyl, however, which is more reactive, there was a 23.9 μg/ml decrease which was produced by the mycelial mat during the 12-hr incubation period. Also, by comparing the fourth fraction "with a mat" and "without a mat," there was 3.8 μg more V-P reactants in the distillate of the diacetyl substrate which had the mycelial mat. The 2.8 μg in the diacetyl "without mat" is probably due to the diacetyl which had not completely distilled over. At the level of diacetyl used (5 μg/ml and since 200 ml were distilled), this is entirely possible. Since the V-P reactants increased in the fourth fraction with the mycelial mat, apparently the mat was converting diacetyl to AMC, which has a constant distillation rate and would be the dominant V-P reactant present in the fourth fraction.

The mycelial mat converted some of the 2,3-butanediol to a V-P reactant, as shown in Table 4. The change in the substrates was least with 2,3-butanediol, next with AMC, and most with diacetyl.

Table 4. Effect of mycelial mats of *R. nigricans* on AMC, 2,3-butanediol and diacetyl.[a]

Substrate[b]	Replicate flasks	Voges-Proskauer Reactants[c] (μg/ml)	
		1st 25 ml	4th 25 ml
AMC with mat	4	11.9	
AMC no mat	3	13.6	
diacetyl with mat	4	23.8	6.6
diacetyl no mat	3	47.7	2.8
2,3-butanediol with mat	4	0.4	
2,3-butanediol no mat	3	0.0	
pH 5.6 buffer with mat	3	0.7	
pH 5.6 buffer no mat	2	0.0	

[a] Mats incubated for 12 hr with substrates at room temperature.
[b] AMC and 2,3-butanediol at about 10 μg/ml, in pH 5.6 buffer; diacetyl 5 μg/ml, pH 5.6.
[c] All V-P reactants calculated as AMC.

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YEHUDA LEVANON
Instituto Adolfo Lutz, São Paulo, Brasil
STELA M. O. ROSSETINI
Faculdade de Odontologia de São José dos Campos, São Paulo, Brasil

A Laboratory Study of Farm Processing of Cocoa Beans for Industrial Use

SUMMARY

Two distinct stages of the farm processing of cocoa beans were recognized: biofermentation with living cells, with resulting metabolites which influence the subsequent stage; and chemofermentation due to the enzymes produced in the previous stage, with living cells not involved. The chocolate flavor resulting from fermentation is seemingly due to the enzymatic digestion. The enzymes are not present in the pulp or in the beans but inside the yeast cells autolyzing in the fermentation boxes or piles.

It is a general practice to make small piles of the cocoa beans harvested from the fruits, or to place them in boxes. Contaminating microorganisms, mostly yeasts, provoke fermentation ("sweating"). During this process, large quantities of liquid and heat are liberated. Some little-known changes occur, and, after drying, the agricultural product is ready for the chocolate industry. In the initial stage of the fermentation, there is liberation of carbon dioxide and production of ethanol. This fermentation is seemingly due to the yeast action. A large number of *Drosophila* sp. flies are attracted, and they contaminate the beans with other microorganisms. The latter microorganisms probably contribute to oxidation of the ethanol to acetic acid. The beans become viscous, and there is diffusion of a purple pigment outside of the specific cells, all over the bean. When the beans are dried, the purple pigment becomes brownish and the cotyledons retract. The whole process is called fermentation, preparation, or curing, and it yields the typical chocolate flavor. The present paper attempts to elucidate the nature of the changes involved in the farm processing of cocoa beans for the chocolate industry.

MATERIALS AND METHODS

Beans were collected from fruits of the varieties "Catongo," "Forasteiro," and "Crioulo." The fruits had been obtained from *Theobroma cacau* plants raised at the Cocoa Experimental Station, Urucuca, State of Bahia. 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. These differed from commercial samples of cocoa beans, which, commonly, are mixtures of beans from plants of several varieties. Cocoa pulp (cocoa honey) of each type was collected separately from fruits of each variety in Erlenmeyer flasks and frozen at −45°C. Thawed pulp was used at a pH of approximately 3.8. All experiments were conducted separately in beans and in pulp of each variety.

One hundred beans were cut in halves, and the percentage of "good beans" was estimated according to methods of De Witt (1953) and Levanon and Martelli (1964). The required number of pulp samples, consisting of 200 ml of thawed or freshly collected pulp, were placed in Erlenmeyer flasks and sterilized by flowing steam (100°C) for 60 min. The same number of samples were sterilized by filtration through a Seitz filter. Then, the preparations were incubated for 72 hr at 37°C to disclose an eventual turbidity due to microbial growth.

Beans were thoroughly rinsed with tap water until the pulp was completely eliminated, and the tegument was peeled off with a knife. Homogenization in a Waring blender was carried out in the following preparations: 100 g of beans and 100 ml of saline; 100 g of beans and 200 ml of saline; 100 g of beans and 300 ml of saline; 100 g of beans and 400 ml of saline. Each of the samples was divided into two parts; one part was sterilized by flowing steam, and the other part was filtered through a Seitz filter. The preparations were incubated for 72 hr at 37°C to test for turbidity due to microbial growth.

Beans for fermentation experiments were rinsed with tap water until all the pulp was eliminated. The tegument was peeled off in part of the beans. The incubation and drying of the beans were done at various temperatures (Table 1, Fig. 1). The fermentation was carried out according to Quesnel (1958, 1960). To 500 ml of 2% acetic or tannic acid solution, 100 g of pulpless beans were added and stirred for 8 hr. The bean preparation was subsequently washed with saline.

Table 1. Number of yeast cells in relation to culture medium, time and temperature.[a,b]

Temperature (°C)	Time (hr)	Culture medium: Steam-sterilized pulp	Filter-sterilized pulp	Beans without pulp	Beans without tegument
25	12	10^{3}	10^{3}	0	0
	24	10^{5}	10^{5}	0	0
	48	3×10^{5}	3×10^{5}	rare	0
	60	10^{5}	10^{5}	rare	0
	72	4×10^{4}	4×10^{4}	rare	0
37	12	10^{5}	10^{5}	0	0
	24	10^{7}	10^{7}	rare	0
	48	3×10^{7}	3×10^{7}	rare	0
	60	10^{7}	10^{7}	rare	0
	72	6×10^{5}	6×10^{5}	rare	0
42	12	3×10^{5}	3×10^{5}	0	0
	24	2×10^{7}	4×10^{7}	rare	0
	48	7×10^{7}	8×10^{7}	rare	0
	60	10^{7}	10^{7}	rare	0
	72	6×10^{6}	7×10^{6}	rare	0
48	12	10^{4}	10^{4}	0	0
	24	10^{6}	10^{6}	0	0
	48	3×10^{5}	4×10^{5}	rare	0
	60	10^{5}	2×10^{5}	rare	0
	72	6×10^{4}	6.5×10^{4}	rare	0

[a] Inoculum composed of 0.1 ml of yeast cell suspension whose turbidity corresponded to tube no. 3 of McFarland scale.
[b] Numbers in the columns correspond to yeast cells per ml.

All the preparations were inoculated with 0.1 ml of yeast-cell suspension from a washed culture with turbidity equivalent to tube no. 3 of the McFarland scale. The suspension was prepared with a strain of *Saccharomyces* sp. isolated from fermentation boxes in the Cocoa Experimental Station at Urucuca and maintained in Sabouraud medium. The number of yeast cells obtained in the culturing experiments was determined by colony counting in Sabouraud plates. An extract of yeast cells was also prepared with the same strain. The cells were washed three times with saline. To 1 g of sedi-

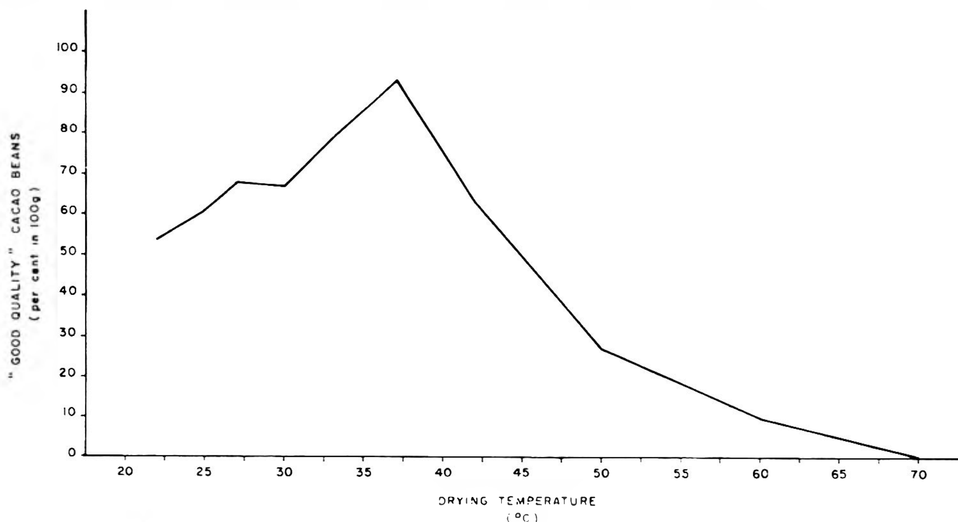

Fig. 1. Yield of "good-quality" cacao beans in relation to drying temperature. Fermentation according to Quesnel's method for small quantities.

mented cells (wet weight) were added 20 ml of saline and 10 g of powdered glass. The mixture was shaken for 1 hr at 4°C in the Mickel disintegrator and then spun for 20 min in a refrigerated centrifuge (0°C) at 6000 rpm. The sediment was washed three times with 20 ml cold saline, and all supernatants were pooled, made up to 100 ml with saline, and stored in a freezer. A portion of this extract was inactivated for 3 hr in a water bath (60°C).

RESULTS

The results obtained with pulp and beans from fruits of the "Forasteiro" variety are presented in Tables 1 and 2 and Fig. 1. The results with the other varieties did not differ significantly in any instance. Separate fermentation of the pulp and the beans showed that pulp inoculated with yeasts favors growth of yeasts (Table 1) and yields ethanol. No growth of yeasts occurred when bean homogenates were used as culture medium, showing that the factors necessary for yeast growth are present not in the bean but in the pulp. The growth factors remained the same after steam or filter sterilization. Little growth of yeasts was obtained when these were inoculated on beans rinsed with tap water without peeling off the tegument. This might be due to the presence of small quantities of pulp still present on the surface of the bean. There was no growth of yeasts on peeled-off beans.

Dipping the beans in a 2% solution of acetic or tannic acid for 12–24 hr made the beans permeable to the enzymes (Table 2). The same was observed when the beans were dipped in 2% acetic or tannic acid and stirred for 8 hr. Filter-sterilized pulp (without any contact with yeast cells), when mixed with washed (pulpless) beans, resulted in beans without chocolate flavor. Furthermore, the germs were not dead. Addition of 12-hr-fermented pulp with yeast cells to washed beans with further drying for 48 hr resulted in the death of germs, retraction of the cotyledons, and appearance of typical chocolate flavor. The mixture of 12-hr-fermented pulp without yeast cells and washed cocoa beans previously treated with acetic or tannic acid, then left for 48 hr and allowed to dry, did not exhibit typical chocolate flavor, although the beans appeared very similar to beans of "good quality." This is probably due to the diffusion of pigment out of their specific cells. Forty-eight hours after beans treated with acetic or tannic acid were added to a solution containing 1% of yeast extract, the beans showed, after drying, typical color, retracted cotyledons, and chocolate flavor.

As can be seen in Fig. 1, the optimum drying temperature was 37°C, which provided the highest yields of "good-quality" beans. When beans were dried at 37–45°C, a medium quality was obtained. When dried at temperatures higher than 46°C the cotyledons retracted and the typical brown color appeared, but the typical chocolate flavor was not present. The optimum temperature for the growth of yeasts was 42°C, but there was also a good cell multiplication at 37°C.

DISCUSSION

It is generally admitted that the preparation of cocoa beans essentially consists of a fermentation process. The work reported here permits recognizing two distinct stages, biofermentation and chemofermentation. The first stage comprises fermentation with living cells and the resulting metabolites, while the second stage comprises the action of enzymes produced in the previous stage, with living cells not involved.

It is clear that the fermentation process of the pulp does not affect the beans but results in a large quantity of yeasts and acetobacteria, with subsequent formation of acetic acid. The autolysis of the yeast cells supplies the endoenzymes necessary for the final chocolate flavor, as indicated by the following evidence: a) typical chocolate flavor was obtained when the yeast-cell extract

Table 2. Yield of "good-quality" cacao beans (beans with chocolate flavor) obtained after fermentation for 48 hr and drying at 37°C.[a]

Previous treatment of beans	Fermentation agent: 12-hr-fermented pulp (without yeast cells)	Seitz-filtered unfermented pulp	Yeast-inoculated pulp	Yeast-cell extract	Inactivated yeast-cell extract[b]
No treatment	0	0	81	3	....
2% acetic acid	0	0	80	96	0
2% tannic acid	0	0	72	83	0

[a] Numbers in the columns are percent of "good-quality" beans in 100 g. "Good-quality" beans determined according to the criteria of De Witt (1953) and Levanon and Martelli (1964).

[b] Heated 3 hr at 60°C.

was used as the fermentation agent; b) no chocolate flavor was obtained when autolysis of yeasts cells did not occur, such as happened when pulp fermented for only 12 hr and then passed through a Seitz filter was inoculated on beans. The enzyme or enzymes responsible for the changes yielding the chocolate flavor are seemingly unable to penetrate the unprocessed bean, probably because the bean tegument is impervious to the enzymes. Acetic acid is a mordant and apparently makes the tegument permeable. A similar action of tannic acid, a common mordant, was also apparent. The effect of acetic acid on the germ is little known. Since this acid denatures proteins, it might prevent the germination. As already mentioned, acetic acid is produced naturally during the common preparation of beans for industrial use.

Yeasts could not be cultivated in pulpless beans, probably because of the absence of growth factors. This would explain the inferior quality of cocoa beans produced by farmers who eliminate the pulp before the fermentation. Absence of pulp decreases yeast cell numbers considerably, and prevents obtaining the adequate amount of enzymes. That the chemofermentation constitutes the most important stage of the cocoa preparation is readily shown by drying the beans at temperatures above 46°C, when the enzymes are rapidly inactivated and the needed water is evaporated.

Recapitulating: The chocolate flavor resulting from the fermentation process is seemingly due to enzymatic digestion. The responsible enzymes are present not in the pulp or in the bean but inside the yeast cells autolysing in the fermentation boxes or piles.

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L. R. HACKLER, J. P. VAN BUREN, K. H. STEINKRAUS, I. EL RAWI,[a] and D. B. HAND

New York State Agricultural Experiment Station
Cornell University, Geneva, New York 14456

Effect of Heat Treatment on Nutritive Value of Soymilk Protein Fed to Weanling Rats

SUMMARY

The effect of heating soymilk at 93 and 121°C for varying periods was evaluated in weanling rats. Also determined were trypsin inhibitor retention and available lysine values. Cooking soymilk 1–6 hr at 93°C had no adverse effect on protein efficiency, growth, or available lysine. With cooking for 32 min at 121°C in contrast, there was a definite decline in protein efficiency ratio, and an indication that available lysine was declining. The drop in available lysine was greater after the soymilk had been heated 40 min at 121°C. The results indicate that the protein efficiency ratio of heat-processed soymilk is dependent upon both time and temperature of treatment. Also evaluated was the effect of spray-drying temperature and drying method on the nutritional quality of soymilk. The results indicate that an inlet temperature of 277°C or higher causes a drop in the utilization of soymilk protein, with a concurrent drop in the available lysine. Various methods of drying (spray, vacuum roll, atmospheric roll, and freeze) soymilk did not alter the nutritional quality of the protein to any great extent, although the data obtained for the freeze-dried soymilk did show a slightly lower growth rate and PER value. Available lysine data obtained for the heat-processed soymilk appear to be a better indication of protein quality in overheated soymilk than in underheated samples. The percentage of trypsin inhibitor retained, on the other hand, appears to be a good criterion for underheated but not for overheated soymilk samples.

INTRODUCTION

Soybeans have been an important source of food in the human diet for centuries. In recent years, research on plant proteins has been stimulated in the United States as a means of furnishing high-quality protein to infants who are allergic to cow's milk (Fomon and May, 1959; Omans *et al.*, 1963; Kay *et al.*, 1960).

[a] Present address: College of Pharmacy, Bagdad, Iraq.

Many countries do not have an adequate supply of high-quality animal protein (such as cow's milk) for infant consumption, and soymilk is widely used in many developing countries. Wide variations have been reported (Mitchell, 1950) in the nutritive value of soybean products other than soymilk. Mitchell *et al.*, as early as 1945, recognized that the most important agency modifying the nutritive value of food proteins during processing was heat. Several years later, Hayward (1959) stated that one of the most common causes of quality variance in soybean products is heat—either too much or too little. This investigation was undertaken to learn to what extent variations in the nutritional quality of the protein in a water extract of soybeans might be caused by differences in heat treatment.

EXPERIMENTAL METHODS

The growth studies reported in this paper were of 4 weeks' duration. In Experiments 1 and 2, male weanling rats of the Holtzman strain were maintained on a basal diet containing 11% casein for 4 days to adjust to their new environment, prior to distributing them into groups of 10 each, according to weight. The groups were then assigned, at random, to the experimental diets. In Experiments 3, 4, and 5 the weanling rats were maintained on a basal diet containing 15% casein for 2 days, and this has become a standard practice in our laboratory. In all studies the rats were handled exactly the same during the 4-week test periods. Additional details of our experimental procedures in handling rats for growth studies have been described by Hackler *et al.* (1963). Criteria used in evaluating the nutritional value of protein in heat-processed soymilks were growth, feed intake, and protein efficiency ratio (PER) in weanling rats. In addition, available lysine was determined by the method of Carpenter (1960), and trypsin inhibitor retention values were determined for the soymilk samples by the procedure described by Van Buren *et al.* (1964).

Preparation of soymilk samples. In all studies, certified Clark variety soybeans were used in

preparing the soymilk samples. The soybeans were soaked overnight at room temperature in three times their weight of tap water. During soaking, the beans absorbed water to approximately 1.2 times their weight. After soaking, the beans were drained and the soak water was discarded. The beans were then ground with water through a .023-in. screen of a disintegrator (Model RA-4K53, Rietz Mfg. Co., Santa Rosa, Calif.), and the resulting slurry was passed through a plate filter to remove the water-insoluble residue. After removal of the residue, the soymilk was immediately cooked in a heat exchanger, concentrated in a vacuum evaporator, and dried.

In Experiment 1 the soymilk samples were cooked 0, 30, 60, 120, 240, and 360 min at 93°C. In Experiment 2 the soymilk samples were cooked 0, 5, 10, 20, 40, 60, and 120 min at 121°C. In Experiments 1 and 2 respectively, the soymilk samples were dried in a vacuum roll dryer (laboratory model, Blaw-Knox Co., Buffalo, N. Y.) and freeze dryer (Model 2004L3, F. J. Stokes Machine Co., Philadelphia, Pa.). The drying conditions were standardized in Experiment 3, and all soymilk samples were dried in a spray dryer (laboratory model No. E13735, Proctor and Schwartz, Inc., Philadelphia, Pa.) with inlet/outlet temperatures of 150/72°C. All samples that were not spray dried were ground through a .050-in. screen in a comminuting mill (Model D, W. J. Fitzpatrick Co., Chicago, Ill.). In Experiment 3 the soymilk samples were heated 15, 30, 60, 120, and 240 min at 93°C, and 2, 4, 8, 16, and 32 min at 121°C.

The soymilk samples in Experiments 4 and 5 were prepared as previously described, except that all of these samples were cooked 10 min at 121°C. In Experiment 4, after the soymilk was cooked it was subjected to the following spray-dryer inlet temperatures: 143, 182, 227, 277, and 316°C. In Experiment 5 the soymilk samples were heat processed as above but were dried by various methods. The drying procedures investigated were spray, freeze, vacuum roll, and atmospheric roll. An inlet spray-drying temperature of 166°C was used in Experiment 5 for the spray-dried sample. A laboratory freeze dryer was used to lyophilize a second sample. A third sample was dried in a roll (drum) dryer using 5 lb of steam and a vacuum of 29 in. Hg. The fourth sample was dried in a roll dryer at atmospheric pressure under 45–50 lb of steam pressure. Additional details on the pilot-plant processing of soymilk have been described by Hand *et al.* (1964).

The soymilk samples were analyzed for protein (N × 6.25) by the micro-Kjeldahl procedure as outlined in AOAC (1960). The fat content was determined by modification of an AOAC method in which the extraction is carried out with ethanol/chloroform. After analyses of soymilk samples were completed, the diets were formulated and mixed, and then subjected to another micro-Kjeldahl analysis prior to starting the experiment. The analyzed protein value was used in determining the protein efficiency ratio.

A commercially available soybean meal was used as a standard for comparison in Experiments 1 and 2. In Experiment 3, casein was used as the standard for comparison. All diets were mixed to contain 10% crude protein and 12% fat. The dietary components were as follows (parts per 100): sucrose, 12; cellulose, 4; mineral mix, 4; vitamin mix, 1; A-D-E oil, 1; choline chloride, 0.4; corn oil, 5.9±.3; dextrose, 50.8±.3; and soymilk, 20.9±.3. The latter three ingredients varied depending upon the results from the analytical data obtained for nitrogen and fat. The source of the dietary components, and the composition of the vitamin mixes have been described (Hackler *et al.*, 1963).

RESULTS AND DISCUSSION

The results obtained in Experiment 1 with soymilk that had been subjected to a processing temperature of 93°C from 0 to 360 min are summarized in Table 1. There

Table 1. Effect of processing soymilk at 93°C for various periods in Experiment 1.

Minutes at 93°C	Av. daily gain (g)[a]	Av. daily feed (g)[a]	PER[a]	Trypsin inhibitor retained (%)	Available lysine (g/16 g N)
0	.29±.05	7.7±.17	.37±.06	100	5.2
30	2.11±.08	12.0±.29	1.76±.06	10	5.2
60	2.33±.09	11.8±.33	1.96±.05	8	5.4
120	2.03±.15	11.3±.53	1.79±.07	4	5.2
240	2.35±.10	12.4±.35	1.86±.05	4	5.2
360	2.33±.10	12.7±.37	1.83±.06	4	5.2
SBM[b]	3.42±.18	14.3±.63	2.30±.05	....	....

[a] Each datum represents the mean value of 10 rats ± SE of mean.
[b] Soybean meal (44% crude protein).

was a strong similarity in the growth, feed intake, and protein efficiency ratio of the rats receiving the diets containing soymilk heated 30 min or longer in this experiment. The rats receiving the diet containing raw soymilk grew much more slowly than did the other rats. The values obtained in this experiment on the amount of trypsin inhibitor retained after processing indicate that if 10% or less was retained in the soymilk there was no interference in growth, feed intake, or PER. In this experiment, available lysine was not altered in the soymilk by cooking it 0–360 min at 93°C.

It should be noted that none of the soymilk samples produced a growth rate or PER value equal to that found for soybean meal (SBM). This supports earlier observations of Hackler *et al.* (1963) on several soybean fractions, in which the soymilk fraction was found to be inferior to dehulled ground soybeans.

The trypsin inhibitor values presented in Table 1 help explain the differences in response to the soymilk-containing diets. Several investigators have reported the "toxic" factor in raw soybeans to be due principally to trypsin inhibitor (Mitchell, 1950). The trypsin-inhibitor retention values observed in this study on soymilk appear to support earlier literature in which the growth rate of rats receiving raw soybeans was reduced.

Since there was no marked change in growth response to the water-extracted soybean preparation cooked for as long as 6 hr at 93°C, it was apparent that this was a mild form of heat treatment insofar as destruction or loss in nutritive value of the protein was concerned. Therefore, Experiment 2 was designed to evaluate the effect of a higher processing temperature on the utilization of soymilk protein. The results for Experiment 2 are summarized in Table 2. In contrast to the previous results on protein efficiency, etc. (Table 1), obtained when the water extract from soybeans was cooked at 93°C, the effects of processing soymilk at 121°C were very striking. In Experiment 2 the peak of nutritional quality was reached in 5-10 min of heating at 121°C, whereas, in the previous study, essentially no change in nutritive value was noted for soymilk protein cooked at 93°C after 90% of the trypsin inhibitor was inactivated.

It should be noted that the rats in Experiment 2 were initially 10 g heavier. This heavier starting weight is, perhaps, an indication that this shipment of weanling rats was more thrifty than the rats used in Experiment 1. The heavier starting weight may account for the greater growth and feed consumption of the rats in Experiment 2. In addition, the rats adjusted to their diets faster and grew more rapidly during the early part of the study. The standard diet containing soybean meal (SBM) as the only source of protein attests to the difference in response of the two groups of rats. The rats receiving the commercial SBM in Experiment 2 grew faster, 4.21 g/rat/day, as opposed to 3.42 g/rat/day for the group in the previous study.

It was apparent from Experiments 1 and 2 that cooking time and temperature are very important considerations in the processing of soymilk. The conclusion might be

Table 2. Effect of processing soymilk at 121°C for various periods in Experiment 2.

Minutes at 121°C	Av. daily gain (g)[a]	Av. daily feed (g)[a]	PER[a]	Trypsin inhibitor retained (%)	Available lysine (g/16 g N)
0	.64±.13	9.2±.59	.65±.11	100	6.0
5	3.51±.17	14.9±.61	2.24±.05	10	6.0
10	3.40±.08	15.3±.56	2.20±.08	6	6.0
20	3.19±.23	15.2±.77	2.00±.08	2	5.9
40	2.53±.15	13.8±.32	1.83±.10	1	5.7
60	2.26±.12	12.7±.46	1.74±.04	1	5.5
120	1.78±.11	12.2±.40	1.37±.06	1	5.0
SBM[b]	4.21±.21	16.6±.67	2.48±.08	....	....

[a] Each datum represents the mean value of 10 rats ± SE of mean.
[b] Soybean meal (44% crude protein).

drawn that 93°C is a mild form of heat treatment, and that 121°C is a severe heat treatment. Secondly, processing soymilk at 121°C resulted in a higher PER value at the optimum processing time (2.24 at 5 min, as opposed to a PER value of 1.96 when soymilk was heated 60 min at 93°C). Therefore, it seemed desirable to conduct another study (Experiment 3) in order to resolve this question of the higher PER to see if there was a real difference in the optimum PER value of soymilk processed at the two temperatures.

The soymilk samples for Experiment 3 were processed exactly alike except for the variables in cooking procedure that were of interest. Since the two shortest times used in the earlier studies were essentially not different from longer times, insofar as optimum growth, feed intake, and PER were concerned, it was decided to add a shorter cooking time at each temperature and to remove the longer cooking times. Secondly, in order to remove the difference observed between the two previous shipments of rats, the soymilk cooked for various periods at 93 and 121°C were fed to rats obtained in a single shipment.

The results obtained in Experiment 3 are summarized in Table 3. These data show very clearly that the optimum cooking time for soymilk at the two different temperatures results in equally high-quality protein as measured by growth in weanling rats. Although there is a slightly higher PER value obtained for soymilk that has been heated for an optimum length of time at 121°C, as opposed to the highest value obtained for soymilk heated at 93°C (2.21 and 2.09, respectively), the values are not statistically different. The data were subjected to analysis of variance and Duncan's multiple-range mean test.

The two shorter cooking times that were added in Experiment 3 (15 min at 93°C and 2 min at 121°C) resulted in less efficient utilization of the protein for growth. Furthermore, in this study, holding the soymilk for 30 min at 93°C did not result in as good growth or as efficient utilization of the protein as did the longer cooking times at the same temperature. The amount of trypsin inhibitor retained in soymilk heated 30 min. at 93°C was 56% in Experiment 3 (Table 3), as opposed to 10% in the earlier study (Table 1). This difference in retention of trypsin inhibitor apparently accounts for the discrepancy in the rat data. The data indicate that the rate of trypsin-inhibitor destruction was different in the three studies. More efficient cooling of the soymilk samples in Experiment 3 may account for the differences.

The data obtained on the effect of spray-drying temperature on the utilization of soymilk cooked 10 min at 121°C are sum-

Table 3. Effect of processing soymilk at 93 and 121°C for various periods in Experiment 3.

Minutes at:	Av. daily gain (g)[a]	Av. daily feed (g)[a]	PER[a]	Trypsin inhibitor retained (%)	Available lysine (g/16 g N)
93°C					
15	1.90±.05	10.8±.43	1.69±.07	66	5.9
30	1.79±.12	10.7±.56	1.67±.07	56	5.4
60	2.54±.19	12.2±.57	2.04±.11	14	5.6
120	2.73±.21	12.6±.72	2.09±.09	4	5.6
240	2.63±.10	12.6±.54	2.09±.08	0	5.5
121°C					
2	1.97±.12	10.9±.53	1.78±.06	36	5.4
4	2.74±.14	12.4±.42	2.21±.05	20	5.8
8	2.61±.17	11.4±.50	2.20±.08	16	5.7
16	2.75±.16	12.5±.48	2.11±.07	12	5.8
32	2.60±.19	12.3±.50	1.97±.08	5	5.6
Casein	2.96±.04	12.3±.67	2.40±.03		

[a] Each datum represents the mean value of 10 rats ± SE of mean.

marized in Table 4. It is apparent from the results that a spray-drying temperature above 227°C interferes with growth and feed intake. In addition, using an inlet temperature of 277°C decreases PER and available lysine values. The available lysine value is approximately 18% lower for soymilk spray-dried with an inlet temperature of 277°C than for soymilk dried with an inlet temperature of 227°C. An inlet temperature of 316°C results in an even greater decrease in growth, PER, and available lysine. Furthermore, there is a marked drop in PER value between inlet temperatures of 227 and 277°C and a more drastic drop between inlet temperatures of 277 and 316°C.

The results obtained with four different drying procedures are summarized in Table 5. A spray-drying inlet temperature of 166°C was used in the preparation of the spray-dried soymilk sample in this study. It is interesting to note that all of the data for the various methods of drying are similar. However, the rats receiving the freeze-dried soymilk grew at a slightly slower rate than the rats in the other groups.

The data on available lysine in Experiment 3 (Table 3) indicate that available lysine is not a good measure of protein quality in soybean products that have been underheated. In Experiment 2, where the soymilk samples were heated long enough to destroy 90% or more of the trypsin inhibitor (Table 2), the available lysine data appear to be a good measure of protein quality. On the other hand, trypsin-inhibitor data from these studies appear to be a valuable tool in assessing the nutritional quality of soymilk that has been underheated.

The data show quite clearly that the length of time soymilk is processed at 121°C is critical. On the other hand, the data for soymilk cooked at 93°C indicate that this is a mild processing temperature, for there was no loss in the nutritive value of soymilk protein cooked for 6 hr.

The PER values obtained in these studies attest to the high quality of the protein in the water extract. Although soymilk has been reported to be inferior to the water-insoluble fraction (discarded in the production of soymilk) when fed to rats (Hackler *et al.*, 1963), it must be realized that for infants the water-insoluble fraction would be of limited value, since it is a very bulky material that contains the insoluble carbohydrates and approximately 12% fiber.

Table 4. Effect of spray-drying temperature (inlet) on the utilization by weanling rats of soymilk cooked 10 min at 121°C.

Spray drying temperature (inlet) (°C)	Av. daily gain (g)[a]	Av. daily feed (g)[a]	PER[a]	Trypsin inhibitor retained (%)	Available lysine (g/16 g N)
143	2.38±.11	12.3±.48	1.90±.03	6	5.3
182	2.87±.16	13.3±.56	2.10±.04	8	5.3
227	2.64±.12	13.2±.45	1.99±.07	4	4.9
277	1.90±.08	11.5±.43	1.63±.03	5	4.0
316	0.13±.06	7.8±.52	0.16±.06	3	1.9

[a] Each datum represents the mean value of 10 rats ± SE of mean.

Table 5. Effect of various drying methods on the utilization by weanling rats of soymilk cooked 10 min at 121°C.

Drying method	Av. daily gain (g)[a]	Av. daily feed (g)[a]	PER[a]	Trypsin inhibitor retained (%)	Available lysine (g/16 g N)
Spray[b]	3.07±.13	13.9±.38	2.22±.04	10	5.4
Vacuum roll	3.06±.15	13.7±.48	2.22±.06	10	5.3
Atmospheric roll	3.20±.14	14.7±.87	2.19±.06	5	5.5
Freeze	2.96±.15	13.8±.31	2.14±.09	10	5.6

[a] Each datum represents the mean value of 10 rats ± SE of mean.
[b] Inlet temperature of 166°C.

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KAYE FUNK, MARY E. ZABIK, and DORIS M. DOWNS
Departments of Foods and Nutrition and Institution Administration, Michigan State University, East Lansing, Michigan 48823

Comparison of Shear Press Measurements and Sensory Evaluation of Angel Cakes

SUMMARY

Procedures were developed for Kramer shear-press measurement of compressibility, tensile strength, and tenderness of angel cakes of three degrees of toughness. Both maximum force and area-under-the-curve values were determined for each. These values were correlated with sensory evaluations of tenderness, of moistness and of texture, defined as cell size, cell distribution, and cell wall thickness.

Maximum-force shear-press values consistently showed differences among the three types of cake in compressibility, tenderness, and tensile strength. Based on area-under-the-curve, compressibility and tenderness differences were detected, but no consistent pattern was noted in tensile-strength readings. Sensory evaluations showed variance in the 3 types of cake for all characteristics except cell distribution.

Correlations between sensory evaluations and maximum-force shear-press measurements were high. Area-under-the-curve values for compressibility and tenderness correlated with sensory evaluation at varying levels of significance. Highly significant correlations were found between the area-under-the-curve value for tensile strength and the following factors: cell size, cell wall thickness, tenderness, and moistness. The correlation between area-under-the-curve for tensile strength and cell distribution was not significant.

INTRODUCTION

The quality of any given food may be defined as the composite of distinctive characteristics which give it unique identity and which contribute to its acceptability to the consumer. Individual quality factors may be considered as separate entities, examined as interrelated parts, or regarded as a composite grouping which conveys a general, overall impression of acceptability.

Quality characteristics may be measured objectively by physical or chemical procedures, or subjectively by means of sensory evaluation. Any type of measurement involves two principles: precision and validity. Precision describes the exactness of method necessary for duplicating results, and validity refers to the degree to which the measurement actually reflects a true quantitative value for the particular quality in question.

Objective measurements of quality characteristics generally offer more possibility for precision than do subjective techniques. Thus an instrument, such as the Kramer shear-press, would be expected to provide reproducible results for identical samples of food under conditions which might affect the reliability of a human judge, such as time of day and environmental conditions. However, the usefulness of objective measurement is, to some extent, contingent upon reasonable agreement with sensory evaluations. Thus, it is of primary importance that objective methods be developed which will provide a true measurement of the particular quality factor under study.

This study was made to: 1) develop procedures to measure the quality characteristics of angel cakes objectively with the Kramer shear-press, and 2) compare data from objective tests with sensory evaluation data on like samples. The investigators theorized that if correlations between data from the two methods were high, the need for sensory evaluation might be eliminated in further studies.

LITERATURE REVIEW

Platt and Kratz (1933) reported procedures for measuring tensile strength and compressibility of sponge cakes, using instruments devised in their laboratory. Tensile strength was measured by the total weight required to break a suspended hour-glass-shaped cake sample. The total weight was determined by weighing the amount of water run at a constant rate of 200 g per min into a suspended vessel attached to the spring clamp holding the cake sample. Compressibility was measured by the amount of deformation produced in a specified time by a plunger 1¼ in. in diameter resting on the sample. Similar measurements were made by Barmore (1936) using angel cake.

Quality characteristics of angel cakes were evaluated by sensory methods in studies by Harnes

et al. (1952) and Carlin and Ayres (1953). In studies by Peet and Lowe (1937), Jordan *et al.* (1954), and Briant and Williams (1956), high correlations were obtained between sensory evaluations and tensile strength measurements. Tensile-strength measurement procedures were modifications of methods used by earlier workers.

The Kramer shear-press was developed by Kramer *et al.* (1951) to measure the tenderness of fruits and vegetables. It has since been used quite extensively for measuring meat and poultry tenderness. High coefficients of correlation between the shear-press measurements and sensory evaluations of meat or poultry have been obtained by Shannon *et al.* (1957), Bailey *et al.* (1962), Burrill *et al.* (1962), Bramblett and Vail (1964), Rogers *et al.* (1964), and Tuomy and Lechnir (1964). Kramer shear-press measurements of fruit firmness have been correlated with sensory evaluations in studies by Sweeney *et al.* (1962) and Shallenberger *et al.* (1963). No work has been reported to date using the Kramer shear-press to measure quality characteristics of angel cakes.

EXPERIMENTAL METHODS

Preparation of cakes. Angel cakes of three degrees of toughness, designated as standard, tough, and very tough, were prepared from household-size packages of commercial cake mix. Each package of cake mix contained two separate packets: a flour-mix portion and a dried-egg-white mix portion. The contents of 2 packages were combined for each batch of cake batter, which was divided into 2 equal portions for baking. To provide enough slices for objective and subjective tests, two cakes were required from the batter for each replication. Each of the 3 variables was replicated 5 times.

To produce cakes of varying degrees of toughness, specified amounts of all-purpose flour were added to the flour-portion of the cake mix. To produce batters for the tough cake, 50 g flour was added to 560 g of the mix; for the very tough cakes, 100 g flour was added to 560 g of the mix. Standard cakes were prepared without added flour. Liquid for all cakes was kept constant.

To each 298 g of the dried-egg-white portion of the cake mix was added 640 ml water at 30°C. This mixture was blended 1 min with the whip attachment of a Hobart Mixer, model A-200, set at speed 1. The sides and bottom of the 12-quart bowl were scraped down and mixing was then continued at the same speed for 1 min. The mixture was then whipped at speed 3 for 1 min.

The flour portion of the cake mix (plus added flour when involved) was then added to the beaten egg whites during a 30-sec period with the mixer on speed 1. The sides and bottom of the bowl were again throughly scraped down, after which beating was continued for 45 sec for the standard cakes, 2½ min for the tough cakes, and 3½ min for the very tough cakes.

Each batch of standard cakes made without adding extra flour, was poured into ungreased aluminum loaf pans, 16 × 3¼ × 4 in., using 700 g/pan. Because of the weight of added flour, 720 g batter was used for each tough cake and 740 g for each very tough cake. Approximately 7% of the batter was lost in handling. All cakes were baked for 35 min at 177°C in an ETCO forced-convection oven, model 186.

Cakes were inverted and cooled for 1½ hr before removal from the pans. After an additional 30-min cooling period on wire racks, cakes were coded, wrapped in Saran, and put into polyethylene bags. They were frozen and held at −23°C for later testing.

Sensory and shear-press evaluation. For sensory and shear-press evaluation, cakes were sliced in the frozen state with a Hobart Slicer, model 410, set at 70. The end pieces of each cake comprising a replication were discarded and the remaining portion of each loaf was cut into 15 slices. The slices from one cake in each replication were numbered consecutively, 1 through 15, and from the second cake, 16 through 30. For each of the six panelists and each of the four methods of shear-press measurements, one slice was taken from every 10th position to permit triplicate evaluations of the quality characteristics from the 30 slices of each replication. The position from which the slice was taken from the cake was moved forward by one for each successive replication. For example, Judge 1 was given slices 1, 11, and 21 for the first replication; slices 2, 12, and 22 for the second replication; and so on. This rotation plan permitted evaluation of samples from the different positions within the cake by each taste panelist and shear-press method. Four cakes, comprising two replications, were randomly selected for evaluation each testing day. Evaluations of the triplicate samples from each replication were averaged.

Samples for sensory evaluation were cut from the frozen cake slices with a 5.08-cm-diameter cutter and placed on plates coded with random numbers. The cake samples were covered with Saran wrap and allowed to warm to room temperature for 1–1½ hr before evaluation. Judges were asked to evaluate the quality characteristics of: 1) texture, defined as cell size, cell distribution, and cell wall thickness, 2) tenderness, and 3) moistness. Descriptive terminology formed the basis of the evaluation. A 7-point numerical scale, designated to correspond with descriptive terms, was used for the analysis of the data.

Compressibility. For measuring compressibility a Kramer shear-press, model SP12, was used with the piston from the succulometer cell and with a fixed-blade upper assembly of a standard shear-compression cell. For both measurements, the 100-lb electronic proving ring, the 5-lb range, and 20-lb pressure were used.

Extra weight was added to the piston from the succulometer cell by winding solder around the shaft. This decreased the need for adjusting the instrument after changing the attachments. A round cake sample, 5.08-cm diameter × 2.02 cm thick, was depressed on a platform, 3.1 cm in thickness, which had been inserted on the support plates at the bottom of the columns (Fig. 1). The distance between the piston in its lowest position and the base plate was measured. An average of nine readings was subtracted from the thickness of the cake slice to obtain the amount of depression to each sample. A downward stroke timed at 30 sec was used. Compressibility was calculated as the maximum force necessary to depress the cake sample 1.25 cm.

For compressibility measurements with the fixed-blade upper assembly of a standard shear-compression cell, a 5.72-cm-sq × 2.02-cm-thick cake sample was depressed 1.41 cm on a platform 2 cm in thickness. The amount of depression was calculated in the same manner as stated above. The maximum force necessary for depression was calculated as compressibility.

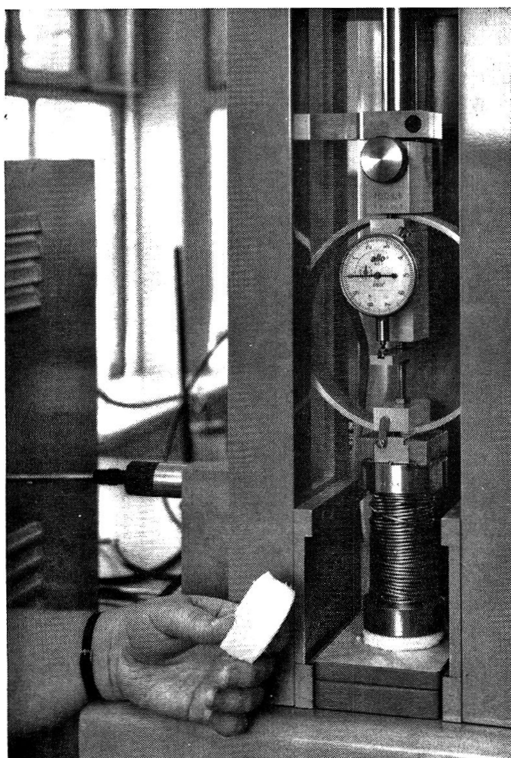

Fig. 1. Measuring compressibility of angel cake with the piston from the succulometer cell of the Kramer shear press.

Tensile strength. Tensile strength was measured by using a specially designed attachment for the shear-press (Fig. 2). Clamps were attached to a U-shaped base plate and to an upper assembly just large enough to attach to the proving ring. An Acco Clamp No. 325 was modified by substituting a lighter spring which would not cut the cake samples when they were placed against the cake sample. A set screw was also added to regulate further the face placed against the cake sample. The 100-lb electronic proving ring, the 5-lb range, and 20-lb pressure were used. By rewinding the chart-drive cable of the shear-press, a reading on the ram upstroke was obtained.

Samples used for tensile strength were cut from frozen cake slices, 2.02 cm thick, with an hourglass-shaped cutter measuring 2.54 cm across the center (Fig. 3). The width of the break was measured with vernier calipers. The formula

$$\frac{\text{Maximum graph reading} \times \text{range}}{\text{Area of the break (in cm}^2\text{)}}$$

was used to calculate tensile strength.

Tenderness. The standard shear-compression cell was used for tenderness measurements. The 3000-lb electronic proving ring, the 300-lb range, and 20-lb pressure were used. A weighed 5.72-cm-sq × 2.02-cm-thick cake sample was sheared during a 30-sec downstroke. Tenderness was calculated as:

$$\frac{\text{Maximum graph reading} \times \text{range.}}{\text{Sample weight}}$$

The cell was washed in tepid water and thoroughly dried with an unheated blower after each measurement.

The area-under-the-curve was calculated for all shear-press measurements. After the area under each graphed curve was carefully cut out, it was weighed on a Mettler balance, type H15. A conversion factor of 174.2 was obtained from a linear calibration curve arrived at by weighing 5 replicates of 8 different squares varying in area from 1 to 64 sq cm cut from randomly selected portions of similar chart paper.

The data were analyzed for variances and coefficients of correlation.

RESULTS AND DISCUSSION

Sensory evaluation. Analysis of variance showed that cell wall thickness provided a very precise method for distinguishing differences in the quality characteristics of angel cakes (Table 1). Judges evaluated the cell

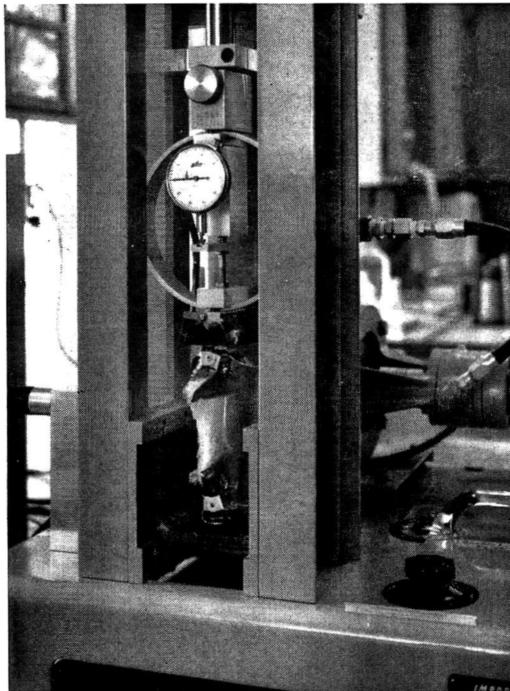

Fig. 2. Measuring tensile strength of angel cake.

walls of standard cakes as thin. Cell walls of tough cakes were considered moderately thick, whereas very tough cakes were characterized by thick cell walls.

Panelists were consistently able to detect differences, which were found to be highly significant, in cell size in the 3 types of cakes. As the toughness of the cake increased, the cells became smaller and more compact. Panelists were asked to evaluate cell distribution on the basis of uniformity; the average values obtained show they were able to distinguish slight differences (Table 1). However, cell distribution was not found adequate to measure differences in quality among the 3 types of cake. All samples had slightly to moderately irregular distribution of cells, regardless of type of cake.

Sensory tenderness evaluations were found to be a reliable means of detecting differences in angel cakes (Table 1). Standard cakes were described as tender. The cakes intended as tough were designated slightly tough by panelists, and cakes intended as very tough were evaluated by them as moderately tough.

Differences detected in cake moistness by the panel were also found to be very highly significant (Table 1). They considered the standard cake moist, the tough cake slightly dry, and the very tough cake moderately dry.

Shear-press measurements. Based on maximum force (Table 2) and area-under-the-curve (Table 3), as respectively determined with the piston from the succulometer cell and with the fixed-blade upper assembly of the standard shear-compression cell, measures of compressibility showed differences among the cake types which were very highly significant. As the toughness of the cake increased, more force was required for the same amount of depression of the cake samples. As the maximum compression force increased, the area-under-the-curve value became larger.

Maximum force per g of cake (Table 2) and area-under-the-curve (Table 3) indicated highly significant differences in the tenderness of the 3 types of angel cakes. Less force was required to shear tender cakes than was necessary for shearing tough or very tough cakes.

Tensile strength, as indicated by maximum force per sq cm, provided a sufficiently precise measure of the toughness to show highly significant differences among the types of angel cakes (Table 2). However, area-under-the-curve measurements of tensile strength did not show significant differences among the cakes (Table 3).

Difficulties were encountered in performing the tensile-strength measurements. Because of these difficulties, 5 of the 15 averages presented in Tables 2 and 3 are based on 2 observations, and it was possible to ob-

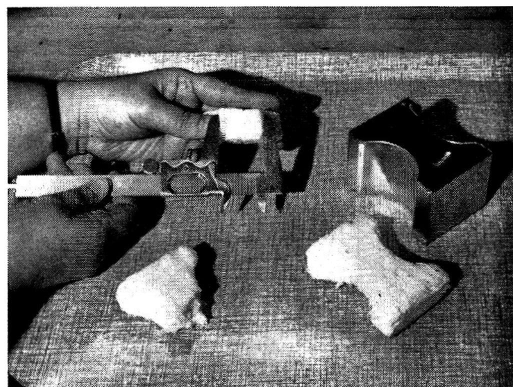

Fig. Hourglass-shaped cutter, angel cake sample, and area of the break measurement used to determine tensile strength.

Table 1. Sensory evaluation (average values for 18 observations) of standard, tough, and very tough angel cakes based on a 7-point scale.

Quality characteristic	Replication 1	2	3	4	5	Av.		Standard deviation
Texture								
Cell size								
Standard	5.0	5.4	5.1	3.8	5.6	5.0	**	.70
Tough	3.9	2.7	4.1	4.5	3.6	3.8		.68
Very tough	2.4	2.0	2.9	2.9	2.7	2.6		.38
Cell distribution								
Standard	5.1	4.7	5.2	4.2	5.9	5.0		.63
Tough	4.6	4.0	4.6	4.9	4.4	4.5		.33
Very tough	4.0	4.1	4.1	4.7	4.2	4.2		.27
Cell wall thickness								
Standard	5.7	5.4	5.2	5.1	5.2	5.3	***	.24
Tough	4.4	4.4	4.1	4.7	4.7	4.5		.25
Very tough	3.5	3.2	3.7	3.6	3.8	3.6		.23
Tenderness								
Standard	6.5	6.1	5.9	5.9	5.8	6.0	***	.27
Tough	4.8	4.3	4.5	4.6	5.0	4.6		.27
Very tough	3.2	3.1	3.3	3.2	3.7	3.3		.23
Moistness								
Standard	5.4	5.6	5.5	5.3	5.2	5.4	***	.16
Tough	4.8	4.1	4.6	4.7	4.8	4.6		.29
Very tough	4.0	3.6	4.1	3.7	3.9	3.9		.21

*** Differences significant at 0.1% level.
** Differences significant at 1% level.

Table 2. Kramer shear-press measurements (average values for 3 observations) of standard, tough, and very tough angel cakes based on maximum force.

Quality characteristic	Replication 1	2	3	4	5	Av.		Standard deviation
Compressibility (lb force) (piston measurement)								
Standard	.35	.19	.26	.23	.39	.28	***	.08
Tough	.73	.89	.55	.56	.32	.61		.21
Very tough	1.94	1.93	2.01	1.94	1.52	1.87		.20
Compressibility (lb force) (fixed-blade measurement)								
Standard	.36	.47	.33	.63	.40[a]	.44	***	.12
Tough	1.44	1.23	1.08	1.20	1.12	1.21		.14
Very tough	3.22[a]	3.65	3.29	3.16	2.67	3.20		.35
Tensile strength (lb force/cm^2)								
Standard	.009	.004[a]	.007[b]	.008	.010	.008	**	.002
Tough	.010	.035	.018	.017	.015	.019		.009
Very tough	.042[a]	.045[a]	.020[a]	.032[a]	.020	.032		.012
Tenderness								
Standard	2.03	1.81	2.09	2.05	1.92	1.98	***	.11
Tough	2.40	2.72	2.74	2.16	2.64	2.53		.25
Very tough	3.21	3.15	3.37	3.15	3.36	3.25		.11

[a] Average values for 2 observations.
[b] Average value for 1 observation.
*** Differences significant at 0.1% level.
** Differences significant at 1% level.

Table 3. Kramer shear-press measurements (average values for 3 observations) of standard, tough, and very tough angel cakes based on area-under-the-curve in sq cm.

Quality characteristic	Replication 1	2	3	4	5	Av.		Standard deviation
Compressibility (piston measurement)								
Standard	.383	.198	.279	.186	.441	.297	***	.112
Tough	.639	1.016	.430	.412	.197	.539		.309
Very tough	1.568	1.759	2.009	1.911	1.544	1.758		.205
Compressibility (fixed-blade measurement)								
Standard	.302	.482	.186	.505	.366[a]	.368	***	.132
Tough	1.272	1.179	1.005	.749	1.033	1.048		.199
Very tough	2.430[a]	3.397	2.625	3.263	2.085	2.760		.557
Tensile strength								
Standard	.099	.079[a]	.139[b]	.046	.192	.111		.056
Tough	.116	.679	.288	.285	.203	.314		.216
Very tough	.400[a]	.698[a]	.192[a]	.267[a]	.267	.365		.201
Tenderness								
Standard	.255	.290	.331	.157	.331	.273	***	.072
Tough	.314	.476	.488	.331	.337	.397		.081
Very tough	.772	.749	.807	.813	.766	.781		.028

[a] Average values for 2 observations.
[b] Average value for 1 observation.
*** Differences significant at 0.1% level.

tain only 1 observation in one case. Although every attempt was made to prevent it, a few cake samples were damaged in the cutting process.

The low sensitivity of the Kramer shear-press recording device used in this investigation may also have been responsible for some of the difficulties encountered in obtaining tensile-strength measurements. All of these readings were extremely low, and it seems feasible that greater accuracy might be obtained in future testing by using a Recorder Indicator, Model E2, with the Kramer shear press to amplify the portion of the scale used.

Of the four Kramer shear-press measurements used in this investigation, compressibility was obtained most easily. Tenderness measurements necessitated washing and drying the cell for standardized conditions after each measurement. Rewinding the chart drive (which was necessary for taking tensile-strength measurements since the Kramer shear-press used for this experiment was not equipped with a switch for automatically reversing the chart drive) was somewhat tedious and time consuming.

Correlations between sensory and objective measurements. Very highly significant correlation coefficients were obtained between maximum-force Kramer shear-press values for measures of compressibility, tensile strength, and tenderness, and sensory evaluations of cell size, cell wall thickness, tenderness, and moistness. Even though cell distribution did not provide an adequate measure of differences in quality among the 3 types of cake, highly significant correlation coefficients were obtained between this sensory evaluation and maximum-force values of compressibility, as measured with the piston from the succulometer cell, and between the same sensory evaluation and objective tenderness measurements. Significant correlations were also obtained between panel evaluations of cell distribution and maximum-force values of compressibility, as measured with the fixed-blade upper assembly of the standard shear-compression cell, and between tensile strength measurements and sensory evaluations of cell distribution (Table 4).

Area-under-the-curve shear-press measurements of compressibility and tenderness correlated at a very high level of significance with sensory evaluations of cell size, cell wall thickness, tenderness, and moistness. Correlation coefficients for tensile strength and sensory evaluations of cell size, cell wall

Table 4. Coefficients of correlation between sensory and Kramer shear-press evaluations of standard, tough, and very tough angel cakes.

Sensory evaluation	Maximum force				Area-under-the-curve			
	Compressibility (piston measurement)	Compressibility (fixed-blade measurement)	Tensile strength	Tenderness	Compressibility (piston measurement)	Compressibility (piston measurement)	Tensile strength	Tenderness
Texture								
Cell size	*** −.82	*** −.86	*** −.85	*** −.90	*** −.80	*** −.85	** −.67	*** −.78
Cell distribution	* −.54	** −.61	* −.58	** −.68	* −.51	* −.57	−.46	* −.50
Cell wall thickness	*** −.91	*** −.94	*** −.83	*** −.93	*** −.89	*** −.93	** −.62	*** −.90
Tenderness	*** −.93	*** −.95	*** −.84	*** −.93	*** −.89	*** −.93	** −.63	*** −.92
Moistness	*** −.90	*** −.91	*** −.88	*** −.93	*** −.89	*** −.92	** −.72	*** −.90

*** Significant at 0.1% level.
** Significant at 1% level.
* Significant at 5% level.

thickness, tenderness, and moistness were highly significant. The cell-distribution evaluation and shear-press measurements of compressibility and tenderness were significantly correlated. The correlation between tensile strength and cell distribution was not significant.

From the data of this study, the investigators concluded that the Kramer shear-press, with appropriate attachments and techniques, can be used to evaluate the quality characteristics of angel cakes with sufficient precision to show significant differences among variables. Lower standard deviations (Tables 1, 2, 3) indicate that greater precision was obtained with objective measurement than with sensory evaluations. Kramer shear-press measurements indicated that a high degree of validity was obtained with the objective measurements. Maximum-force values showed greater validity than did area-under-the-curve values for measuring tensile strength.

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