# Journal of
# FOOD SCIENCE

(formerly Food Research)

PUBLISHED BY THE INSTITUTE OF FOOD TECHNOLOGISTS





CALVERT L. WILLEY, *Publisher*
221 North LaSalle Street
Chicago, Illinois 60601 U. S. A.

VOLUME 31

JANUARY-DECEMBER, 1966

printed by

R. F. Colwell Printing Corp.

CHAMPAIGN, ILLINOIS U. S. A.

Journal of FOOD SCIENCE

JANUARY-FEBRUARY, 1966 Volume 31, No. 1

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An Official Publication of the Institute of Food Technologists
Suite 2120, 221 North LaSalle St., Chicago, Illinois 60601, U.S.A.

C. E. BODWELL,[a] A. M. PEARSON, J. WISMER-PEDERSEN,[b] and L. J. BRATZLER
*Department of Food Science, Michigan State University, East Lansing, Michigan 48823*

# Post-Mortem Changes in Muscle
# II. Chemical and Physical Changes in Pork

## SUMMARY

**The chemical changes occurring from 0 to 24 and/or 48 hr post-mortem were followed in longissimus dorsi muscle from 18 pork carcasses. In an attempt to induce soft, watery pork, one side from each of 13 carcasses was placed at 37°C immediately after slaughter, and the other side of each carcass was subjected to -29°C. Values for pH, glycogen, total reducing sugars, and lactic acid differed markedly at 0 time. Carcasses from Poland China pigs showed a stoichiometric relationship between the post-mortem decrease in glycogen and the corresponding accumulation of lactic acid and total reducing sugars. This relationship was less apparent in Hampshire pigs. The 37°C treatment did not consistently result in soft, watery and pale muscle, as was expected from other reports. This suggests that exposure of muscle to a low pH at a high temperature per se does not necessarily cause the soft, watery, and pale appearance. A loss in fibrillar water-binding capacity as a result of low pH values and high muscle temperatures confirmed earlier investigations. However, the decrease in fibrillar water-binding capacity as a result of the low pH at high muscle temperatures did not usually make the muscle appear soft and watery. Results indicate that even though a loss in fibrillar water-binding capacity occurs as a consequence of a low pH and high temperature, and is a characteristic frequently encountered in soft, watery muscle, low fibrillar water-binding capacity by itself is not the primary causal factor in making pork muscle appear soft and watery. Post-mortem levels of ATP, creatine phosphate, lactic acid, total reducing sugars and glycogen are reported and compared with literature values.**

## INTRODUCTION

Distinct variations in the rate and extent of certain chemical changes during the post-mortem prerigor and rigor stages in pork muscle have been shown to have considerable influence on the quality of the resulting meat (Lawrie, 1962; Bendall, 1960; Briskey, 1963, 1964). Although ultimate pH and rate of muscle glycolysis have been strongly implicated (Wismer-Pedersen, 1959; Lawrie, 1960; Wismer-Pedersen and Briskey, 1961a,b; Briskey and Wismer-Pedersen, 1961a,b), some exceptions have been reported (Lawrie, 1960; Sayre *et al.*, 1961). It has been generally implied or concluded that a low pH ($< 5.7$–$6.0$) at a high muscle temperature ($> 35°$) resulting from rapid post-mortem glycolysis (Ludvigsen, 1953; Wismer-Pedersen, 1959; Wismer-Pedersen and Briskey, 1961a,b; Briskey and Wismer-Pedersen, 1961a,b; Bendall *et al.*, 1963; McLoughlin and Goldspink, 1963; Sayre *et al.*, 1963 a,c; Briskey, 1963) or a low pH at onset of rigor (Sayre *et al.*, 1964; Sayre and Briskey, 1963; Briskey, 1964) is associated with the development of soft, watery and pale pork muscle. Numerous workers (Wismer-Pedersen and Briskey, 1961a,b; Briskey and Wismer-Pedersen, 1961a; Bendall and Wismer-Pedersen, 1962; Bendall *et al.*, 1963) have reported that muscle held post-mortem at 37°C develops the characteristics of soft, watery pork. Accordingly, it has been suggested (Wismer-Pedersen and Briskey, 1961b; Bendall *et al.*, 1963; Goldspink and McLoughlin, 1964; Borchert and Briskey, 1964) that rapid post-mortem cooling to attain muscle temperatures of less than about 30°C before the pH level falls below 5.8–6.0 would be expected to retard or prevent the development of the soft, watery condition.

The current study investigated the effect of post-mortem temperature treatments on various physical and chemical characteristics of the longissimus dorsi muscle from the pig.

## EXPERIMENTAL

**Animals.** Ten Poland China gilts, acquired from a private breeder, were kept for 2–3 weeks at the Michigan State University Farm prior to slaughtering. These gilts and one Yorkshire gilt from the

[a] Laboratory of Biophysical Chemistry, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland.
[b] Royal Veterinary and Agricultural College, Copenhagen, Denmark.

University herd were slaughtered in groups of 2 or 3 animals upon reaching 190–220 lb. The animals were brought to the Meat Laboratory 2½ days before slaughter, and provided with feed and water until slaughter in order to avoid stress. The carcasses from the 10 Poland China gilts and one Yorkshire gilt were respectively designated 1–10 and 11Y.

Six Hampshire gilts were obtained from the University Farm and housed at the Meat Laboratory for 3–6 days. They were allowed free access to feed and water up to slaughter. Carcasses from these gilts were designated A-G.

**Slaughtering and sampling procedures.** The pigs were electrically stunned, shackled, hoisted, bled, and handled in the usual manner. Initial samples were removed from the uneviscerated but scalded and dehaired carcasses at an average of 11½ min after sticking. The maximum interval between death and initial sampling was 16 min.

Carcasses 1–5 were dressed in the usual manner with the carcasses split down the backbone and the fatback left intact. On all other carcasses a similar procedure was followed except that on the side to be placed at −29°C the backfat over the longissimus dorsi muscle was trimmed to less than ¼ inch thickness to facilitate rapid cooling.

Samples were removed and treated as previously described (Bodwell *et al.*, 1965) except that chloroform was added to the TCA extracts prior to storage. In addition, samples were removed for determination of fibrillar water-binding capacity in carcasses 1–10 and 11Y. The sampling locations and sequence of removal of samples are listed in Table 1. The sequence of sample removal was randomized for the first 5 carcasses (1–5), but was standardized in all other carcasses (6–10, 11Y and A–G). The standardized procedure eliminated the side to side variation due to location.

Table 1. Sampling locations[a] on pork carcasses.

| Carcass | Right side | Left side |
|---|---|---|
| 1 | 1L, 9T, 6L, 13T, 3L, 4/5L, 2T, 11T[b] | 3L, 11T, 6L, 13T, 9T, 7T, 1L, 4/5L |
| 2 | 7T, 9T, 6L, 13T, 1L, 11T, 8T | 5L, 13T, 4/5L, 3/4L, 11T, 1L, 7T |
| 3 | 6L, 9T, 11T, 4/5L, 7T, 3L, 13T, 1L, 5L | 1L, 3L, 11T, 4/5L, 6L, 13T, 7T, 9T, 7T |
| 4 | 4/5L, 2/3L, 1L, 6L, 7T, 9T, | 2/3L, 4/5L, 1L, 6L, 7T, 9T |
| 5 | 4/5L, 11T, 1L, 9T, 2/3L, 14T | 2/3L, 11T, 1L, 9T, 4/5L, 14T |
| 6 | 2/3L, 11T, 1L, 9T, 4/5L, 14T | Same as in right side |
| 7 | " | " |
| 8 | 4/5L, 7T, 1L, 6L, 2/3L, 9T | " |
| 9 | " | " |
| 10 | 6L, 4/5L, 2/3L, 1L, 13/14T, 9T | " |
| 11Y | " | " |
| A–G | 2/3L, 11T, 1L, 9T, 4/5L, 14T | " |

[a] L = lumbar vertebra; T = thoracic vertebra. Numbers refer to vertebra numbers, i.e., 1 = 1st, 2 = 2nd, etc.
[b] Listed in order of sampling.

Table 2. Temperature treatments.

| | Treatment | | | |
|---|---|---|---|---|
| Carcass no. | Right side | Hours[a] | Left side | Hours |
| 1 | 4°C | — | 37°C | 4½–5 |
| 2 | 4°C | — | −29°C[b] | 1 |
| | | | 25°C | 1½ |
| 3 | 37°C | 4½–5 | 4°C | — |
| 4 | 4°C | — | −29°C | 4–4½ |
| 5 | 4°C | — | −29°C | 4–4½ |
| 6–10, 11Y | −29°C | 4–4½ | 37°C | 4½–5 |
| A–G | −29°C | 4–4½ | 37°C | 4½–5 |

[a] Number of hours a side was subjected to a given treatment prior to being placed in the 4°C cooler.
[b] The left side of carcass 2 was first placed in the −29°C treatment for 1 hr then moved to room temperature for 1½ hr prior to being placed in the 4°C cooler.

**Temperature treatments.** The various temperature treatments to which the individual sides were subjected are listed in Table 2. All sides were placed at a given temperature within 13–27 min of death. One side of carcasses 1–5 was subjected to so-called "normal cooling conditions" (3–4°C), while the opposite side was subjected to one or more of three different temperature treatments (Table 2). Since the procedures used for carcasses 1–5 did not result in marked differences in ultimate muscle condition between the two sides from a given carcass, the two extremes in temperature (+37°C, −29°C) were subsequently employed for the remaining carcasses (6–10, A–G).

**Determination of fibrillar water-binding capacity.** The water-binding capacity of the fibrils was determined according to the general procedure of Bendall and Wismer-Pedersen (1962). A sample weighing approximately 2 g was homogenized in 25 ml of a 0.04*M* $KH_2PO_4$ buffer (pH 7.0; approximate ionic strength = 0.09) with a VirTis homogenizer, which was taken to full speed in 3 bursts of 10-sec duration so as to avoid excessive heating of the homogenate. Furthermore, the homogenizing flask was placed in an ice-bath during homogenization. The homogenate was centrifuged for 15 min at 200 × G and the supernatant discarded. The precipitated fibrils were remixed with 25 ml of buffer and centrifuged as before. The supernatant was discarded. The concentration of nitrogen in the washed fibrils was determined by a micro-Kjeldahl method. Samples removed at 24 hr post-mortem were weighed on an analytical balance before homogenization, and the weight of the precipitated fibrils was also determined prior to nitrogen measurements. Grams of water retained per g of fibrillar protein was calculated for all samples. Fibrillar protein precipitated per g of sample was also calculated for the 24-hr samples. Calculations were made according to the procedure of Bendall and Wismer-Pedersen (1962).

**Temperature, pH, and chemical determinations.** Temperature was recorded and pH determinations made according to procedures previously described (Bodwell *et al.*, 1965). Temperature measurements were recorded only during the initial 6–8 hr post-mortem. The levels of creatine phosphate, glucose (i.e. total reducing sugars), glycogen, and lactic acid were determined and the data analyzed as previously described (Bodwell *et al.*, 1965). The initial levels of ATP (adenosine triphosphate) for carcasses 6–10 and 11Y were determined by an acid-molybdate and an enzymic method (Bodwell *et al.*, 1965). On all other samples, ATP analyses were made only by the enzymic method.

**Panel evaluation.** The extent of the soft, watery, and pale condition of the cut loin surface of carcasses 6–10, 11Y, and A–G was evaluated subjectively at 48 hr post-mortem by a four-member panel. The panel rated each loin as being either not, slightly, moderately, or extremely soft, watery and pale (0, 1, 2, or 3, respectively).

## RESULTS AND DISCUSSION

Individual values for some of the various characteristics or chemical constituents which are not included herein have been tabulated elsewhere (Bodwell, 1964).

**Carcasses 1–5.** The various temperature treatments utilized for carcasses 1–5 did not result in any appreciable differences between the two sides of a given carcass. The longissimus dorsi muscle surface for both sides of all five carcasses was considered normal at 48 hr post-mortem.

**Muscle temperature.** Fig. 1 depicts two typical temperature curves for two sides of the same carcass, one subjected to −29°C and the other to >37°C. The initial temperature in both sides was approximately 41°C prior to being subjected to the two different temperatures. The temperature of the side held at −29°C decreased to about 30°C by approximately 65 min post-mortem and had decreased to 19–20°C at 2 hr. The side subjected to the higher temperature was still more than 35°C at 3½ hr post-mortem.

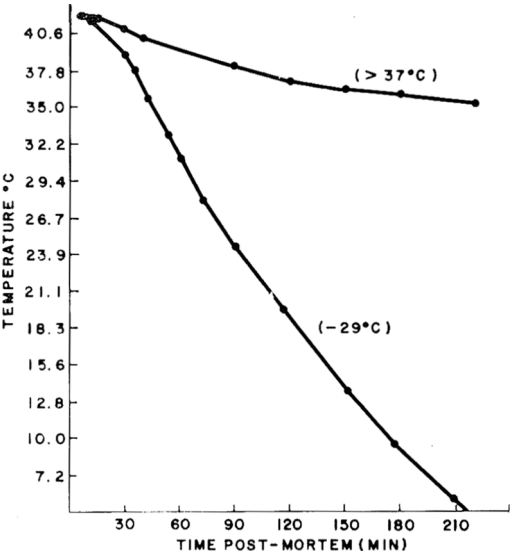


Fig. 1. Post-mortem temperature of pork longissimus dorsi muscle of representative sides subjected to −29 or 37°C temperatures immediately after death.

**Muscle condition, pH and temperature.** The effect of the two temperature treatments on pH value and ultimate muscle condition is given in Table 3 for carcasses 6–10, 11Y, and A–G. Previous reports (Wismer-Pedersen, 1959; Lawrie, 1960; Briskey and Wismer-Pedersen, 1961b; Bendall *et al.*, 1963; Briskey, 1963; McLoughlin and Goldspink, 1963) suggest that the extremely low initial pH value for carcass 6 would be expected to result in the soft, watery muscle condition in both sides of this carcass. However, the side cooled at −29°C appeared nearly normal, while the other side (>37°C) was rated a little lower than moderate. This is a case where rapid cooling would not have been expected to greatly alter the muscle condition of the side placed at the −29°C treatment, since the prevailing conditions believed to have a causal relationship to the development of the soft, watery condition were already developing within 16 min post-mortem. These conditions were a low pH level (less than pH 6.0) while muscle temperature was still high, i.e., above 30–35°C (Wismer-Pedersen, 1959; Briskey and Wismer-Pedersen, 1961a,b; Bendall *et al.*, 1963; McLoughlin and Goldspink, 1963).

The muscle condition of the two sides of carcass 7 was evaluated as normal, and slightly to moderately soft and watery for the −29°C and 37°C treatments, respectively. The rate of glycolysis immediately after death was not greatly different in the two sides as evidenced by 3-hr pH levels, but ultimate pH differed (Table 3). The high 3-hr pH value of 6.2 for the side in the 37°C treatment was reflected in high levels of $ATP_2$ (enzymic method of analysis) and glycogen. The relatively slow rate of glycolysis was expected to result in normal muscle (Briskey and Wismer-Pedersen, 1961b; Bendall *et al.*, 1963). However, development of the soft, watery condition in the 37°C treatment supported previous observations as to the role of the low pH-high temperature relationship post-mortem, and/or the suggestion that rapid cooling to below 30°C before the pH level decreased to 5.9–6.0 or less would be expected to prevent development of the soft, watery condition (Briskey and Wismer-Pedersen, 1961b, Wismer-Pedersen and Briskey, 1961b; Bendall *et al.*, 1963; Borchert and Briskey, 1964).

Similar differences (Table 3) in ultimate muscle condition were apparent in the two sides of carcass 8. The identical pH levels in the two sides of this carcass at 3 hr post-mortem would not necessarily preclude a faster rate of glycolysis in the side at 37°C. However, the initial pH of 6.1 suggested

Table 3. Post-mortem pH and ultimate muscle condition as affected by temperature treatments of carcasses 6–10, 11Y and A–G.

| | | 3 hr | | 48 hr | | Degree of soft, watery condition[a,b] | |
|---|---|---|---|---|---|---|---|
| Carcass no. | Initial | −29°C | >37°C | −29°C | >37°C | −29°C | >37°C |
| 6 | 5.6 | 5.4 | 5.3 | 5.3 | 5.3 | 0.75 | 1.75 |
| 7 | 6.7 | 6.5 | 6.2 | 5.6 | 5.3 | 0.00 | 1.25 |
| 8 | 6.1 | 5.3 | 5.3 | 5.3 | 5.3 | 0.00 | 1.75 |
| 9 | 6.4 | 5.7 | 5.4 | 5.3 | 5.5 | 0.00 | 0.75 |
| 10 | 6.3 | 6.3[c] | 5.5[c] | 5.5 | 5.5 | 0.00 | 0.00 |
| 11Y | 6.6 | 6.3[d] | 5.4[d] | 5.7 | 5.5 | 0.00 | 0.00 |
| A | 6.7 | 6.4 | 5.4 | 5.7 | 5.3 | 0.00 | 1.25 |
| B | 6.6 | 6.1 | 5.7 | 5.8 | 5.7 | 0.00 | 0.50 |
| C | 6.9 | 6.5 | 5.4 | 5.7 | 5.3 | 0.00 | 0.25 |
| D | 6.8 | 6.6 | 5.5 | 5.7 | 5.4 | 0.00 | 0.00 |
| E | 6.7 | 6.6 | 5.4 | 5.6 | 5.3 | 0.00 | 1.00 |
| F | 6.8 | 6.7 | 5.3 | 5.7 | 5.2 | 0.00 | 0.25 |
| G | 6.8 | 6.7 | 5.4 | 5.6 | 5.3 | 0.00 | 0.75 |

[a] 48 hr post-mortem.
[b] Evaluated on a scale of 0 = none, 1 = slight, 2 = moderate and 3 = extreme.
[c] Approximately 2 hr post-mortem.
[d] Approximately 4 hr post-mortem.

that the pH in the side at −29°C would have declined to below 5.9–6.0 prior to any significant drop in muscle temperature.

Marked differences were observed in 3-hr pH levels between treatments for the two sides of carcasses 9, 10, and 11Y; this was particularly evident for opposite sides from carcasses 10 and 11Y. Results on these three carcasses (Table 3) suggested that a low pH at a high muscle temperature cannot be equated with routine development of the soft and watery condition. This observation was further supported by data obtained with carcasses A–G (Table 3). All of these carcasses had an initial pH of 6.6 or higher. Ultimate 48-hr pH levels varied from 5.5 to 5.8 in muscles held at −29°C and from 5.2 to 5.7 in the muscles at 37°C. In general, the 37°C treatment greatly lowered the 3-hr pH values, but alteration of the ultimate appearance of the muscles was less apparent.

Results of carcass A followed the pattern that would be expected from the literature (Table 3). The initial pH value was 6.7 (Table 3). At 3-hr post-mortem, the muscle at −29°C had a pH of 6.4, while the side at 37°C had a pH of 5.4. The 48-hr pH values were respectively 5.5 and 5.3 for the muscle from the low and high temperature treatments. No evidence of the soft, watery condition was found in the muscle held at −29°C, while a slight to moderate degree was observed at 37°C. Results were similar with carcass E.

In the remaining five carcasses from the Hampshire gilts (Table 3), no significant changes in ultimate muscle condition were apparent. The data from carcass F were of particular interest. The initial pH value was 6.8. The 3-hr pH values were respectively 6.7 and 5.3 for the −29 and 37°C treatments. The eventual condition of the muscles from both treatments, however, was assessed as being normal.

From the results on the two groups of carcasses, it is concluded that a low pH at a high muscle temperature was not a causal factor *per se* in producing soft, watery and pale muscle. This conclusion does not preclude the occurrence of low pH-high temperature conditions immediately post-mortem as a normally encountered characteristic of muscle eventually termed soft, watery and pale. These conclusions are in disagreement with observations of previous investigators (Wismer-Pedersen and Briskey, 1961b; Bendall *et al.*, 1963), who reported that soft, watery, pale muscle could be induced by holding excised muscle from Danish Landrace pigs at 37–41°C until rigor ensued, and/or by merely heating a minced sample of normal muscle for 1½ hr at 37°C. Likewise, the current observations do not support the conclusion (Wismer-Pedersen and Briskey, 1961a,b; McLoughlin and Goldspink, 1963; Sayre *et al.*, 1963a,c) that a low pH at a high temperature results in the development of soft, watery and pale muscle in pork carcasses.

Conversely, the current results are similar to a report by Lawrie (1960), in which excised muscle strips from the longissimus dorsi muscle of Large White × Middle White pigs were held at 37°C under anaerobic conditions. The time required to reduce pH from 6.7 to 5.5 varied 3-fold, indicating a wide range in glycolytic rates. Although the author noted that rate of pH decline and degree of exudation were related, all muscles were implied to be normal, even though they had been held at a high temperature until a low muscle pH was attained. Similarly, Briskey (1964) in a recent review has referred to unpublished research which is in agreement with results of the current study.

**Properties of fibrillar protein.** Table 4 gives the fibrillar protein content/g of muscle and the water retained/g of fibrillar protein from 24-hr post-mortem samples of carcasses 6–10 and 11Y. The means and standard deviation for the g of fibrillar protein/g of muscle were respectively 0.0635±0.007 and 0.0970±0.0045 for the −29 and 37°C treatments. Corresponding values for g of water retained/g of fibrillar protein were 34.77± 11.98 and 13.25±2.91 g. The data for individual sides paralleled the observations on alterations in pH (Table 4). The −29°C treatment was ineffective in significantly altering either the fibrillar protein content or the water retention of the washed fibrils in carcass 6. The muscle from both sides of this carcass was ultimately somewhat soft and watery, as previously discussed. A pro-

Table 4. Fibrillar protein content and water binding of washed fibrils from 24-hr post-mortem samples of carcasses 6–10 and 11Y.

| | Grams of fibrillar protein per g of muscle | | Grams of $H_2O$ retained per g of fibrillar protein | | Ultimate degree of soft, watery condition[a] | |
|---|---|---|---|---|---|---|
| | −29°C | >37°C | −29°C | >37°C | −29°C | >37°C |
| 6 | 0.0745 | 0.0763 | 14.1 | 10.1 | 0.75 | 1.75 |
| 7 | 0.0579 | 0.1135 | 36.5 | 14.5 | 0.00 | 1.25 |
| 8 | 0.0642 | 0.0901 | 29.8 | 13.8 | 0.00 | 1.75 |
| 9 | 0.0525 | 0.0888 | 45.6 | 18.2 | 0.00 | 0.75 |
| 10 | 0.0621 | 0.1059 | 35.8 | 11.7 | 0.00 | 0.00 |
| 11Y | 0.0698 | 0.1072 | 46.8 | 10.9 | 0.00 | 0.00 |

[a] 0 = none; 1 = slightly; 2 = moderately; and 3 = extremely.

nounced response to the temperature treatments was observed in carcass 7, while an intermediate effect was obtained for carcass 8. The g of water retained/g of fibrillar protein in the 37°C treatment was only 39.7 and 46.3% of that retained in the −29°C treatment for carcasses 7 and 8, respectively.

The effect of the two treatments was as marked in carcasses 9, 10, and 11Y as in carcasses 7 and 8. This was especially apparent in carcass 11Y, in which the water bound/g of fibrillar protein in the muscle from the 37°C treatment was only 23.3% of that for the corresponding muscle from the −29°C treatment. In contrast to carcasses 6 and 7, both sides of carcasses 9, 10, and 11Y were assessed as normal or almost normal (Table 4).

In both carcasses 7 and 8, the 37°C treatment appeared to induce the soft, watery condition. Results on these two carcasses support observations of Bendall and Wismer-Pedersen (1962), who found that the washed fibrils from muscle exhibiting the soft, watery condition had a lower water retention and that the fibrils showed a gain in protein content over that of fibrils from normal muscle. They further observed that the fibrils from soft, watery muscle were covered with a layer of denatured sarcoplasmic protein, which appeared to be firmly bound to the surface of the myofilaments. Bendall and Wismer-Pedersen (1962) also theorized that this was caused by the occurrence of a low pH while muscle temperature was still high. They observed a similar phenomenon when muscle samples were allowed to pass into rigor at 37°C. The effect of the 37°C treatment in increasing fibrillar protein content and in decreasing fibrillar water-binding capacity in the carcasses is in agreement with observations of Bendall and Wismer-Pedersen (1962) suggesting that this phenomenon *per se* resulted from the occurrence of a low pH at a high muscle temperature. However, results from carcasses 9, 10, and 11Y do not support the common belief that the decrease in fibrillar water-binding capacity resulting from a low pH at a high temperature necessarily makes the meat appear soft and watery. These results do not preclude the occurrence of decreased fibrillar water-binding capacity and the concurrent gain in protein by the fibrils in soft, watery muscle. It is suggested that factors other than the low-pH high-temperature phenomenon may also contribute to the amount of protein gained by the fibrils, and thus decrease water retention by the fibrils.

The values for grams of water retained/g fibrillar protein by Bendall and Wismer-Pedersen (1962) were respectively 11.22 and 6.47 for normal and for soft, watery muscles. These and similar values (Vold and Wismer-Pedersen, 1963) are even lower than the values found for the muscles from the 37°C treatment in the current study (Table 4). It is possible that the muscles studied by Bendall and Wismer-Pedersen (1962) may have undergone a much sharper decline in pH immediately after death than the carcasses in the present study, with a resultant accentuated decrease in fibrillar water-binding capacity. However, observations in the current study do not support such a suggestion. For example, the side of carcass 6 (initial pH of 5.6) subjected to the 37°C treatment had a 24-hr water-binding

capacity of 10.1 g of water/g of fibrillar protein (Table 4). This value is similar to those for normal muscle from Danish Landrace pigs (Bendall and Wismer-Pedersen, 1962; Vold and Wismer-Pedersen, 1963). It is also possible that slight differences in the efficiency of separating the precipitate and centrifugate may account for the difference in the level of fibrillar water-binding capacity between the present and the Danish studies. This does not appear likely, however, since the fibrils in the current study were precipitated under a force of 2000 × G, compared to 1500 × G for the Danish studies (Bendall and Wismer-Pedersen, 1962; Vold and Wismer-Pedersen, 1963). It seems more probable that the muscles utilized in the current study exhibit a greater water-binding capacity at a high temperature and low pH than was true for the muscles from Danish Landrace carcasses.

Likewise, results of the current study are in agreement with the decrease in sarcoplasmic protein solubility observed by Sayre and Briskey (1963) in post-mortem muscle having a low pH concurrently with a high temperature. In contrast to the present study, an apparent relationship was reported by Sayre and Briskey (1963) between the resulting appearance of the muscle and the solubility of the sarcoplasmic protein. The same authors also reported that pH and temperature at the onset of rigor as determined on samples removed immediately after slaughter were related to the solubility of the sarcoplasmic protein. However, pH and temperature at the onset of rigor accounted for only 41 and 18%, respectively, of the variability in the proportion of soluble sarcoplasmic protein at 24 hr post-mortem.

**Chemical components and ultimate muscle condition**. Changes in the chemical components generally reflected the changes in pH levels associated with the temperature treatments. Aside from this general observation, the large variations from carcass to carcass did not reveal any apparent relationship to ultimate muscle condition. Accordingly, the ensuing discussion of individual and/or groups of chemical constituent(s) involves comparisons with previously reported values whenever possible.

**Creatine phosphate.** Creatine phosphate (CP) was not present in detectable amounts ($>0.3\mu M/g$ of tissue) in any of the initial samples of the 18 carcasses. The absence of CP may have resulted from electrical stunning, which was used. Electrical stunning has been shown to sometimes accelerate the rate of post-mortem pH decline (McLoughlin, 1964 a,b), which may be due in part to a rapid depletion of all CP within a few minutes of death.

The absence of CP in the present study is in contrast to results reported by Bendall *et al.* (1963), who found average CP concentrations of 8.0 and $5.5\mu M/g$ of longissimus dorsi muscle for two groups of Danish Landrace pigs which were stunned electrically. Lawrie (1960) reported 1-hr post-mortem levels of CP equivalent to about $7.2\mu M/g$ of longissimus dorsi muscle in Large White × Middle White pigs. He did not report the method of stunning. Kastenschmidt *et al.* (1964) reported "low creatine phosphate levels" in the longissimus dorsi muscle from Poland China pigs immediately after death, but did not give any values. Bendall *et al.* (1963) and Lawrie (1960) used an acid-molybdate procedure to determine CP, which has been shown to generally overestimate CP (Ennor and Rosenberg, 1952; Weil-Malherbe and Green, 1951).

**ATP**. The averages and standard deviations of $ATP_2$ (enzymic assay) and the corresponding pH levels for various times post-mortem are given in Table 5 for the two groups of carcasses, i.e., group 1 (carcasses 6–10) and group 2 (carcasses A–G). Average initial pH values for groups 1 and 2 were respectively 6.2 and 6.7. The initial $ATP_2$ value for both groups was $5.6\mu M/g$ of tissue. In the −29°C treatment, more than 50 and 25% of the initial $ATP_2$ was present in both groups at approximately 3 and 4½–5 hr post-mortem, respectively. No $ATP_2$ was detected at approximately 3 hr post-mortem in the sides held as 37°C, except in carcass 7. The levels of ATP for this carcass were 8.7 and $8.9\mu M/g$ of tissue for the 37°C and −29°C treatments, respectively. In support of these observations, the corresponding pH values were 6.22 and 6.51.

The average initial $ATP_1$ value (acid hydrolysis) of 6.9$\mu$M/g of tissue for the carcasses in group 1 was over 20% higher than the corresponding $ATP_2$ value (Table 5, enzymic assay). Two of the carcasses had $ATP_2$ levels of less than 2$\mu M$/g of tissue ($\bar{X} = 1.6\mu M$) while the average corresponding $ATP_1$ value for the same two carcasses was 6.3$\mu M$/g. Hence, the $ATP_1$ values probably reflect levels of acid-hydrolyzable phosphate compounds other than ATP, as suggested by Bendall and Davey (1957) and Marsh (1959). However, the initial $ATP_1$ values are in general agreement with the values of 7.1 and 6.8$\mu$M/g of longissimus dorsi muscle obtained by a similar method of analysis by Bendall *et al.* (1963) for two groups of Danish Landrace pigs, with corresponding pH values of 6.78 and 6.66, respectively.

An ATP value equivalent to about 2$\mu M$/g longissimus dorsi muscle with a pH level of about 6.3 at 0 hr can be estimated from the data of Kastenschmidt *et al.* (1964) with Poland China pigs. This is in good agreement with a value of 1.6$\mu M$/g longissimus dorsi muscle from Hampshire pigs with a similar pH level in the current study (group 2, Table 5), but is at variance with the results obtained for the Poland China carcasses (group 1, Table 5).

In disagreement with the current results, Lawrie (1960) reported acid-labile phosphate levels equivalent to about 6.9$\mu M$/g of longissimus dorsi muscle from Large White × Middle White pigs at 110 min post-mortem. The corresponding pH level was 6.5. However, Lawrie (1960) considered the ATP levels found in muscle from these pigs to be relatively high.

The almost complete absence of detectable ATP at 12 and/or 24 hr post-mortem in any of the carcasses (Table 5) is in disagreement with observations of Fredholm (1963) that 17% of the initial ATP in gracilis muscle was present after 3–5 days of storage under refrigeration.

**Glycogen, glucose (total reducing sugars), and lactic acid.** The means and standard deviations of pH, glycogen, glucose, and lactic acid at various times post-mortem for the sides of the two groups of carcasses are given in Table 6. The initial levels of glycogen in groups 1 and 2 were respectively 28.3 and 75.8$\mu M$/g of tissue. Wismer-Pedersen (1959) reported a comparable value equivalent to 31.1$\mu$/Mg of longissimus dorsi muscle from Danish Landrace pigs at 45 min post-mortem. The corresponding pH level was 6.24. This is in good agreement with the value for group 1 carcasses (pH 6.2) in the current study, but is considerably lower than the comparable value for group 2 (Table 6). Conversely, Kastenschmidt *et al.*, (1964) reported initial pH values of 6.5 and glycogen levels of about 41$\mu M$/g in longissimus

Table 5. Post-mortem levels of ATP and pH.

| Carcasses | Treatment | Av. time post-mortem (min) | pH | $ATP_2$[a] (umoles/g) |
|---|---|---|---|---|
| Group I (6–10) | — | 13 | 6.2±0.4 | 5.6±4.1 |
| | −29°C | 166 | 5.8±0.6 | 3.2±3.7 |
| | | 286 | 5.8±0.5 | 2.6±2.4 |
| | | 518 | 5.7±0.4 | 0.5±0.7 |
| | | 745 | 5.4±0.3 | 0.4±0.9 |
| | 37°C | 166 | 5.5±0.4 | –[b] |
| | | 285 | 5.4±0.1 | 0.0 |
| Group 2 (A–G) | — | 11 | 6.7±0.1 | 5.6±2.2[c] |
| | −29°C | 177 | 6.5±0.2 | 3.7±2.8 |
| | | 323 | 6.4±0.1 | 1.6±2.1 |
| | | 1440 | 5.7±0.1 | –[d] |
| | 37°C | 170 | 5.4±0.1 | 0.0 |
| | | 317 | 5.4±0.1 | 0.0 |

[a] ATP(2) determined by enzymic assay.
[b] The value for carcass 7 was 8.7$\mu M$/g; all others, 0.0.
[c] This value based on 6 carcasses.
[d] 1 carcass contained 0.3$\mu M$/g; All others, 0.0.

Table 6. Means and standard deviations of pH, glycogen, glucose, and lactic acid at various times post-mortem as related to temperature treatments for carcasses of groups 1 and 2.

| Carcass group | Av. time post-mortem (min) | pH | | | Glycogen ($\mu M$/g) | | |
|---|---|---|---|---|---|---|---|
| | | Initial | −29°C | >37°C | Initial | −29°C | >37°C |
| 1 (6–10) | 13 | 6.2±0.4 | | | 28.3±12.5 | | |
| | 166 | | 5.8±0.6 | 5.5± 0.6 | | 20.0±19.6 | 14.0±15.8 |
| | 286 | | 5.8±0.5 | 5.4± 0.1 | | 15.7±16.2 | 2.4± 1.7 |
| | 1440 | | 5.5±0.2 | 5.4± 0.1 | | 6.3± 8.7 | 0.5± 0.2 |
| | 2880 | | 5.4±0.1 | 5.4± 0.1 | | 2.8± 3.5 | 0.8± 0.2 |
| 2 (A-G) | 11 | 6.7±0.1 | | | 75.8±36.6 | | |
| | 174 | | 6.5±0.2 | 5.4± 0.1 | | 73.8±41.1 | 27.6± 2.48 |
| | 320 | | 6.4±0.1 | 5.4± 0.1 | | 57.7±39.6 | 20.6±19.2 |
| | 1440 | | 5.7±0.1 | 5.4± 0.1 | | 40.5±32.2 | 11.1±13.4 |
| 1 (6–10) | 13 | 12.6±3.7 | | | 54.8±21.4 | | |
| | 166 | | 15.2±7.6 | 17.1± 5.9 | | 67.6±28.0 | 83.6±18.3 |
| | 286 | | 13.5±5.2 | 20.9± 2.8 | | 79.8±19.1 | 88.4± 1.4 |
| | 1440 | | 20.4±6.2 | 24.4± 6.7 | | 82.6±13.8 | 86.0± 7.9 |
| | 2880 | | 20.2±4.2 | 25.3± 4.3 | | 88.7± 9.7 | 86.0± 5.4 |
| 2 (A-G) | 11 | 11.6±1.5 | | | 31.4± 9.7 | | |
| | 174 | | 10.7±4.9 | 19.4± 5.1 | | 39.6±16.1 | 80.4± 5.5 |
| | 320 | | 16.1±3.5 | 27.0± 9.2 | | 46.4± 5.6 | 80.3± 6.8 |
| | 1440 | | 15.0±3.5 | 35.4±15.9 | | 75.9± 6.6 | 80.8± 4.7 |

dorsi muscle from Poland China pigs. This is considerably higher than the values found in Poland China muscle in the current study. Also, in disagreement with current results, Lawrie (1960) reported a value equivalent to about 34.9$\mu M$/g of longissimus dorsi muscle from Large White × Middle White pigs. The corresponding pH value was 6.7 at 1 hr post-mortem. However, the average initial glycogen level for the group 1 carcasses was in good agreement with an equivalent level of 33.9$\mu M$/g of longissimus dorsi muscle from Danish Landrace pigs, with a corresponding pH of 6.2 at 15 min post-mortem (Briskey and Wismer-Pedersen, 1961b). Those workers classified this type of muscle as having a "sharp" post-mortem pH decline.

The average level of glycogen (11$\mu M$/g) at 1440 min post-mortem in the muscles from the group 2 carcasses is in contrast to the lack of detectable glycogen reported by Lawrie (1960) in muscle strips treated in a similar manner, as well as similarly low levels found in the group 1 carcasses (Table 6). These observations are in agreement with results of Sayre *et al.* (1963a), who found 3 times as much initial glycogen in the longissimus dorsi muscle of Hampshire pigs as in Poland China pigs.

The initial glucose levels (total reducing sugars) in groups 1 and 2 were respectively 12.6 and 11.6$\mu M$/g of tissue, considerably lower than the equivalent values of 28.3 and 15.5 reported by Wismer-Pedersen (1959) for longissimus dorsi muscle from Danish Landrace carcasses, with respective pH levels of 6.24 and 6.76 at ¾ hr post-mortem. However, the initial glucose values (Table 6) are higher than the level of 7.4$\mu M$/g of longissimus dorsi muscle immediately after death as calculated from data of Sharp (1962). The 24- and 48-hr glucose values varied from 24.4 to 35.4$\mu M$/g of muscle (Table 9). In general agreement with these values, Sharp (1962) reported glucose levels equivalent to 32.2$\mu M$/g of longissimus dorsi muscle held at 18°C for 26 hr and then a further 144 hr at 1°C.

The initial lactic acid levels for groups 1 and 2 were respectively 54.8 and 31.4$\mu M$/g of tissue, with corresponding pH levels of 6.2 and 6.7. These results are in general agreement with those of Wismer-Pedersen (1959), who reported values equivalent to 67.8 and 37.8$\mu M$/g of longissimus dorsi muscle from Danish Landrace pigs when the respective pH levels were 6.2 and 6.7 at 45 min post-mortem. However, the initial lactic

acid values (Table 6) were lower than the values of 68.9, 68.9, and 97.8$\mu M$/g of longissimus dorsi muscle from the Danish Landrace as calculated from data of Briskey and Wismer-Pedersen (1961b). The corresponding pH levels were 6.4, 6.6, and 6.2 at 15 min post-mortem.

The 48-hr lactic acid values in the current study varied from 75.9 to 88.7$\mu M$/g of longissimus dorsi muscle (Table 6). These levels are much lower than the 124.4–128$\mu M$/g for longissimus dorsi muscle from Danish Landrace pigs 24 hr after death as calculated from data of Briskey and Wismer-Pedersen (1961b).

**Effect of temperature on glycogen, glucose (total reducing sugars), and lactic acid levels.** The effects of the two temperature treatments on the average levels of glucose, glycogen, and lactic acid for the group 2 carcasses are shown in Fig. 2. As expected, in the carcasses held at 37°C the glycogen level decreased rapidly. At 3 hr post-mortem, the accumulation of glucose was evident, while the lactic acid concentration had reached a constant level. In the muscles from the −29° treatment, over 90% of the initial glycogen was present at 3 hr post-mortem, and over 70% was present at 5 hr. For the same group, glucose accumulation was not appreciable until after 3 hr. Lactic acid had attained a level of approximately 50% of that in the 37°C treatment at 5 hr post-mortem.

**Degradation of glycogen.** Table 7 shows the means and standard deviations of the sums of glycogen, glucose (total reducing sugars), and lactic acid expressed as $\mu M$ of glucose equivalents, and the estimates of the errors in measurement for each constituent.

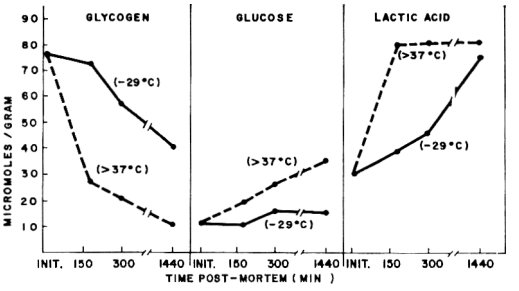


Fig. 2. Effect of temperature treatment on levels of glycogen, glucose (total reducing sugars), and lactic acid in carcasses A–G. Values are expressed in glucose equivalents.

Table 7. Means and standard deviations of sums of glycogen, glucose, and lactic acid, and estimates of measuring error for each constituent.

| | Group 1 | Group 2 |
|---|---|---|
| Glycogen[a] + glucose[b] + lactic acid | | |
| Initial | 68.3±7.3 | 103.2±33.9 |
| −29°C treatment | 67.5±2.9 | 98.3±35.8 |
| 37°C treatment | 68.5±4.2 | 87.3±27.3 |
| Estimated error of measurement | | |
| Glycogen | ±1.9 | ±4.6 |
| Glucose | ±1.4 | ±2.0 |
| Lactic acid | ±4.8 | ±4.3 |

[a] All values are expressed in micromoles of glucose equivalents/g of tissue. Means are based on sums calculated for each individual sample in all sides within treatments. The values for glucose used included total reducing sugars.
[b] Total reducing sugars expressed in glucose equivalents.

The values for the muscles of the carcasses in group 1 are very similar to those obtained for beef muscle (Bodwell *et al.*, 1965). The overall mean for beef was approximately 69$\mu M$/g muscle for these same chemical constituents. Hence, on the basis of the data for the group 1 carcasses (Table 7), there is nothing to suggest that glycogen was not broken down to glucose and lactic acid in a stoichiometric manner similar to that observed in beef muscle.

However, the values from the group 2 carcasses do not completely support the above conclusion. In these carcasses, there was a general tendency for the sums of the three components to decrease from the initial value to the −29°C value, and to the 37°C value. This may suggest that some undetected glycolytic intermediate(s) were accumulating, and that the level(s) were influenced by the temperature treatments used.

**Effect of breed on pH.** Although the lack of data between initial sampling and 3 hr post-mortem in the current study precludes any extensive comparisons with previous reports, some general observations can be made. Lawrie (1960) reported a typical pH of about 6.7 in the longissimus dorsi muscles removed from English pigs (primarily Large White × Middle White) which had been held at about 37°C for 60 min post-mortem. Initial pH levels in the current study were 6.2 and 6.7 for Poland China and Hampshire

muscle, respectively. It would therefore appear that post-mortem glycolysis in these muscles was more rapid than that found with the English pigs. Bendall *et al.* (1963) likewise concluded that post-mortem glycolytic rate in muscle from Danish Landrace pigs was much more rapid than that found by Lawrie (1960).

Sayre *et al.* (1963a) conducted an extensive study involving 99 pigs, including equal numbers of animals from the Poland China, Hampshire, and Chester White breeds. Althuogh 48 (Sayre *et al.*, 1963b) and 36 (Sayre *et al.*, 1963c) of these pigs had been used to study effects of various ante-mortem treatments, these workers reported that Poland China muscle was consistently lower in initial pH than Hampshire muscle and remained lower during the first 3 hr post-mortem. Muscle from Hampshire pigs, however, had the lowest ultimate 24 hr pH. The initial levels of pH in the current study (Table 6) are in agreement with the above observations. Conversely, the Poland China muscle in the −29°C treatment also had the lowest ultimate pH, while in the carcasses exposed to the 37°C treatment after 3 hr post-mortem almost identical pH levels were constantly present.

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Ms. rec'd 3/24/65.

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J. C. FORREST, M. D. JUDGE,[a] J. D. SINK,[b] W. G. HOEKSTRA, and E. J. BRISKEY
*University of Wisconsin, Madison, Wisconsin 53706*

# Prediction of the Time Course of Rigor Mortis Through Response of Muscle Tissue to Electrical Stimulation

## SUMMARY

**A technique based on muscle response to electrical stimulation was shown to be useful in predicting, within 10 min of exsanguination, the time course of rigor mortis, rate of post-mortem glycolysis, and ultimate color-morphology rating. The response of an excised muscle to electrical stimulation was highly associated with post-mortem muscle properties. The excitability threshold (lowest voltage at which contraction resulted) was found to be high in muscles which had a short time course of rigor mortis, fast post-mortem glycolysis, and pale, soft, and exudative (PSE) ultimate gross morphology; and low in muscles with a long time course of rigor mortis, slow post-mortem glycolysis, and ultimately normal color-morphology. Strength of contraction (at 5, 10, 25, and 50 volts) was observed to he highest in muscles which exhibited long rigor, slow glycolytic rate, and normal color-morphology. The duration of contractility (maintained under repeated stimulation at 2 cycles/sec) was also noted to he longer in this type of muscle. Multiple-regression analysis indicated that up to 87% of the variability in color-morphology rating could be predicted by combining the various parameters of muscle response to electrical stimulation. Breed differences were observed which must be taken into account when using prediction equations.**

## INTRODUCTION

The time course of rigor mortis development has been observed to vary from a few minutes to 8 hr post-mortem in porcine skeletal muscle (Briskey *et al.*, 1962; Sayre and Briskey, 1963; Kastenschmidt *et al.*, 1964, 1965). Many of the physical characteristics of 24-hr post-mortem muscle may be predicted by considering the variability in time course of rigor mortis and the rate of post-mortem pH decline (Briskey and Wismer-Pedersen, 1961) or pH and temperature at the onset of rigor mortis (Sayre and Briskey, 1963). However, in the laboratory, and conceivably even under commercial conditions, an immediate post-mortem identification of the potential properties of muscle would facilitate: 1) the initiation of intensive studies on the biochemistry of rigor mortis, as well as the development of various associated muscle properties; and 2) the application of measures to control or regulate post-mortem changes in muscle (Borchert and Briskey, 1964).

During preliminary studies on the response of excised muscle tissue to electrical stimulation, muscles which ultimately became pale, soft, and exudative (PSE) differed widely in response from muscles which became normal, or dark, firm, and dry (DFD). The present experiment was undertaken to ascertain the association of electrical stimulation response, measured shortly after death, with the time course of rigor mortis and other related properties of porcine striated muscle.

## MATERIALS AND METHODS

Twenty-one Poland China and 11 Chester White market barrows, weighing approximately 90 kg, were used. Preslaughter conditions were standardized as much as possible by holding all animals in a constant-temperature room for at least one week prior to experimentation. Immediately after exsanguination, samples were excised from the left longissimus dorsi muscle in the area posterior to the last thoracic vertebra. These samples were used for determining pH, electrical stimulation response, and time course of rigor mortis; portions were also frozen in liquid nitrogen for subsequent lactic acid determination. The 24-hr samples were also given a color-morphology rating according to the 5-point scale of Forrest *et al.* (1963).

**Specific aspects of procedures for electrical stimulation.** Adjacent strips of parallel muscle fibers (1 $cm^2 \times 6$ cm) were prepared. One strip was placed between two sets of Lucite jaws fitted with

[a] Present address: Department of Animal Science, Purdue University, W. Lafayette, Indiana.

[b] Present address: Department of Nutrition and Animal Industries, Pennsylvania State University, University Park, Pennsylvania.

pin electrodes as described by Briskey *et al.* (1962). The upper set of jaws was attached to a fixed support which had provision for adjustment of tension on the muscle strip, and the other set was fastened to a myographic force transducer (E & M Physiograph, Myograph "C"). Fig. 1 shows the muscle strip as positioned in this apparatus (room temperature, 22°C). The myograph was calibrated in grams to measure strength of muscle contractions. A permanent record of the muscle contractions was made with a polygraph. A tension equivalent to approximately 30 g was applied to each muscle before stimulation was initiated in order to compensate for loss of tonus in the muscle strips as they became exhausted during experimentation.

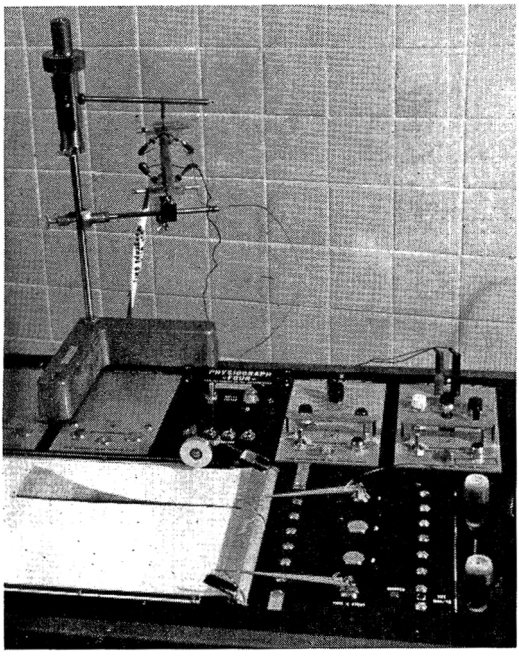

Fig. 1. Apparatus for stimulation of muscle strips.

Single electrical stimulations of 1, 3, 5, 10, 25, and 50 volts at stimulus duration of 0.1 millisecond were administered consecutively at approximately 3-sec intervals. The voltage at which the first contractile response was observed was termed the "excitability threshold." The "strength of contraction" in grams was measured for the "excitability threshold" and for each succeeding stimulus voltage (Fig. 2).

A continuous series of stimulations was then administered at 50 volts, 2/sec frequency, and 0.1-millisecond stimulus duration until the strength of each contraction was less than 10 g. "Duration of contractility" was measured to two arbitrarily defined points, the time (in seconds) during which the muscle was able to sustain a contractile strength of (1) $\geqslant$ 200 g, and (2) $\geqslant$ 10 g. The latter point represents virtual exhaustion of the muscle under the above defined conditions (Fig. 2).

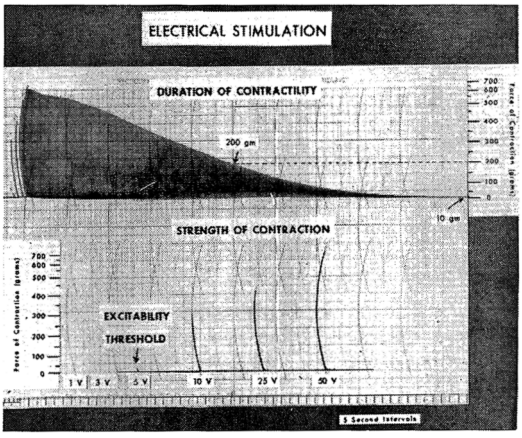


Fig. 2. Typical record of muscle response to electrical stimulation. Duration of contractility is the time in seconds during which the muscle strip, when stimulated with 50 volts at a frequency of 2 stim/sec responded with contractions $\geqslant$ 200 g or $\geqslant$ 10 g. Strength of contraction is shown at stimulations of 1, 3, 5, 10, 25, and 50 volts. Excitability threshold indicates the lowest voltage that elicited a contractile response.

**Associated muscle properties.** Time course of rigor mortis (37°C) was determined using the second strip of parallel muscle fibers according to the procedure described by Briskey *et al.* (1962), (Fig. 3). The delay phase represents the duration of time from death to point of onset (no change in relative extensibility), and onset phase represents a continuous reduction in extensibility (from the point of onset to completion). When the muscle has lost its extensibility it is considered to be at the point of completion.

pH was determined at 5 min, 45 min, and 24 hr by placing a combination electrode directly on the freshly cut cross-section surface of the muscle fibers.

Lactic acid was determined by the enzymatic method of Bergmeyer (1963).

A subjective color-morphology rating was made 24 hr post-mortem on the longissimus dorsi and gluteus medius muscles with the 5-point scale of Forrest *et al.* (1963).

**Statistical analysis.** The data were subjected to simple correlation analysis, analysis of variance, *t*-test, and multiple-regression analysis according to methods outlined by Steel and Torrie (1960).

## RESULTS AND DISCUSSION

Excitability threshold, strengths of muscle contraction at 5, 10, 25, and 50 volts, and

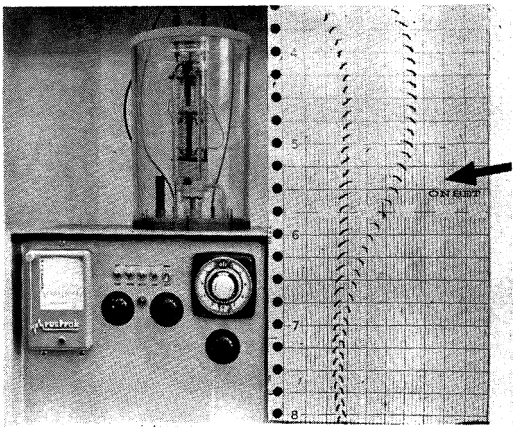

Fig. 3. Rigorometer and typical record of time course of rigor mortis. (Briskey *et al.*, 1962).

duration of contractility maintained $\geq$ 10 g were evaluated to determine their relationship to the time course of rigor mortis, rate of post-mortem glycolysis, and subjective color morphology ratings.

**Relation of electrical stimulation response to time course of rigor mortis.** Fig. 4 shows specific examples of muscles which sustained a contractile response over a long duration (425 sec) and/or exhibited strong contractions and which were observed to have a long duration (405 min) of both the delay and onset phases of rigor mortis. Conversely, muscle which exhibited an intermediate duration (285 sec) of contractility and/or intermediate contractile force were observed to have an intermediate (135 min) time course of rigor mortis. Furthermore, the bottom portion of Fig. 4 shows that extremely weak contractile response and short duration of contractility (105 sec) were associated with a very short time (52 min) to completion of rigor mortis. In the latter example the onset of rigor mortis occurred within 15 min of exsanguination.

Table 1 shows the correlations between muscle response to stimulation and the time course of rigor mortis. In the Poland China pigs, strength of contraction at 10, 25, and 50 volts and duration of contractility was significantly related ($P < 0.01$) to the delay, onset, and completion phases of rigor mortis. The strongest relationships with the rigor phases were those for duration of contractility $\geq$ 200 g, and for strength of contraction

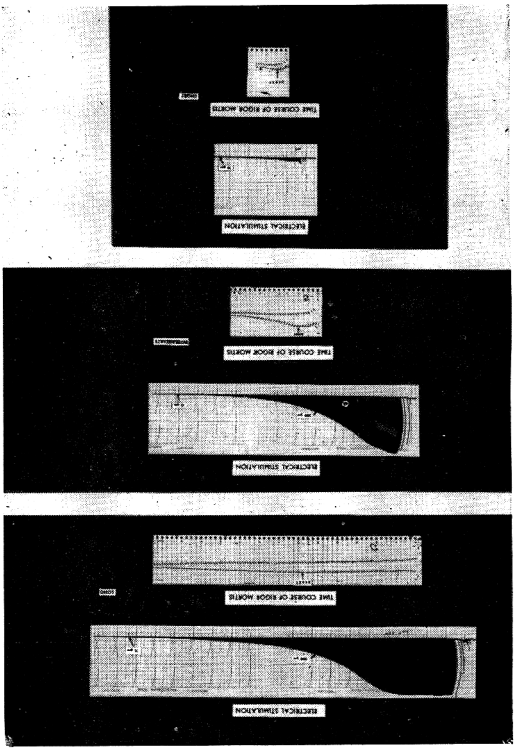

Fig. 4. Comparison of electrical stimulation and rigor mortis patterns in muscles with long, intermediate, and short durations of rigor mortis. The long stimulation response represents 425 sec, and the long rigor mortis duration represents 405 min. The intermediate stimulation response represents 285 sec, and the intermediate rigor mortis duration represents 135 min. The short stimulation response represents 105 sec, and the short rigor mortis duration represents 52 min.

after a 10-volt stimulation. When all seven parameters were combined, multiple correlations of 0.85, 0.92, and 0.90 were respectively observed for delay, onset, and completion of rigor mortis in the muscles of the Poland China pigs. Simple correlations of the data from the muscles of the Chester White pigs tended to indicate less significant relationships between the individual parameters of electrical stimulation and those of the time course of rigor mortis; however, when these parameters were combined, multiple correlations of 0.92, 0.96 and 0.92, respectively, were observed for delay, onset, and completion of rigor mortis. The overall simple correlations indicate that a close relationship existed between all three phases of rigor mortis and strength of contraction and duration of contractility. However, the over-

Table 1. Simple and multiple correlations showing the relationship between response of muscle to electrical stimulation and time course of rigor mortis.

| | Rigor mortis-phases | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Delay[e] | | | Onset[f] | | | Completion[g] | | |
| | Poland China | Chester White | Overall | Poland China | Chester White | Overall | Poland China | Chester White | Overall |
| Excitability threshold [a] | −.46* | .11 | −.31 | −.46* | −.21 | −.37* | −.47* | .06 | −.37* |
| Strength of contraction [b] | | | | | | | | | |
| 5 volts | .40 | .72* | .55** | .50* | .37 | .41* | .46* | .67* | .53** |
| 10 volts | .77** | .68* | .52** | .84** | .44 | .47** | .82** | .68* | .54** |
| 25 volts | .68** | .60* | .49** | .63** | .46 | .42* | .67** | .64* | .50** |
| 50 volts | .60** | .22 | .41* | .55* | .24 | .40* | .59** | .28 | .44* |
| Duration of contractility [c] | | | | | | | | | |
| 200 g | .78** | .35 | .49** | .85** | .70* | .65** | .83** | .63* | .61** |
| 10 g | .75** | .23 | .47** | .71** | .29 | .48** | .75** | .31 | .52** |
| Multiple correlation [d] | .85 | .92 | .62 | .92 | .96 | .73 | .90 | .92 | .69 |

[a] Minimum voltage required to produce a contractile response.
[b] Contractile response in grams to a single stimulus of 0.1 msec duration at each of the given voltages.
[c] Time in seconds during which the muscle has the ability to sustain the indicated contractile force with a 50-volt repetitive stimulation at a frequency of 2 cycles/sec and a stimulus duration of 0.1 msec.
[d] Combination of all seven parameters of response to stimulation (see text).
[e] Time in min during which there is no change in extensibility (Briskey *et al.*, 1962).
[f] Time in min during which there is a gradual to sharp decrease in extensibility (Briskey *et al.*, 1962).
[g] Time (min) to complete loss of extensibility.
* $P < 0.05$.
** $P < 0.01$.

all multiple correlations of 0.62, 0.73, and 0.69 for delay, onset, and completion of rigor, respectively, were much lower than those which were observed within breeds. This may be explained by the fact that strength of contractions appeared to contribute more to prediction of the time course of rigor mortis in the muscles of the Chester White pigs while duration of contractility appeared to be more important in the muscles of the Poland China pigs. It is concluded that this method can be used to predict the time course of rigor mortis but that separate prediction equations must be used for each breed. Prediction equations are not presented in this paper, because the authors feel that the equations should be developed under the conditions and with the breeds and/or strains of animals with which they are to be used.

**Relation of electrical stimulation response to rate of glycolysis.** Muscles were divided into categories with fast or slow glycolytic rates, according to the rate of pH decline in the unstimulated muscle. Muscles with a pH of less than 5.9 at 45 min postmortem were considered to have a fast glycolytic rate, while muscles with a pH greater than 6.0 were considered to have a slow glycolytic rate. Fig. 5 shows that muscles with a fast glycolytic rate required a significantly higher voltage to reach the excitability threshold than muscles with a slow glycolytic rate. It is also observed that muscles with a slow glycolytic rate had the ability to maintain contractility over a longer duration than muscles with a fast glycolysis. Fig. 6 shows that strengths of contraction at stimulus voltages of 5, 10, 25, and 50 were significantly ($P < 0.05$) stronger in muscles with slow glycolysis.

Multiple correlations between the 7 parameters of electrical stimulation and pH of unstimulated muscle at 45 min post-mortem were 0.94, 0.89, and 0.60 for Poland Chinas, Chester Whites, and overall, respectively. Thus it appears that electrical stimulation response was highly associated with rate of post-mortem glycolysis or 45-min post-mortem pH, although breed effect should be taken into account in using this as a method of prediction.

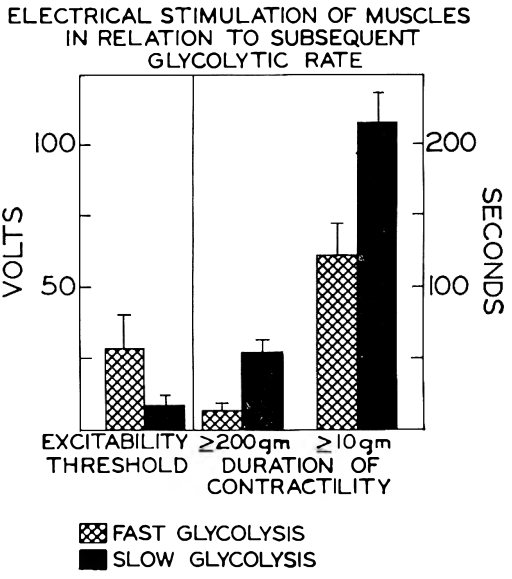


Fig. 5. Relation of excitability threshold and duration of contractility to post-mortem glycolysis. Fast glycolysis represents 45-min post-mortem pH of $< 5.9$, and slow glycolysis represents 45-min post-mortem pH of $\geq 6.0$.

**Relation of electrical stimulation response to initial pH value and lactic acid concentration.** Table 2 shows that the correlations between the initial pH of the longissimus dorsi and the various parameters of electrical stimulation are considerably higher in the Poland China than the Chester White pigs. The difference in stimulatory response is particularly marked with respect to duration of contractility. The low nonsignificant ($P > .05$) correlations of these parameters in the muscles of the Chester White pigs may be due, in part, to their lower variability in pH, compared to Poland pigs ($6.56 \pm 0.17$ vs. $6.33 \pm 0.26$). Table 3, however, shows that the correlations between stimulatory response and initial lactic acid content of the muscles tended to be high and significant ($P < .05, < .01$) in both breeds. These findings indicate that the quantity of lactic acid in the muscle may be extremely important in influencing its response to electrical stimulation. Although the lactic acid content is lower and less variable in the muscles of Chester White pigs ($3.36 \pm 0.99$) than in Poland China pigs ($4.50 \pm 1.3$), the differences in lactic acid appear to influence the stimulatory response of the muscle.

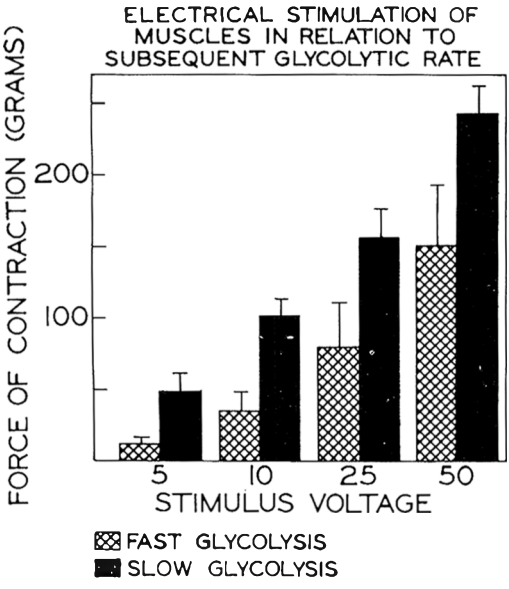


Fig. 6. Relation of strength of contraction to post-mortem glycolysis. Fast glycolysis represents 45-min post-mortem pH of $< 5.9$, and slow glycolysis represents 45-min post-mortem pH of $\geq 6.0$.

**Relation between electrical stimulation response and color-morphology ratings.** Since the response of muscle tissue appeared to be highly related to the objective indices of post-mortem porcine muscle properties, determinations were made on the relation of this response to the subjective color-morphology rating.

The muscles were classified into 3 groups according to their 24-hr post-mortem color-morphology rating. Extremely PSE muscles had a rating of less than 1.5, slightly PSE had a rating of 1.5–2.0, and normal a rating of >2.0. There were no dark (>3.5) longissimus dorsi or gluteus medius muscles in the carcasses of this study. Fig. 7 shows that the excitability threshold was higher in muscles which ultimately became PSE than in muscles which were ultimately normal. Normal muscles tended to have the ability to sustain contractility longer than PSE muscles. Fig. 8 shows that normal muscles contracted more strongly at stimulus voltages of 5, 10, 25, and 50 volts than muscles that ultimately became PSE.

Table 4 shows the simple and multiple correlations between the parameters of electrical stimulation response and color-morphology rating. In the Poland Chinas each of the electrical-stimulation response parameters was significantly ($P < 0.01$) related

Table 2. Correlations between initial pH values (longissimus dorsi) and electrical stimulation response of muscle.

| | Poland China | Chester White | Overall |
|---|---|---|---|
| Excitability threshold [a] | —.50* | —.08 | —.47** |
| Strength of contraction [b] | | | |
| 5 volts | .27 | .38 | .28 |
| 10 volts | .53* | .38 | .38* |
| 25 volts | .52* | .25 | .40* |
| 50 volts | .45* | .11 | .39* |
| Duration of contractility [c] | | | |
| 200 g | .56** | .10 | .41* |
| 10 g | .66** | .16 | .52** |

[a] Minimum voltage required to produce a contractile response.
[b] Contractile response in grams to a single stimulus of 0.1 msec duration at each of the given voltages.
[c] Time in seconds during which the muscle has the ability to sustain the indicated contractile force with a 50-volt repetitive stimulation at a frequency of 2 cycles/sec and a stimulus duration of 0.1 msec.
* $P < 0.05$.
** $P < 0.01$.

Table 3. Correlations between initial lactic acid values (longissimus dorsi) and electrical stimulation response of muscle.

| | Poland China | Chester White | Overall |
|---|---|---|---|
| Excitability threshold [a] | .69** | .86** | .70** |
| Strength of contraction [b] | | | |
| 5 volts | —.46* | —.63 | —.47** |
| 10 volts | —.62** | —.74* | —.54** |
| 25 volts | —.52* | —.76* | —.49** |
| 50 volts | —.57** | —.71* | —.56** |
| Duration of contractility [c] | | | |
| 200 g | —.66* | —.55 | —.57** |
| 10 g | —.75** | —.79** | —.73** |

[a] Minimum voltage required to produce a contractile response.
[b] Contractile response in grams to a single stimulus of 0.1 msec duration at each of the given voltages.
[c] Time in seconds during which the muscle has the ability to sustain the indicated contractile force with a 50-volt repetitive stimulation at a frequency of 2 cycles/sec and a stimulus duration of 0.1 msec.
* $P < 0.05$.
** $P < 0.01$.

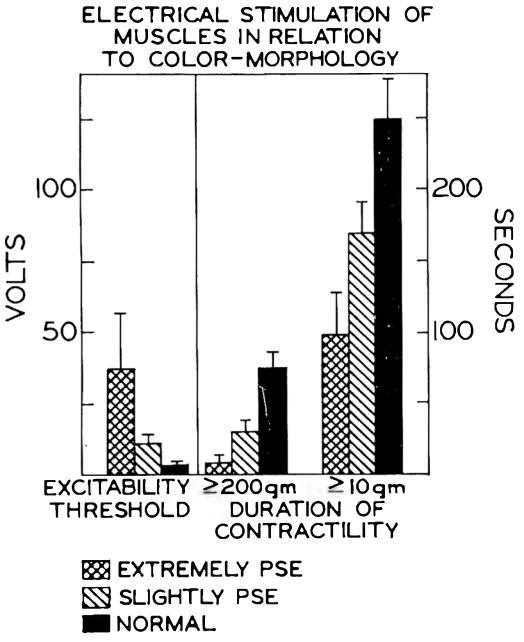


Fig. 7. Relation of excitability threshold and duration of contractility to color-morphology rating. Extremely PSE muscles scored less than 1.5, slightly PSE 1.5–2.0, and normal > 2.0 on the 5-point scale of Forrest *et al.* (1963).

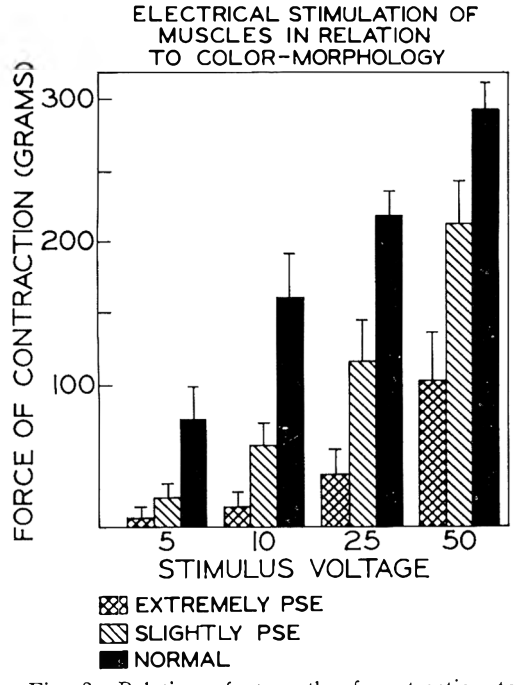


Fig. 8. Relation of strength of contraction to color-morphology rating. Extremely PSE muscles scored less than 1.5, slightly PSE 1.5–2.0, and normal > 2.0 on the 5-point scale of Forrest *et al.*

to the color-morphology rating, the strongest relationship being between duration of contractility and color-morphology rating. When the 7 electrical-stimulation response parameters were combined, a multiple correlation of 0.93 was observed. In the Chester Whites, all individual correlations of electrical-stimulation response with color-morphology ratings were low and nonsignificant. However, it is pertinent to note that the strongest relationships were with the strength of contraction parameters and that variation in postmortem color-morphology rating in Chester Whites is much less than with Poland Chinas with PSE muscle occurring rarely. Also important is the fact that, when the seven electrical-stimulation parameters were combined, a multiple correlation of 0.90 with color-morphology rating was observed in the Chester Whites. The lower multiple correlation observed when both breeds were combined again emphasizes the importance of

Table 4. Correlations between color-morphology rating (longissimus dorsi) with electrical-stimulation response of muscle.

| | Color-morphology rating[e] | | |
|---|---|---|---|
| | Poland China | Chester White | Overall |
| Excitability threshold [a] | —.64** | —.04 | —.59** |
| Strength of contraction [b] | | | |
| 5 volts | .59** | .29 | .43* |
| 10 volts | .84** | .25 | .62** |
| 25 volts | .72** | .49 | .61** |
| 50 volts | .68** | .30 | .61** |
| Duration of contractility [c] | | | |
| 200 g | .87** | —.01 | .63** |
| 10 g | .75** | .02 | .58** |
| Multiple correlation coef.[d] | .95 | .90 | .74 |

[a] Minimum voltage required to produce a contractile response.

[b] Contractile response in grams to a single stimulus of 0.1 msec duration at each of the given voltages.

[c] Time in seconds during which the muscle has the ability to sustain the indicated contractile force with a 50-volt repetitive stimulation at a frequency of 2 cycles/sec and a stimulus duration of 0.1 msec.

[d] Combination of all seven responses to stimulation parameters.

[e] Rated on a 5-point scale with a higher rating indicating a darker color (Forrest *et al.*, 1963).

* $P < 0.05$.

** $P < 0.01$.

taking breed into account when using electrical-stimulation response as a method of studying post-mortem muscle properties.

**Interrelations between time course of rigor mortis, glycolytic rate and color-morphology ratings.** In this study the three phases of rigor mortis combined were observed to account for 70% of the variability in color-morphology rating in the Poland Chinas and 40% in the Chester Whites, and pH at 45 min accounted for 55% of the variability in Poland Chinas and a negligible amount in the Chester Whites. Probably of more significance to the prediction of the color-morphology ratings is the rate of pH decline rather than actual pH value at a particular time post-mortem. Table 5 shows that muscles with a slow post-mortem glycolytic rate have a significantly higher 24-hr color-morphology rating than muscles with a fast glycolytic rate. Table 6 shows that each of the three phases of rigor mortis are significantly longer in muscles exhibiting a slow glycolytic rate than in muscles with a fast rate. These results are in agreement with those of Briskey and Wismer-Pedersen (1961) and Kastenschmidt *et al.* (1964, 1965).

## DISCUSSION

The reason that muscle which ultimately becomes PSE does not respond as vigorously to electrical stimulation appears to be partially due to its high lactic acid content. There are numerous other differences between PSE and normal muscle which could influence stimulatory response, and these characteristics are the subject of further study.

Ranvier (1874) demonstrated that the stimulation frequency required to produce tetany was lower for red muscle fibers than for white fibers. Denny-Brown (1929) used response to electrical stimulation to distinguish between muscles with predominantly red fibers and muscles with predominantly white fibers. However, white muscles exhibited a longer time course of rigor mortis than red fibers (Briskey *et al.*, 1962). In this study, PSE muscles had a significantly ($P < 0.01$) shorter time course of rigor than normal muscles.

Karpatkin *et al.* (1964) reported a significant build-up of lactic acid in muscle as a result of electrical stimulation. Hallund and Bendall (1965) recently reported an acceler-

Table 5. Color-morphology ratings in relation to glycolytic rate.

| Muscle | Fast glycolysis[a] | | Slow glycolysis[b] | | |
|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | $T$ |
| Longissimus dorsi (color morphology)[c] | 1.3 | .12 | 2.5 | .09 | −7.8** |
| Gluteus medius (color morphology)[c] | 1.0 | .11 | 1.8 | .09 | −5.5** |

[a] pH < 5.9 at 45 min post-mortem.
[b] pH ≥ 6.0 at 45 min post-mortem.
[c] Rated on a 5-point scale with a higher rating indicating a darker color (Forrest *et al.*, 1963).
** $P < 0.01$.

Table 6. Time course of rigor mortis in relation to glycolytic rate.

| | Fast glycolysis[a] | | Slow glycolysis[b] | | |
|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | $T$ |
| Delay phase[c] (min) | 51.1 | 6.3 | 103.1 | 10.6 | −4.16** |
| Onset phase[d] (min) | 45.5 | 4.1 | 114.0 | 11.6 | −5.46** |
| Completion phase[e] (min) | 96.6 | 8.9 | 217.6 | 19.2 | −5.60** |

[a] pH < 5.9 at 45 min post-mortem.
[b] pH ≥ 6.0 at 45 min post-mortem.
[c] Time during which there is no change in extensibility (Briskey *et al.*, 1962).
[d] Time during which there is a gradual to sharp decrease in extensibility (Briskey *et al.*, 1962).
[e] Time to complete loss of extensibility.
** $P < 0.01$.

ation of the rate of pH decline in electrically stimulated muscles. In this study a correlation coefficient of .58 ($P < 0.01$) was observed between 0-hr lactic acid and duration of contractility. Preliminary results from another study in this laboratory indicate that there may be a significant accumulation of lactic acid in certain muscles stimulated under the conditions used in this study. The technique described in this paper can be used to predict, within a few minutes, the rigor mortis and associated muscle characteristics, which otherwise may require up to several hours for direct determination.

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Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported, in part, by Public Health Service Research Grant EF-81 (C7) from the Division of Environmental Engineering and Food Protection, and by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. Department of Meat and Animal Science (Paper No. 417) and Biochemistry cooperating.

S. D. DHARKAR, K. A. SAVAGAON, U. S. KUMTA, and A. SREENIVASAN
*Atomic Energy Establishment Trombay, Bombay, India*

# Development of a Radiation Process for Some Indian Fruits: Mangoes and Sapodillas

## SUMMARY

**A combination heat-radiation process has been evolved for sterilizing mango and sapodilla slices in cans. The fruit, after peeling and slicing, is packed in sanitary cans with sucrose syrup, and the respiratory enzymes are inactivated by heating for 10 min at 70°C. The cans are next evacuated for 6 min at a vacuum of 28 inches Hg, vacuum doubled-seamed, and irradiated with a total dose of $4 \times 10^5$ rads at room temperature.**

**Organoleptic evaluation, vitamin retention, and sterility tests show that these processing conditions are optimum and give an acceptable product. Canned mangoes showed progressively lower acceptability with radiation doses above $4 \times 10^5$ rads, compared with the thermally processed product. Sapodillas, on the other hand, did not show appreciable deterioration up to $1.2 \times 10^6$ rads.**

## INTRODUCTION

Spoilage microorganisms associated with fruits require very high doses of radiation for inactivation (U.S. Army, 1957). Yeasts and molds, for example, require 1.5 megarads, nonsporulating bacteria about 2 megarads, and bacterial spores over 2 megarads (U.S. Army, 1957). These are high doses which bring about extensive organoleptic and other changes (Pratt and Ecklund, 1956; Salunkhe, 1961) and would point to the desirability of bringing down the final sterilizing dose by suitable combination with conventional methods of food preservation.

Recently, much work has been carried out on the use, in combination, of radiation with heat (Morgan and Read, 1954; Kempe, 1960), radiosensitizing chemicals (El-Tabey Shehata, 1961; Bridges, 1962), or ultrasonic treatment (Dharkar 1964a) for control of food-borne organisms. Such methods have potential applications in that safe sterilizing doses could be employed consistent with palatability of the products. Indeed, as has been shown by Kempe (1960), Licciardello and Nickerson (1962), and others, there could actually be a potentiation of the radiation effect by postirradiation heat treatments. This principle has been utilized in evolving a radiation process for orange juice (Dharkar, 1964b).

In the present work, a low-dose radiation process was sought to be evolved, utilizing both radiation and heat, to conserve some Indian fruits like Alphonso mangoes and Sapodillas (*Achras zapota*) as slices after being packed in hermetically sealed sanitary cans.

## MATERIALS AND METHODS

Used for these experiments were Alphonso mangoes, grown extensively on the Indian West Coast, and Sapodillas (sapotas), common in many parts of the country.

The fruits, of table-ripe quality, were peeled and sliced, and the slices were filled in sanitary cans (301 × 206), topped with 40% sucrose syrup, and processed as described in the text. Some of the packs were also inoculated with known amounts of standard suspensions of *Saccharomyces cerevisiae* cells and *Bacillus subtilis* spores (as detailed later) prior to sealing and processing.

**Thermal sterilization.** This was achieved by exhausting the filled cans for 10 min at 80–85°C, double seaming, and steam-heating for 15 min at 100°C. It was ascertained that under these conditions, the cans, including the inoculated packs, were sterile.

**Irradiation of cans.** The cans were irradiated in a gamma cell 220 (Atomic Energy of Canada Ltd., 22,500 curies) at an average dose rate of $1.5 \times 10^6$ rads/hr. The irradiation chamber (6 × 8-inch cylinder) could hold 3 cans of the size employed at a time. The observed small variation in dose rate in the chamber along the vertical axis was evened out by rotating the cans every 1/3 of the irradiation time, so that each can got the same dose of radiation.

**Sterility tests.** Sterility tests were made, after storing the cans at room temperature (25–30°C) for 2, 4, and 6 weeks, by plating 1-ml portions from the aseptically opened containers, which were thoroughly shaken earlier, on medium made up of (percentages) of peptone 2, glucose 2, yeast extract 1, and agar 2. Colony counts were taken after incubating the Petri dishes at both 30 and 37°C. Based on counts per ml, the total microbial load carried by each can was calculated.

**Inactivation of respiratory enzymes.** Respiration of mangoes or sapodillas was measured by studying oxygen uptake by the fruit slices in a Warburg apparatus. For evaluating the optimum heat treatment for inactivation of respiratory enzymes, whole fruits were heat-treated in a constant-temperature bath at 50, 60, or 70°C for 10, 30, 60, or 120 min, cooled, and then cut into small pieces. One-gram lots of these small bits were transferred into the main chamber of the respiration flasks and covered with 2 ml of 40% sucrose syrup, 0.2 ml of 40% KOH being placed in the center well with a filter-paper strip roll to absorb the $CO_2$ evolved. All flasks were maintained at 37°C, and manometric readings were taken at half-hour intervals. Calculations were made of the $\mu$l of oxygen consumed per 100 g of the fruit tissue, and respiration rates were plotted. Mango juice as well as disintegrated slices did not show any respiration.

**Organoleptic evaluation.** This was done by a panel of 12 judges on a nine-point hedonic scale (1, extreme dislike; 9, extreme like). The judges were to compare the irradiated with heat-sterilized products and also compare both with natural fruit in taste and flavor. The average acceptability score was found by totaling scores of the different tasters and averaging out, with 9 as the maximum score. For simplifying the histogram, the total rating of 9 was subdivided into 2¼ each for the different parameters of color, flavor, texture, and taste. The term *flavor* connotes aroma, and is an expression commonly used by the consumer in evaluating quality, especially in mangoes.

**Vitamin estimations.** Ascorbic acid was assayed by 2:6-dichloro-phenol-indophenol titration (AOAC) (1960) and xylene extraction methods (Robinson and Stolz 1945). It was necessary to make use of a specific method for ascorbic acid estimation to eliminate the effect due to other reducing substances, and xylene extraction assay was found to be useful.

Thiamine was estimated by oxidizing to thiochrome and measuring the fluorescence on an Aminco-Bowman spectrophotofluorometer at 365 m$\mu$ activating and 435 m$\mu$ fluorescence wavelengths (AOAC, 1960).

Riboflavin and niacin were respectively estimated by microbiological methods with *Lactobacillus casei* and *Lactobacillus arabinosus* (Barton-Wright, 1952).

The total carotenoids were extracted by blending mango pulp, methanol, and petroleum ether, (1:1:2) in a Waring blender, washing the ethereal layer after separation by centrifugation with water, and reading the optical density at 440 m$\mu$ after making up to a known volume (Ramkrishnan *et al.*, unpublished).

## RESULTS AND DISCUSSION

**Inactivation of respiratory enzymes.** Sterilization by irradiation alone would require a high dose of radiation and even then would not satisfy requirement for enzymatic inactivation. Hence, a radiation-sterilized product, apart from causing undesirable organoleptic changes, would, during storage, deteriorate further because of the action of various enzymes. A food product can therefore be stabilized best, both microbiologically and enzymatically, only by a combination process of heat and radiation.

Fruit slices, when placed in hermetically sealed containers, use up the oxygen available during respiration. When the oxygen content goes below 3%, a process of anaerobic respiration sets in (Biale, 1960), giving rise to a respiratory quotient greater than unity, or an evolution of carbon dioxide more than the free oxygen used. This, in turn, will cause the ends of the can to bulge and even to burst. It is therefore important to inactivate these respiratory enzymes for developing any combination process aimed at reducing heat and radiation treatments. The optimum heating conditions for such enzyme inactivation can be assessed from the results shown in Fig. 1.

It would seem that mangoes heated 1 or 2 hr at 50°C do not show any decrease in respiration; on the contrary, there is a slight increase, suggesting that this may be the optimum temperature for enzymes to act. Treatment at 60°C for ½, 1, and 2 hr caused a steady decrease in respiratory rates, while with 70°C for 10 min the respiration is nil. This would mean that the respiratory enzymes can be completely inactivated at 70°C in 10 min.

Similar experiments were carried out with Sapodillas, which showed the respiratory pattern to be different although inactivation was accomplished at 70°C in 10 min in the same way as with mangoes; Table 1 shows the respiratory rate to be much slower in this case (1000 $\mu$l/100 g, compared with 8000 $\mu$l/100 g for mangoes). Also, there was inactivation of the respiratory enzymes at 50°C, in contrast to the observed activation of these enzymes in mangoes at this temperature. For working out an optimum proce-

Table 1. Heat inactivation of respiratory enzymes in Sapodillas (oxygen uptake $\mu$l/100 g of fruit).

| Time (min) | No heat treatment | 50°C 1 hr | 60°C 1 hr | 70°C 10 min |
|---|---|---|---|---|
| 10 | — | — | — | Nil |
| 30 | 260 | 110 | 40 | Nil |
| 60 | 490 | 215 | 90 | Nil |
| 90 | 740 | 300 | 130 | Nil |
| 120 | 990 | 410 | 170 | Nil |

Sapodilla (control and heated at 50, 60, and 70°C) were cut into small pieces, and respiration of 2-g samples was studied in a Warburg apparatus during the time intervals shown. Inactivation of respiratory enzymes is seen to result with 70°C treatment for 10 min.

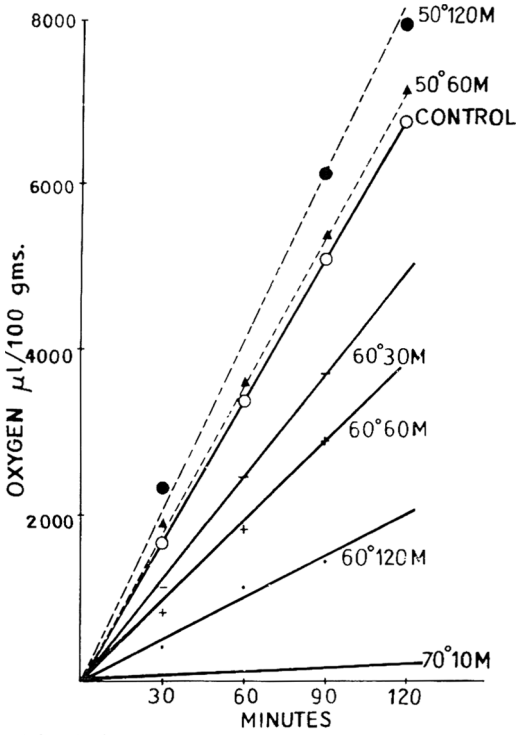


Fig. 1. Heat inactivation of respiratory enzymes in mangoes. Mangoes, control and heated for different periods at 50, 60 and 70°C, were cut into small pieces, and the respiration of 1-g samples was studied in a Warburg apparatus. Data show inactivation of respiratory enzymes after heat treatment at 70°C for 10 min.

dure for heat-cum-radiation process, therefore, the mangoes or sapodillas were peeled and sliced, the slices were filled into 301 × 206 plain sanitary cans, 40% sucrose syrup was added, and the containers were kept in a water bath at 70°C for 10 min.

**Exhausting.** In conventional canning practice, it is customary to exhaust fruit cans by subjecting them to 80–85°C for the double purpose of enzyme inactivation and removal of occluded air from inside the fruit slices. This latter purpose is not met by treatment at 70°C for 10 min as described above. It was found necessary, therefore, to subject the cans to a vacuum of 28 inches Hg for about 6 min, this procedure being found sufficient to get rid of most of the air from inside the fruit tissues. Alphonso mango, in particular, is very susceptible to radiation effect, especially in the presence of air. There are extensive flavor and other organoleptic changes due to oxidative free radicals during irradiation. The vacuumizing process has the desired effect of oxygen removal and replacement of the tissue interstices with syrup. The vacuumized cans were next sealed with a vacuum double seamer at a vacuum of 20 inches Hg.

With a few cans, the feasibility of irradiating under nitrogen atmosphere was studied by first evacuating cans at a vacuum of 20–22 inches Hg and flushing the can-holding chamber with nitrogen gas. This step was repeated 3–4 times to effect maximum replacement of oxygen with nitrogen.

**Irradiation.** Irradiation was carried out after the cans were cooled to room temperature (25–30°C) and with different doses of radiation from $10^5$ to $8 \times 10^5$ rads. Some of the cans were also irradiated by higher doses up to $1.2 \times 10^6$ rads to study organoleptic changes. Table 2 shows that cans were

Table 2. Sterility of cans after various treatments (incubation at 30°C).

| Radiation dose ($\times 10^5$ rads) | Total count per can after incubation for 2 wk | 4 wk | 6 wk |
|---|---|---|---|
| 1 | $10^6$ | $10^6$ | — |
| 2 | $10^6$ | $10^6$ | — |
| 3 | 4000 | 8960 | — |
| 4 | Nil | Nil | Nil |
| 3(+$N_2$) | — | 12320 | — |
| 4(+$N_2$) | Nil | Nil | Nil |
| 5 to 12 | Nil | Nil | Nil |
| Heat treatment | | | |
| 100°C 15 min | Nil | Nil | Nil |
| 70°C 10 min | $10^6$ | — | — |

Cans of mango slices, treated either with various radiation doses or at different temperatures, were incubated at 30°C for different periods as stated, and their sterility determined. A radiation dose of $4 \times 10^5$ rads gives a sterile product.

sterile at a total absorbed dose of $4 \times 10^5$ rads.

Alongside these experiments, another batch of cans inoculated with 10,000 *Saccharomyces cerevisiae* yeast cells and approximately 500 *Bacillus subtilis* spores were also irradiated with the same doses. Such cans irradiated with $4 \times 10^5$ rads were sterile at the end of 2, 4, or 6 weeks of incubation at 30° or 37°C. Results were similar with incubation at either temperature. The combination process with low heat treatment followed by sterilization with a low radiation dose was therefore effective even with an initially high microbial load of the product.

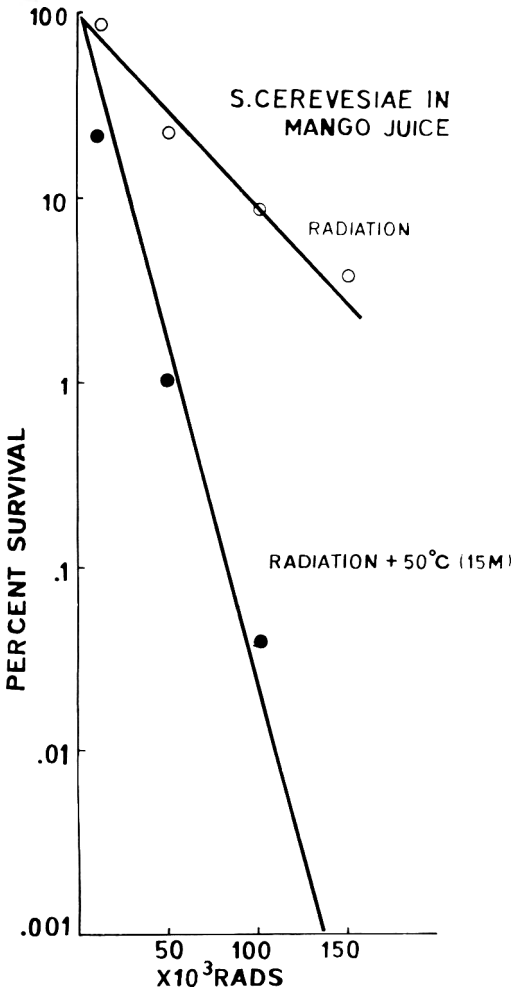


Fig. 2. Radiation sensitivity of *Saccharomyces cerevisiae* suspended in mango juice. Mango juice with $10^5$ yeast cells per ml after irradiation with different radiation doses or irradiated and heat-shocked at 50°C for 15 min, was plated on yeast agar medium by pour plate method. The colonies were counted after incubation for 48 hr at 30°C. From the slope of the curve *B*, it is seen that radiation sensitizes the organism to a sublethal temperature of 50°C.

Postirradiation heat treatment of spoilage organisms sensitizes them to even sublethal temperatures. This may be seen from Fig. 2, which shows the survival of *Saccharomyces cerevisiae* cells, suspended in mango juice (mango pulp dispensed in twice its volume of water), with or without heat treatment at 50°C for 15 min, after irradiating with different doses of radiation. There is an appreciable drop in the $D_0$ values (90% mortality dose) when radiation effect alone is compared with the combination of irradiation and heat. It seems that the organism becomes very much sensitive to this low temperature after irradiation. This would suggest therefore that, if heat treatment (70°C), which was carried out in the beginning, were done after irradiation, sterilization would have been possible even at lesser radiation doses. However, the presence of other organisms, especially bacillary spores, would make this postirradiation heat treatment less effective. The effect of different radiation doses and subsequent heat treatments at 50, 60, or 70°C on spores of *Bacillus subtilis* suspended in phosphate buffer at pH 3.5 is shown in Fig. 3. It would appear from these results that there is no appreciable post-irradiation heat-sensitization of these spores, since the $D_0$ values for this organism at various treatments are the same. Because of this difference in the behavior of the two organisms studied with respect of their sensitivities to heat (70°C) after irradiation, and since with the yeast about 90% of the cells are killed at 70°C, the rest being inactivated by the radiation treatment, the relative order of heat treatment in the combination process may not be important. For practical reasons, therefore, especially since efficient evacuation of the cans has to be done prior to irradiation, it is advantageous to heat-treat the product first and then to irradiate.

**Organoleptic evaluation.** The heat sterilization process for preserving mango slices in sanitary containers is a standard procedure and is practised extensively. The combination-processed product was therefore

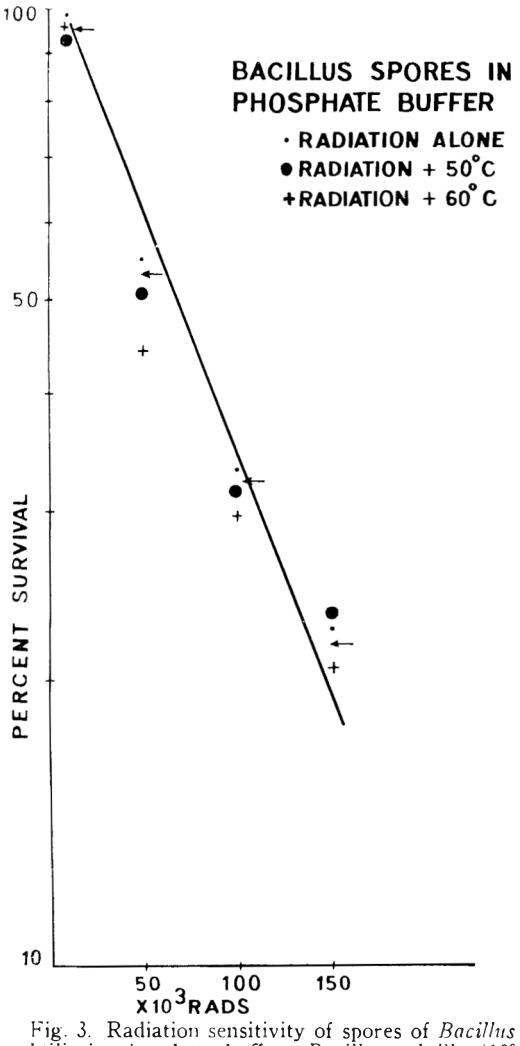


Fig. 3. Radiation sensitivity of spores of *Bacillus subtilis* in phosphate buffer. *Bacillus subtilis* ($10^5$ spores) was suspended in phosphate buffer at pH 3.5 and, after irradiation with different radiation doses or treatment with radiation followed by heat shocking at 50, 60, and 70°C as given in text, was plated on yeast agar medium by pour plate method. The colonies were counted after incubation at 37°C for 48 hr. It is seen that no sensitization to heat treatment at 50, 60, or 70°C is obtainable after irradiation for the spores of this organism.

compared with the conventionally canned product for consumer preference (Fig. 4) on a 9-point scale. It may be seen that the combination processed product (70°C followed by $4 \times 10^5$ rads) is as much acceptable as the heat-sterilized one. The acceptability decreases progressively as the total radiation dose increases to $1.2 \times 10^6$ rads.

Similar canning procedures were followed in the case of Sapodillas, where organoleptic evaluation (Fig. 4) showed a marked contrast with mangoes, since there was no deterioration in quality even up to a maximum dose of 1.2 megarads. The data show that Alphonso mangoes are more radiosensitive than Sapodillas. It is of interest that the texture of the irradiated mango and the Sapodillas was better than that of the thermally sterilized samples, which showed loss to some extent. It was consistently observed that the heat-sterilized product had a slightly brownish tinge, presumably due to caramelization of sugars. This was more pronounced in Sapodillas and could also be seen from the scoring (Fig. 4).

These differences in the radiation-processed and heat-sterilized products, although small, were observable in all experiments carried out over three different seasons.

**Irradiation under nitrogen.** Irradiation of mangoes under nitrogen atmosphere did not improve the quality of the mangoes. The acceptability of this product when irradiated ($4 \times 10^5$ rads) under nitrogen decreased sharply as compared with irradiation without nitrogen by the same dose, due to the formation of some malodorous substances (Fig. 4). Irradiation of proteins in nitrogen and oxygen atmospheres has been found to give different end products (Hansen, 1964). Attack at the peptide bond is the same during irradiation under either nitrogen or oxygen, but attack at the sulfur bond in a nitrogen atmosphere results in the production of malodorous compounds like mercaptans and hydrogen sulfide, etc. Presumably, similar reactions occur with mangoes, rendering use of nitrogen impractical for this process. Sapodilla slices, on the other hand, did not show any off-flavor effect, and in fact gave a much better and brighter appearance when irradiation was done under nitrogen (Fig. 4).

**Vitamin retention.** Vitamin retention values in the variously treated mango slices are given in Table 3. Vitamin losses due to the irradiation process are not more than those resulting from thermal sterilization. It was observed, however, that with higher radiation doses there was progressively more destruction of the vitamins. Radiation with $4 \times 10^5$ rads dose, evolved as described, is the optimum from all points of view.

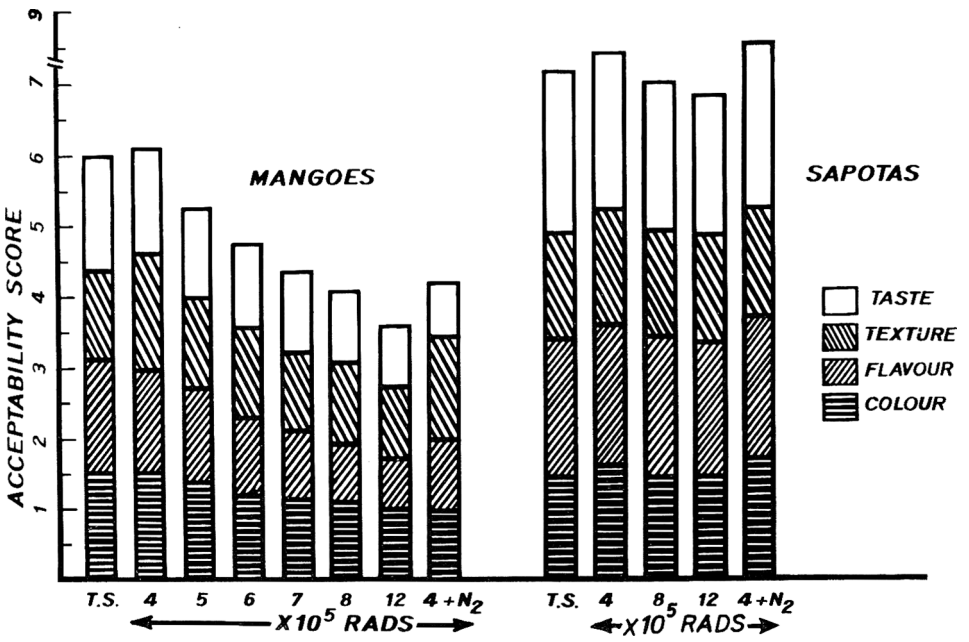


Fig. 4. Organoleptic evaluation of irradiated and thermally processed mangoes and sapodillas. Histograms show acceptability of mango and sapodilla slices after various radiation or heat treatments. As can be observed, a dose of $4 \times 10^5$ rads gives an acceptable product in both the fruits. $4 + N_2$ shows irradiation by $4 \times 10^5$ rads under nitrogen atmosphere. T.S. stands for thermally sterilized. The total rating of 9 has been subdivided into 2¼ each, for the different parameters of color, flavor, texture, and taste.

Table 3. Vitamins retained in 100 g canned mango product, irradiated or thermally processed.

| Treatment | Niacin (mg) | Riboflavin (μg) | Ascorbic acid (mg) | Thiamine (μg) | Total carotenoids (mg) |
|---|---|---|---|---|---|
| Irradiated | | | | | |
| $4 \times 10^5$ rad | 1.815 | 40 | 24.05 | 52 | 6.94 |
| Thermally processed | | | | | |
| 100°C 15 min | 1.78 | 42.2 | 24.37 | 38.9 | 6.49 |

It may be concluded that although irradiation at $4 \times 10^5$ rads for mangoes and at 4, 8 and 12 $\times$ $10^5$ rads for Sapodillas did not harm the products, these treatments conferred no appreciable advantage over conventional thermal processing with respect of organoleptic qualities or vitamin retention. However, as stated above, a definite though small improvement in texture with both fruit products and in color with Sapodillas was observed with the combination treatment.

Since susceptibility to irradiation of desirable attributes may vary with the nature of the fruits and vegetables, it may be expected that a combination of radiation and thermal processing may be of advantage in certain cases. In studies with guavas, apples, and peas (to be published), it has, in fact, been observed that the combination procedure results in products distinctly superior to heat-sterilized ones.

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Ms. rec'd 6/26/65.

**J. ILANY-FEIGENBAUM**
*Department of Biochemistry, Bar Ilan University, Ramat-Gan, Israel*

# Complexes of Dye and Soy Protein as Substrate for Proteolytic Enzymes, and Simple Colorimetric Determination of Their Activity

## SUMMARY

**Insoluble dye-protein complexes like carmine-fibrin, were prepared from soybean oil meal and various dyestuffs. Soy-carmine and especially soy-amido black were the most suitable substrates for proteolytic enzymes, tinting the test mixtures only after digestion of the proteins. They are easier and simpler to prepare than carmine-fibrin. These soy-dye complexes may be used in qualitative, semi and quantitative colorimetric determination of the activity of proteolytic enzymes.**

Insoluble dye protein complexes have been used for many years for qualitative and approximate quantitative determination of proteolytic enzymes. Nelson *et al.* (1961) recently described a quantitative assay for proteolytic enzymes with these complexes used as substrates. Investigating the soybean trypsin inhibitors, we (Ilany-Feigenbaum, 1964) found soybean oil meal a more convenient protein component than fibrin (in carmine-fibrin) to use in dye protein complexes. (Since the quality of the soymeal may change with the variety, composition, and processing of soybeans, we used a product No. 100 of General Mills, U.S.A., which is of a uniform and constant quality.)

This paper describes the preparation of dye-soy protein complexes and a simple colorimetric determination of the activity of proteolytic enzymes by using these protein complexes as a substrate.

## EXPERIMENTAL

**Preparation of the dye protein complexes.** *Soy-carmine.* Ten grams of toasted soybean oil meal (General Mills No. 100) was mixed into about 250 ml of a freshly prepared dye solution. This solution is obtained by dissolving 50 mg carmine (Eastman Chemicals) in 25 ml 10% ammonia solution to which are added 225 ml of 0.1*N* hydrochloric acid.

The mixture of the soymeal and dye solution was shaken for 1 hr for maximum absorption of the dye. The colored product was then centrifuged and washed several times with distilled water until no more color was given up. It was dried on a water bath (50–60°C) and ground. A mauve-colored powder was obtained.

*Soy-phthalein dyes.* Because of the tendency of phthalein dyes to combine with toasted proteins (Fraenkel-Conrad and Cooper, 1944; Frolich, 1954; Olomucki and Bornstein, 1960), we prepared soy-phthalein compounds: soy-phenophthalein, soy-bromthymol blue, soy phenol red, and soy cresol red. These substrate indicators were prepared similarly to soy-carmine by combining the phthalein dyes with toasted soybean oil meal. Ten g of toasted soymeal of General Mills No. 100 were mixed into 250 ml of a freshly prepared dye solution of 50 mg of the dye in 25 ml 96° alcohol, instead of the ammonia solution, and mixed into 225 ml of 0.1*N* hydrochloric acid. Thereafter, the operation resembles that with soy-carmine, described above.

Among these soy-phthalein dye complexes the most stable and suitable as substrate in the determination of proteolytic enzymes is soy bromthymol blue complex. The indicator was obtained from Coleman and Bell Co., U.S.A.

*Soy-amido black.* Following the method for estimation of protein content of milk by its binding with orange G (Gruner *et al.*, 1961) and with Buffalo Black (Vanderzant and Tennison, 1961), we used for our purposes a sample of Amido Black 10B of E. Gurr Ltd. London, Microme No. 670. The method of preparation was as follows: 10 g of soymeal samples of General Mills No. 100 were mixed into 100 ml of the dye solution. This solution was prepared by dissolving 50 mg of the dye in distilled water. Then the mixture of soymeal and dye solution was shaken for 1 hr. The subsequent procedure was like that with soy-carmine. A dry substance that was hard to grind was obtained.

**Determination of enzyme activity.** Enzyme activity was tested in test tubes with a mixture consisting of 100 mg soy-dye powder; 4 ml distilled water; and 1 ml of a solution containing 10 mg of each of the following enzyme preparations: trypsin Difco 1:250, papain NBC, and pepsin NBC. Five ml of 0.1*M* citric acid and 0.2*M* disodium phosphate buffer mixture according to McIlvaine (1921), with pH 8.0 for trypsin, pH 7.0 for papain and pH 2.2 for pepsin.

The test mixtures were prepared as follows: to the dry soy substrate were added 4 ml distilled water, 1 ml enzyme solution, and 5 ml buffer mixture, completing the volume to 10 ml in the control

tests. The tube contents were thoroughly shaken and after the addition of a few drops of toluene to prevent alteration, the test mixtures were incubated at 35°C.

Usually during 3-6 hr, a distinct tinting was observed, increasing with the progress of the enzyme digestion of the substrate proteins in the mixtures. A longer period of incubation (24 hr) was, however, necessary, as the enzymes used were commercial samples of more or less slow activity.

A slight tint may be observed, especially with carmine in the enzyme-free control test, when trypsin is tested. This is due to the dissolving of some dye in the alkaline buffer solution. The deep tint that develops in tubes containing active trypsin is more intense than in control tubes.

The activity of the enzymes was followed by reading the color with a Klett-Summerson photoelectric colorimeter. In the tests of carmine and amido black, a green filter No. 54 was used.

The colorimetric readings were converted to micrograms of the free dye according to a specially designed curve established from various known concentrations of the dye solution.

To check the colorimetric readings and the effect of the dyes used on the enzymes, we carried out parallel tests with the same enzyme concentrations and medium on substrates of the same soybean meal treated similarly to the soy-dye complexes as described above but without addition of the dyes. Digestion of the substrate was followed by formol titration and by the copper amino complex method (Pope and Stevens, 1939).

The colorimetric readings were also compared with the results obtained by using Anson's procedure (Anson, 1938) for determining proteolytic enzyme activity on soymeal as substrate. This comparison will be discussed in another paper on the same subject, to be published later.

## RESULTS

The tables show the results when soy-dye complexes and soybean meal are used as substrates for the proteolytic enzymes trypsin, papain, and pepsin. The "digestion ratio" was calculated in each case by division of the digestion values of these enzymes during 24 hr by those during 6 hr.

## CONCLUSION

According to the tables, soy-carmine and soy-amido black complexes seem to be suitable substrates for simple and practical determination of proteolytic activity. Comparing the "digestion ratios" obtained by different methods of determination of the proteolytic activity, we found soy-amido black a better substrate than soy-carmine.

Table 1. Soy carmine as substrate.

| | | 6 hr | 24 hr | Digestion ratio |
|---|---|---|---|---|
| Colorimetric readings, in mg carmine | Trypsin | 0.67 | 4.45 | 6.64 |
| | Papain | 0.38 | 1.35 | 3.60 |
| | Pepsin | 0.30 | 0.60 | 2.00 |
| Formol titration ml 0.1*N* NaOH | Trypsin | 0.45 | 2.66 | 5.95 |
| | Papain | 0.15 | 0.50 | 3.33 |
| | Pepsin | 0.75 | 1.35 | 1.80 |

Table 2. Soy-amido black as substrate.

| | | 6 hr | 24 hr | Digestion ratio |
|---|---|---|---|---|
| Colorimetric readings, in mg amido black | Trypsin | 0.10 | 1.28 | 12.80 |
| | Papain | 1.08 | 1.98 | 1.83 |
| | Pepsin | 0.27 | 0.53 | 2.00 |
| Formol titration | Trypsin | 0.12 | 1.50 | 12.50 |
| | Papain | 2.70 | 4.90 | 1.81 |
| | Pepsin | 0.15 | 0.30 | 2.00 |

Table 3. Soymeal-copper amino complex.

| | | 6 hr | 24 hr | Digestion ratio |
|---|---|---|---|---|
| Copper amino complex g Amino-N | Trypsin | 5.2 | 63.6 | 12.23 |
| | Papain | 2.8 | 5.1 | 1.81 |
| | Pepsin | 33.6 | 64.8 | 1.92 |

Soy-amido black, besides being the simplest to prepare, does not yield any color, either in acid medium of pepsin, of papain, or in alkaline medium of trypsin, unless the protein component of the dye substrate complex is digested by the enzymes.

Using as a substrate, soymeal treated similarly to the soy-carmine complex preparation, and the copper amino complex for the determination of the proteolytic activity, the "digestion ratios" differed from those obtained with the soy-carmine complex. The results of these experiments are not given, because of large discrepancies.

Soy-amido black, of all the soy-dye complexes, seems to be especially suitable for use as a substrate in simple qualitative, semi- and quantitative evaluation of proteolytic enzyme activity. Its preparation and the enzymatic test procedure are simple, practical, and do not require special laboratory facilities as do other methods.

The figures of amino-N obtained with the substrate treated as in the case of soy-amido black determined by the copper amino complex are given in Table 3. The "digestion

ratios" are almost similar to those obtained with other methods.

Using these soy-dye complexes as substrates we could find a simple method for direct determination of the antitryptic factor in soybeans and soybean oil meal, egg white, and lentils.

We could also find an alkaline protease in Japanese koji (*Aspergillus oryzae* grown on rice) and in taka diastase samples of Parke Davis Co.

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Ms. rec'd 7/12/65.

The author thanks Mrs. Fr. Shor, Mrs. T. Boger-Leipziger, and Mr. A. Netzer for assistance.

N. I. MONDY, S. BOND GEDDE-DAHL, and E. OWENS MOBLEY
*Graduate School of Nutrition, New York State College of Home Economics*
*Cornell University, Ithaca, New York 14850*

# Effect of Storage Temperature on the Cytochrome Oxidase and Polyphenol Oxidase Activities and Phenolic Content of Potatoes

## SUMMARY

**Ontario and Pontiac potatoes, respectively representing varieties susceptible and resistant to precooking blackening, were examined for phenolic content and for cytochrome oxidase and polyphenol oxidase activities following storage at 40 and 50°F. The study was conducted for two consecutive years. In each of the two years the phenolic content of both varieties was significantly higher in potatoes stored at 40°F than in potatoes stored at 50°F. Both varieties had higher cytochrome oxidase and polyphenol oxidase activities when stored at 50°F than when stored at 40°F. The lower enzyme activities at 40°F may be responsible, in part, for the accumulation at that temperature of phenolic substances which serve as substrates for the enzymes. The higher phenolic content at lower temperatures may account for the greater discoloration of the potatoes.**

## INTRODUCTION

Storage conditions have been shown to affect the susceptibility of potatoes to the type of precooking discoloration known as "black spot" which appears as gray or black lesions just under the peel. This type of discoloration, an abnormal physiological condition which occurs when potatoes are bruised, is one of the most serious and costly problems of the potato industry.

Storage temperature has been shown to affect the degree of blackening in potatoes. Massey (1952) found that up to 4 months of storage at 50°F resulted in less blackening than storage at 40°F. Potatoes stored at 50°F however, became flaccid and were more susceptible to black spot following lengthy storage. Jacob (1959) and Scudder (1951) reported that after four months of storage the black-spot index was lower at 40°F than at 35 or 50°F, but by this time the potatoes had begun to sprout. Wiant *et al.* (1951), using Long Island and Red River Valley potatoes, found that tubers stored up to 18 weeks at 50° had a much lower black-spot index than potatoes stored at 33 or 40°F.

Wiant *et al.* (1951) reduced black spot significantly by holding tubers previously stored at 40°F at a conditioning temperature of 50° for three days prior to bruising. Greater reduction in blackening was obtained by increasing the conditioning temperature to 70°F. Massey (1952) found that a two-day conditioning period at 70°F, before bruising, reduced black spot significantly.

Potato discoloration has been associated with the action of polyphenol oxidase on the phenolic constituents of the potato, resulting in the formation of the black pigment melanin (Lerner and Fitzpatrick, 1950; Mondy *et al.*, 1960). The role of polyphenol oxidase in the metabolism of the potato has not been clearly defined. Early workers associated this enzyme with terminal oxidation in the tuber (Baker and Nelson, 1943; Boswell, 1945; Boswell and Whiting, 1938), but more recent studies have indicated that cytochrome oxidase activity is high enough to account for the total respiration of the tuber (Goddard and Holden, 1950; Hopkins, 1924; Schade and Levy, 1949; Thimann *et al.*, 1954). Hopkins (1924) reported that the respiration rate of the potato is at a minimum at 37°F and increased with increasing temperature. Joslyn and Ponting (1951) pointed out that cytochrome oxidase reacts with certain phenols to convert them into pigmented compounds, and under certain conditions may be involved in darkening. Relatively high concentrations of phenolic substances such as tyrosine, chlorogenic acid, and caffeic acid, which could serve as substrates for enzymes involved in discoloration, have been found in potatoes (Cheng and Hanning, 1955; Johnson and Schaal, 1952, 1957). Mondy *et al.* (1960) observed that at 40°F the concentration of phenolic substances in the cortex tissue increased with storage duration. Craft *et al.* (1958) did not find an increase in phenolic content with storage, but they sampled the entire tuber including the pith, the section known to be relatively low in phenolic content.

This study was made to determine whether storage temperature affected the enzymatic activity and the phenolic content of potatoes.

## EXPERIMENTAL

Two varieties of potatoes—Pontiac, which is resistant to, and Ontario, which is susceptible to, precooking darkening—were grown on the same farm near Ithaca, New York, in each of two consecutive years, 1959 and 1960. Potatoes were planted so that soil variation would be randomly distributed among the plots. In each of the two years, potatoes were harvested 19 weeks after planting, and one-half of the potatoes were stored at 40° and the remainder at 50°F. Phenolic content and cytochrome oxidase and polyphenol oxidase activities were determined after 3, 4, 5, and 6 months of storage in 1959, and after 1, 2, 3, and 6 months of storage in 1960. In the 1960 study, phenolic content was determined after 1, 2, 3, 5, and 6 months.

**Determination of total phenols.** Four tubers of each variety were selected at random for each extract used in phenol determinations. Two extracts were made at each storage temperature at each storage period in 1959, and four extracts at each storage period in 1960. A 50-g sample of cortex tissue, taken longitudinally from the bud to stem end of the potatoes, was blended with 150 ml of 95% ethanol for 5 min. The total extract was measured and filtered, and 35 ml of the filtrate was used for phenol determination. Total phenolic constituents were determined by the method of Rosenblatt and Peluso (1941) with tannic acid used as the standard. Since this method is not specific for phenols but also includes ascorbic acid, corrections were made for ascorbic acid. Potato tissue was extracted with meta-phosphoric acid, and analyzed for ascorbic acid by the indophenol dye method simultaneously with the analyses for total phenols.

**Determination of enzyme activity.** Three tubers of each variety were selected at random for each extract. Two extracts were made for each variety at each storage temperature. A 50-g sample of cortex tissue, taken longitudinally from the bud to stem end of the potatoes, was blended with 50 ml of 0.05$M$ sodium barbiturate for 1 min, and filtered through three layers of cheesecloth for 3 min. The filtrate was then centrifuged for 3 min at 600 $\times$ G, and the supernatant liquid was used for enzyme activity determinations. All reagents and containers were chilled before and during preparation of the extract. Cytochrome oxidase and polyphenol oxidase activities were determined manometrically by a modification of the method described by Goddard and Holden (1950). For the determination of cytochrome oxidase, the main compartment of the Warburg flask contained 1 ml of 0.5$M$ phosphate buffer at pH 7.1 plus 0.6 ml of $2.25 \times 10^{-4}$ $M$ cytochrome $c$; the center well 0.2 ml of 2$N$ sodium hydroxide; one sidearm 0.3 ml of the potato extract; and the second sidearm 0.8 ml of $6.82 \times 10^{-2}$ $M$ hydroquinone. Water was added to the main compartment to bring the total volume of the liquid in the flask to 3.2 ml. The sidearm contents were added after a 5-min equilibration period at 30°C; stopcocks were closed, and readings were taken every 10 min over a 1-hr period. Polyphenol oxidase activity was determined in a similar manner, using 0.2 ml of extract in the sidearm and 0.2 ml of $4.55 \times 10^{-3}$ $M$ catechol in place of cytochrome $c$ in the main part of the flask. Cytochrome oxidase and polyphenol oxidase activities were determined simultaneously on the same potato extract. All readings were corrected for endogenous respiration.

## RESULTS AND DISCUSSION

**Phenolic content.** The effect of storage temperature on the phenolic content of Pontiac and Ontario potatoes is given in Figs. 1 and 2. In each of the two years the phenolic content of both varieties was significantly higher in potatoes stored at 40°F than in potatoes stored at 50°F. The greater difference was observed in the Ontario variety, the variety which is more susceptible to discoloration. In the 1960 study the phenolic content dropped noticeably at 6 months storage. This may have been due to changes occurring during storage. The phenolic content of potatoes increased during storage at both storage temperatures but the greater increase generally occurred at 40°F. The potatoes grown in 1960 exhibited a high incidence of scab disease, and some of the difference may be attributed to this factor.

These findings are in agreement with those of Mondy *et al.* (1960), who observed that the phenolic content of potatoes stored at 40°F increased with storage duration, indicating synthesis during storage. Zucher and Levy (1959) showed that chlorogenic acid was formed in disks of potato tissue which were initially almost devoid of this compound, and found that the availablity of oxygen was an important factor in the synthesis of chlorogenic acid.

**Enzyme activity.** Potatoes stored at 50°F had higher cytochrome oxidase activity than potatoes stored at 40°F (Figs. 3, 4). These results were highly significant when data were pooled from the two varieties of potatoes for the two years of the study.

Polyphenol oxidase activity was higher in potatoes stored at 50° than in potatoes stored

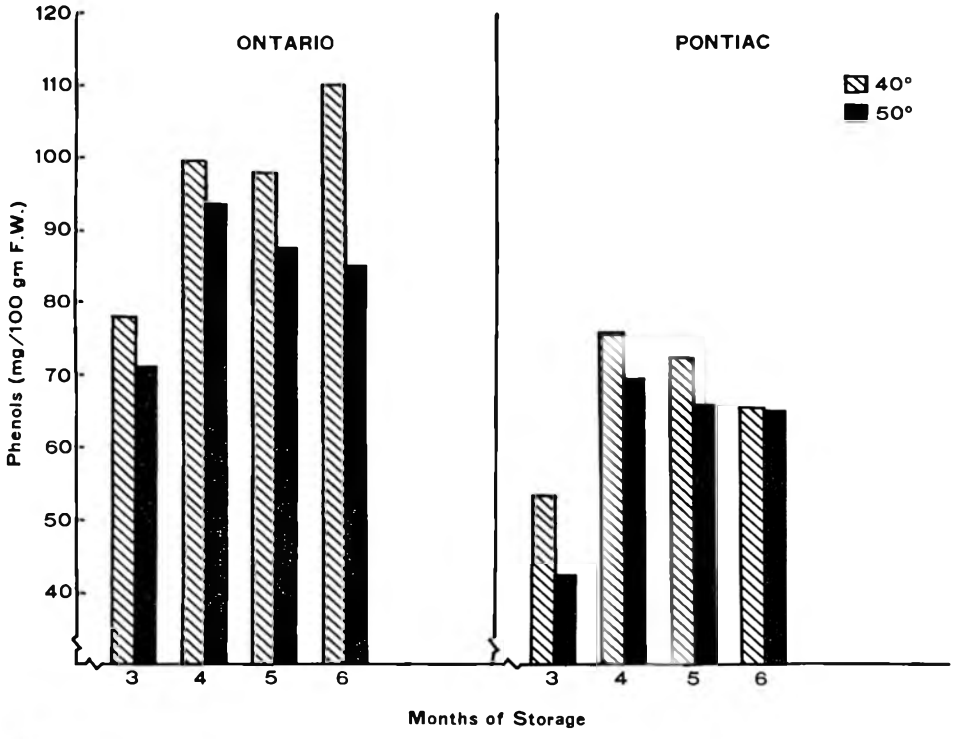


Fig. 1. The effect of storage temperature on the phenolic content of potatoes. 1959 study.

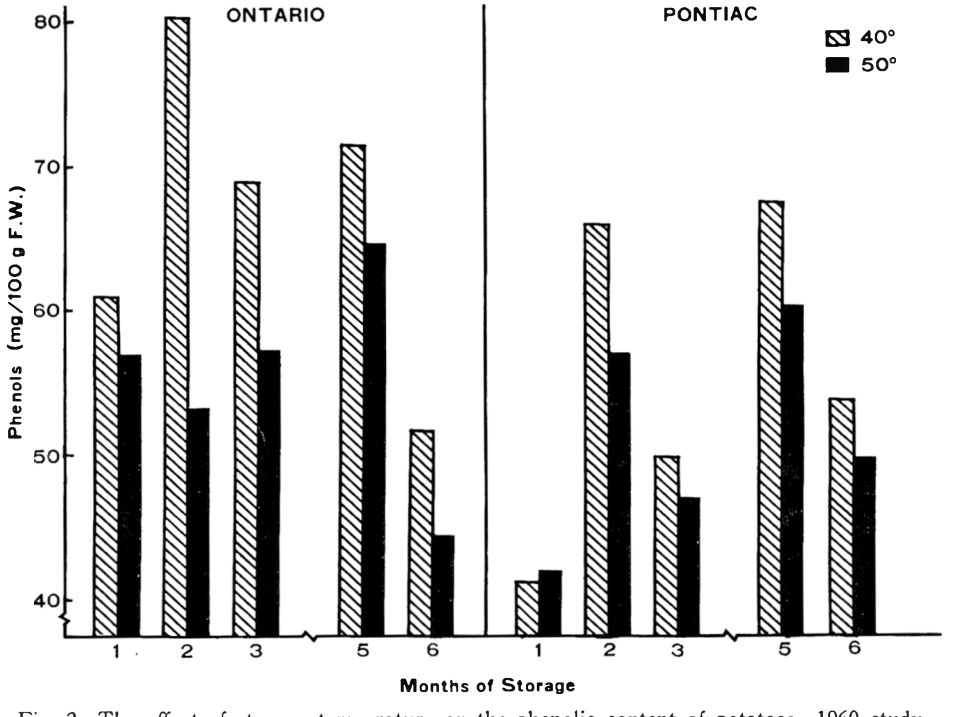


Fig. 2. The effect of storage temperature on the phenolic content of potatoes. 1960 study.

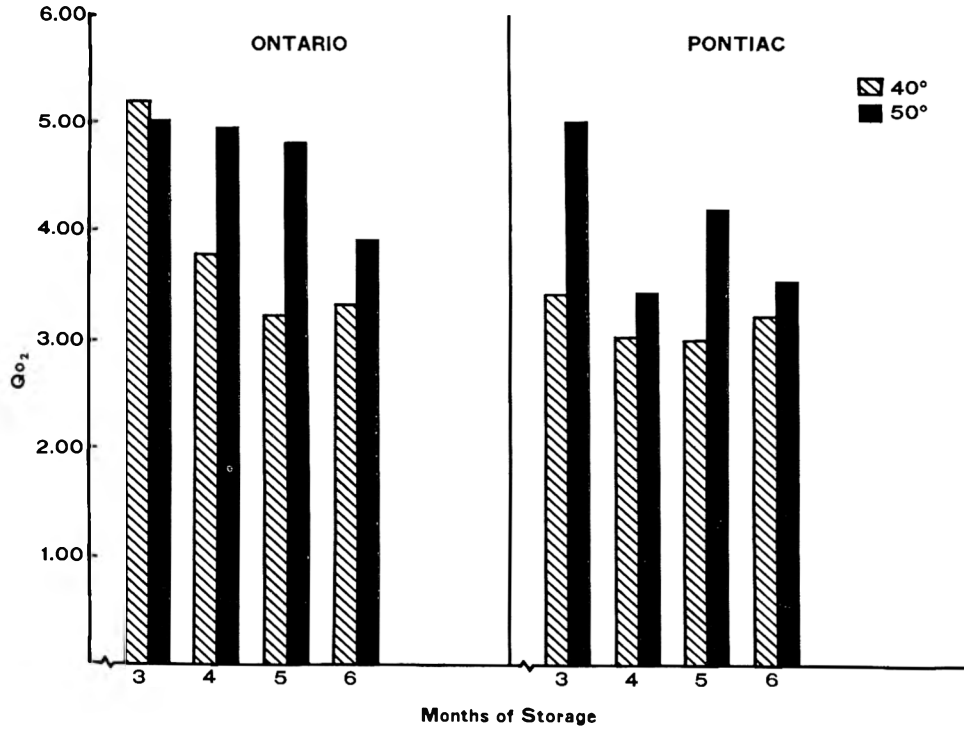


Fig. 3. The effect of storage temperature on cytochrome oxidase activity of potatoes. 1959 study. ($Q_{O_2}$: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.)

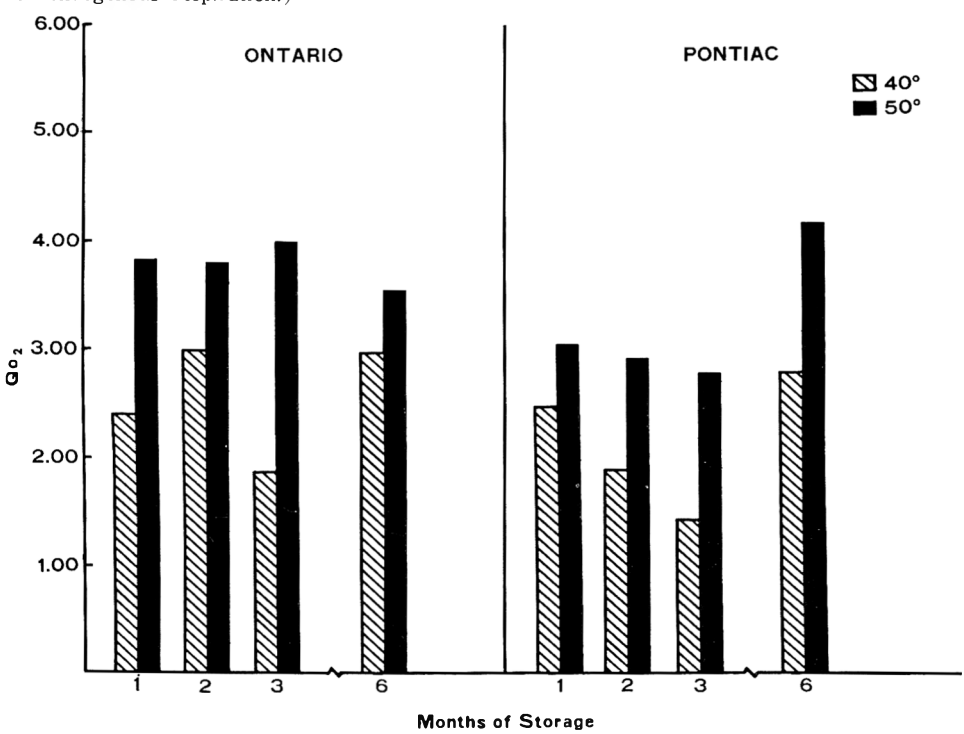


Fig. 4. The effect of storage temperature on cytochrome oxidase activity of potatoes. 1960 study. ($Q_{O_2}$: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.)

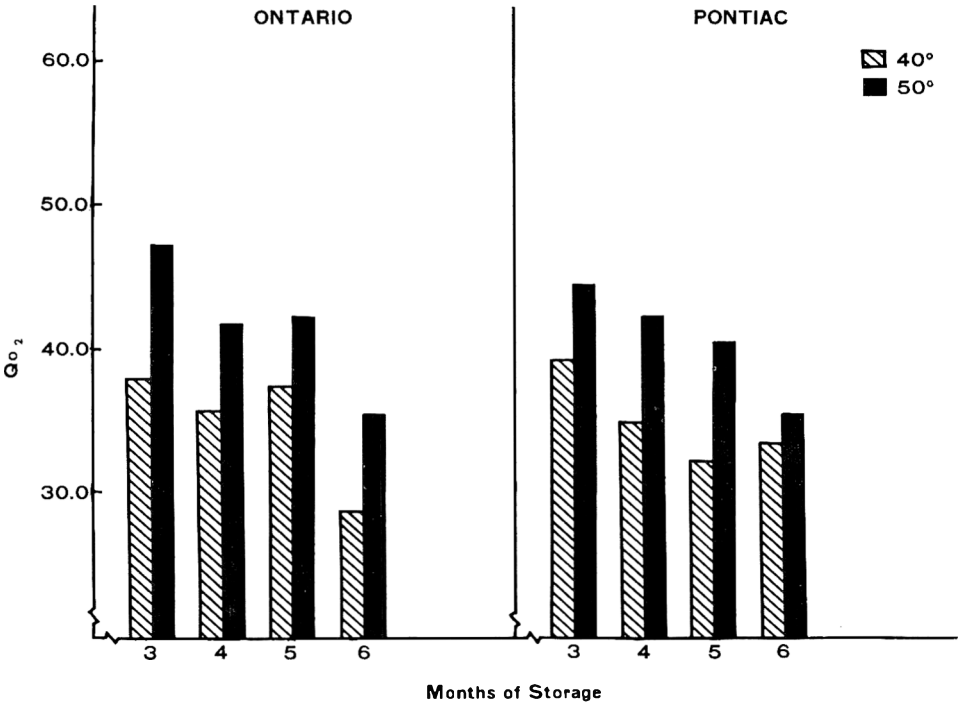


Fig. 5. The effect of storage temperature on the polyphenol oxidase activity of potatoes. 1959 study. ($Q_{O_2}$: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.)

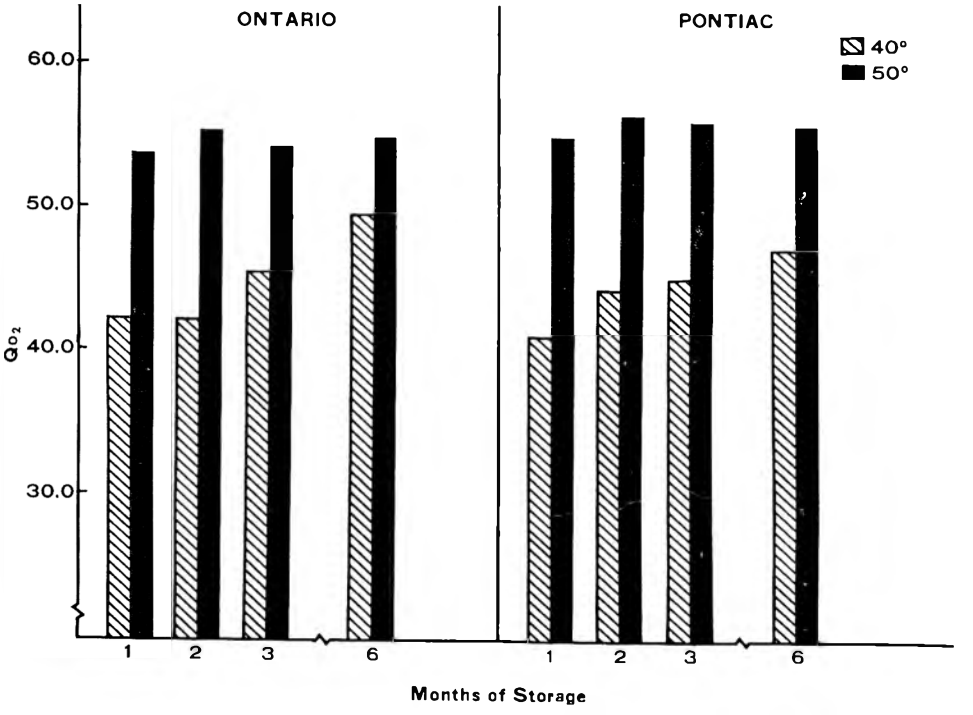


Fig. 6. The effect of storage temperature on the polyphenol oxidase activity of potatoes. 1960 study. ($Q_{O_2}$: microliters oxygen uptake/hr/mg dry weight of tissue. All values were corrected for endogenous respiration.)

at 40°F (Figs. 5, 6). In 1959 and 1960 the differences in polyphenol oxidase activities were significant in both varieties. Potatoes stored at 50° were higher in both cytochrome oxidase and polyphenol oxidase activities but were lower in phenolic content than potatoes stored at 40°F. In general, enzyme activity might be expected to increase with increasing temperature within the biological temperature range. The lower enzyme activities which were observed at 40°F storage may be responsible in part for the accumulation at that storage temperature of phenolic substances which serve as substrates for the enzymes.

Bond (1961) found that discoloration increased with increasing phenolic content of potatoes. In the present study, potatoes stored at 40°F were higher in phenolic content than potatoes stored at 50°F, and the greater phenolic content may account for the greater discoloration of potatoes stored at 40°F.

Several workers (Jacob, 1959; Massey, 1952; Scudder, 1951; Wiant *et al.*, 1951) have observed more discoloration in potatoes stored at low temperatures. These differences may have been due to the increase in phenolic substances that occurs at the lower temperatures, accompanying the lowered cytochrome oxidase and polyphenol oxidase activities.

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The authors acknowledge the assistance of Drs. Richard Sawyer and Ora Smith, Department of Vegetable Crops; Dr. Robert Plaisted, Department of Plant Breeding; and Dr. L. C. Peterson, Department of Plant Pathology, for the arrangements made in growing, harvesting, transporting, and storing the potatoes. Grateful acknowledgment is also made to Dr. John Skory for guidance and assistance in the statistical analyses.

D. C. ABBOTT and J. A. JOHNSON

*Department of Biochemistry, Oklahoma State University, Stillwater, Oklahoma 74075 and Department of Flour and Feed Milling Industries, Kansas State University, Manhattan, Kansas 66504*

# Gel Filtration of the Water-Soluble Protein Fraction of Wheat Flour

## SUMMARY

**The usefulness of gel filtration through cross-linked dextrans as a means of separating protein components in the water extracts of flour was investigated. Dextrans employed included Sephadex G-25, G-50, G-75, G-100, and G-200, while distilled water and several concentrations of lactic acid were used as eluting solvents. The most effective combination was Sephadex G-100 gel with 0.5*N* lactic acid as eluant. Although single protein components could not be isolated by this technique, it was possible to effect a simplification of the protein mixture and a concentration of some components in relatively small fractions of the eluate from gel columns. The combination of gel filtration and preparative starch-gel electrophoresis was utilized to isolate a fraction which migrated electrophoretically as a single component.**

## INTRODUCTION

The important role of the proteins of wheat and flour in bread baking has been recognized for many years. The voluminous literature pertaining to investigation of these proteins was recently reviewed by Sullivan (1965). Much progress has been made, particularly within the past five years, toward the elucidation of the composition and properties of major fractions of the wheat proteins. Study of the function of the individual protein components, however, has been hampered by the lack of suitable means for isolating single components from the complex mixture of proteins in wheat.

Because of the readily apparent importance of the gluten proteins and their role in baking, investigation of the soluble proteins has been largely neglected until recent years. Examination of distilled water and dilute salt extracts by a number of workers (Pence and Elder, 1953; Woychik *et al.*, 1961; Elton and Ewart, 1962; Grabar *et al.*, 1962; Kaminski, 1962; Holme, 1962; Kelly and Koenig, 1962; Nimmo *et al.*, 1963; Oh and Gehrke, 1965) has demonstrated that both are highly heterogeneous. Attempts by Pence and Elder (1953) and others to isolate individual components by classical fractional precipitation methods have resulted in multicomponent mixtures. More recent work employing ion-exchange chromatography (Grabar *et al.*, 1962; Kelley, 1964; Oh and Gehrke, 1965) or preparative starch-gel electrophoresis (Elton and Ewart, 1963) has resulted in the isolation of nearly pure single components from water or dilute salt extracts of wheat flour.

Ultracentrifugal analysis of the albumin fraction of wheat flour by Holme (1962) showed a high degree of heterogeneity with respect to particle size in this fraction. These results suggested that resolution of the water-soluble proteins on the basis of molecular size might be possible. Gel filtration studies reported (Jones *et al.*, 1963; Wright *et al.*, 1964; Godon *et al.*, 1964) were concerned either with gluten or with the total acid extract of flour. The present investigation was undertaken to study the application of gel filtration using cross-linked dextrans for resolution or simplification of the protein mixture in the water-soluble fraction of flour. It was also of interest to study the combination of gel filtration with preparative starch-gel electrophoresis for the isolation of single protein components.

## EXPERIMENTAL

**Flours.** Straight-grade experimentally milled flours from two Hard Red Winter wheat varieties, SuperKing and Kaw, were employed. Respective protein contents (N × 5.7) were 16.6 and 15.9%.

**Protein solutions.** Solutions of water-soluble proteins for electrophoresis and gel filtration experiments were prepared by stirring flour with water at the ratio of 1:1.5 (w/v) at room temperature. The slurry was stirred at 5-min intervals for 30 min, then centrifuged at 13,000 × G for 30 min at 2°C. The clear supernatant solution was decanted and either used immediately or lyophilized for later use.

**Electrophoresis apparatus and procedures.** Starch-gel electrophoresis was conducted on a commercial horizontal unit, Model EC-305, EC Appa-

ratus Company, Swarthmore, Pa. The starch employed was a commercial product from Connaught Medical Research Laboratories, Toronto, Canada. Starch gels were held either in plexiglass trays or on glass plates. The trays had inside dimensions of $\frac{1}{4} \times \frac{3}{4} \times 11$ in. Six thicknesses of filter paper (S & S no. 470) $\frac{3}{4}$ in. wide by 4 in. long were inserted $\frac{1}{2}$ in. into the open ends of the trays to close the ends and at the same time served as wicks to connect the gels to the buffer compartment. For experiments employing thin starch-gels, glass plates $1/16 \times 8 \times 10$ in. were employed to support the gels. A 1/16-in.-thick plexiglass frame of the same size as the glass plate was laid on the plate to contain the gelatinized starch and fix the gel thickness. Wicks for the thin gels were single thicknesses of filter paper $7\frac{1}{2}$ in. wide by 4 in. long.

The electrophoresis system employed, essentially as described by Woychik *et al.* (1961), used an aluminum lactate–lactic acid buffer containing urea (0.008*M* aluminum lactate, 3*M* urea, adjusted to pH 3.3 with lactic acid). Starch gels contained 15 g of starch per 100 ml of buffer. Trays were overfilled with the gelatinized starch, covered, and allowed to cool. Excess starch was cut off, and the gel was held no longer than 24 hr prior to use. For the thin gels on glass plates the 1/16-in. frame was taped to the plate, the gelatinized starch poured on in excess, and a cover plate laid over the gel to force out the excess. After cooling, the cover plate was removed and the gel covered with thin plastic and placed in the refrigerator until used. Protein samples were introduced into the gels by inserting small sections of filter paper impregnated with protein solution into slits in the gels 1 inch from the anode end. The anode is to the left on all starch-gel photographs. During electrophoresis experiments the voltage was checked periodically and maintained at approximately 5 v/cm as measured directly on the gels with an external voltmeter. All electrophoresis studies were conducted at 2–4°C.

Protein bands in starch gels were detected by staining with a saturated solution of Amido-black 10B in 5% acetic acid. The $\frac{1}{4}$-in.-thick gels were sliced into 1/16-in. thick layers prior to staining, while the thin gels on glass plates were stained directly without further treatment. After 20–30 min of immersion in the stain, excess stain was removed by allowing the gels to stand in 5% acetic acid wash solution for about 24 hr with occasional agitation. The solvent was changed occasionally, and washing was continued until the gels were suitable for photography.

**Gels and columns for gel filtration experiments.** The cross-linked dextran gels used for gel filtration studies were commercial Sephadex preparations obtained from Pharmacia, Uppsala, Sweden. The types employed were G-25, G-50, G-75, G-100, and G-200. The Sephadex gels were prepared for column packing by equilibrating an appropriate quantity of dry Sephadex for 4–24 hr with the eluant to be employed. The shorter equilibration times were used for the G-25 and G-50. Two sizes of columns were employed in the gel filtration experiments: small glass columns, $2.2 \times 40$ cm, were used for exploratory experiments, while larger columns, $4.2 \times 80$ cm, were employed for small-scale preparatory experiments. To minimize the dead volume beneath the gel the columns were constructed without stopcocks at the bottom. The lower ends were nearly flat and were fitted with 2-mm capillary tips for passage of effluent from the column. Gels were retained in the column by a thin layer of glass wool covered by a layer of small glass beads to keep the glass wool in place. For the small columns the total gel volume was about 100 ml, while for the large columns the gel volume was 900 ml. Gels were washed 8–12 hr before use.

**Gel filtration of flour extracts.** In experiments in which the eluting solvent was water or dilute lactic acid, the flour extract was added to the column by careful layering of the extract over the gel under a layer of the eluant. When more concentrated lactic acid (0.50*N*) was used as eluant, the procedure for adding the extract had to be modified because the lactic acid solution and the extract had nearly the same density. For these experiments, therefore, a circle of filter paper was placed carefully over the gel and the column allowed to run until the solvent level was at the gel surface; then the extract was layered carefully on the gel, and sample collection was begun as the extract entered the gel. After all the extract had entered the gel, additional solvent was added above the gel to a depth of about 3 inches; then the reservoir was connected and the elution continued. Effluent from the columns was collected by means of a Packard drop-counting fraction collector. For small columns, 4-ml fractions were collected, while for the large columns the fractions were approximately 12 ml.

**Analysis of fractions from Sephadex columns.** Protein appearing in the effluent from columns was detected by measuring absorbance at 280 and 260 mμ for each tube. Used for absorbance measurements was a Beckman DU spectrophotometer equipped with a GME transferator. Tubes containing major fractions were pooled, dialyzed, and lyophilized. The dried products were weighed, and portions were used for protein assay by the method of Lowry *et al.* (1951). In some

cases, protein was determined by a micro-Kjeldahl procedure. In some of the early experiments the correspondence of the 280-mμ absorbance with protein was checked by analysis of selected fractions by the Lowry method. The concentration of carbohydrate in fractions from some experiments was assayed by the anthrone procedure (Mokrasch, 1954).

## RESULTS AND DISCUSSION

**Preliminary experiments.** Used for initial experiments was Sephadex G-100 with a nominal particle exclusion size of 100,000 molecular weight. When a water extract of SuperKing flour was applied to a large column, and water was the eluant, the elution pattern was as shown in Fig. 1. The extremely high absorbance for the first fraction reflects the fact that the eluate in this fraction was turbid. Examination of the pooled fractions and crude extract by starch-gel electrophoresis produced the patterns shown in Fig. 2. These patterns indicated that the second fraction contained essentially all the components present in the original extract. The first fraction, although badly blurred, showed the presence of several of the same bands. Some of the faster-moving components, however, appeared to be absent from this fraction. The blurring shown in this gel, together with the impaction and heavy staining at the origin, has been associated by Woychik *et al.* (1961) and Cunningham (1963) with the presence of glutenin, the extremely high-molecular-weight fraction of gluten. Cunningham also showed that the 4 or 5 slowest-moving bands under these conditions were gliadin. Gel filtration, under conditions of this experiment, effected a limited separation of the protein components present in the extract. When this experiment was repeated with dilute (0.04*N*) lactic acid used as the eluant to prevent precipitation in the first fraction, the general shape of the elution curve was the same as when water was the eluant. Starch-gel electrophoresis also showed essentially the same distribution of components as found in the earlier experiment. Reading the absorbance of the eluate at 260 mμ as well as 280 mμ showed that the second peak contained material with higher absorbance at 260 mμ than at 280 mμ. The significance of this observation is discussed in greater detail in a later section.

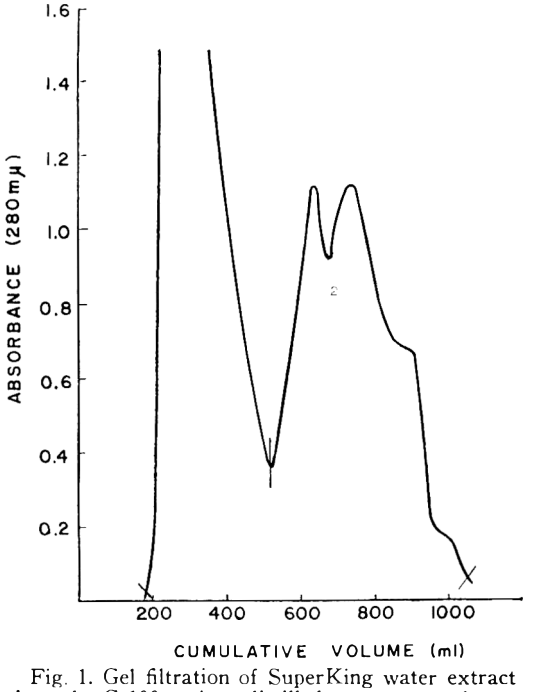


Fig. 1. Gel filtration of SuperKing water extract through G-100 using distilled water as eluant. Sixty ml of extract containing 398 mg of protein were added to 4.2 × 75-cm column.

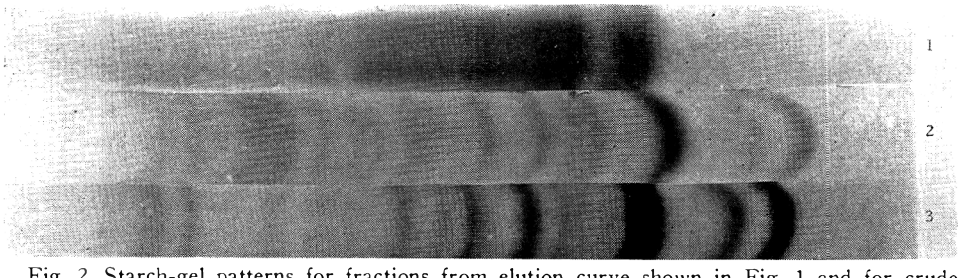


Fig. 2. Starch-gel patterns for fractions from elution curve shown in Fig. 1 and for crude water extract of SuperKing flour. 1) Fraction 1; 2) crude extract; and 3) fraction 2.

**Influence of gel porosity, sample size, and lactic acid concentration on resolution.** Although the preliminary experiments with G-100 indicated that, under the conditions employed, there was a partial separation of protein components according to molecular size, the two fractions obtained were still very heterogeneous. Therefore, dextran gels with differing porosities were investigated, with the thought that some separation might be achieved by selective exclusion. Sephadex G-25, G-50, G-75, and G-100, with nominal exclusion limits of 4, 10, 50, and 100 thousand molecular weight, were used, and 0.01*N* lactic acid was the eluant. Small columns were used for these comparisons. The elution patterns obtained are shown in Fig. 3. Part of the protein was apparently excluded by each of these gels, the amount decreasing with increasing porosity. Since the G-100 appeared to be the most effective, its use was investigated further. Fig. 4 shows the elution patterns obtained when two different volumes of extract were eluted with 0.01*N* lactic acid (curves E, F) and when the same volume of extract was eluted with different concentrations of lactic acid (curves F, G, H). The two levels of extract applied to the column resulted in no difference in the general appearance of the elution curve except for concentration differences. Increasing the concentration of lactic acid in the eluant, however, produced a marked effect. The exclusion of some components appeared to increase with increasing acid concentration. With 0.50*N* lactic acid as eluant the material absorbing with a maximum at 280 mμ was resolved into two fractions.

Eluates from the last three experiments (F, G, H) were pooled as indicated in the elution diagrams, dialyzed to remove lactic acid, and lyophilized. The fractions were examined by starch-gel electrophoresis, with the results shown in Fig. 5. The distribution of components on the gel strips indicated that glutenin and gliadin components were present mainly in fractions representing the forepart of the first absorbance peak. The more rapidly migrating components, presumably albumin and globulin, were present

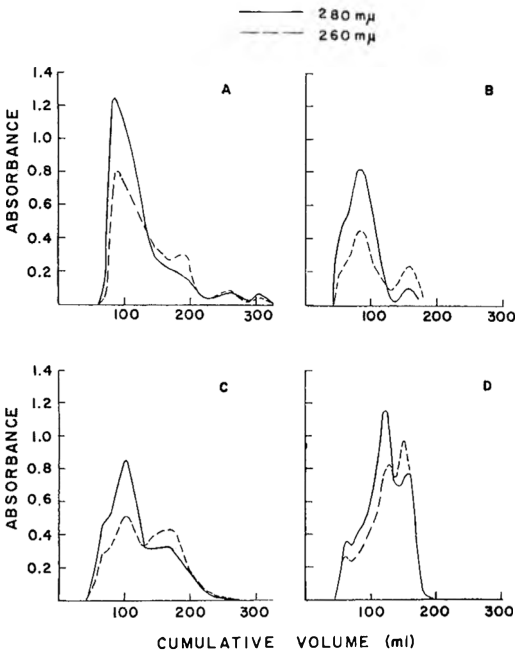


Fig. 3. Gel filtration of SuperKing water extract using different grades of Sephadex. Columns were 2.2 × 30 cm, and 0.01*N* lactic acid was the eluant. Seven ml of extract containing about 65 mg protein were applied to columns. A) G-25; B) G-50; C) G-75; D) G-100.

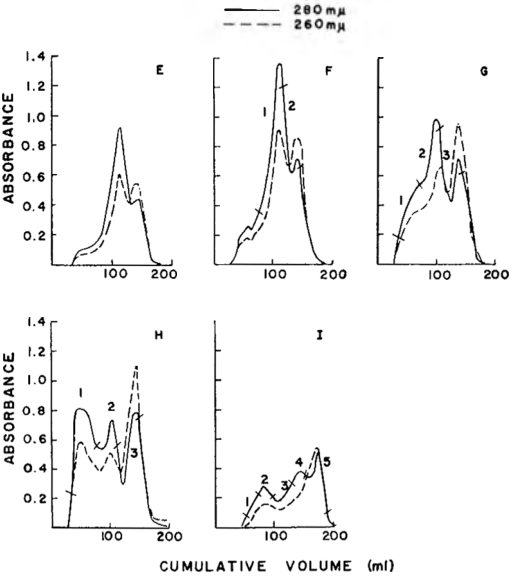


Fig. 4. Gel filtration of SuperKing water extract under various conditions. E) 4 ml of extract (38 mg protein), Sephadex G-100, 0.01*N* lactic acid eluant. F) 7 ml of extract (58 mg protein), Sephadex G-100, 0.01*N* lactic acid eluant. G) 7 ml of extract (69 mg protein), Sephadex G-100, 0.04*N* lactic acid eluant. H) 7 ml of extract (82 mg protein), Sephadex G-100, 0.50*N* lactic acid eluant. I) 5 ml of extract (36 mg protein), Sephadex G-200, 0.50*N* lactic acid eluant.

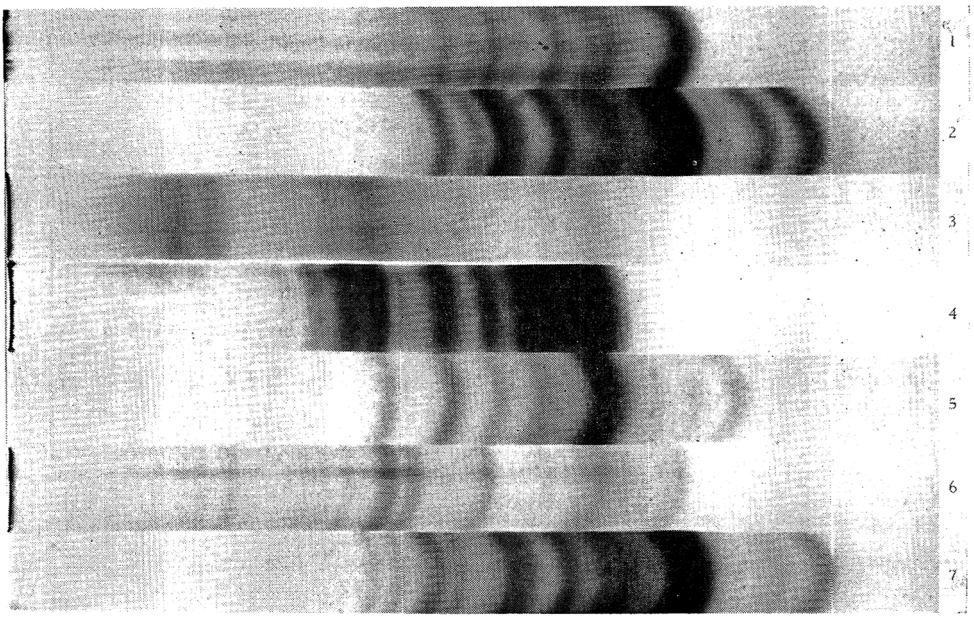


Fig. 5. Starch-gel patterns for fractions from elution curves shown on Fig. 4 for Sephadex G-100. 1) fraction 1, curve F; 2) fraction 2, curve F; 3) fraction 1, curve G; 4) fraction 2, curve G; 5) fraction 3, curve G; 6) fraction 1, curve H; 7) fraction 2, curve H.

in both parts of this peak, but were more concentrated in the rear portion or in the second peak in the case of the 0.50*N* lactic acid elution curve. The third fraction of the latter curve was so low in protein that no electrophoretic pattern could be obtained.

The possibility of obtaining better separations by the use of Sephadex G-200, having a nominal exclusion limit of about 200,000 molecular weight, was explored with 0.50*N* lactic acid used as eluant. The elution curve is shown in Fig. 4, curve I. Starch-gel patterns for the fractions indicated in the curve showed that Sephadex G-200 provided no advantage over G-100.

It was of interest at this point to determine whether the last absorbance peak appearing in the elution diagrams represented protein or some other absorbing species, or both. The high 260-m$\mu$ absorbance for this fraction suggested the presence of nucleic acids. Therefore, protein assays by the Lowry procedure (1951) and carbohydrate assays by the anthrone method (Mokrasch, 1954) were performed on selected fractions from the filtration experiments. The results of a typical assay are shown in Fig. 6 for the filtration experiment in Fig. 4, Curve H. These data show that the first absorbance peaks were, indeed, protein peaks, with only very low levels of carbohydrate present. The third peak, however, with a higher absorbance at 260 m$\mu$ than at 280 m$\mu$, contained very small quantities of protein with relatively high concentrations of carbohydrate.

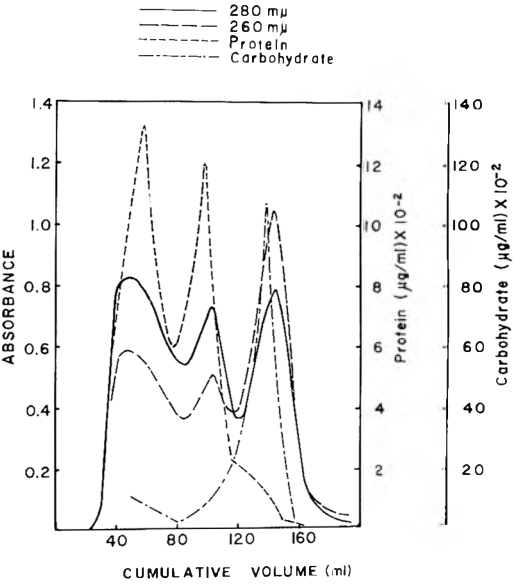


Fig. 6. Comparison of absorbance at 280 and 260 m$\mu$ with protein and carbohydrate content of fractions eluted from G-100, with 0.50*N* lactic acid the eluant.

These data, while not proving the presence of nucleic acid in this peak, strongly suggested that it was there. Since the interest in this study was in the protein portion of the flour extracts, identification of the constituents of this fraction was not pursued further. In later gel filtration experiments, however, the collection of samples was usually discontinued when absorbance at 260 mμ exceeded that at 280 mμ.

Because of the partial success in fractionation achieved with Sephadex G-100 in the 0.50*N* lactic acid system, use of this system was investigated further. To permit examination of pooled fractions representing smaller portions of the elution curve, the large columns were employed in the remaining studies. Typical of the diagrams resulting with this system is the elution diagram in Fig. 7, representing the first experiment with the large column with 0.50*N* lactic acid used as eluant. The eluate was

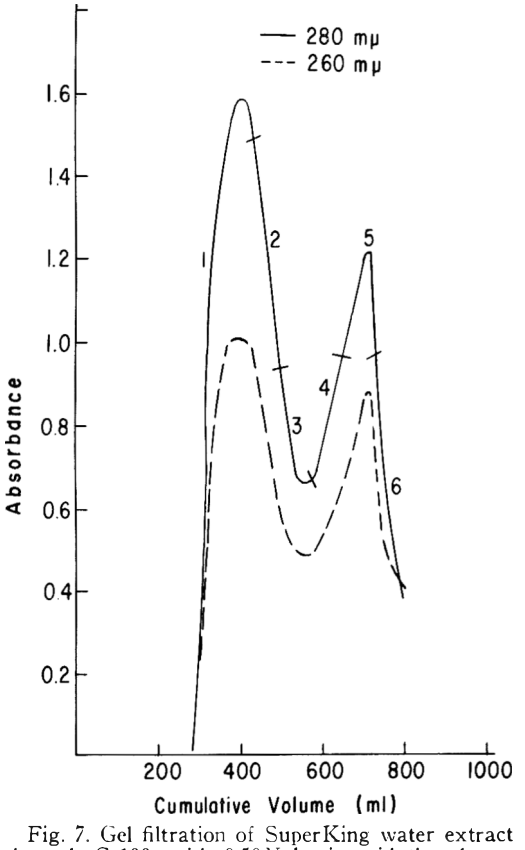


Fig. 7. Gel filtration of SuperKing water extract through G-100, with 0.50*N* lactic acid the eluant. Sixty-six ml of extract containing 605 mg protein were added to 4.2 × 75-cm column.

pooled in six fractions as indicated in the diagram. The electrophoretic analysis of the last five of these fractions is shown in Fig. 8. Fraction 1 was accidentally lost during lyophilization. The gel for fraction 2 shows the presence of glutenin, as well as most of the highly mobile components, while fraction 3 was very similar to fraction 2 except for the presence of a group of slow-moving components and the absence of glutenin. Fractions 4 and 5 were considerably less complex, showing the presence of 7 or 8 bands with faint traces of 1 or 2 others. In fraction 5 a marked concentration of some components occurred, particularly the major component, which showed the second-fastest migration. Fraction 6 contained only 5 prominent components, with 1 weak band among these, and 2 very faint bands which located almost off the gel on the cathode end. The most striking feature of these gels was the demonstration that some of the components were concentrated in the fractions of the second peak, and that the actual physical separation of bands during electrophoresis of these fractions was considerably greater than in the original extract.

**Reproducibility of gel filtration experiments, and the comparison of two flours.** To check the reproducibility of separations shown in the preceding experiment, several additional gel filtration experiments were conducted on water extracts of SuperKing flour. In addition, for comparative purposes, several experiments were performed on Kaw flour extracts. In some of these experiments the procedure was modified. It had been noted that, when crude water extract was used for filtration experiments, there was a pronounced and progressive shrinkage of the gel column as the extract entered and progressed through the gel. This resulted in considerable reduction in flow rate during the experiment. It was found that dialyzing the extract for about 24 hr against 0.50*N* lactic acid before placing it on the column markedly reduced the shrinkage and retardation of flow rate. The dialyzed extract was centrifuged before being placed on the column. About 5% of the protein was lost in the process. The general elution pattern for these dialyzed extracts was very similar

Table 1. Recovery of protein from gel filtration of water extract of flour through Sephadex G-100 using 0.50*N* lactic acid eluant. Protein added to column was 269.0 mg (N × 5.7).

| | Region | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | Total |
| Protein in region (mg) | 13.2 | 36.1 | 53.9 | 49.0 | 47.8 | 34.5 | 17.1 | 251.6 |
| Percent of total protein | 4.9 | 13.4 | 20.0 | 18.2 | 17.8 | 12.8 | 6.4 | 93.5 |

to those for the undialyzed extract, but the first peak was reduced in height, probably the result of loss of protein. Typical elution diagrams for the SuperKing and Kaw dialyzed extracts are presented in Fig. 9. The numbers on these curves indicate the regions usually included in pooled fractions which were subsequently examined by starch-gel electrophoresis. Protein recovery for a typical experiment is shown in Table 1. In this particular experiment, the 6.5% of the total protein (N × 5.7) not recovered in the 7 fractions was accounted for in the material having high absorbance at 260 mμ.

To conduct electrophoretic analysis of a large number of fractions available in limited

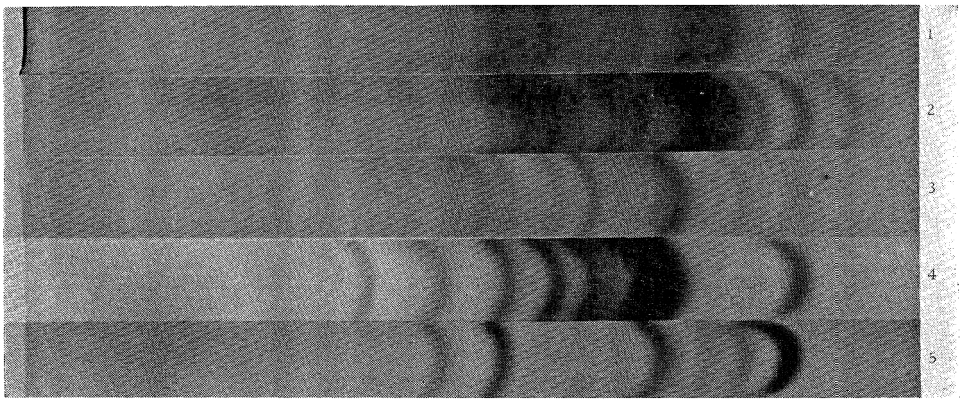


Fig. 8. Starch-gel patterns for fractions from elution curve shown on Fig. 7. 1) fraction 2; 2) fraction 3; 3) fraction 4; 4) fraction 5; 5) fraction 6.

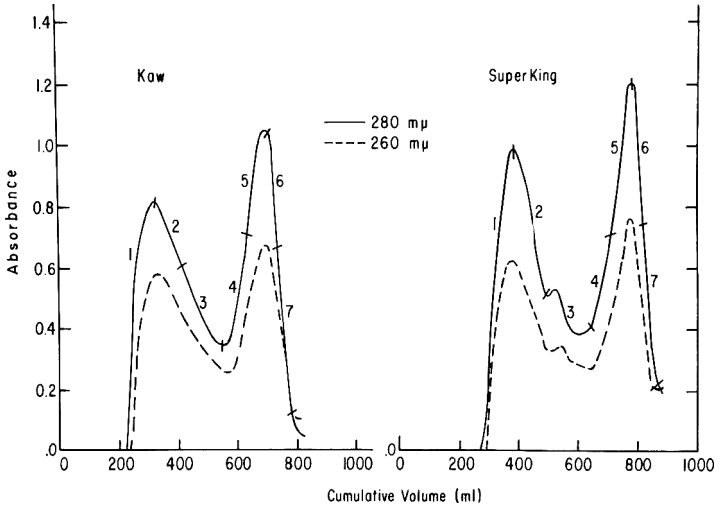


Fig. 9. Sephadex G-100 gel filtration of SuperKing and Kaw water extracts previously dialyzed against 0.50*N* lactic acid. For SuperKing, 75 ml of dialyzed extract containing 367 mg protein were added to a 4.2 × 75-cm column, while for Kaw, 57 ml containing 381 mg protein were used. The eluant was 0.50*N* lactic acid.

quantity, the electrophoresis procedure was modified to utilize starch-gels 1/16 in. thick and 7½ in. wide. By using sample wicks ¼ in. wide spaced at ¼-in. intervals, up to 18 samples could be analyzed simultaneously. Good starch-gel patterns were obtained using 0.2–0.4 mg of protein in 10–15 μl of buffer, about 1/10 the quantities required for the larger trays.

Fig. 10 shows the starch-gel patterns obtained for the last six fractions of three separate gel filtration experiments with water extracts of SuperKing and Kaw flours. Fraction one was omitted from these comparisons since previous starch-gel analysis of this fraction showed little resolution. It was apparent that there was a general similarity among the patterns for a given fraction when compared for different experiments with the same flour or with different flours. The variation from one experiment to another for a given flour was probably due chiefly to differences in the actual pooled tubes going into a given fraction, since the elution patterns were not identical. These starch-gel patterns showed that fractions 2 and 3 contained mainly slow-moving components, with fast-moving components absent or very weak. The patterns were generally diffuse. Fractions 4 and 5 both contained 10 components. Of these, 1 was very strong, 5 were strong, and 4 weak. The electrophoretic resolution of fraction 5 was superior to that of fraction 4. Fraction 6 contained six strong components corresponding in mobility and concentration with those in fractions 4 and 5. Slow-moving components were absent or very weak in fraction 6, but four weak components of high mobility were present. Fraction 7 contained five well-resolved components of intermediate mobility and four weak components having high mobility. These components corresponded with those present in fraction 6, but one of the major components present in fraction 6 was absent from fraction 7.

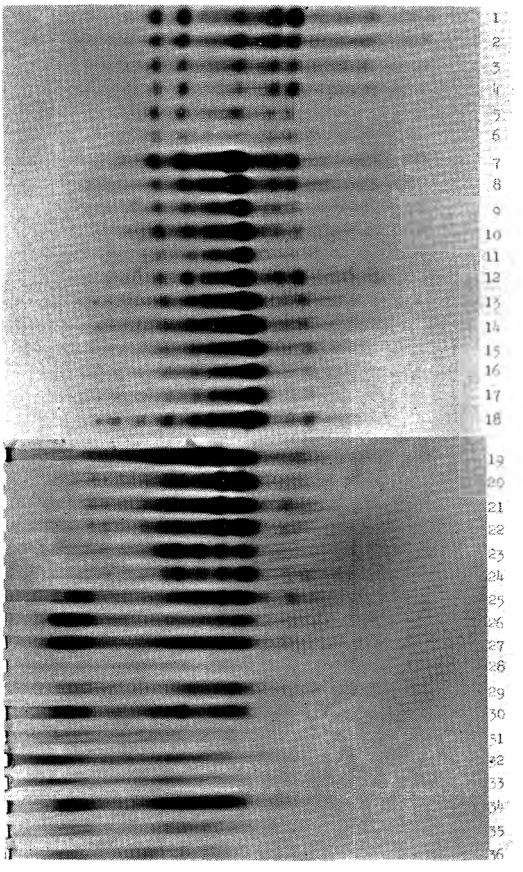

Fig. 10. Starch-gel patterns for fractions from replicated G-100 gel filtration experiments with water extracts of SuperKing and Kaw flours. Extracts were dialyzed against 0.50*N* lactic acid before gel filtration, and the eluant was 0.50*N* lactic acid. 1–3, fraction 7, Kaw; 4–6, fraction 7, SuperKing; 7–9, fraction 6, Kaw; 10–12, fraction 6, SuperKing; 13–15, fraction 5, Kaw; 16–18, fraction 5, SuperKing; 19–21, fraction 4, Kaw; 22–24, fraction 4, SuperKing; 25–27, fraction 3, Kaw; 28–30, fraction 3, SuperKing; 31–33, fraction 2, Kaw; 34–36, fraction 2, SuperKing.

**Isolation of a single component from a fraction obtained by gel filtration.** While gel filtration of flour extracts on Sephadex G-100 did not resolve the mixture into fractions containing single components under the conditions employed, a considerable simplification of the mixture and a concentration of some components in selected fractions was effected. These results, together with the high degree of resolution of such fractions by starch-gel electrophoresis, suggested the possibility of employing a combination of gel filtration and starch-gel electrophoresis for the isolation of single components.

About 70 mg of fraction 5 from the gel filtration experiment shown in Fig. 7 were available. Since this fraction exhibited a rather high concentration of one component which was well separated from other components on starch gel, an attempt was made

to isolate this material. A starch gel, ¼ × 7½ × 11½ in., was poured on a glass plate, and 70 mg of fraction 5 dissolved in 0.7 ml of aluminum lactate-urea buffer was applied near one end of the gel by insertion of a ¼ × 7½-in. wick impregnated with the solution. Electrophoresis was performed for about 13 hr at approximately 5 v/cm. Strips 1/16 in. wide were then cut lengthwise from the gel at about 1-inch intervals. These were stained and reassembled in the same relative positions they had occupied in the gel, and a template was marked to correspond with the position of the component to be isolated. The main gel, which had been held in the freezer, was then thawed, and, using the template, a band of gel was cut out corresponding to the position of the desired component. The gel was centrifuged at low speed in tubes fitted with fritted-glass discs about an inch above the bottom of the tube. Most of the liquid was forced from the gel by the centrifugation and was collected below the glass disk. The filtrates were combined, dialyzed to remove urea and lactic acid, and lyophilized. There appeared to be an appreciable quantity of insoluble starch-degradation products in the lyophilized final product, as shown when a portion of it was dissolved for re-electrophoresis. Therefore, the dried material was extracted with water and lyophilized again. The process was repeated a second time. The final product was not pure protein, but was probably contaminated with some carbohydrate and possibly with aluminum lactate, which is very difficult to remove by dialysis. The electrophoretic homogeneity of this isolated product with regard to its protein components was determined by electrophoresis in the same system as used for its isolation. A sample of the fraction from which it had been isolated was subjected to electrophoresis on the same gel for comparative purposes. The resulting starch-gel patterns are shown in Fig. 11. The protein in the isolated sample migrated as a single component in this system. This, although not conclusive, was strong evidence that this was one molecular species, because of the high resolution possible with starch-gel electrophoresis.

The results of these experiments indicated that the combination of gel filtration with other methods of fractionation may provide a suitable means of isolation of several of the individual protein components present in the soluble fraction of flour. Preliminary simplification of the mixture by gel filtration was of major assistance to the final isolation by other means. Since the components present in fractions 6 and 7 also occurred together in other fractions from the column, these components were probably of about the same molecular weight. Their electrophoretic behavior, however, indicated that they differed appreciably in their ionic character. It might be expected, therefore, that ion-exchange chromatography of such fractions would result in isolation of individual components rather than the mixture obtained when crude extracts were employed (Nimmo *et al.*, 1963). With fractions containing only 5

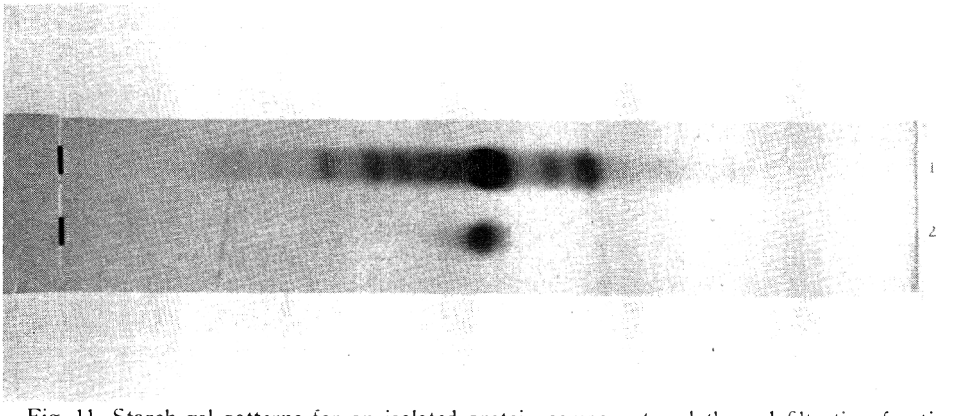


Fig. 11. Starch-gel patterns for an isolated protein component and the gel filtration fraction from which it was isolated. 1) Fraction 5 from elution curve shown on Fig. 8; 2) protein component isolated from fraction 5.

or 6 components it may be possible to achieve a preparatory separation by column electrophoresis, while this would be virtually impossible with the crude, unfractionated extract. Further work will be required to evaluate these possibilities.

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A joint contribution: No. 1213. Department of Biochemistry, Oklahoma Agricultural Experiment Station, Stillwater, Oklahoma and No. 524, Department of Flour and Feed Milling Industries, Kansas Agricultural Experiment Station, Manhattan, Kansas. Part of a Ph.D. thesis by D. C. Abbott, Kansas State University.

G. LAMBERTSEN, H. MYKLESTAD, and O. R. BRAEKKAN
*Government Vitamin Laboratory,*
*Norwegian Fisheries Research Institute*
*Bergen, Norway*

# The Fatty Acid Composition of Commercially Hydrogenated Fish Oils

## SUMMARY

**Five samples of industrially hydrogenated fats of marine origin were analyzed for fatty acid composition by the combined methods of group-separation by thin-layer partition chromatography followed by gas-liquid chromatography of each group.**

**The general fatty acid composition was 30–45% saturated acids, 45–55% monoenoic acids, and 10–20% polyenoic acids. A substantial part of the polyenoic acids were trienoic acids, the rest dienoic. The presence of tetraenoic acids could be ascertained in the low-melting samples (mp 30–32°C).**

## INTRODUCTION

The bulk of the world production of marine oils is hydrogenated for human consumption as margarine and shortening. Since the marine oils are characterized by a high content of polyenoic acids with 4–6 double bonds, extensive hydrogenation is necessary. The process results in a fat of specific, desired melting point, usually in the range of 30–45°C. The ultimate melting point is a function not only of the degree of saturation, but also of the degree of trans-isomerization during the process. An extensive hydrogenation may therefore lead to a fat with a melting point approaching that of lard but still containing substantial amounts of polyenoic acids.

The literature on this problem is concerned almost entirely with hydrogenated vegetable oils. The literature on hydrogenated marine oils seems limited. Pedersen (1938) studied partly hydrogenated whale oils by iodine values, thiocyanogen values, bromine absorption, etc., and stated that polyenoic acids of up to four double-bonds were present. Schilling (1959) has discussed problems of determination of fatty acids in hydrogenated marine oils. Pokorny *et al.* (1963) determined the fatty acid composition of hydrogenated vegetable and marine oils and in margarines. Their results, based on gas-liquid chromatography, show a complicated pattern including several polyenoic acids.

Analytical methods available for the determination of fatty acid composition are all of limited value when applied to hydrogenated fats. Particular problems result from the extensive isomerization which takes place during catalytic hydrogenation. These isomerization reactions have been studied in detail by Dutton (1963) and his group. Besides the formation of trans-isomers there is also found a complicated mixture of positional isomers. An example of this is given by Scholfield *et al.* (1963) for hydrogenated methyl linolenate. The polyenoic acids include isomers containing conjugated double bonds and isomers with more than one methylene group between the double bonds. The technique of alkaline isomerization followed by spectroscopy thus fails to give a correct picture of the content of polyenoic acids in hydrogenated fats.

Methods of gas-liquid chromatography presently dominate fatty acid analysis. Unfortunately, positional isomers show small differences in retention times and cannot be satisfactorily separated by direct gas-liquid chromatography (Scholfield *et al.*, 1961). Two closely related acids, i.e. $C_{20}$ with 2 and 3 double bonds, have each broad bands of elution which overlap and cannot be quantitated (Fig. 1).

The partition chromatographic methods described by Kaufmann (1958) will separate fatty acids into groups of "critical partners." The principle can be applied to thin-layer chromatography of methyl esters of fatty acids, thus giving separation into groups which can be directly resolved by GLC. The group separation depends upon the major differences in the over-all unsaturation and the molecular size, and is not influenced by the presence of isomers. The principles are illustrated by Fig. 1 and 2.

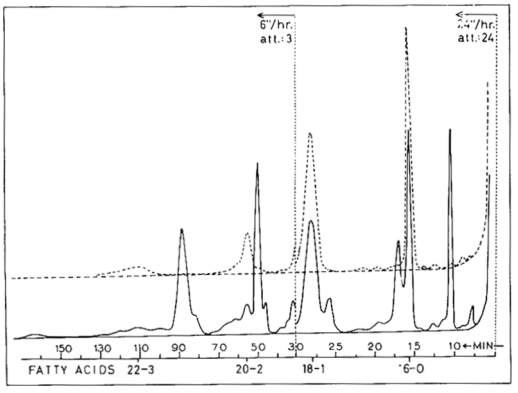


Fig. 1. Gas-chromatogram of the methyl esters from a hydrogenated fat (mp 30–32°C), full line, and of fraction 4 from thin-layer chromatography of these esters, broken line 15% BDS; column, 190°C.

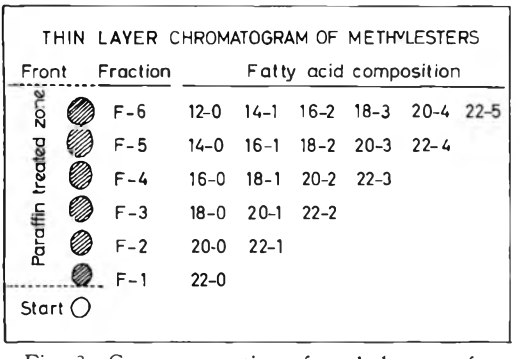


Fig. 2. Group separation of methyl esters from hydrogenated fats by thin-layer chromatography. System: paraffin/acetonitril-isopropanol-water 80:15:5.

## METHODS

**Sampling and preparation of methyl esters.** Samples of hydrogenated fats of marine origin were received from three different Norwegian refineries. Two samples had specified melting points of 30–32°C, and three had 31–33, 36, and 38–40°C. Five-gram portions were saponified for 30 min with 40 ml methanol (aldehyde free) + 5 ml 60% (w/w) KOH in the presence of pyrogallol and ascorbic acid for protection. The methanol was quickly removed under vacuum, thereby cooling the mixture. The saponification mixture was transferred to a separating funnel with 100 ml ethyl ether (peroxide-free) and 100 ml distilled water. The unsaponifiable matter was extracted into the ethyl ether, and the extract washed with 30 ml distilled water to which was added 1 ml KOH solution. The water phase was added to the soap solution, and the ether phase discarded. The fatty acids were set free by 50 ml of 15% (v/v) HCl and extracted three times with 50 ml ethyl ether. The combined extracts were washed four times with 50 ml distilled water. The ethyl ether was evaporated in vacuum at approx. 40°C. Twenty ml of 12% (v/v) borontrifluoride (Fluka) in methanol was added quickly, and the solution boiled for 5 min. The methyl esters were transferred to a separating funnel with 25 ml distilled water and extracted three times with 25 ml ethyl ether. The combined extracts were washed four times with 25 ml distilled water. The ethyl ether was evaporated at low temperature in vacuum. The methyl esters were dissolved in a few ml of hexane (bp 67–72°C). Oxydation products were removed by passing the solution through a column (ID 12 mm) of soft alumina (40 g $Al_2O_3$ Brockmann + 10% water). The methyl esters were eluted with 50 ml of hexane, which was removed in vacuum at low temperature. The esters were dissolved in ten times their weight of methylhexanoate, and stored in the freezer.

**Hydrogenation.** To 3 ml of the hexanoate solution of the methyl esters was added a pinch of palladium on activated carbon (Fluka) as a catalyst. The mixture was shaken in a small flask for 3–4 hr under approx. 20 psi of hydrogen, and then filtered.

**Preparative thin-layer chromatography.** Sets of nine glass plates (20 × 10 cm) were prepared with 5 g Kieselguhr G (Merck) in 15 ml distilled water. The plates were dried for 3–4 hr at 120°C. The plates were impregnated by allowing a solution of 10% paraffin (bp 180–200°C) dissolved in pentane (bp 45–50°C) to ascend to within 2.5 cm of the top edge. The pentane was evaporated by drying in air. On each plate was applied 7–8 spots each of 5 $\mu$l of the methyl ester solution on the unimpregnated strip. The elution was carried out with a mixture of acetonitrile (Merck)–isopropanol–water (80:15:5). The mixture was saturated with paraffin prior to use. Normal elution time was 45 min. The plates were dried under nitrogen at room temperature, and developed by spraying with a solution of 0.001% of sodium fluoresceinate in water. The spots were plotted by UV-light (360 m$\mu$). The rows of spots were carefully scraped off the plates and transferred to centrifuge tubes containing 20 ml hexane and 10 ml distilled water. The tubes were shaken, centrifuged, and frozen. The hexane phases were removed, concentrated in vacuum, and transferred to small tubes with conical bottoms. The rest of the hexane was removed by a current of nitrogen, and the methyl esters were left in the accompanying paraffin solution.

**Gas-liquid chromatography.** The fractions were chromatographed in a PYE-argon chromatograph with $Sr^{90}$ ionization detector. The columns were 120 cm long, with an internal diameter of 4 mm.

They were provided with a flash heater (230°C). The stationary phases were 10% of SE-30 silicone gum on silanized Kieselguhr (80–100-mesh), and 15% of butanediol succinate polyester on the same adsorbent. The first column was run at 205°C, the second at 190°C, both with a gas velocity of 40 ml per min. The hydrogenated fractions were chromatographed on the silicone column, whereas the original methyl esters and the fractions from the thin-layer chromatography were chromatographed on the polyester column. Each analysis consisted of three parallel runs.

**Calculation of results.** Each peak was quantitated by calculation of the area from the formula height × width at half height. The polyenoic acids of groups $C_{20}$ and $C_{22}$ were generally of a shape corresponding to two overlapping peaks. This was probably caused by the presence of several positional isomers. Such composite peaks were calculated as one.

Chromatograms from the fully hydrogenated fraction were used for calculation of chain-length percentages and the percentages of odd-numbered and branched fatty acids. The sum was calculated to 99.5%, thus giving 0.5% as the sum of uncalculated peaks.

From each of the six fractions (Fig. 2) obtained by thin-layer chromatography, a major fatty acid peak was chosen as representing the fraction. In the present investigation these fatty acids were: $C_{29}$:0 for fraction 1; $C_{22}$:1 for fraction 2; $C_{20}$:1 for fraction 3; $C_{18}$:1 for fraction 4; $C_{14}$:0 for fraction 5; $C_{14}$:1 for fraction 6.

These six peaks were quantitated on the chromatograms of the original methyl esters to obtain relative values between the thin-layer fractions. The peaks from the chromatograms of each of the thin-layer fractions could then be calculated on the basis of their representative fatty acid. All areas summed up according to the relations found in the chromatogram of the original methyl esters.

Lastly, the acids from each chain length were summed up and corrected according to the values found in the chromatograms of the fully hydrogenated fraction.

Table 1. Fatty acid composition of hydrogenated oils of marine origin. Complete list of fatty acids recorded and calculated (percentages).

| Fatty acid designation | Samples A-1 30—32° | A-2 31—33° | A-3 30—32° | B 38—40° | C 36° |
|---|---|---|---|---|---|
| 14:0 | 7.8 | 7.8 | 9.7 | 8.0 | 7.9 |
| 14:1 | 0.2 | 0.5 | 0.2 | 0.4 | 0.4 |
| 15:br | 0.6 | 0.3 | 0.4 | 0.7 | 0.4 |
| 15:0+1 | 0.6 | 0.3 | 0.4 | 0.6 | 0.4 |
| 16:br | — | — | — | 0.1 | 0.1 |
| 16:0 | 12.6 | 13.8 | 18.4 | 12.4 | 17.7 |
| 16:1 | 6.8 | 8.5 | 11.0 | 8.7 | 6.2 |
| 16:2 | 0.8 | 1.5 | 0.5 | 1.1 | 0.2 |
| 17:brI | 0.4 | 0.1 | 0.1 | 0.4 | 0.2 |
| 17:brII | 0.4 | 0.2 | 0.1 | 0.4 | 0.2 |
| 17:0+1 | 0.9 | 0.4 | 0.4 | 0.7 | 0.5 |
| 18:br | — | 0.3 | 0.1 | 0.3 | 0.3 |
| 18:0 | 3.5 | 3.8 | 3.2 | 6.8 | 7.8 |
| 18:1 | 19.9 | 15.5 | 17.0 | 14.8 | 15.3 |
| 18:2 | 2.0 | 2.5 | 2.5 | 1.5 | 1.2 |
| 18:3 | 0.1 | 0.6 | 0.2 | 0.1 | — |
| 19:0+1 | 0.4 | 0.4 | 0.2 | 0.3 | 0.5 |
| 20:0 | 1.2 | 1.9 | 0.8 | 3.4 | 3.9 |
| 20:1 | 10.5 | 11.4 | 9.8 | 10.3 | 10.3 |
| 20:2 | 4.0 | 4.2 | 4.4 | 4.4 | 3.5 |
| 20:3 | 4.5 | 3.2 | 4.5 | 2.3 | 1.6 |
| 20:4 | 0.1 | 0.3 | 0.2 | — | — |
| 21:0+1 | 0.4 | 0.6 | 0.2 | 0.5 | 0.6 |
| 22:0 | 1.1 | 2.0 | 1.0 | 3.9 | 4.1 |
| 22:1 | 13.5 | 13.6 | 8.0 | 11.9 | 10.7 |
| 22:2 | 1.6 | 2.8 | 1.7 | 2.9 | 2.8 |
| 22:3 | 4.3 | 2.2 | 2.7 | 1.8 | 1.9 |
| 22:4 | 0.8 | — | 1.1 | — | — |
| 24+ | 0.5 | 0.7 | 0.6 | 0.8 | 0.8 |
| Others[a] | 0.5 | 0.6 | 0.6 | 0.5 | 0.5 |
| Totals | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |

[a] By difference.

## RESULTS AND DISCUSSION

The results of the analyses are reported in Tables 1 and 2. The methods described for the preparation of methyl esters gave recoveries above 95% by weight. The methylation by boron trifluoride was performed mainly as described by Metcalfe and Schmitz (1961). The thin-layer chromatography was adopted from Mangold (1962). The principle of calculation of gas-liquid chromatograms on the basis on fully hydrogenated samples is generally adopted, and has been used by Ackman and Burgher (1963) and Gruger *et al.* (1964). The areas are summarized as weight-percentages, and this has been controlled by calculation and determination of the mean molecular weight of the fully hydrogenated methyl esters. The results agreed within ±2.5%. Identification of the fatty acid peaks was carried out by the principles discussed by Ackman (1963), and by Iverson *et al.* (1965). The different principles of analyses used in the present work are discussed in detail by Lambertsen (1965).

Table 2. Fatty acid composition of hydrogenated oils of marine origin. Summation of groups according to chain lengths and unsaturation.

| | Samples | | | | | |
|---|---|---|---|---|---|---|
| | A-1 30—32° | A-2 31—33° | A-3 30—32° | B 38—40° | C 36° | Av. |
| $C_{14}$ | 8.0 | 8.3 | 9.9 | 8.4 | 8.3 | 8.58 |
| $C_{16}$ | 20.2 | 23.8 | 29.9 | 22.2 | 24.1 | 24.04 |
| $C_{18}$ | 25.5 | 22.4 | 22.9 | 23.2 | 24.3 | 23.66 |
| $C_{20}$ | 20.3 | 21.0 | 19.7 | 20.4 | 19.3 | 20.14 |
| $C_{22}$ | 21.3 | 20.6 | 14.5 | 20.5 | 19.5 | 19.28 |
| $C_{24}$ | 0.5 | 0.7 | 0.6 | 0.8 | 0.8 | 0.68 |
| Others | 4.2 | 3.2 | 2.5 | 4.5 | 3.7 | 3.62 |
| Totals | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| Saturated | 29.1 | 31.5 | 34.9 | 38.0 | 44.1 | |
| Monoenoic | 52.7 | 51.2 | 47.3 | 47.9 | 44.7 | |
| Polyenoic | | | | | | |
| Dienoic | 8.4 | 11.0 | 9.1 | 9.9 | 7.7 | |
| Trienoic | 8.9 | 6.0 | 7.4 | 4.2 | 3.5 | |
| Tetraenoic | 0.9 | 0.3 | 1.3 | — | — | |
| Totals | 18.2 | 17.3 | 17.8 | 14.1 | 11.2 | |
| Iodine value (Wijs) | 83.9 | 73.6 | 87.6 | 66.0 | 63.6 | |

Tables 1 and 2 show that the general fatty acid composition as well as the iodine values classify these hydrogenated oils as rather similar in unsaturation to many vegetable oils (palm oil: 40% sat., 50% oleic, 10% linoleic I.V. 65; olive oil: 20% sat., 70% oleic, 10% linoleic I.V. 80). This can be seen in relation to the experiments on the effect of different oils on blood cholesterol levels. Hydrogenated rats fall in the "neutral" group in this connection (Nicolaysen and Ragård, 1961). The Tables give all the details with regard to the distribution of the different fatty acids, but certain relations may be emphasized.

The monoenoic acids constitute about half of the total fatty acids in the present samples. The same concentration is normally present in the corresponding nonhydrogenated marine oils. Evidently hydrogenation from polyenoic to monoenoic acids balances the hydrogenation from monoenoic to saturated acids. Somewhat higher values for $C_{22}:1$ and somewhat lower values for $C_{16}:1$ characterize the hydrogenated oils as compared with the raw oils (Lambertsen, 1965).

The saturated acids increase during hydrogenation and constitute 30–45% of the total fatty acids. The main increase falls on $C_{16}:0$. It is of particular interest to note that the $C_{20}:0$ and the $C_{22}:0$ are present in relatively small concentrations. Only in oils hydrogenated to higher melting points is a notable increase of these acids observed to about 4%.

The major polyenoic acids of marine oils are $C_{18}:4$, $C_{20}:5$, $C_{22}:5$, and $C_{22}:6$ (Gruger *et al.*, 1964). These acids have all completely disappeared during the hydrogenation process. Up to 1.3% of the tetraenes could be found in the low-melting samples, and none in the higher-melting samples. Trienes, however, are important constituents of these oils. They were found in concentrations from 3.5 to 8.9% in the samples. These acids are partly masked by the dienes in methods applied for the analysis of fatty acid composition because of the many positional isomers after hydrogenation. They can be clearly identified by thin-layer partition chromatography, as in the present study. The percentage of trienes is notably lower in the high-melting fats than in the low-melting ones, respectively 3.9 and 7.4%. The dienes are present in total quantities from 7.7 to 11.0%. These acids show no relation to the degree of hydrogenation, the

average value being 9.5% for low-melting fats and 8.8% for high-melting fats.

Generally, the polyenoic acid may be considered an important part of hydrogenated marine oils, with values ranging from 11.2 to 18.2 in the present samples.

Finally may be mentioned that the gas chromatographic methods have firmly established the presence of many acids of odd-numbered and branched carbon chain. Such acids are particularly prominent in marine oils, many in quantities of the order from 0.5 to 1% of the total fatty acids. The group of $C_{17}$-acids must be given particular attention in analyses of marine oils. Small quantities of $C_{12}$ and lower acids may be present, but the methods applied in the present study did not record these short-chain compounds.

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Ms. rec'd 5/25/65.

This study was made possible by a grant from the Research Fund of Norwegian Margarine Producers.

S. R. TANNENBAUM
*Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

# Protein Carbonyl Browning Systems: A Study of the Reaction Between Glucose and Insulin

## SUMMARY

**The interaction between glucose and insulin in the dry state was studied at a relatively low glucose concentration with carbon-14-labeled glucose. The conditions of the investigation were that the equilibrium relative humidity was 0.74, pH range 4–7, and temperature range 35–55°C. Under these conditions, the rate of the reaction decreased with increasing pH and had an activation energy of approximately 2.7 Kcal/mole. Reasons are given for believing that the reaction may be the formation of a Schiff base.**

## INTRODUCTION

The literature on nonenzymatic browning is quite extensive and is not reviewed here since the general chemistry of Maillard-type of browning has recently been summarized (Reynolds, 1963). The reactions between aldoses and proteins in the "dry" state were intensively investigated over a decade ago by Lea and co-workers (Hannan and Lea, 1952; Lea and Hannan, 1949, 1950; Lea and Rhodes, 1952; and Lea *et al.*, 1951) with respect to the effect of moisture content and pH on the rate of loss of free amino-groups on the protein, and by Haugaard and Tumerman (1956) on the rate of reaction of amino groups in proteins in solution. The reaction between insulin and glucose has been studied (Schwartz and Lea, 1952) with particular reference to the relative reactivity of the different amino groups in insulin.

Each of these studies was performed under conditions in which the aldose was in molar excess with respect to the reactive sites on the protein. The method of measurement of the rate constants was necessarily indirect, since the unreacted amino groups were determined either by Van Slyke analysis or through the use of fluoridinitrobenzene. With these methods, the possibility exists that the more labile derivatives of amino groups formed during the browning reaction might be liberated by the treatment in the analysis.

This paper introduces a new approach to the general problem of the reaction of aldoses and proteins in which it is feasible to study the reaction at very low aldose concentrations with respect to the reactive groups on the protein, a situation which is of importance with respect to many dehydrated protein foods. As will be seen, it was also possible to isolate from the complex browning reaction pathway the very earliest stages involved in the binding of glucose to insulin.

## EXPERIMENTAL

**Materials.** Crystalline bovine pancreas zinc insulin (3.9 mg Zn/g protein) was obtained from Calbiochem, Los Angeles, California.

Glucose-2-$C^{14}$, specific activity 2.05 $\mu C/\mu M$, was obtained from New England Nuclear Corp., Boston, Mass., and was chromatographically pure.

**Preparation of system.** Insulin and glucose were mixed in the appropriate buffer (0.05$M$ acetate) to give 9.4$M$ insulin/$M$ glucose, or approximately 28 Eq. insulin amino groups/Eq. glucose. The system was frozen in liquid nitrogen and freeze-dried to constant weight. This system could be stored without any detectable changes for at least one month at 4°C.

**Method of procedure.** Approximately 2 mg of the insulin-glucose system was weighed into a 2-ml centrifuge tube under anhydrous conditions. The centrifuge tube was then placed in a large, sealed test tube containing saturated sodium chloride (relative humidity 74%) and incubated at the appropriate temperature. At the end of a given reaction time, the centrifuge tube was removed, 0.5 ml of cold 5% trichloroacetic acid (TCA) was mixed into the insulin-glucose preparation, and the mixture was immediately centrifuged. An aliquot of the supernatant was then taken for counting of radioactivity in a liquid scintillation counter (Packard Instrument Co., La Grange, Ill.). The percentage of unreacted glucose was then plotted versus time, and the rate constant determined from the slope of the rate curve. No visible browning was detectable in any of the samples.

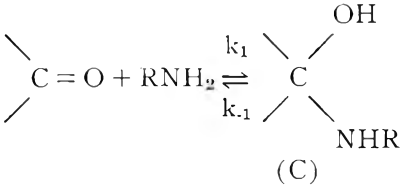
**Measurement of pH.** As far as is known, there is no method for accurately determining the hydrogen ion activity of a relatively dry system (17% water for insulin at an equilibrium relative humidity

of 0.74). Therefore, as an approximation of the hydrogen ion activity of the system, the insulin-glucose preparation was wetted with the minimum quantity of distilled water necessary to obtain a stable reading on a pH meter equipped with micro glass-calomel electrodes (*ca.* 20 $\mu$l $H_2O$/mg insulin-glucose).

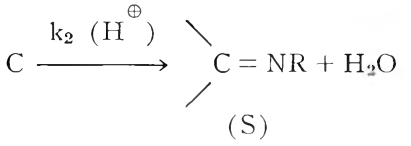
**Chromatography of the 5% TCA supernatant.** The TCA supernatant was spotted onto a glass plate coated with a 300-$\mu$ layer of cellulose powder CC41 (Scientifica, Clifton, New Jersey) and chromatographed to a distance of 10 cm with *n*-propanol-water (2:1). The cellulose powder was then subdivided into 1-cm sections, the material scraped off into liquid scintillation counting vials, and the radioactivity determined as previously described.

## RESULTS AND DISCUSSION

The following reactions describe the overall formation of Schiff base from a carbonyl compound and an amine (Cordes and Jencks, 1962; Jencks, 1964):



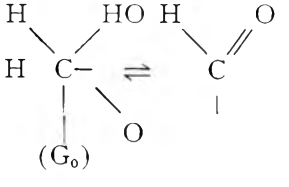

[1]




[2]

The same authors have shown that a pH-rate maximum occurs for the overall formation of Schiff base (S) because of the rate-limiting attack of amine on the carbonyl compound at acid pH, and the rate-limiting rehydration of carbinolamine (C) near neutral or alkaline pH. For the reaction of glucose with an amino acid or an amino group on a protein we must also consider equilibria 3 and 4 since the attacking species is the free amine, and only the open-chain form of glucose enters into the reaction.


[3]




[4]

Under the conditions employed for studying the reaction of glucose with insulin, the insulin is present in large excess with respect to glucose, and the pH of the system is in a region where dehydration of the carbinolamine (reaction 2) may be expected to be rate-limiting. That this condition exists in the system studied is shown by the agreement of the experimental data with the theoretical curve for the rate of the dehydration reaction as a function of pH (Fig. 1).

It is also of interest that the data follow the rate curve for an amine of pK 6.9, since this agrees within experimental error with the pK of N-terminal phenylalanine, which Schwartz and Lea (1952) found to be the most reactive site in insulin. Although the formation of a Schiff base on the protein may be the rate-limiting reaction in this system, it is considered more likely that the structure of the isolated reaction product would be the cyclic glycosylamine or even the Amadori product (Reynolds, 1963). The mild conditions and short time required for the extraction of unreacted glucose would not be expected to hydrolyze any of these structures, since Schiff base hydrolysis is more generally promoted by base catalysis (Jencks, 1964). This indication of the formation of a Schiff base is, however, largely circumstantial, and proof of structure via additional physical or chemical evidence is required.

If, in addition, we consider that the rate of mutarotation of glucose (Bronsted and Guggenheim, 1927) is much greater than that of the dehydration reaction, we can assume a

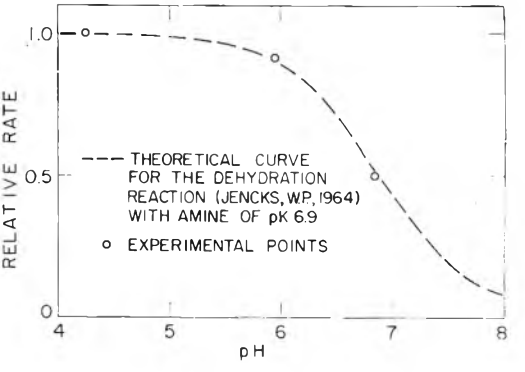



Fig. 1. Effect of pH on the relative rate of the insulin-glucose reaction.

high steady-state concentration of carbinolamine (C) during the reaction. Since the method for recovery of unreacted glucose from the system involves conditions under which the very labile carbinolamine would be rapidly hydrolyzed (reverse of reaction 1), we can further assume that the observed velocity of the reaction would be related to the disappearance of glucose. This assumption is partially verified by the observation that 100% of the glucose can be recovered from the system directly after freeze-drying or after short periods of storage at moderate temperature and zero moisture content. The observed rate of the reaction would be:

$$dS/dt = k_2 [C\text{-}S] [H^{(+)}] = k' [G_o\text{-}S] \quad [5]$$

Since S undergoes further reactions (Amadori Rearrangement, Hodge, 1955) and the rate constants are determined from the initial velocity of the reaction, the concentration of S is initially small with respect to C, and the reverse of reaction 2 need not be considered. Thus, the final form of the rate expression would be:

$$v = k' [G_o\text{-}S] = k' [G_t] = -\frac{dG}{dt} \quad [6]$$

which, when integrated, would be

$$-\ln G_t/G_o = k't \quad [7]$$

where $G_t$ is the concentration of unreacted glucose at any time $t$, and $k'$ is the observed pseudo-first-order rate constant for the reaction. The experimental verification of this equation is given in Fig. 2.

A possible source of error in the method of measurement of $G_t$ would be the release of glucose from the insulin as 3-deoxyglucosone or similar compounds (Anet, 1960). Thin-layer chromatrography of the TCA extract of a glucose-insulin system which had been incubated 3 hr at 50°C indicates that at that time 8.5% of the radioactivity could not be accounted for as glucose (Fig. 3). Since the identity of this material is not known, no estimate of the error in the method can be attempted at this time, but further investigation is warranted.

The rate of reaction between insulin and glucose as a function of temperature is shown in Fig. 4.

The activation energy for the reaction is

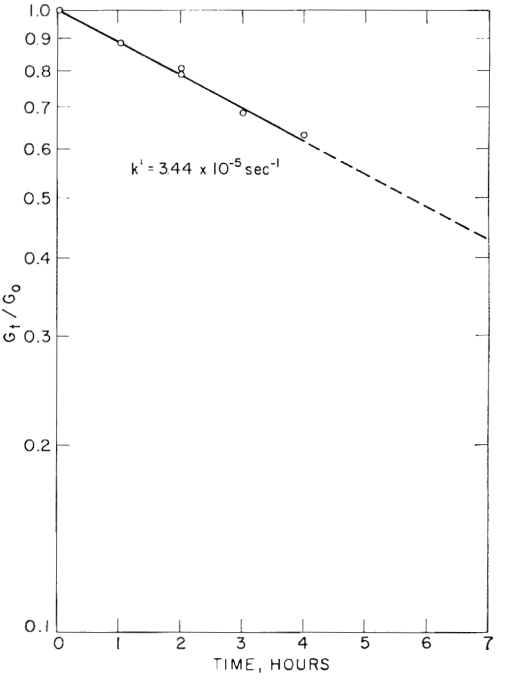



Fig. 2. The rate of reaction of glucose with insulin at 48°C, pH 5.95, and a water activity of 0.74.

2.7 Kcal/mole. This is much lower than might have been expected, considering that previously reported values for the browning reaction overall, and for the Amadori rearrangement, are one order of magnitude above the presently reported value (Reynolds, 1963). An activation energy of 2–3 Kcal/mole is typical, however, of reactions leading to the production of Schiff base, such as in semicarbazone formation (Price and Hammett, 1941).

The relatively low value of the activation energy for the initial interaction product of glucose and insulin may be of technological significance, since the formation of this intermediate predisposes the system to browning. The fact that the reaction proceeds at a significant rate at ambient temperatures indicates that the condition of storage of a processed material would be of greater importance than the conditions of processing. The magnitude of the activation energy would indicate that in processes involving the application of heat to a food material, the use of higher temperatures for shorter times would be more effective than lower temperatures for longer times. Since the predictable

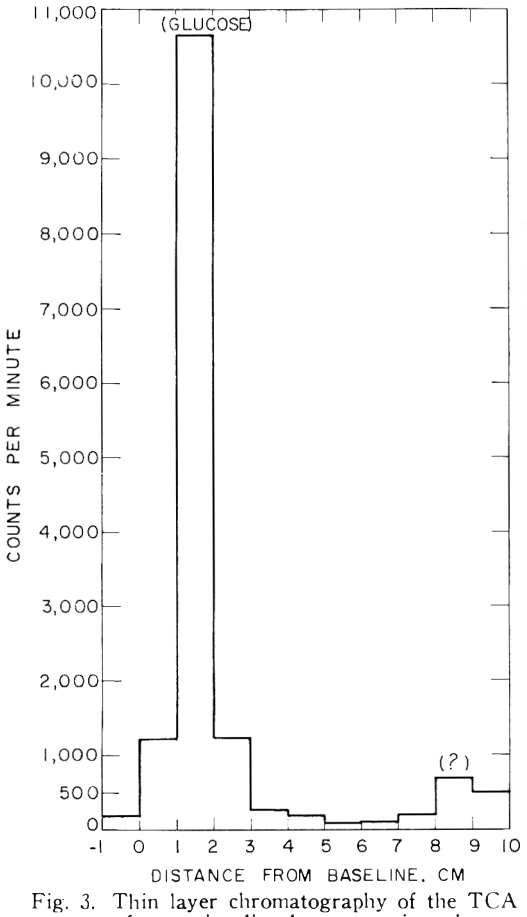



Fig. 3. Thin layer chromatography of the TCA supernatant from an insulin-glucose reaction mixture.

conditions are those commonly employed in commercial practice, this study lends an additional theoretical basis to this practice.

## CONCLUSIONS

The reaction between glucose and insulin to form what may be the Schiff base was found to proceed at a significant rate at ambient temperatures and an equilibrium relative humidity of 0.74. In agreement with theory, the rate of the reaction decreases with increasing pH over the range 4–7. The activation energy for the reaction appears to be less than that for other steps in the browning sequence.

The study of the reactions between sugars and proteins is amenable to a method employing carbon-14-labeled sugars, and in combination with chromatographic procedures might be used to study other steps in the browning sequence. The method is particularly attractive because of the difficulty and expense associated with obtaining relatively pure proteins.

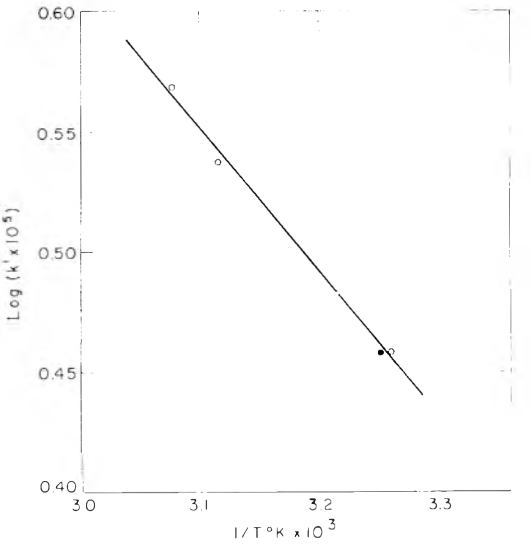



Fig. 4. Temperature dependence of the rate of interaction of glucose and insulin at pH 5.95 and a water activity of 0.74.

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Ms. rec'd 4/17/65.

Presented in part at the 24th Annual Meeting of the Institute of Food Technologists, Washington, D. C., May 1964. Contribution number 679 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139.

The author acknowledges the generous advice of Dr. Marcus G. Karel throughout this investigation.

CARL E. VANDERCOOK, LAURENCE A. ROLLE[a]

*Fruit and Vegetable Chemistry, 263 South Chester Avenue, Pasadena, California 91106, Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture and*

H. L. POSTLMAYR and R. A. UTTERBERG

*Minute Maid Company, 1226 North Olive, Anaheim, California 92803*

# Lemon Juice Composition. V. Effects of Some Fruit Storage and Processing Variables on the Characterization of Lemon Juice

## SUMMARY

**The variables included storage prior to processing, heavy and light juice-extraction pressures, and various methods of juice finishing. Lemons stored for 15 weeks dropped 64% in *l*-malic acid but increased 34% in the total amino acids. Total polyphenolics showed no significant change. Extremes of extraction pressure had a significant effect on the amino acid (increased), total polyphenolic (increased), and *l*-malic acid (decreased) values but not on the citric acid, sterol, carotenoid, and soluble pectin values. The finishing process affected only the pectin content. Changes in some of the constituents with extraction pressure were small in comparison with the natural range of values. The prediction of citric acid by the multiple-regression approach, reported earlier, is independent of commercial fruit storage and processing practices.**

## INTRODUCTION

Previous papers on lemon juice composition from this laboratory (Vandercook *et al.*, 1963; Vandercook and Rolle, 1963; Rolle and Vandercook, 1963) established a relationship between total amino acids, *l*-malic acid, and total polyphenolics such that a theoretical citric acid value could be predicted. The method, to be generally useful, must apply over a broad range of cultural and commercial processing practices. Although there was a high correlation ($r = 0.921$) between the combined variables and citric acid in the commercial samples studied, the levels of the individual constituents fluctuated over a fairly wide range. Two possible explanations for these variations (the length of fruit storage before extraction and the method of processing) are studied in this paper. Other variables, such as growing area, cultural practices, climatic conditions, and varietal differences, will be dealt with later.

There have been a number of investigations on lemon storage conditions as related to changes in juice content, acidity, soluble solids, ascorbic acid (Eaks, 1961), and malic acid (Sinclair and Eny, 1945). Unfortunately, not all of these constituents were measured on the same fruit samples. No data were found on the chemical composition of lemon juice as related to the method of processing. This study was made to investigate variations in sterols, carotenoids, soluble pectin, total amino acids, total polyphenolics, and *l*-malic acid which may be a function of fruit storage period or method of extracting and finishing the juice.

## METHODS

**Storage study.** Ten boxes of mature green lemons (approximately 1000 fruit) from the Ventura (California) area were picked on March 13, 1962. The fruit was waxed, sized, and electronically color-sorted under commercial packing-house conditions. The fruit was stored at 58°F and 85% relative humidity in boxes of 50 lemons each with biphenyl pads. Every two weeks for 15 weeks, 50 lemons were taken at random for analysis. Spoiled fruits were discarded. The fruit was lightly reamed, the seeds and rag strained off, and the juice filtered through Celite and analyzed immediately. The constituents measured were: a) total acidity as citric acid, determined by titration to pH 8.4; b) soluble solids, determined by index of refraction; c) total amino acids and amino acid pattern (Vandercook *et al.*, 1963); d) *l*-malic acid (Vandercook *et al.*, 1963); and e) total polyphenolics (Vandercook and Rolle, 1963).

**Processing variables.** Twelve boxes of lemons were obtained from the Saticoy California area (15 miles from the coast). The sample, which was a mixture of fruit stored 60 days and washer culls, was completely randomized. Fig. 1 shows a flow

[a] Present address: Western Regional Research Laboratory, 800 Buchanan Street, Albany, California 94710.

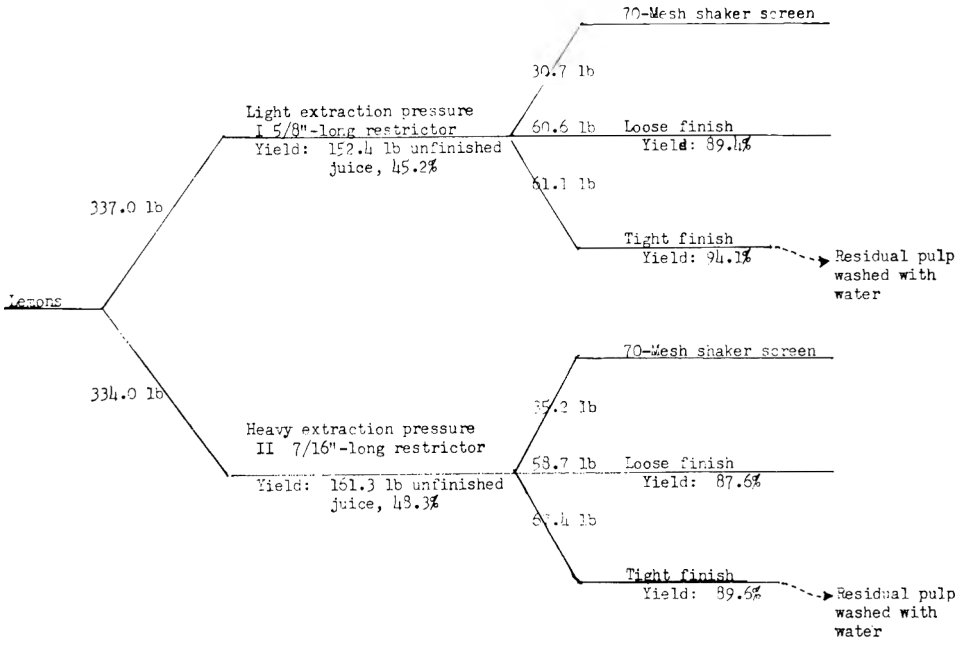


Fig. 1. Flow chart of extraction and finishing procedure.

diagram of the extraction and finishing process. The fruit was washed and weighed, and the juice was extracted with an FMC 75-rpm inline juice extractor (Table 1). Two extractor combinations

Table 1. Juice extractor components.

| | |
|---|---|
| Upper cup | 2-3/8" × 1-3/16" |
| Upper cutter | 3/4" × 1" |
| Lower cup | 2-3/8" profiled to 3" |
| Lower cutter | 3/4" |
| Restrictor | I 5/8" long |
| | II 7/16" long |
| Strainer tube | 0.040" long |

were used (I and II, in Table 1). The one with a 5/8-inch long restrictor in the orifice tube resulted in the lightest pressure. The other used the 7/16-inch-long restrictor for the heaviest pressure. The extracted juice was weighed, thoroughly stirred, and split into three portions. One was passed over a 70-mesh shaker screen. The other two were finished with an FMC 35-C finisher (0.020-inch screens) adjusted for a tight finish in one case and a loose finish in the other (depending upon how tightly the juice and pulp were squeezed through the screen). The tightly finished pulp from both extractor combinations was mixed with twice its weight of water and passed over a 70-mesh shaker screen. The finished juices were pasteurized at 190°F, canned hot, then frozen and stored at 0°F until analyzed.

Measured in addition to the above constituents were the carotenoids and sterols (Vandercook and Yokoyama, 1965) and soluble pectin (McCready and McComb, 1952).

## RESULTS AND DISCUSSION

**Storage.** The optimum storage conditions for lemons have been well established (Bartholomew and Sinclair, 1951), so that the only concern in this study was to determine the changes in juice composition during storage. Although the constituents might vary in total amounts with season, growing area, or variety, the relative changes in composition should be typical for any lemon sample.

During the first 7 weeks the lemons gradually lost their green color. By the 8th to 9th week they were completely yellow and had lost some turgidity. From the 12th through the 15th weeks most of the lemons were still of acceptable retail quality but were becoming somewhat soft. An increasing number of fruits were spoiled, had button browning, or showed surface discoloration.

The most striking change in composition of the fruit during storage (Table 2) was a large drop in the *l*-malic acid content of the juice. Sinclair and Eny (1945) observed a similar decrease. Conversely, the total amino acid content increased significantly through

Table 2. Analyses of lemons during storage.

| Weeks stored | Amino acids[a] | Malic acid[a] | Total polyphenolics[b] | Citric acid[c] | Predicted citric acid[d] |
|---|---|---|---|---|---|
| 0 | 1.66 | 3.88 | 0.755 | 90.3 | 89.7 |
| 1 | 1.72 | 3.88 | 0.545 | 99.8 | 84.1 |
| 3 | 1.69 | 3.17 | 0.601 | 101.0 | 83.5 |
| 5 | 1.97 | 2.74 | 0.681 | 99.6 | 88.1 |
| 7 | 2.07 | 1.90 | 0.671 | 99.8 | 86.7 |
| 9 | 2.05 | 1.66 | 0.613 | 104.4 | 84.1 |
| 11 | 2.09 | 1.70 | 0.613 | 106.3 | 84.7 |
| 13 | 2.27 | 1.39 | 0.668 | 104.9 | 87.6 |
| 15 | 2.18 | 1.44 | 0.603 | 106.3 | 84.8 |

[a] Milliequivalents/100 ml lemon juice.
[b] Absorbance at 330 mμ of lemon juice diluted 1:20 with 95% ethanol.
[c] Titratable acidity as citric acid meq/100 ml lemon juice.
[d] Rolle and Vandercook, 1963.

the 13th week and then dropped as the fruit began to deteriorate. The relative amounts of the individual amino acids as determined by paper chromatography did not change. The total polyphenolics content, as measured by ultraviolet absorbance, showed no definite trend. A least-squares calculation of the citric acid vs. storage time data gives a small positive slope ($b = 0.262$, $\sigma = \pm 0.195$), which, however, is not significantly different from zero at the 95% confidence level. However, when considered as two groups, the citric acid values were significantly higher during the last 8 weeks of storage than in the first 7 weeks. Since there is no corresponding increase in the other constituents, it must be assumed that this is a physiological change rather than an increase in concentration due to loss of water.

Although some of the juice constituents changed during storage, the citric acid predicted ($CA_p$) by the multiple-regression equation (Rolle and Vandercook, 1963) remained essentially constant. With these particular lemons, the $CA_p$ was unusually low with respect to titrated citric acid ($CA_t$) from the beginning of the experiment. Thus, after the 9th week, when the citric acid increased, the $CA_t - CA_p$ values increased an average of 5.7 meq/100 ml, which put the difference just outside of the limits. The limiting difference between the $CA_t$ and $CA_p$ at the 99% confidence level in the original data was 19.1 meq/100 ml.) Analysis of many commercial samples has shown that the value of $CA_t - CA_p$ is normally close to zero. Therefore, the data indicate that under normal commercial storage conditions the values of $CA_t - CA_p$ will be within acceptable limits.

**Processing.** The many types and makes of commercially available extracting and finishing equipment and their many possible adjustments allow almost an infinite number of pressure combinations. The plan of this study was to choose one widely used extractor and juice finisher adjusted to bracket the industrially feasible ranges of extracting and finishing. In commercial practice the juice yield is unacceptably low if the extraction and finishing pressure is too light, and if the pressure is too great the juice acquires a bitter taste. Furthermore, at tight finishing adjustments the resulting juice concentrate has a milky appearance and a higher viscosity. It is also common practice in the lemon industry to wash the pulp with water after the finishing step to remove any remaining juice solids. The pulp wash effluent is then added to the lemon juice as it goes to the low-temperature vacuum evaporator. The pulp wash was examined to determine if anything was being extracted from the pulp.

It can be seen from the data in Fig. 1 that juice yields increase both with a smaller diameter restrictor in the orifice tube and with increased finishing pressure. An analysis of variance (Youden, 1951) of the data in Table 3 between the extraction and finishing conditions for all of the constituents measured shows that at the 95% confidence level there are no significant differences for citric acid, pH, soluble solids, sterols, and carotenoids. The amino acid concentration increased significantly with higher extraction pressure, although paper chromatography indicated that the relative individual amino acid composition did not change. The total polyphenolics also increased significantly with extraction pressure. The ratios of the peak absorbances ($A/B$) in the ultraviolet spectrum of the juice indicate that there was no major change in polyphenolic composition at the heavier pressure. The effect of the finishing variables was not significant for the amino acids or the polyphenolics. There was a barely sig-

Table 3. Processing vs. lemon juice composition.

| | Light extraction pressure (I) 5/8"-long restrictor | | | Heavy extraction pressure (II) 7/16"-long restrictor | | |
|---|---|---|---|---|---|---|
| | 70-mesh shaker screen | Loose finish | Tight finish | 70-mesh shaker screen | Loose finish | Tight finish |
| Acidity[a] | 94.6 | 92.9 | 93.5 | 88.4 | 92.4 | 90.8 |
| pH | 2.2 | 2.0 | 2.1 | 2.2 | 2.0 | 2.1 |
| Amino acids[a] | 1.98 | 2.01 | 2.04 | 2.06 | 2.17 | 2.18 |
| Absorbance 330 mμ[a] | 0.824 | 0.845 | 0.829 | 0.906 | 0.996 | 0.959 |
| *A/B* | 1.91 | 1.89 | 1.90 | 1.93 | 1.95 | 1.92 |
| Malic acid[a] | 2.96 | 2.92 | 2.94 | 2.37 | 2.65 | 2.55 |
| Pectin[b] | 29.2 | 30.2 | 28.0 | 40.6 | 50.5 | 62.5 |
| Sterols[c] | 7.44 | 6.89 | 6.87 | 6.89 | 7.74 | 7.37 |
| Carotenoids[d] | 65 | 45 | 49 | 61 | 67 | 61 |

[a] Same units as in Table 2.
[b] mg anhydrouronic acid/100 ml lemon juice.
[c] mg β-sitosterol/100 ml lemon juice.
[d] μg β-carotene/100 ml lemon juice.

nificant drop in *l*-malic acid with the higher pressure, but, again, no effect of finishing was observed.

The soluble pectin seemed to be affected by both extraction pressure and finishing pressure. Since the pectin changed with changed finishing pressure only when the fruit was extracted under heavy pressure, it would seem that the additional pectin came from an increase in the albedo particles in the juice rather than from the pulp.

Table 4 shows the composition of the pulp wash water. The increase in total citric acid was calculated to be 3.16 for the 5/8-inch-long restrictor plus tight finish, and 7.75% for the 7/16-inch-long restrictor plus tight finish. The total amounts of the other constituents also increased in about the same proportion. That the pulp wash would make only a small change in the relative concentration of the original juice constituents is also shown in Table 4. Furthermore, the amino acid paper chromatograms and the relative peak absorbances for the polyphenolics of the pulp wash indicated no major change in composition of these fractions.

Table 5 shows the citric acid values predicted from the multiple-regression equation. The differences between the predicted and titrated citric acid values were greater with heavier extraction pressure, showing again the increase of substances contributing to the equation. However, the values were still well within the acceptable limits in each case. On the basis of this study and since the commercial practice will generally be within the extremes tested here, it is reasonable to conclude that, except for pectin, the extraction and finishing will affect the juice composition only slightly.

Table 4. Composition of the pulp wash and the effects of its addition to lemon juice.

| | 5/8"-long restrictor and tight finish | | 7/16"-long restrictor and tight finish | |
|---|---|---|---|---|
| | Measured[a] | Change on addition to juice[b] | Measured[a] | Change on addition to juice[b] |
| Citric acid | 25.1 | | 30.4 | |
| Amino acids | 0.630 | 0.5 | 0.862 | 1.4 |
| Total polyphenolics | 0.323 | 1.4 | 0.457 | 2.9 |
| Malic acid | 0.707 | −0.3 | 0.932 | 0.8 |

[a] Same units as Table 2.
[b] The change in percent that would occur if the pulp wash were concentrated to a citric acid value equal to that of the corresponding finished juice, then mixed with that juice.

Table 5. Effects of processing variables on predicted citric acid.

| | Predicted citric acid[a] | Measured citric acid[a] |
|---|---|---|
| 5/8"-long restrictor | | |
| Shaker screen | 93.4 | 94.6 |
| Loose finish | 95.3 | 92.9 |
| Tight finish | 95.2 | 93.5 |
| 7/16"-long restrictor | | |
| Shaker screen | 96.5 | 88.4 |
| Loose finish | 99.8 | 92.4 |
| Tight finish | 98.5 | 90.8 |

[a] meq citric acid/100 ml lemon juice.

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The authors thank the Ventura Coastal Lemon Company for donation and color sorting of the lemons used in the storage study, and Drs. Vincent P. Maier and E. A. Beavens for assistance in preparation of the manuscript.

This study was supported in part by the Lemon Products Technical Committee, Los Angeles, California.

Reference to a company or product does not imply endorsement.

D. E. HEINZ,[a] M. R. SEVENANTS,[b] and W. G. JENNINGS
*Department of Food Science and Technology, University of California, Davis 95616*

# Preparation of Fruit Essences for Gas Chromatography

## SUMMARY

**A large-scale continuous liquid-liquid extractor, designed for efficiently extracting large volumes of extremely dilute essence with minimal quantities of solvent, is described. Chromatograms are compared that were obtained on Bartlett pear essences prepared by: 1) direct fruit extraction; 2) extraction of laboratory steam distillates; and 3) extraction and 4) adsorption techniques applied to a commercial deaerator effluent.**

## INTRODUCTION

Gas chromatography has proved to be a valuable tool for the separation of complex volatile essences, but the technique of preparing samples from fruits and fruit products suitable for gas chromatographic injections is rarely interchangeable from fruit to fruit. It is desirable that this concentrated sample represent, qualitatively and quantitatively, the volatiles originally present, without the production of additional compounds. The injected sample must contain enough of each individual component (to be detected) to activate the detector. Each such component should enter the detector at maximum concentration, diluted as little as possible by the carrier gas. Ideally, each chromatographic component should exist, as it passes through the column, as a concentrated mass occupying as short a linear length of column as possible. To achieve these conditions, the size of the injected sample must be small with relation to the volume of carrier gas flowing into the column, and this small sample must be concentrated enough to contain detectable quantities of the components of interest.

Two general methods have been used for preparing chromatographic samples that satisfy these conditions: one involving a direct solvent extraction of the material to be studied, and the other some type of steam distillation, and subsequent isolation of the volatile constituents. This can involve solvent extraction and concentration, or adsorption techniques. In almost every case, solvents are used in some step of the preparation. This can pose an additional problem, because the use of large volumes of solvent may increase solvent-borne impurities to a point where they can be confused with the volatile components under investigation.

A large number of extraction devices and techniques have been described in the literature (*cf.* Morrison, 1964), but none of these are too satisfactory for efficiently extracting large volumes of extremely dilute aqueous material with minimum quantities of solvent. Some workers (e.g. Ralls *et al.*, 1965) have utilized carbon adsorption to isolate the compounds of interest in these very dilute aqueous systems.

This study compares essences of Bartlett pear prepared by direct fruit extraction, extraction of steam distillates with different solvent systems, and the use of activated carbon to isolate volatiles from steam distillates, followed by Soxhlet extraction of the adsorbent.

## METHODS

**Gas chromatography.** Used for gas chromatography was an Aerograph Hy-Fi 600B with flame ionization detection, containing a 200-ft × 0.02-in. stainless-steel capillary column coated with Carbowax 20M. Nitrogen carrier gas was supplied at 4 ml/min, and runs were programmed from 60 to 195°C.

**Sample preparation.** Four different techniques were used: 1) direct solvent extraction of fruit; 2) extraction of laboratory steam distillates of fresh fruit; and 3) extraction and 4) adsorption techniques utilizing an aqueous essence obtained from a large commercial concern processing select, ripe Bartlett pears to pear purée. This aqueous essence, which proved a rich source of pear volatiles, comprised the effluent from the vacuum deaeration of heated pear purée.

**Direct fruit extraction.** A selected ripe pear (36 days at 0°C, 12 days at 20°C) was blended with an equal weight of iced water and immediately extracted by shaking with an equal volume of freshly distilled reagent-grade diethyl ether (Allied Chemicals). This mixture was centrifuged, and the ether layer was removed from the aqueous pear residue. The ether was separated from the

[a] Present address: Department of Chemistry, Ventura College, Ventura, Calif.
[b] Present address: Winton Hill Research Laboratories, Procter & Gamble Company, Cincinnati, Ohio.

higher-boiling lipid fraction by fractional distillation in an 0.8 × 30.0-cm concentric tube Podbelniak column, with an efficiency of 75 theoretical plates and an operating holdup of 0.7 ml. 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 which collected on the cold finger possessed a desirable pear aroma. It was removed and collected from the cold finger by rinsing with a small amount of ether.

**Steam distillation.** Whole, ripe Bartlett pears were used for isolation of the volatile compounds by steam distillation. Ten lb of Bartlett pears were chilled to approximately 5°C and then were blended under a nitrogen atmosphere with an equal weight of iced water. These light-yellow blended pears showed almost no browning and possessed a characteristic desirable aroma. This material was then placed in a 12-L round-bottom flask, and steam was passed through the heated material for 4 hr. A total of 1 L of distillate was collected, using two ice traps. A thin, oily layer appeared on the top of the water distillate in the first trap. The distillate was extracted with isopentane, and the volatiles were concentrated by passing nitrogen over the solvent. After most of the solvent was removed, a slightly yellow, oily residue remained. The volatile compounds were separated from the yellow residue by utilizing the one-plate molecular still as before. The distillate possessed a desirable pear aroma.

**Continuous liquid-liquid extraction.** The continuous counter-current liquid-liquid extractor (Fig. 1) was devised to extract the large amounts of aqueous material obtained from the commercial plant. The partition coefficient, $K$, is quite large for the process of extracting organic volatiles consisting mostly of esters from water, where

$$K = \frac{\text{concentration of solute in organic phase}}{\text{concentration of solute in aqueous phase}}$$

This represents, however, an equilibrium condition which is difficult to obtain in a high-throughput continuous extractor. Consequently, the extractor was designed to mix the two phases thoroughly for 1–2 min or longer. Solvent recirculation permits the system to use small quantities of solvent and minimize impurities due to solvent contaminations. The extractor proved capable of handling up to 15 gallons of aqueous distillate per hour. This permitted large volumes of essence to be extracted promptly, minimizing the possibility of degradative chemical reactions. The extraction process was as follows:

1) The glass extractor was filled with chilled aqueous material (5–10°C) to the desired level (Fig. 1).

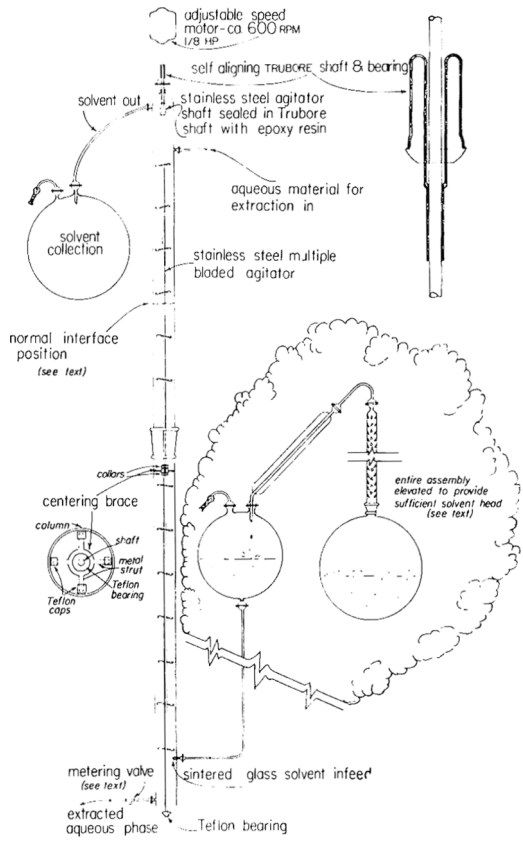


Fig. 1. Continuous liquid-liquid extractor.

2) The solvent was then passed through this water phase by providing a sufficient solvent head in the solvent-reservoir to push the solvent through the sintered-glass infeed.

3) Once the solvent started bubbling up through the cold aqueous phase, the speed of the stirrer motor was adjusted to achieve considerable mixing.

4) After a solvent layer formed above the water layer, more chilled aqueous material was metered into the top of the extractor at a rate of 10–15 gal/hr.

5) At this time a metering valve at the bottom of the apparatus was opened and adjusted so that the position of the interface remained constant. This occurred when the extracted aqueous phase was expelled at the same rate that the fresh aqueous phase was added.

The rate of addition of the solvent was controlled by either adjusting the solvent head or by utilizing the rate of distilling fresh solvent to the high-solvent reservoir. The solvent, which contained the extracted volatile compounds, accumulated above the water layer, and spilled into a solvent collection reservoir. This reservoir was

drained periodically, and transferred to the distillation pot where the organic volatiles were then concentrated by distilling the lower-boiling solvent to the solvent reservoir for recycling.

One hundred and forty gallons of aqueous material containing pear volatiles were collected in 10-gallon milk cans. The cans were placed in a cold room (−25°C) until they were chilled to 5°C (approximately 2 hr). The chilled materials were promptly extracted, with isopentane the solvent. The isopentane solvent (Matheson, Coleman, and Bell, practical-grade) had been purified by passing it slowly through silica gel. Three gallons of purified isopentane were used to extract the 140 gallons of aqueous pear material.

Concentrations of the dienoate esters in the aqueous system before and after a single extraction were measured from their absorption at 260 mμ. The efficiency is reported as percent extracted, calculated as:

$$\% \text{ extracted} = \frac{100\ (C_i - C_f)}{C_i} = \frac{100\ (A_i - A_f)}{A_i}$$

from the relationship $A = l\epsilon C$

where $C_i$ = initial concentration
$C_f$ = final concentration
$A_i$ = initial absorption
$A_f$ = final absorption
$l$ = length of cell (1 cm)
$\epsilon$ = extinction coefficient (*ca.* 26,000)

The extraction efficiency of this method varied from 86% to 90%.

The isopentane extract was concentrated to a final volume of approximately 700 ml by distilling-off the excess solvent for recyclization. This isopentane fraction was concentrated further by utilizing the semimicro Podbelniak fractionation column maintaining a 5:1 reflux ratio. The distillation pot was heated with a 45°C water bath, and the distillate was collected (28°C fraction) until the head temperature dropped to 25°C. The 8-ml sample thus obtained had an odor characteristic of acetates. A small sample of this material gave a desirable pear aroma when exposed to air, allowing the lower-boiling compounds to escape.

**Charcoal adsorption.** The maximum amounts of concentrated volatiles were obtained utilizing charcoal adsorption of the pear volatiles from the aqueous material obtained from the commercial plant. Four pounds of activated coconut charcoal (Matheson, Coleman, and Bell), 8–12 mesh, were secured in a two-gallon perforated canister. This canister was placed in the processing line so that the aqueous material containing volatiles passed through the charcoal for a two-day period. The charcoal was then freeze-dried to remove residual water. Two pounds of this dry charcoal was extracted for 40 hr with freshly distilled reagent-grade ether utilizing a 2.3-L Soxhlet extractor. The excess ether was removed from the pear volatile extract by employing a semi-micro Podbelniak fractionating column. The 60 ml of the pear volatiles thus obtained had an odor characteristic of acetates. A desirable pear aroma was obtained when a small sample of this material was allowed to evaporate for 10–15 min, allowing the lower-boiling components to dissipate.

## RESULTS AND DISCUSSION

Figs. 2–5 respectively show chromatograms of pear volatiles obtained by direct ether extraction of blended pears, extraction of a laboratory steam distillate, extraction of the commercial deaerator effluent, and charcoal adsorption from the deaerator effluent. In each case the chromatograph was programmed from 60 to 195°C in 35 min, and operated isothermally at 195°C for the remaining periods.

Peaks on all chromatograms were numbered with reference to the order of their appearance on Fig. 5, the charcoal adsorption essence chromatogram. The compounds which have been tentatively identified are listed in Table 1. All of the compounds listed have been identified as being present in the adsorption essence. Identification of peaks on the capillary Carbowax 20M col-

Fig. 2. Chromatogram of essence obtained by direct fruit extraction.

Fig. 3. Chromatogram of essence obtained by extraction of a laboratory steam distillate.

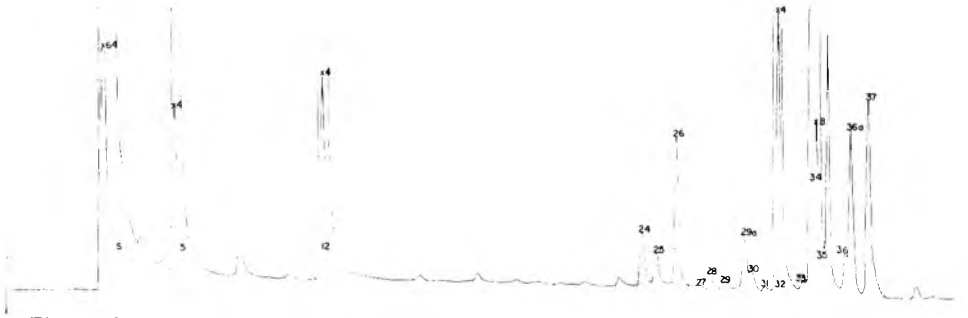

Fig. 4. Chromatogram of essence obtained by extraction of commercial deaerator effluent.

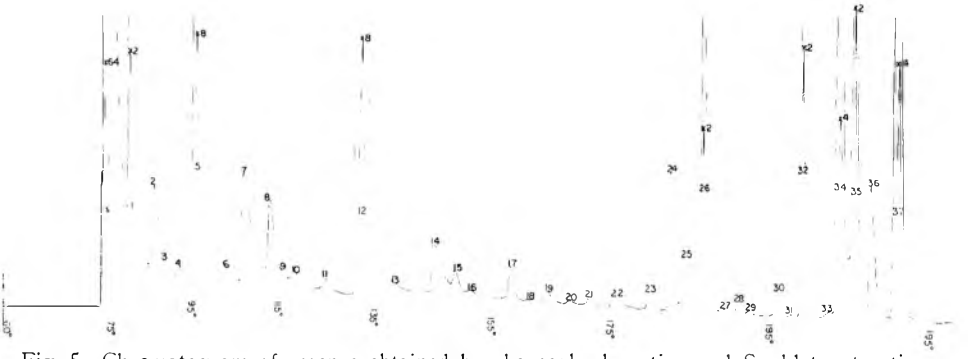

Fig. 5. Chromatogram of essence obtained by charcoal adsorption and Soxhlet extraction.

umn was based on: 1) comparing retentions to pear samples to which the known compounds were added; 2) comparing to chromatograms of ⅛-inch 10-ft Carbowax 20M packed columns where the retention of the compounds have been established by trapping and obtaining ultraviolet and infrared spectra (Heinz and Jennings, 1966); and 3) comparing relative peak size to chromatograms of the same pear material run on Triton X305 where the peaks were established (Heinz and Jennings, 1966).

Qualitatively, the major difference in the chromatograms was the relatively larger proportions of high-boiling or less-volatile compounds obtained by the laboratory steam-distilled and ether-extracted fractions. However, this relationship between the high- and low-boiling compounds was probably due to the final purification step, vacuum-distillation, and not to the actual source of samples. The four chromatograms in Figs. 2–5 show a similarity in compounds in the high-boiling regions, especially the range of compounds numbered in Fig. 5. Peaks 29a and 36a in Figs. 2–4 were the only observed peaks different from those of the charcoal adsorption essence.

The most significant feature of the ether-extracted volatile fraction represented by Fig. 2 is the dominance of peaks 32 and 34, respectively methyl and ethyl *trans*:2-cis:4-

Table 1. Volatile compounds of Bartlett pears, identified on a 200-ft by 0.02-in. Carbowax 20M capillary column.

| Peak no. | Name of compounds present |
|---|---|
| S | solvent (ether) |
| 1 | ethyl acetate |
| 2 | propylacetate |
| 5 | butylacetate |
| 7 | butanol |
| 8 | amylacetate |
| 12 | hexyl acetate |
| 14 | hexanol |
| 23 | methyl decanoate |
| 24 | methyl cis :4-decanoate |
| 25 | ethyl decanoate |
| 26 | ethyl cis :4-decenoate |
| 32 | methyl trans :2-cis :4-decadienoate |
| 34 | ethyl trans :2-cis :4-decadienoate |
| 35 | methyl trans :2-trans :4-decadienoate |
| 37 | ethyl trans :2-trans :4-decadienoate |

decadienoate. Jennings and Sevenants (1964) reported that esters of this acid appear to be character impact compounds for Bartlett pear flavor. The synthesized homologous series of decadienoates had an odor that was remarkably pear-like (Jennings *et al.*, 1964). The odors of the methyl, ethyl, n-propyl, and n-butyl esters could be described as pear-peel, pear-core, or fresh pear. The odors of the n-amyl and n-hexyl esters appear to be slightly less desirable. Previous work (Heinz *et al.*, 1964) indicated that ultraviolet absorption measurements of these diunsaturated esters correlate well with the sensory odor intensity of pears. The production of these esters in the ripening fruit increased rapidly during the climacteric and continued to increase as both ethlyene and $CO_2$ production declined (Heinz *et al.*, 1965). For these reasons, methyl and ethyl *trans*:2-*cis*:4-decadieonate appear to be important to the aroma of ripe Bartlett pears.

The two samples from the commercial deaerator (Figs. 4, 5) showed only quantitative differences. More obvious is the relative scarcity or lack of peaks on Fig. 4, as, for example, peak numbers 1, 7, 14, 35, and 37. This may be due to their relative solubilities in isopentane. Butanol and hexanol, respectively peaks 7 and 14, are not as soluble in isopentane as in ether. Their presence in the charcoal essence might also be explained by the possibility of hydrolysis of the butyl and hexyl acetates (respectively peaks 5 and 12) occurring on the activated charcoal; however, acetic acid was not detected in this sample. Other differences in relative amounts of volatiles isolated by the two methods might be explained by isomerization of the adsorption essence. For example, if peaks 32 and 34, the methyl and ethyl *trans*:2-*cis*:4-decadienoates, were isomerized to the more stable *trans*:2-*trans*:4-decadienoates while on the activated charcoal, peaks 32 and 34 in Fig. 3 would decrease while peaks 35 and 37, the *trans-trans* isomers, would become relatively larger. This was observed. Peaks 29a and 36a, appearing in Figs. 2, 3, and 4 but not in Fig. 5, may also have reacted or changed during adsorption on the charcoal. These compounds may, however, have entirely different adsorption properties, such that they are not eluted by ether from the charcoal once they are adsorbed. It is doubtful that they are solvent impurities, since they do appear in chromatograms which contain both ether and pentane.

Ethyl acetate (peak 1) was a major component in the adsorption essence, but Fig. 4 shows it was almost completely lacking in the isopentane extract. It is difficult to explain this observation by considering solubility, chemical, or adsorption properties of the systems involved, because similar compounds (butyl and hexyl acetate) are present to approximately the same extent in both systems. One possible explanation is that the pears which were being processed when essence was collected for the isopentane extraction may not have produced a substantial concentration of ethyl acetate. Lim (1963) has shown that the butyl and hexyl acetates are produced by the pear during ripening before production of ethyl acetate commences, while ethyl acetate is of major significance in an overripe pear. The sample collected for isopentane extraction was collected from the processing line over a period of approximately 20 min. The sample collected by charcoal adsorption for subsequent ether extraction was collected from the same processing line, but over a period of two

days, thus representing a larger average of maturity levels of pears.

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The authors are grateful to the Gerber Products Company for cooperation; one of the authors (DEH) was supported during this period by a pre-doctoral fellowship from the Campbell Soup Company. Portions of this paper are from a Ph.D. thesis by DEH, and others from a Ph.D. thesis by MRS, both in Agricultural Chemistry. This research program is supported by Research Grant EF0092 from the Division of Environmental Engineering and Food Protection.

D. E. HEINZ [a] and W. G. JENNINGS
*Department of Food Science and Technology*
*University of California, Davis 95616*

# Volatile Components of Bartlett Pear. V.

## SUMMARY

**The volatile essence of fresh, ripe Bartlett pears was separated into individual compounds by repetitive gas-liquid chromatography. Techniques used for characterization included retention data; infrared, ultraviolet, nuclear magnetic resonance, and mass spectroscopy; melting points, and formation of appropriate derivatives. The compounds identified include: ethanol, *n*-propanol, *n*-butanol, *n*-pentanol, *n*-hexanol, *n*-octanol; methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl; *n*-hexyl, *n*-heptyl, and n-octyl acetate; and the methyl and ethyl esters of *n*-octanoic, 3-hydroxyoctanoic, *trans*:2-octenoic, n-decanoic, *cis*:4-decenoic, *trans*:2-decenoic, *trans*:2-*cis*:4-decadienoic, and *trans*:2-*trans*:4-decadienoic acids. Possible biosynthetic pathways for the formation of the higher-boiling methyl and ethyl esters are discussed.**

## INTRODUCTION

Until the advent of gas chromatography, studies concerned with non-ethylenic emanations from pears were seriously handicapped. Harley and Fisher (1927) studied pear scald and breakdown and reported that Bartlett pear volatiles included acetaldehyde. Tindale *et al.* (1938) followed the alcohol and acetaldehyde content during the ripening of Bartlett pear, and observed a continuous increase in the alcohol content, while acetaldehyde increased during cold storage and subsequent ripening and achieved maximum concentration at the onset of core breakdown. During maturity studies of both pears and apples, Antoniani *et al.* (1954), Antoniani and Serini (1955), and Serini (1956) reported that the amounts and ratios of 2,3-butylene glycol and acetoin varied during the ripening process. They suggested that these variations might serve as an index of ripeness, but because of difficulties in determining the concentrations of these compounds the method has not found acceptance. It is interesting to note that these compounds were not isolated in the present study. Gerhardt (1954) studied the rates of emanation of volatiles from pears and apples by bubbling the evolved gases through sulfuric acid and then oxidizing these with ceric sulfate. However, none of the volatiles were identified during the study, which was limited to the low-boiling volatiles capable of oxidation under these conditions. Similar work was done by Luh *et al.* (1955) while studying the volatile reducing substances in Bartlett pears. They reported that, as the pears ripened at 20°C, methyl alcohol, total carbonyl compounds, acetoin, diacetyl, and ester content gradually increased. Mehlitz and Matzik (1956), while studying the volatile acids of several pear varieties not including the Bartlett, identified formic and acetic acids by paper chromatographic techniques.

The development of gas-liquid chromatography has vastly improved the possibilities of obtaining separations of complex volatile mixtures. Jennings *et al.* (1960) fractionated an essence obtained from fresh ripe Bartlett pears into 32 components by gas chromatography. Each of these was submitted to a trained aroma panel, and the results indicated that five of these fractions contributed significantly to the desirable pear aroma, while four possessed atypical and undesirable aromas. On the basis of gas chromatographic retentions, Drawert (1962) tentatively identified ethyl formate, methyl acetate, ethyl acetate, ethanol, isopropanol, 2-butanol, 2-methylpropanol, n-butanol, pentanol, 3-methylbutanol, n-hexanol, and other ester in pears. Lim (1963) utilized gas chromatography in following the production of the lower-boiling volatiles from Bartlett pears and other fruits during the ripening process. Based on retention data, she tentatively identified ethylene, acetaldehyde, and the six normal acetates from methyl to hexyl acetate in Bartlett pears. Jennings (1961) and Jennings and Creveling (1963) studied the hydrolysis products of Bartlett pears utilizing gas chromatography and, in the latter study, infrared and ultraviolet spectroscopy. They reported acetic, propionic, butyric, caproic, caprylic,

[a] Present address: Department of Chemistry, Ventura College, Ventura, California.

nonanoic, and 2,4-decadienoic acids, and the alcohols ethyl, n-propyl, n-butyl, n-amyl, and n-hexyl. Jennings and Sevenants (1964) separated the essences of Bartlett pears by gas chromatography and recovered this essence without losing its desirable aroma characteristics. By infrared spectroscopy, they identified hexyl acetate and methyl *trans*:2-*cis*:4-decadienoate. The former, they described as a "contributory flavor compound," and the latter as a "character impact compound" for Bartlett pear. Later, Jennings *et al.* (1964) identified ethyl *trans*:2-*cis*:4-decadienoate as a flavor component of Bartlett pear. The methyl, ethyl, n-propyl, n-butyl, n-amyl, and n-hexyl *trans*:2-*cis*:4-decadienoates were synthesized, and found to have strong pear-like aromas.

Heinz *et al.* (1964) studied the relation of instrumental and sensory techniques as methods of evaluating the intensity of pear aroma. The instrumental method, utilizing ultraviolet spectroscopy, estimated 2,4-decadienoate concentration by measuring the absorption intensities of aqueous pear samples at 263–267 mμ. The results of these measurements were shown to correlate well with the aroma intensities. Heinz *et al.* (1965) followed the production of these decadienoate esters by the ripening Bartlett pear, and found that the rate achieved a maximum immediately following the climacteric point.

The cumulative results of all these studies resulted in identifying (sometimes tentatively) a few of the major volatile compounds of Bartlett pear. This present study was directed toward definitive studies on this complicated essence and the development for their separation, isolation, and identification.

## METHODS AND PROCEDURES

**Pear essence.** The pear volatiles were obtained from a commercial processing company which was producing pear puree from fresh, ripe, peeled Bartlett pears, free from defects and blemishes, and possessing desirable and characteristic aroma. The volatile compounds were collected from the processing line by employing charcoal adsorption followed by ether elution as described previously (Heinz *et al.*, 1966).

**Gas chromatography.** Preliminary separations were achieved on an Aerograph Autoprep 700 utilizing an 18-ft by ¼-inch stainless-steel column packed with 60–80-mesh Gas Chrom Q coated with 10% Triton X305 and helium carrier gas. Fractions isolated from this unit were further resolved to individual compounds by employing a modified Beckman Thermotrac chromatograph using a Carle thermistor micro-detector (Carl Instruments, Anaheim, California). This instrument utilized two 10-ft by ⅛-inch stainless-steel columns, one containing 15% Apiezon L on 60–80-mesh HMDS treated Chromosorb W, and the other 10% Carbowax 20M on 40–60-mesh Gas Pack-F. The injection chambers for both chromatographs were maintained between 165 and 185°C, and the collectors and detectors were maintained at 200–230°C.

The Aerograph Autoprep was operated with a flow rate of 50 ml/min at 65°C. For the separation on Triton X305, the starting temperature was kept constant at 65°C for 10 min, and increased to 230°C over the next 30 min. For this initial separation of the pear volatiles, injection sample sizes varied from 15 to 150 μl.

Fractions were collected in 1-ft thin-walled glass capillaries inserted in the outlet as described by Kratz (Jennings *et al.*, 1964). One- to 5-μl fractions so collected were further purified by rechromatographing on the Apiezon L or Carbowax 20 M columns utilizing the Beckman Thermotrac. All runs on the Thermotrac used linear-programmed temperatures (65–230°C) and a He flow rate of 20 ml/min. Two linear programs were used; one spanned the temperature range in 30 min, and the other required 1 hr. The individual compounds were then recollected from at least a third dissimilar column to verify their purity. These samples were then subjected to spectral methods of identification.

Retention data of identified compounds were determined by adding known compounds to the pear samples and chromatographing these samples under the conditions of the original pear sample. When applicable, homologous series of compounds, i.e. acetates, were added simultaneously to the pear sample. To obtain approximate retentions, the compounds were also chromatographed individually, under the same conditions used for the pear samples.

**Spectroscopy.** Infrared spectra were determined on a Beckman spectrophotometer, Model IR-5, fitted with a Beckman beam condenser. Spectra were taken on thin films between two NaCl plates or in a CIC type-C micro-cavity cell (0.05-mm pathlength). Infrared spectra were also determined on solutions in spectral grade $CCl_4$.

Ultraviolet spectra were determined on a Beckman DB spectrophotometer in spectral-grade isooctane, methanol, or water, using 10-mm silica cells.

The nuclear magnetic resonance spectrum (60 Mcs) was determined with a Varian A-60 on a dilute carbon tetrachloride solution.

The mass spectral determinations were performed by the Department of Chemistry, University of California, Berkeley, utilizing a CEC 103 instrument.

**Knowns and derivatives.** Used for comparison with fractions isolated from the Bartlett pear volatiles were authentic samples of esters and alcohols purchased from Allied Chemicals, General Chemical Division, New York; Eastman Kodak Company, New York; Matheson, Coleman and Bell, Cincinnati, Ohio; and K and K Laboratories, Plainview, New York. An authentic methyl cis:4-decenoate was obtained from the Western Regional Research Laboratory, Albany, California (Buttery *et al.,* 1963). Other saturated esters which were not readily available were synthesized. The esters of the *trans*:*2-cis*:4-decadienoic acid were synthesized as described previously (Jennings *et al.,* 1964). Methyl 2-octenoate, methyl 2-decenoate, and ethyl 2-decenoate were prepared by allowing 0.25 ml 2-decenoic acid and 0.25 ml ethyl 2-octenoate in 0.10 ml of methanol to react at 100°C for 30 min. The desired products were isolated by gas chromatography.

The methyl hydroxy ester was characterized by oxidation, hydrolysis, decarboxylation, and derivative formation. To 1 $\mu$l of the ester in a 0.6-mm × 10-cm glass tube was added 0.5 ml of 0.2*N* $K_2Cr_2O_7$ in 2*N* $H_2SO_4$. The tube was sealed and placed in a boiling $H_2O$ bath for 3 hr. 5% $FeSO_4$ was added dropwise to the contents until the yellow solution turned green. A saturated solution of 2,4-dinitro phenylhydrazine in 1*N* HCl was added, and the yellow 2,4-dinitrophenylhydrazone (DNPH) precipitate which was formed was recrystallized from ethanol-water.

## RESULTS

Table 1 lists the compounds which have been identified or tentatively identified in pear essence. The peak numbers are the same as the assignments in Fig. 1, a chromatogram obtained with the 18-ft by ¼-inch Triton X305 column. Each apparent peak is numbered from 1 through 45; however, each peak does not necessarily represent a single compound (see Table 1).

Table 1. Volatile compounds of Bartlett pear.

| Fraction[a] | Compounds(s) present[b] |
|---|---|
| S | ether solvent |
| 1 | methyl acetate[c,d] R |
| 2 | ethyl acetate[c] R, IR |
| 3 | ethanol[c,d] R |
| 4 | n-propyl acetate[c] |
| 5 | n-propanol[c] R |
| 6 | unidentified |
| 7 | unidentified |
| 8 | unidentified |
| 9 | n-butylacetate[c] R, IR |
| 10 | n-butanol[d,f] R, IR |
| 11 | unidentified |
| 12 | n-amyl acetate[c] R, IR |
| 13 | unidentified |
| 14 | n-pentanol[d,e] R, IR |
| 15 | unidentified |
| 16 | n-hexyl acetate[c,g] R, IR |
| 17 | n-hexanol[d,f] R, IR |
| 18 | unidentified |
| 19 | n-heptylacetate R, IR |
| 20 | methyloctanoate R |
| 21 | methyl 4-oxy*trans*butenoate IR, UV, D, MP<br>n-heptanol R |
| 22 | unidentified |
| 23 | ethyl octanoate R, IR |
| 24 | ethyl 4-oxy*trans*butenoate IR,UV, D |
| 25 | octyl acetate R, IR |
| 26 | methyl *trans*:2-octenoate IR |
| 27 | n-octanol R, IR |
| 28 | ethyl *trans*:2-octenoate R, IR |
| 29 | unidentified |
| 30 | methyl decanoate R, IR |
| 31 | methyl *cis*:4-decenoate R, IR, NMR, D |
| 32 | ethyl decanoate R, IR |
| 33 | ethyl *cis*:4-decenoate IR, MS, D |
| 34 | methyl *trans*:2-decenoate R, IR |
| 35 | unidentified |
| 36 | methyl *trans*:*2-cis*:4-decadienoate[g] R, IR, UV<br>methyl 3-hydroxyoctanoate IR, MS, D<br>ethyl *trans*:2-decenoate R, IR |
| 37 | methyl *trans*:2-*trans*:4-decadienoate IR<br>ethyl *trans*:2-*cis*:4-decadienoate[h] R, IR, UV<br>ethyl 3-hydroxyoctanoate, IR, MS |
| 38 | unidentified |
| 39 | ethyl *trans*:2-*trans*:4-decadienoate IR |
| 40–45 | unidentified |

[a] Fractions represent peak numbers of chromatogram in Fig. 1, obtained on Triton X305.

[b] R, retention data. IR, infrared spectral data. UV, ultraviolet spectral data, D, derivative, MP, melting point, MS, mass spectral data, NMR, nuclear magnetic resonance spectral data.

[c] Previously reported by Lim (1963) on the basis of retention data.

[d] Previously reported by Drawert (1962) on the basis of retention data.

[e] Previously reported by Jennings and Creveling (1963).

[f] Previously reported by Jennings (1961).

[g] Previously reported by Jennings and Sevenants (1964).

[h] Previously reported by Jennings *et al.* (1964).

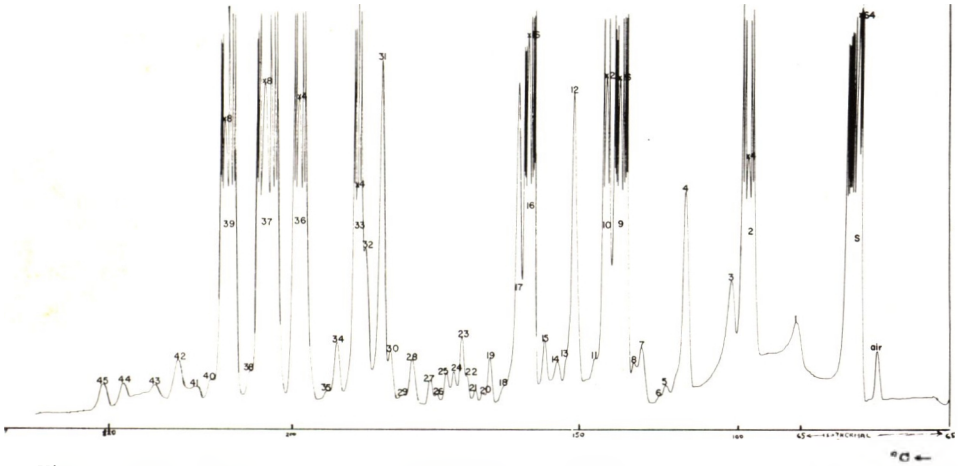


Fig. 1. Chromatogram of 15 $\mu$l of Bartlett pear volatiles on an 18-ft by ¼-inch Triton X305 column.

The peak marked *S*, due mostly to solvent, may mask other very volatile compounds (e.g. acetaldehyde, reported in Bartlett pear volatiles by Harley and Fisher, 1927; Lim, 1963).

Peaks 1, 3, 4, and 5 probably represent methyl acetate, ethanol, propyl acetate, and n-propanol, respectively, since the work of other investigators (Lim, 1963; Drawert, 1962) indicates that these compounds are present, and their retentions would agree with those of these peaks. Not enough of these materials were isolated to obtain infrared spectra.

The infrared spectra of peaks 26, 28, 34 and a component of peak 36 (Figs. 2–5) are respectively

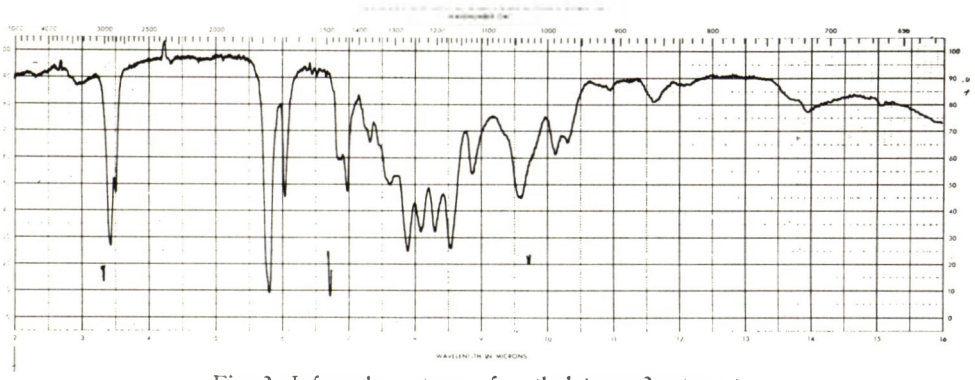


Fig. 2. Infrared spectrum of methyl *trans*:2-octenoate.

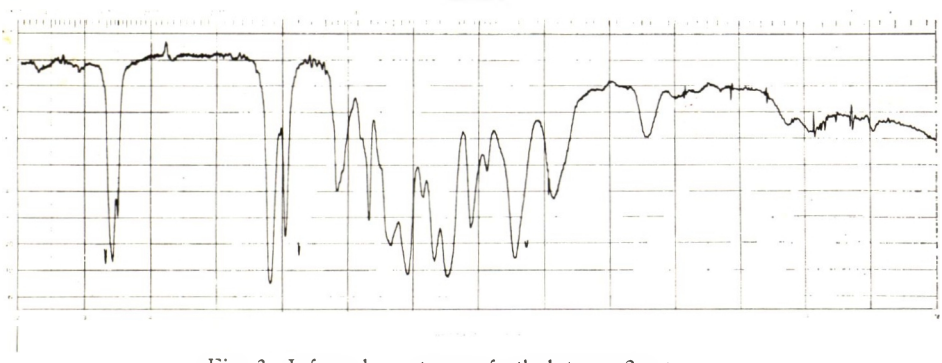


Fig. 3. Infrared spectrum of ethyl *trans*:2-octenoate.

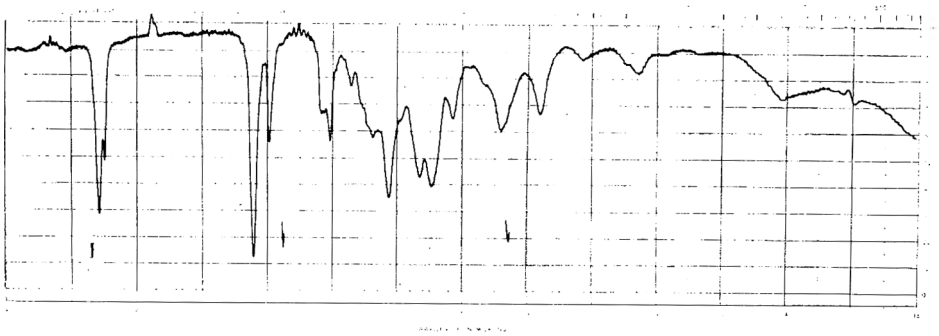


Fig. 4. Infrared spectrum of methyl *trans*:2-decenoate.

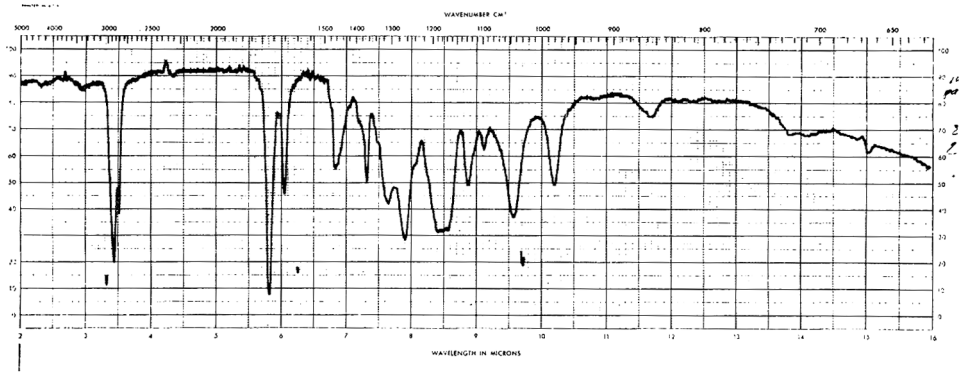


Fig. 5. Infrared spectrum of ethyl-*trans*:2-decenoate.

in agreement with methyl and ethyl *trans*:2-octenoate and methyl and ethyl *trans*:2-decenoate. Strong absorptions at 1725, 1660, and 980 $cm^{-1}$ are characteristic of the conjugated unsaturated esters containing the *trans* double bond (Nakanishi, 1962; Bellamy, 1958). The retention and the infrared spectra of the methyl and ethyl 2-octenoate and the methyl and ethyl 2-decenoate match those of the authentic samples. The infrared spectrum of the methyl 2-octenoate agrees with that reported for the *trans* isomer (Mercuri *et al.*, 1964).

The infrared spectra of peaks 30 and 32 (Figs. 6, 7) respectively match those of methyl and ethyl decanoate. The retentions of these compounds also agree with those of saturated esters.

Peaks 31 and 33 were identified as the methyl and ethyl *cis*:4-decenoate by their infrared (Figs. 8, 9), nuclear magnetic resonance (NMR), and mass spectra. The compounds were separated from their saturated homologs by successive injections on several chromatographic columns.

The mass spectrum of the ethyl *cis*:4-decenoate showed a monounsaturated ester with a molecular weight corresponding to $C_{12}H_{22}O_2$ (parent peak, P, m/e 198) and a peak at m/e 88 indicated the ethyl ester (Biemann, 1962; Silverstein and Bass-

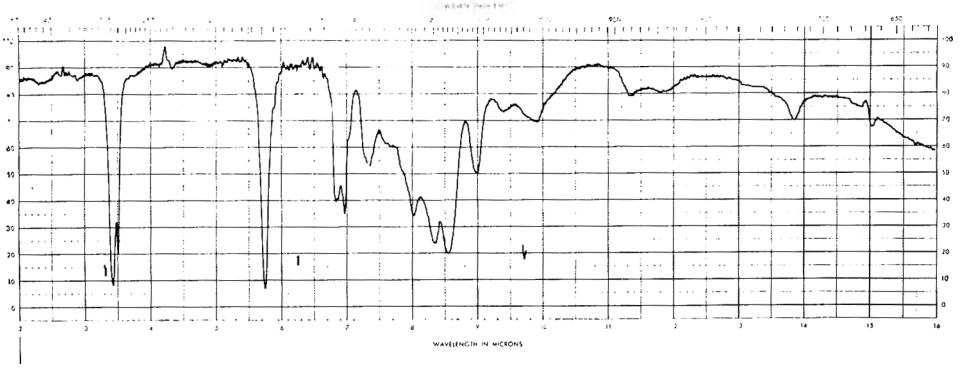


Fig. 6. Infrared spectrum of methyl decanoate.

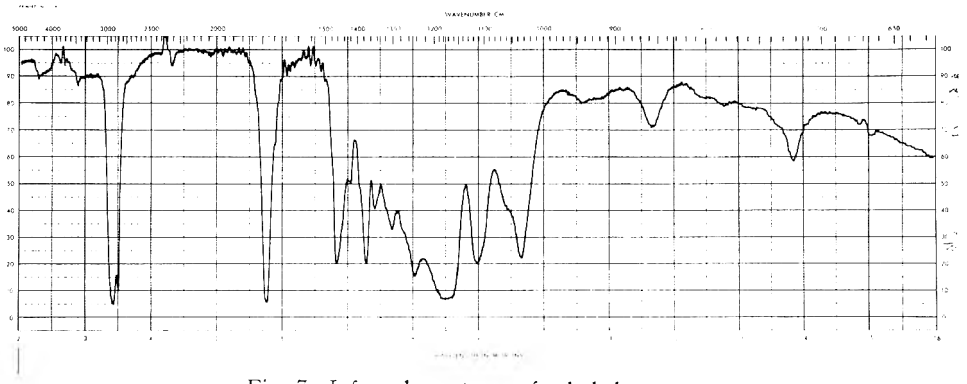


Fig. 7. Infrared spectrum of ethyl decanoate.

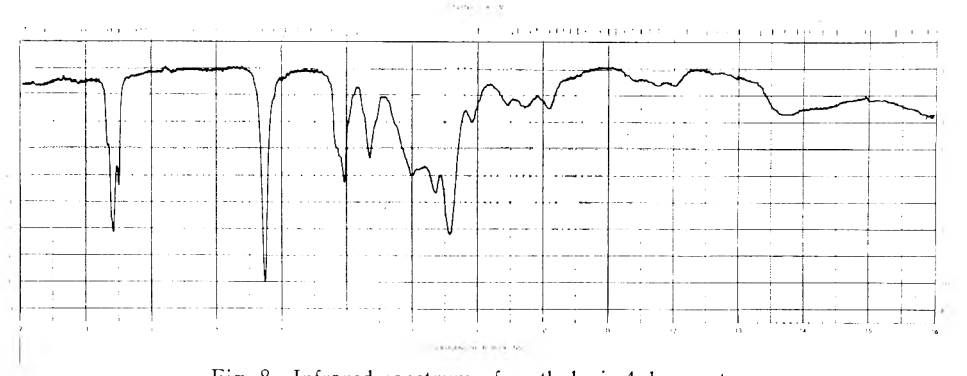


Fig. 8. Infrared spectrum of methyl *cis*:4-decenoate.

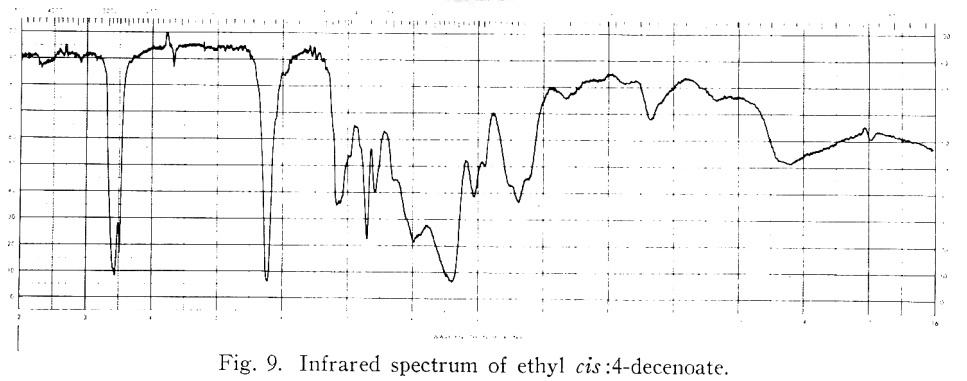
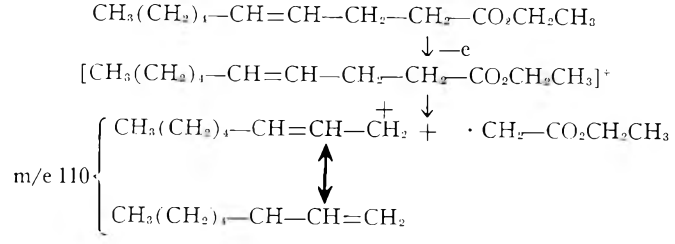
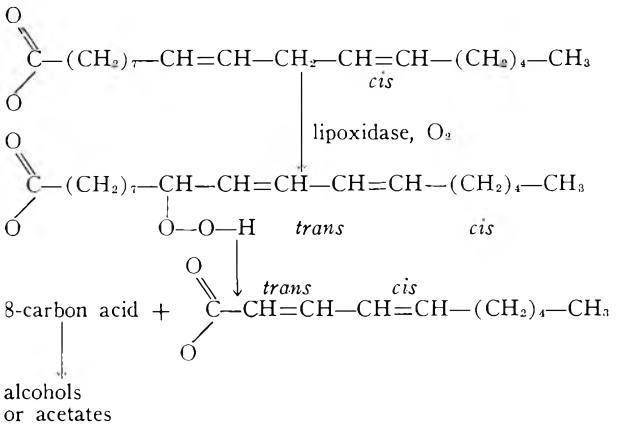


Fig. 9. Infrared spectrum of ethyl *cis*:4-decenoate.

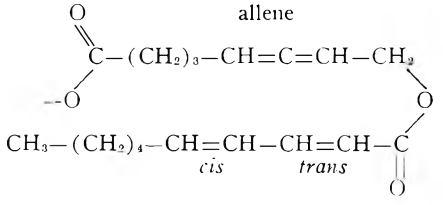
ler, 1963). The largest mass above m/e 88 occurred at m/e 110. This intense peak can be accounted for by fragmentation of the double bond (Biemann, 1962) at the 4 position:

Biemann (1962) pointed out that double bonds can migrate with extreme ease via successive shifts of hydride ions and hydrogen radicals, which would suggest that cleavage of this type would also pro-






The lipoxidase reaction leading to the peroxide is well established (Tappel, 1961; Holman and Bergstrom, 1951). A related, optically active compound




has been found in higher plants (Sprecher *et al.*, 1964) as the tetraestertriglyceride.

Considerably more attention should be directed toward studies to elucidate the source and function of these compounds in fruit. If the volatile esters isolated in this study do arise from unsaturated fatty acids, it seems possible that the superior flavor developed by low-temperature storage could be attributed to increased production of the unsaturated acids.

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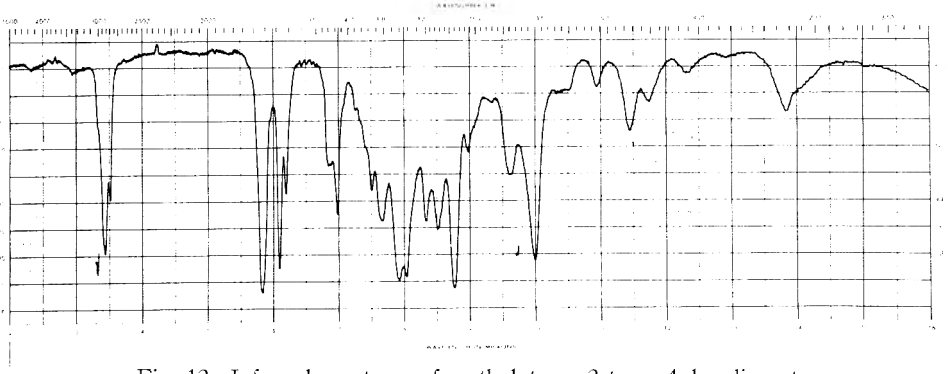


Fig. 12. Infrared spectrum of methyl *trans*:2-*trans*:4-decadienoate.

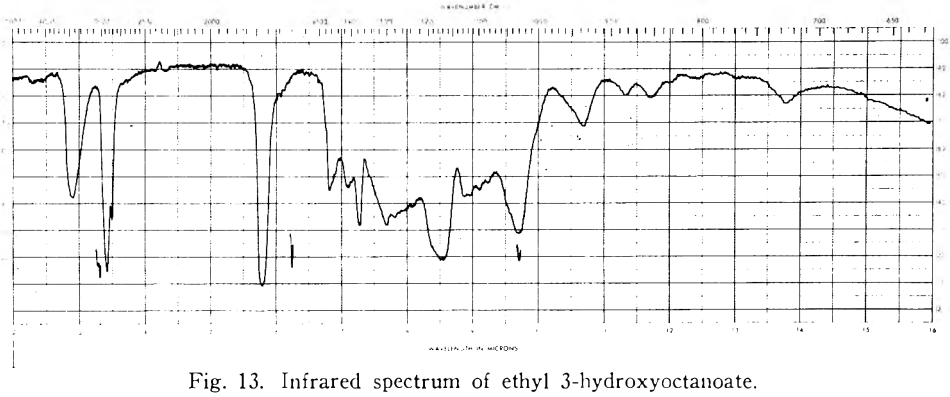


Fig. 13. Infrared spectrum of ethyl 3-hydroxyoctanoate.

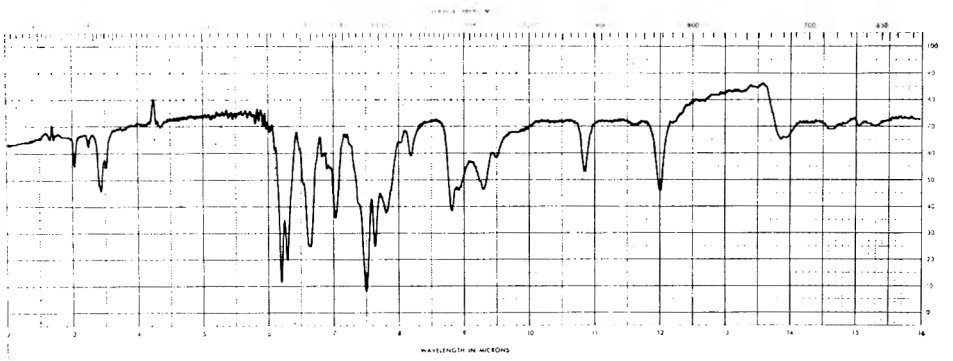
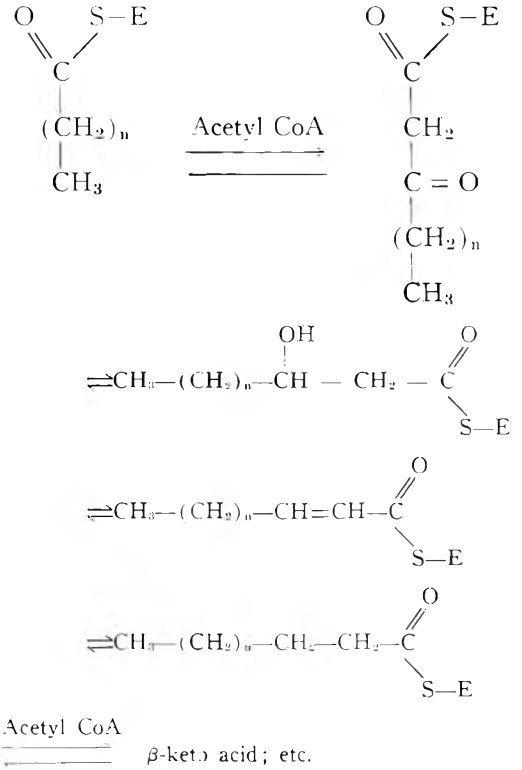


Fig. 11. Infrared spectrum of 2,4-dinitrophenylhydrazone of 2-heptanone (10% in $CCl_4$).

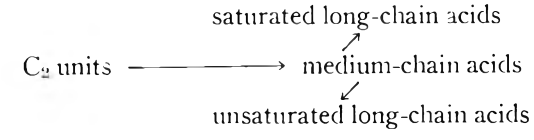
pounds, and possibly the other 8- and 10-carbon acid esters found in the pear volatiles, may be intermediates in lipid oxidation or biosynthesis.

Condensation reactions involving acetyl-S-CoA and malonyl-S-CoA for fatty acid synthesis are proposed to be enzyme-bound with no free intermediates (Bressler and Wakil, 1961). The following scheme (Lynen, 1962; Vagelos, 1964) represents a repeating cycle which ceases at some certain chain length, when the acyl group separates from the enzyme E:




All of these proposed enzyme-bound intermediates can be found among the pear volatiles as esters when $n = 6$, except for the beta-keto acid. This suggests the possibility that these enzyme-bound intermediates may be released from the cycle through the senescing of the fruits. The biosynthesis of the unsaturated fatty acids may likewise lead to the other 10-carbon esters with the *cis* double-bond 6 carbons removed from the methyl-terminal end of the molecule.

Goldfine and Block (1961) have found that octanoate and decanoate are precursors of both saturated and unsaturated long-chain fatty acids, suggesting the pathways may be




Again, these last steps may be interrupted through the senescing fruits releasing the intermediate medium-chain acids.

It is doubtful that $\alpha$-oxidation or $\omega$-oxidation of fatty acids is involved in the formation of the pear volatiles, because only $\beta$-hydroxy acid esters and even-numbered carbon mono-acids are found among the pear volatiles. $\beta$-oxidation, however, could account for both the volatile pear esters and the acetates.

Similary, a plausible case could be constructed for the production of these $C_8$ and $C_{10}$ esters by oxidation of the longer-chain polyunsaturated *cis,cis* unconjugated fatty acids by lipoxidase. One such pathway could follow the scheme:

duce peaks at P −29, P −43, P −57, P −71, and P −85 as well as the peak at 110. These peaks are observed at m/e 169, 155, 141, 127, and 113.

The NMR spectrum of the methyl *cis*:4-decenoate shows a sharp peak at 6.40 $\tau$, a complex multiplet centered at 4.69 $\tau$, a doublet at 7.72 $\tau$, a complex peak centered at 8.71 $\tau$ with a shoulder at 8.55 $\tau$, a triplet centered on 9.10 $\tau$. A large sharp singlet peak occurred at 7.93 $\tau$ which may have been due to acetone impurities. With this exception, the spectrum agrees with that reported (Buttery *et al.*, 1963) for the methyl *cis*:4-decenoate.

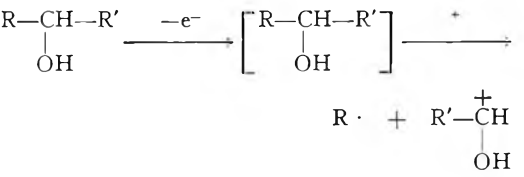
The infrared absorption spectra of both the methyl (Fig. 8) and ethyl *cis*:4-decenoate (Fig. 9) are characterized by ester absorptions at 1735, 1190, 1160 $cm^{-1}$ (Nakanishi, 1962; Bellamy, 1958). Weak absorptions at 3005, 1650, and 725 $cm^{-1}$ and the lack of strong absorptions near 980 $cm^{-1}$ indicate a *cis* double bond not conjugated with the carbonyl (Nakanishi, 1962; Bellamy, 1958). The infrared spectrum of the methyl *cis*:4-decenoate and its retention exactly match those of authentic sample.

The esters were finally hydrogenated using a gas chromatograph employing $H_2$ carrier gas and a platinum catalyst bed in the injector system (Sevenants and Jennings, 1966). The products, identified by infrared spectroscopy, were methyl and ethyl decanoate. The spectra agree with those shown in Figs. 6 and 7.

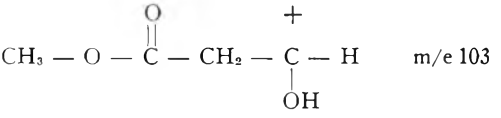
Peak 36 was separated on Apiezon L into three compounds. One of these, the ethyl 2-decenoate, has been discussed previously. It occurs in quite small amounts when compared with the other two compounds found in peak 36. The infrared spectrum and retention of the compound present in the largest amount match those of the synthesized methyl *trans*:2-*cis*:4-decadienoate (Jennings *et al.*, 1964).

The second compound isolated from peak 36 was shown to be the methyl 3-hydroxyoctanoate by its infrared (Fig. 10) and mass spectra, and the formation of a derivative. The infrared spectrum of the hydroxyl ester has a strong band at 3460 $cm^{-1}$ due to the hydroxy stretching, and at 1725 $cm^{-1}$ due to the ester carbonyl (Nakanishi, 1962). The band at 1120 $cm^{-1}$ could be attributed to the secondary hydroxyl group (Nakanishi, 1962), while the bands at 1165 $cm^{-1}$ and 1440 $cm^{-1}$ are consistent with the methyl ester (Nakanishi, 1962; Bellamy, 1958).

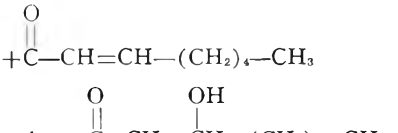
The mass spectrum of this ester shows a molecular weight corresponding to $C_9H_{18}O_3$ (small peak at m/e 174). A large peak at m/e 74 shows the methyl ester, while the large peak at m/e 156 (parent peak, 18) is consistent with a secondary or primary hydroxide (Biemann, 1962). The largest peak of the spectrum, at m/e 103, can be used to locate the hydroxyl group on the $\beta$-carbon. Biemann (1962) pointed out that secondary alcohols show strong peaks due to the following fragmentation:



For the methyl 3-hydroxy octanoate, the fragment which is consistent with this process is:



Other peaks consistent with the hydroxy ester are at m/e 125 and 143, which can be accounted for by the fragments:



(Biemann, 1962).

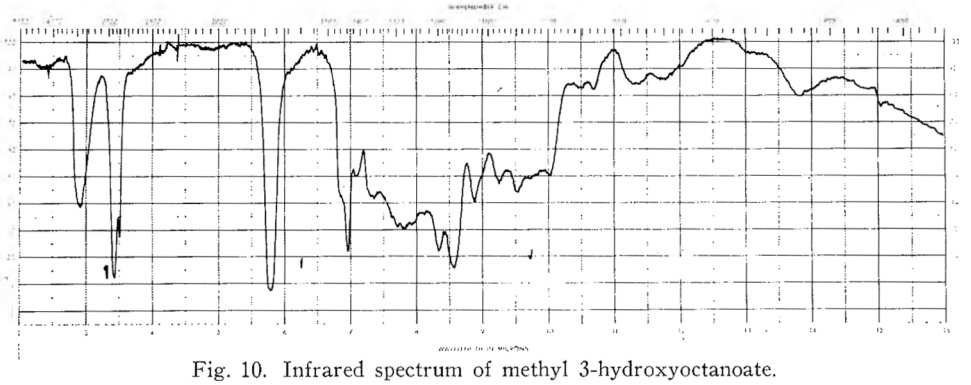
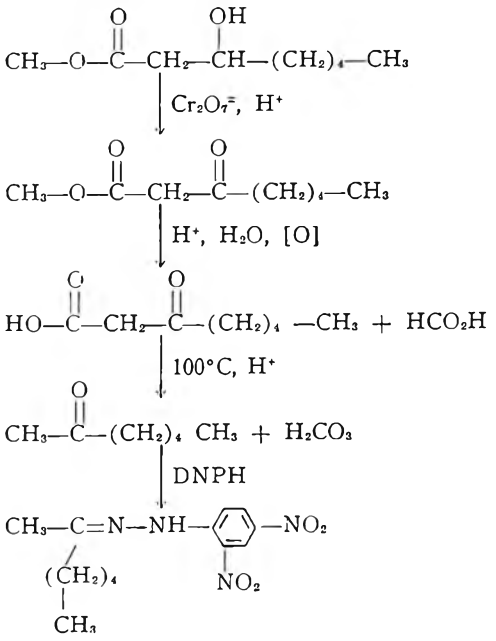


Fig. 10. Infrared spectrum of methyl 3-hydroxyoctanoate.

Evidence for assigning the location of the hydroxyl group to the β-carbon of methyl 3-hydroxyoctanoate was obtained by oxidation, hydrolysis, decarboxylation and formation of the DNPH from the methyl ester. The reaction sequence can be represented as follows:




The infrared spectrum (Fig. 11) of the DNPH matched the infrared spectrum of the DNPH of 2-heptanone. The spectrum shows no carbonyl stretching or acid function, which would be expected if the hydroxyl were in any position except β. Moreover, the decarboxylation step would be expected to proceed under these reaction conditions only if the hydroxy were in the β-position.

When rechromatographed on Apiezon L, peak 37 was also found to contain three compounds. The latter two compounds, collected from Apiezon L, were shown to be ethyl *trans*:2-*cis*:4-decadienoate and methyl *trans*:2-*cis*:4-decadienoate. The infrared spectra and retention of the major compound obtained from peak 37 match precisely those of the synthesized ethyl *trans*:2-*cis*:4-decadienoate reported by Jennings *et al.* (1964). The infrared spectrum of the methyl diunsaturate (Fig. 12) agrees with that reported by Crombie (1955) for methyl *trans*:2:*trans*:4-decadienoate.

The third compound isolated from peak 37 was shown to be the ethyl 3-hydroxyoctanoate by its infrared and mass spectra. The infrared spectrum of the hydroxyl ester (Fig. 13) was quite similar to that of the methyl 3-hydroxyoctanoate (Fig. 10), with the hydroxyl stretching appearing at 3460 $cm^{-1}$ and the ester carbonyl appearing at 1725 $cm^{-1}$. Some differences do occur in the regions 1500–1300 $cm^{-1}$ and 1100–900 $cm^{-1}$. Katritzky *et al.* (1960) pointed out that absorptions in these regions are due to the scissoring, rocking, and skeletal vibrations of the alcohol moieties.

The mass spectrum of this hydroxyl ester shows a molecular weight corresponding to $C_{10}H_{20}O_3$ (small peak at m/e 188). A large peak at m/e 88 is in agreement with the ethyl ester, and the peak at m/e 170 is consistent with a typical hydroxide, parent peak —18 (Biemann, 1962). The largest peak at m/e 117 is in agreement with the 3-hydroxyl ester (Biemann, 1962) and represents the fragment:



m/e117

Peak 39 was tentatively identified as the ethyl *trans*:2,*trans*,4-decadienoate by its infrared spectrum (Fig. 14), characterized by absorptions at 1720, 1647, 1617, 1250, 1204, 1177, 1142, 1112, 1041, and 1002 $cm^{-1}$, all of which are in exact agreement with the infrared spectrum for the methyl *trans*:2,*trans*,4-decadienoate (Fig. 12). The absorption bands which do not match the methyl trans:2-trans:4 ester occur in the regions of 1500–1300 $cm^{-1}$ and in the fingerprint regions which were reported by Katritzky *et al.* (1960) to be due to the alcohol moieties.

Peaks 6, 7, 8, 11, 13, 15, 18, 22, 29, 35, 38, and 40 through 45 could not be collected in sufficient amounts in order to obtain suitable spectral evidence for their identifications. Peaks 21 and 24 were shown to be degradation products arising from air oxidation of 2,4-decadienoate and are elsewhere identified as the methyl and ethyl 4-oxy*trans*butenoate (Heinz and Jennings, 1966).

## DISCUSSION

To produce fruit of maximum flavor, Bartlett pears are ideally stored at −1 to 0°C for approximately 60 days and ripened at 20°C (Claypool *et al.*, 1958). Zill and Cheniae (1962) reported that low temperatures encourage the production of unsaturated fatty acids rather than their saturated counterparts. Romani *et al.* (1965) have shown that although long-chain fatty acids ($C_{16}$, $C_{18}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$) exist in pears, the unsaturated fatty acids decrease with relation to their saturated homologs during ripening at 20°C. Heinz *et al.* (1965a) found that the decadienoates increase rapidly during the ripening process. These observations suggest that these com-

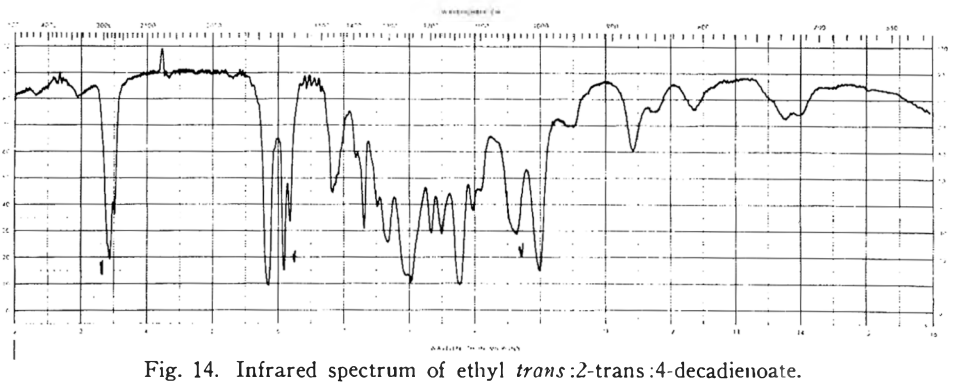


Fig. 14. Infrared spectrum of ethyl *trans*:2-trans:4-decadienoate.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The authors are grateful for the cooperation of the Gerber Products Company, who supplied the aqueous essence used in this study, and to the Campbell Soup Company for a pre-doctoral Fellowship which supported DEH during a portion of this period. This project was supported by Research Grant EF0092 of the Division of Environmental Engineering and Food Protection, USPHS.

From a Ph.D. thesis by D. E. Heinz in Agricultural Chemistry.

**M. R. SEVENANTS** [a] **and W. G. JENNINGS**
*Department of Food Science and Technology, University of California*
*Davis, California 95616*

# Volatile Components of Peach. II

## SUMMARY

**A volatile-essence concentrate isolated from the Red Globe variety of freestone peaches and already partially characterized, was further fractionated by newly developed gas chromatographic techniques of repetitive ultra micro collections and reinjections. An adapted microcavity infrared cell permitted infrared analysis of these minute fractions, which were then identified by comparison with known compounds. Synthesis and ultra-micro vapor-phase hydrogenation were used to characterize compounds for which standards could not be obtained. Compounds identified include hexyl formate, hexyl acetate, *trans*-2-hexenyl acetate, hexyl alcohol, *trans*-2-hexene-1-ol, isovaleric acid, ethyl benzoate, benzyl acetate, caproic acid, *gamma*-heptalactone, *gamma*-nonalactone, hexyl benzoate, and an *alpha*-pyrone.**

## INTRODUCTION

In earlier investigations on the volatile components of peaches, Daghetta *et al.* (1956) and Lim (1963) reported a series of low-molecular-weight compounds, including acetaldehyde, methyl acetate, ethyl acetate, ethyl alcohol, hexyl acetate, and acetic acid. Power and Chesnut (1921, 1922) reported that peach volatiles included linalool, and suggested that the aroma of freestone peaches was due primarily to linalyl esters of formic, acetic, valeric, and caprylic acids. Their findings have not been confirmed, and the present study found no traces of linalyl esters or other terpene derivatives in Red Globe peach essence.

## METHODS

**Peach essence.** The peach essence investigated in this work was the same material described earlier (Jennings and Sevenants, 1964).

**Gas chromatography.** The gas chromatograph and columns used were the same as those in the previous work (Jennings and Sevenants, 1964). The two columns, composed of 5% Carbowax 20*M* on Gas Pack-F and 20% Apiezon L on silanized Chromosorb W, retained their characteristics over two years of repeated use and exhibited no noticeable change in resolution or relative retentions.

[a] Present address, Winton Hill Technical Center, Procter and Gamble Co., Cincinnati, Ohio.

Initial separations were accomplished on the Carbowax 20*M* column, which gave good resolution with a 1-hr linear program from 50° to 230°C. Injections of 5–10 $\mu$l yielded fractions ranging from a barely detectable mist adhering to the capillary wall to *ca.* 1 $\mu$l of the more abundant compounds. The individual fractions were trapped in thin-walled glass capillaries. Fractions not immediately utilized were sealed and stored at −15°C. Fractions initially collected from Carbowax 20*M* were usually rechromatographed on Apiezon L.

Because the very small amounts of material available precluded the use of micro-syringes for reinjections, special techniques were devised. The fractions contained in the glass capillaries were reinjected by inserting the capillary tube containing the trapped fraction into an external injector consisting of ⅛ × 6-inch stainless-steel tube fitted with a side-opening syringe needle and connected to the gas shunting valve (Fig. 1). A 500-watt

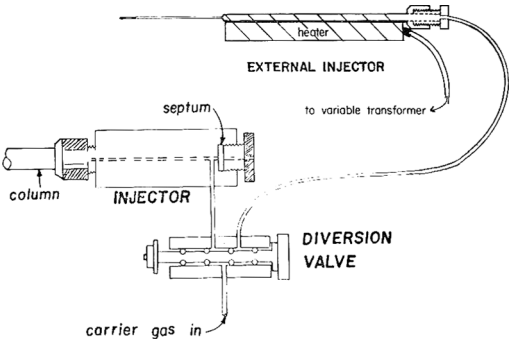


Fig. 1. External injector and two-way carrier-gas shunting valve.

cartridge heater was used to heat the system. The external injector was loaded, inserted through the injector gasket of the chromatograph, and heated to volatilize the sample, and the carrier gas was shunted by means of the two-way valve. The fraction flashed onto the chromatographic column and the temperature program was started. Reinjection and purification of very minor components was accomplished by collecting the same fractions from a series of injections and packing the several capillary sections into the external injector. Programs requiring 60 min from 50° to 230°C at a linear rate of increase were usually sufficient, depending on the retention range of the fraction being examined. Resolved fractions which, when rechromatographed on this dissimilar column, still ap-

peared as single components were then subjected to infrared analysis.

**Hydrogenation.** Further modifications of the external injection system permitted hydrogenation of ultra-micro quantities of material with essentially complete recovery. The apparatus described by Mounts and Dutton (1965) was simplified for use with the two-way shunting valve. A small quantity of 60–80-mesh Gas Pack-F was mixed with sufficient chloroplatinic acid in methanol to give a 3% w/w coating of platinum. The methanol was evaporated and the fresh catalyst packed into the external injector between loose plugs of glass wool. Hydrogen carrier gas was allowed to saturate the catalyst and purge the chromatographic column for 2 hr before use.

A glass capillary containing the compound to be hydrogenated was placed in the void before the catalytic chamber and heated rapidly (not exceeding 200°C), and the hydrogen was shunted to sweep it onto the column.

**Infrared analysis.** Samples were prepared as thin films in a Beckman ultra-micro NaCl cell or an adapted ultra-micro NaCl CIC cavity cell, and spectra were determined on the Beckman IR-5 spectrophotometer with a Beckman beam condenser. The modifications carried out on the CIC cavity cell consisted of cleaving the cell, grinding down one inside face, and resealing the two halves with epoxy resin. The modified cavity cell possessed a tapered, or wedged-shaped, sample compartment, as compared to the original parallel compartment of uniform 0.05-mm pathlength. The pathlength varied from a thin film to *ca.* 0.03 mm, and by varying the position in the cell holder the desired transmission readings were readily obtained. The desirable pathlength for oxygenated compounds ranged from a thin film to 0.025 mm and up to 0.03 mm for hydrocarbons. The sample volume of the adapted cell was 0.11 $\mu$l, but suitable spectra could be obtained with less, provided that part of the sample compartment passing energy was flooded.

Filling of the adapted cell, especially with very volatile fractions and fractions of less than 0.1 $\mu$l, was achieved by sealing off one end of the glass capillary containing the collected fraction about 2 cm from the liquid, and drawing out the other end (as close as possible to the liquid) to a very fine, quickly tapering point, which was then sealed. When the larger end was warmed and the fine capillary tip cooled, the liquid moved into the fine tip, where it formed a liquid plug. The tip was then broken off and touched to the cell cavity. Capillary forces conducted the fraction smoothly into the cell.

**Synthesis and stock chemicals.** Trans-2-hexenyl acetate was synthesized from trans-2-hexenal (purchased from K&K Laboratories, Plainview, New York). Excess sodium borohydride in methanol was used to reduce a 20-$\mu$l sample of the aldehyde to trans-2-hexenol, whose structure was verified by comparing its infrared spectrum with that taken of a sample supplied by Dr. A. D. Webb of this campus (this same sample was used to confirm the presence of trans-2-hexenol). Esterification of the alcohol was carried out with excess acetic anhydride (50 $\mu$l) and a drop of pyridine as catalyst. The reaction mixture, in a stoppered 2-ml conical test tube, was placed for 10 min in a hot-water bath in an ultrasonic generator (Acustica Associates, Los Angeles, California).

Purification of the trans-2-hexenyl acetate was carried out by gas chromatography utilizing a ¼-inch × 10-ft SE-30 column. An isothermal temperature of 130°C separated the ester from the acetic acid and unreacted acetic anhydride; unreacted alcohol was not detected on the chromatogram.

Chemicals used to verify the structures of the essence constituents and to make up a composite synthetic peach essence were obtained from the following sources: hexyl formate, hexyl acetate, gamma-caprolactone, gamma-heptalactone, gamma-octalactone, gamma-nonalactone, and isovaleric acid were purchased from K&K Laboratories, Plainview, New York; benzyl alcohol, benzaldehyde, acetic acid, n-caproic acid, acetaldehyde, methyl acetate, ethyl acetate, ethyl benzoate, hexyl benzoate, and benzyl acetate were purchased from Eastman Organic Chemicals, Rochester, New York; gamma-decalactone and delta-decalactone were graciously supplied by Hubert M. Cole for Firmenich Inc., New York, N. Y. All were purified by gas chromatography.

## RESULTS AND DISCUSSION

Fig. 2 is a typical chromatogram obtained from the ⅛-inch Carbowax 20M column used for preparative analysis of the peach essence. It is probably misleading to attempt to draw quantitative conclusions from this chromatogram. The relative ratios of compounds in the concentrated essence extract are almost certainly different from those in the original peach. To minimize heat-produced volatile components and encourage recovery of aroma molecules entrapped in and adhering to flesh particles, the aqueous essence was produced by vacuum steam distillation (Jennings and Sevenants, 1964). Recovery efficiencies of the compounds included in this broad spectrum undoubtedly vary. The aqueous distillate recovered did possess a peach aroma, somewhat more in-

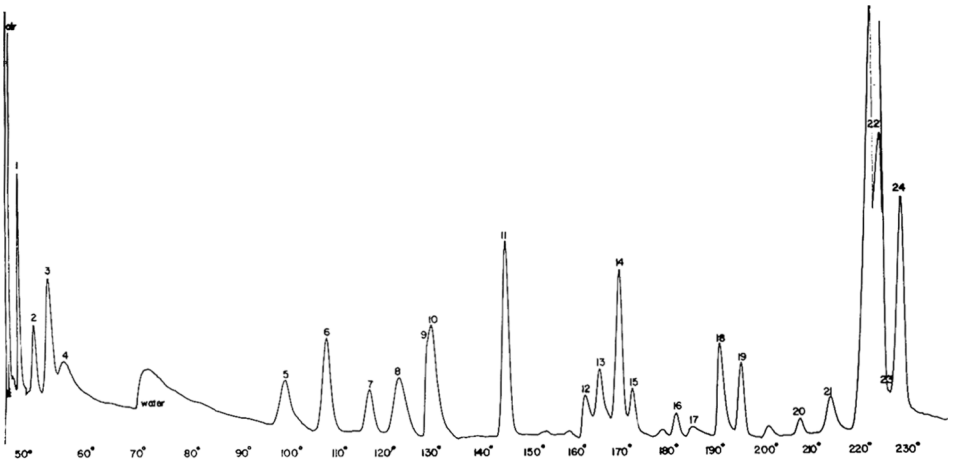


Fig. 2. Chromatogram of Red Globe peach essence.

tense than that of the fresh fruit, but also had a discernible cooked quality. This cooked aroma could have resulted from production of additional odorous compounds, changes in the ratios of volatile compounds, loss of the characteristic fresh odor by exclusion of molecules possessing very low vapor pressure, or chemical changes. Further changes in relative concentrations were probably caused by the extraction of the aqueous distillate, since the varied partition coefficients would have influenced the final ratios of compounds migrating to the ether phase.

The peaks identified are numbered and correspond to the compounds listed in Table 1. Peaks 1–4 represent compounds too volatile to confine in the ultramicro IR cell, and were tentatively identified by retention times on two dissimilar columns; these values are given in Table 2.

The Carbowax 20M column gave the best resolution, resulting in chromatograms which were more definitive than those obtained from numerous other liquid phases. The temperature program was interrupted or slowed at specific times to achieve greater resolution for the collection of particular fractions.

Preliminary infrared examination of fractions collected directly from Carbowax 20M showed impurities to be present in almost all cases, indicating the need for further purifications. Attempts to recover samples from glass capillaries by a micro syringe, with or without solvent additions, were unsatisfactory because of losses due to syringe hold-up. By placing the capillary-tube trap directly in the external injector, essentially complete reinjection was achieved. This permitted individual fractions trapped from one chro-

Table 1. Peaks and volatile compounds of peach.

| Peak no. | Compound [a] |
|---|---|
| 1 | Acetaldehyde (R)[b] |
| 2 | Methyl acetate (R)[b] |
| 3 | Ethyl acetate (R)[b] |
| 4 | Ethyl alcohol (R)[b] |
| 5 | Hexyl formate (IR) |
| 6 | Hexyl acetate (IR)[c] |
| 7 | *trans*-2-hexenyl acetate (IR, D,S) |
| 8 | Hexyl alcohol (IR) |
| 9 | Acetic acid (IR) |
| 10 | *trans*-2-hexene-1-ol (IR,S) |
| 11 | Benzaldehyde (IR)[c] |
| 12 | Isovaleric acid (IR) |
| 13 | Ethyl benzoate (IR) |
| 14 | gamma-caprolactone (IR)[c] |
| 15 | Benzyl acetate (IR) |
| 16 | gamma-heptalactone (IR) |
| 17 | Caproic acid (IR) |
| 18 | Benzyl alcohol (IR)[c] |
| 19 | gamma-octalactone (IR)[c] |
| 20 | gamma-nonalactone (IR) |
| 21 | Hexyl benzoate (IR) |
| 22 | gamma decalactone (IR)[c] |
| 23 | alpha-pyrone (IR, D) |
| 24 | delta-decalactone (IR)[c] |

[a] R, retention data; IR, infrared data; D, derivative; S, synthesis.

[b] Previously identified by Lim (1963).

[c] Previously identified by Jennings and Sevenants (1964).

Table 2. Retention times of the essence components not confirmed by infrared analysis.

| Compound as it appears on chromatogram (Fig. 3) | Compound name | Relative retention time in minutes[a] | | | |
|---|---|---|---|---|---|
| | | Carbowax 20M[b] | | Apiezon L[c] | |
| | | Essence | Knowns | Essence | Knowns |
| 1 | Acetaldehyde | 1.17 | 1.11 | 0.09 | 0.09 |
| 2 | Methyl acetate | 2.79 | 2.85 | 0.81 | 0.84 |
| 3 | Ethyl acetate | 4.32 | 4.29 | 1.77 | 1.71 |
| 4 | Ethyl alcohol | 5.94 | 5.97 | 0.45 | 0.45 |

[a] Air equal to zero time.
[b] 10-ft × ⅛-in. column 5% stationary phase on 45/60 Gas Pack-F, 60°C, 20 cc/min.
[c] 10-ft × ⅛-in. column, 20% stationary phase on 60/80 silanized Chromosorb W. 50°C, 20 cc/min.

matographic column to be reinjected on a dissimilar column, and each resultant fraction was then subjected to infrared analysis.

The infrared spectra of fractions identified as hexyl formate, hexyl acetate, acetic acid, isovaleric acid, ethyl benzoate, benzyl acetate, *gamma*-heptalactone, caproic acid, *gamma*-nonalactone, and hexyl benzoate were found to match the infrared spectra of the appropriate known.

To further characterize certain peach fractions whose infrared spectra indicated unsaturation (e.g. peak 7, Fig. 3; peak 24, Fig. 5), the external injector was used for microhydrogenation. Beroza (1962) reported that unsaturated fatty acid esters gave fission products with catalyst temperatures maintained at 280°C and a hydrogen flow rate of 20 ml per minute. Mounts and Dutton (1965) observed no fission products at 200°C and a hydrogen flow of 60 ml per minute. Fragmentations were not detected in the present study, which utilized minimum temperatures necessary to volatilize the un-

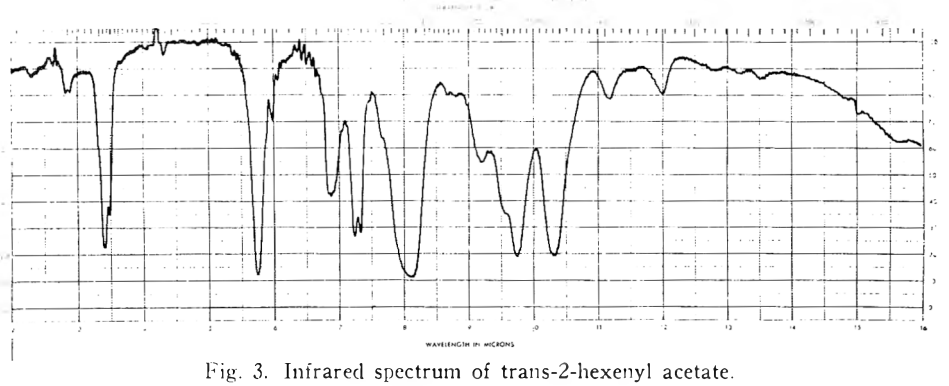

Fig. 3. Infrared spectrum of trans-2-hexenyl acetate.

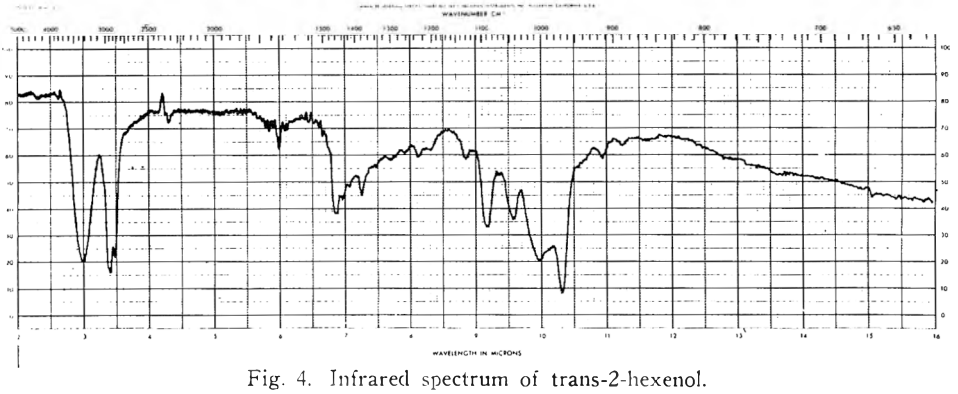

Fig. 4. Infrared spectrum of trans-2-hexenol.

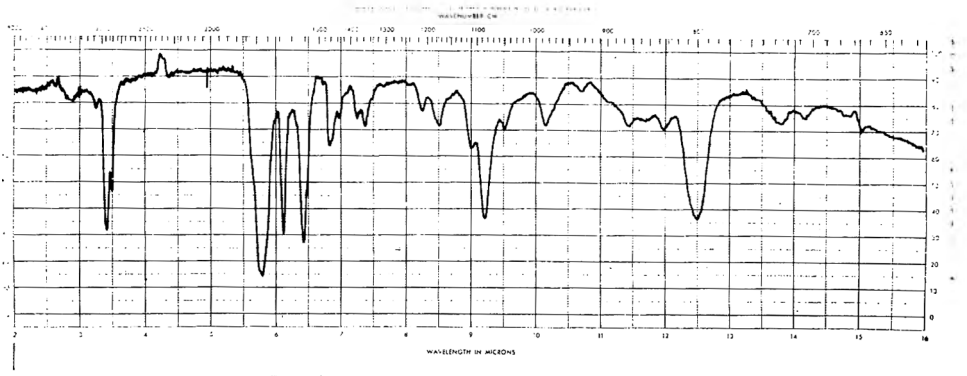

Fig. 5. Infrared spectrum of alpha-pyrone.

saturated compounds and flow rates of 20 ml per minute.

Performance tests were conducted on ethyl trans-2-cis-4-decadienoate and ethyl trans-2-hexeneoate, which require stringent conditions to hydrogenate compared to unconjugated systems. The chromatograms obtained during the hydrogenation reaction and the infrared spectra of the hydrogenated products indicated that the reactions proceeded smoothly and completely, with no apparent side reactions.

Hydrogenation of peak 8 of Fig. 2 gave a product that on infrared analysis proved to be identical to hexyl acetate. Comparison of the IR spectrum of the synthesized trans-2-hexenyl acetate with that of fraction 8 (Fig. 3) showed identical absorptions, thus verifying its structure.

Peak 21 of Fig. 2 consists primarily of gamma-decalactone. In earlier work, Jennings and Sevenants (1964) reported that an unsaturated compound co-chromatographed with gamma-decalactone and interfered with its purification. When the temperature program is halted at 180°C, the two compounds are separated on Carbowax 20M. Fig. 5 is the infrared spectrum of the unsaturate, which is evidently an alpha pyrone. This is supported by the split C=O absorptions at 1740 and 1725 $cm^{-1}$, and the pronounced C=C stretching bands at 1636 and 1553 $cm^{-1}$ (Nakanishi, 1962). The ultraviolet spectrum (in methanol) is not sharply definitive, with a broad absorption ranging from 250 to 340 m$\mu$. 5-Methyl-alpha pyrone exhibits a similar ultraviolet spectrum (Fried and Elderfield, 1941). Micro vapor-phase hydrogenation of the pyrone and infrared analysis of the product indicated an aliphatic carboxyllic acid as the only product. Vapor-phase hydrogenation at temperatures up to 200°C could have caused total opening of the lactonic ring by fission of the delta carbon-oxygen bond. Further structural elucidation will require isolating more of this component.

Acetaldehyde, methyl acetate, ethyl acetate, ethyl alcohol, acetic acid, isovaleric acid, hexyl acetate, and hexyl alcohol have been reported in many fruits; one or more of these compounds occur, e.g., in apples, pear, banana, grape, raspberry, strawberry, coffee, and cocoa. Hexyl formate has not been reported as a common constituent of fruits, although hexyl alcohol and formic acid have. Benzyl alcohol and benzaldehyde have been found in raspberry (Coppens *et al.*, 1939), and benzaldehyde was reported in cherry (Nelson and Curl, 1939), strawberries (McFadden *et al.*, 1965), and black currants (Andersson and von Sydow, 1964). Benzyl acetate has been reported in strawberries (McFadden *et al.*, 1965), and ethyl benzoate in black currants (Andersson and von Sydow, 1964). The gamma-lactones $C_6$—$C_{10}$ and delta $C_{10}$ lactone appear to be unique to peach among fruits. Trans-2-hexenol and its acetate have been reported in strawberry (Dimick and Makower, 1956).

**Olfactory analysis.** None of the compounds identified in this study possess a peach-like aroma in themselves, but some possess aromas of other fruit. The lactones are characterized by a coconut odor; trans-2-hexenyl acetate is reminiscent of pear; the

remaining esters yield strong fruity aroma. A composite mixture of all of the compounds identified, in proportions estimated from chromatograms of peach essence, was prepared and subjected to a panel of 5 judges trained to recognize the odors of various peach products. The composite mixture was diluted to intensities equivalent to the authentic peach products and in some cases added to peach preparations possessing very little aroma. Sample presentation and sensory methodology were the same as reported by Heinz *et al.* (1964). All judges recognized the synthetic mixture and agreed that it imparted an artificial odor reminiscent of peaches but of a definite synthetic nature. When amounts of iso-valeric acid were greatly increased, the diluted essence was more peach-like, but the aroma resembled that of cling rather than freestone peach.

The results indicate that the typical peach aroma is due not to one or two compounds, but is probably an integrated response to a wide spectrum of compounds whose individual aromas are not at all peach-like.

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Ms. rec'd 8/31/65.

Parts of this are from a Ph.D. thesis by MRS in Agricultural Chemistry.

The authors are grateful to Mrs. R. M. Pangborn for invaluable help in the sensory aspects of this study. This project was supported by Research Grant EF00092 of the Division of Environmental Engineering and Food Protection, U.S.P.H.S.

S. S. PAWAR [a] and N. G. MAGAR
*Department of Biochemistry, Institute of Science, Bombay 1, India*

# Chemical Changes During Frozen Storage of Pomphrets, Mackerel, and Sardines

## SUMMARY

**The effects of quick and slow freezing processes, with and without glazes, on the storage life of three commercially important fish from the Bombay Coast were studied. During storage, moisture content decreased, resulting in an apparent increase in total crude proteins. Glazing showed a definite advantage over control fish in the retention of moisture during storage. Sarcoplasmic ATP-ase activity decreased very slowly during storage. Total ribose decreased, whereas Ba-acetate alcohol nonprecipitable ribose increased to a different extent in all three fish. The ratio of Ba-acetate alcohol nonprecipitable ribose to total acid-soluble ribose (denoted as *R*) was found to be higher in slowly as against quickly frozen fish, indicating a greater extent of cell damage in the former. Large quantities of trimethylamine, nonprotein nitrogen and ribose were lost from the fish on thawing. Of all the glazes used, that containing citric acid was most effective in preserving the color of the fish. All glazes prolonged storage life by protecting from rancidity and desiccation.**

## INTRODUCTION

Because of high perishability, fish presents considerable difficulties in preservation in an acceptable condition. Fish has been preserved by dehydration (sun-drying), brining, curing, and fermenting for hundreds of years, but the advent of freezing and cold storage effected a decided improvement on these methods in that they preserve much of the natural fish characteristics such as appearance, color and flavor. However, freezing and cold storage do not necessarily always completely arrest deterioration. Oxidative and enzymatic changes can still proceed at low temperatures as well as protein denaturation reactions. These changes may be affected by factors such as rate of freezing, temperature of storage and protection against desiccation.

Since available data are scanty regarding the effect of freezing with and without glazes on the quality of Indian species of fish, an attempt was made to study the effect of different rates of freezing, with and without glazes, on the shelf-life of a fatty and a low-fat fish during cold storage.

## EXPERIMENTAL

The following fish were selected: pomphrets *(Stromateus cinereus)*; mackerel *(Rastrellager canagurta)*; and sardines *(Sardinella longiceps)*.

Two batches of pomphrets were respectively quick-frozen at −40°C (for 4 hr) and slow-frozen at −18°C (for 12 hr). Each batch was divided into four groups, and one group of each batch was used as a control while the remaining three groups were respectively given the following glazes: 1) ascorbic acid glaze (0.1%); 2) citric acid glaze (1%); and 3) sodium nitrite glaze (1%). These groups were then stored at −18°C, and the fish from each group were analyzed after thawing to determine the quality by chemical and organoleptic tests after 1, 4 and 7 months.

Mackerel were similarly frozen in two batches and divided into four groups, one group from each batch was used as a control, and the remaining three groups were respectively given the following treatments: 1) cold-water glaze. 2) sodium chloride glucose glaze (0.5% sodium chloride + 0.5% glucose); and 3) ascorbic acid glaze (0.1%). All the groups were stored at −18°C and analyzed to determine the quality as above.

Sardines were frozen both by slow-freezing (at −18°C for 12 hr in block form) and quick-freezing (at −40°C for 4 hr in block form) and stored at −18°C. Quality was similarly assessed by chemical and organoleptic tests after 5, 8, 12, 16, 22, and 29 weeks.

Drip was collected by placing the frozen fish in a large funnel and allowing them to thaw at room temperature for 2½ hr. In the case of block-frozen sardines, the fish were allowed to thaw after the adherent ice was scraped from them.

Moisture was determined by the AOAC method (1950, p. 342). Total nitrogen and nonprotein nitrogen (NPN) were determined by the Kjeldahl method (AOAC 1950, p. 13). For NPN, 5 ml of a trichloroacetic acid (TCA 5%) extract of the muscle was used for digestion. The total crude protein was calculated by multiplying total nitrogen (N) with 6.25.

[a] Present address: Department of Biochemistry, University of Texas Southwestern Medical School, Dallas 35, Texas, U.S.A.

The total acid-soluble ribose was estimated from a TCA (5%) extract of the muscle by Mejbaum's method (1939) as follows: To a 3-ml portion were added 3 ml of $FeCl_3$ reagent (0.1% $FeCl_3$ in conc. HCl) and 0.3 ml of orcinol solution (10% in 95% alcohol). The tubes were then heated for 40 min in a boiling-water bath, cooled, made up to 25 ml and the intensity of the green color developed was measured at 670 mμ. The Ba-acetate alcohol nonprecipitable ribose was determined from an aliquot (5 ml) of the supernatant obtained after centrifugation of TCA extract with saturated barium acetate in 30% alcohol at pH 8.2 by the color reaction as described above. The ratio of Ba-acetate alcohol nonprecipitable ribose to total ribose is denoted as $R$. For total amino nitrogen, 2–5 g of the muscle were hydrolyzed with 6$N$ HCl by autoclaving for 8–10 hr at a pressure of 15 psi. The hydrolysates were neutralized, made up to 25 ml and filtered. Amino nitrogen was then estimated by Spies and Chambers' method (1951). Free amino acid nitrogen was determined from neutralized TCA extracts by the above method. The volatile basic nitrogen was measured using 1 ml of TCA extract by the Conway and Byrne (1933) microdiffusion method.

TMA was estimated from TCA extracts by Dyer's method (1945). Thiobarbituric acid value (TBA value) was estimated by Yu and Sinnhuber's method (1957). The ATP-ase activity of aqueous extracts of the muscle was measured by Dubois and Potter's method (1943). For this 1 g of muscle was extracted with 10 ml ice cold water at 0°C and made up to 25 ml.

Samples of the stored fish, together with appropriate fresh samples were subjected to organoleptic appraisal of quality both in the raw and cooked form.

## RESULTS AND DISCUSSION

The results of analysis are in Tables 1–3.

**Moisture and total crude proteins.** The moisture content of all the fish decreased during frozen storage. This loss of moisture content of fish might have been due to desiccation as well as to loss of drip during thawing, causing increased dehydration of the tissues. The loss of moisture content resulted in an apparent increase in total crude protein. It was observed that all the slow-frozen groups showed a greater loss of moisture than the corresponding quick-frozen groups. Sawant and Magar (1961) also reported a decrease in moisture content of fish during frozen storage. The loss of moisture content was minimized by the use of glazes which protected the fish from desiccation during storage. In glazed fish, losses due to desiccation were less because moisture from the tissue was not lost till the glaze on the fish had evaporated. In the present experiment with pomphrets, citric acid was found to be the best of the glazes tested whereas sodium chloride glucose glaze was found more effective with mackerel. Dyer and Morton (1956) and Dyer *et al.* (1956) similarly protected the fish from desiccation by coating with moss gel and keeping in waxed cartons during frozen storage.

**Sarcoplasmic ATP-ase activity.** Sarcoplasmic ATP-ase activity decreased very slowly during frozen storage in both quick- and slow-frozen pomphrets.

**Ribose.** Ribose decreased to a different extent in the various fish groups during frozen storage, which might be due to its unequal leaching in the drip lost during thawing. The ratio of Ba-acetate alcohol nonprecipitable ribose to total acid-soluble ribose ($R$ value) for each group of fish increased during frozen storage. It was noted that $R$ was higher in slow-frozen fish than in corresponding quick-frozen fish, indicating a decrease in phosphorylated compounds that could be precipitated by barium acetate. Jones and Murray (1957), working with codling, reported increases in the Ba-acetate alcohol nonprecipitable ribose and decreases in total ribose during storage.

**Nitrogen compounds.** Trimethylamine content increased quite regularly in quick- and slow-frozen pomphrets. The maximum TMA values were found during the first month of storage, but a gradual decrease was observed thereafter, some part or all of which might have been due to losses in the drip after thawing. Numerous workers have reported the possible use of trimethylamine content in assessing spoilage. In the present investigation it was observed that measurement of TMA content during frozen storage is unlikely to be a reliable method of assessing the quality of frozen fish.

The total amino nitrogen decreased very slowly in the three fish studied.

During storage free amino nitrogen increased in the sardines, and NPN increased in all three species. Both observations indi-

Table 1. Chemical changes during frozen storage of pomphrets and mackerel at 3 storage periods (1, 4, and 7 months).

| | Fresh | Quick-frozen | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | One month | | | | Four months | | | | Seven months | | | |
| | | A | B | C | D | A | B | C | D | A | B | C | D |
| Pomphret | | | | | | | | | | | | | |
| Moisture (g%) | 80.6 | 75.3 | 77.9 | 78.1 | 77.8 | 70.2 | 73.0 | 74.3 | 73.1 | 68.8 | 70.9 | 72.0 | 71.3 |
| Total crude proteins (N × 6.25, g%) | 20.7 | 22.0 | 21.3 | 20.9 | 21.1 | 26.2 | 22.9 | 22.2 | 25.5 | 30.3 | 25.8 | 24.2 | 24.8 |
| Total acid-soluble ribose (mg%) (R2) | 172.2 | 158.1 | 161.3 | 163.7 | 161.5 | 97.0 | 120.3 | 128.6 | 123.8 | 62.1 | 82.8 | 100.0 | 84.20 |
| Ba-acetate alcohol non-precipitable ribose (mg%) (R1) | 5.9 | 8.2 | 6.7 | 6.5 | 6.7 | 25.0 | 18.6 | 16.5 | 17.2 | 34.2 | 23.9 | 22.7 | 23.0 |
| $R = \frac{R1}{R2} \times 100$ | 3.4 | 5.2 | 4.2 | 4.0 | 4.1 | 25.9 | 15.5 | 12.9 | 14.2 | 35.0 | 26.9 | 22.5 | 27.3 |
| Total nonprotein nitrogen (g%) | 0.27 | 0.29 | 0.28 | 0.27 | 0.28 | 0.37 | 0.33 | 0.32 | 0.32 | 0.49 | 0.41 | 0.40 | 0.40 |
| Total amino nitrogen (g%) | 2.48 | 2.18 | 2.28 | 2.32 | 2.32 | 2.16 | 2.28 | 2.31 | 2.36 | 1.78 | 2.01 | 2.14 | 2.17 |
| Trimethylamine nitrogen (mg%) | 0.38 | 0.59 | 0.32 | 0.33 | 0.31 | 0.62 | 0.41 | 0.42 | 0.44 | 1.03 | 0.87 | 0.62 | 0.83 |
| ATP-ase activity[a] | 892.5 | 810.0 | 873.4 | 880.3 | 869.7 | 637.4 | 712.8 | 739.1 | 129.0 | 552.3 | 680.1 | 699.1 | 689.5 |
| TBA value | 0.003 | 0.11 | 0.089 | .089 | 0.075 | 0.121 | 0.094 | 0.083 | 0.079 | 0.160 | 0.123 | 0.132 | 0.097 |
| | Slow-frozen | | | | | | | | | | | | |
| | One month | | | | | Four months | | | | Seven months | | | |
| | A | B | C | D | | A | B | C | D | A | B | C | D |
| Pomphret | | | | | | | | | | | | | |
| Moisture (g%) | 73.1 | 75.2 | 75.5 | 75.3 | | 67.9 | 70.2 | 71.8 | 71.6 | 64.9 | 67.1 | 70.1 | 69.3 |
| Total crude proteins (N × 6.25, g%) | 21.9 | 21.9 | 21.2 | 21.6 | | 27.8 | 23.3 | 22.9 | 24.3 | 31.5 | 27.3 | 26.7 | 26.9 |
| Total acid-soluble ribose (mg%) (R2) | 156.2 | 160.3 | 158.6 | 159.6 | | 95.6 | 113.7 | 120.5 | 120.1 | 60.9 | 75.9 | 90.3 | 80.1 |
| Ba-acetate alcohol non-precipitable ribose (mg%) (R1) | 9.2 | 6.9 | 6.9 | 6.8 | | 25.5 | 18.9 | 17.3 | 17.8 | 34.5 | 25.3 | 23.5 | 24.1 |
| $R = \frac{R1}{R2} \times 100$ | 5.9 | 4.3 | 4.4 | 4.3 | | 26.7 | 16.0 | 15.0 | 14.8 | 56.7 | 33.3 | 26.0 | 30.1 |
| Total nonprotein nitrogen (g%) | 0.30 | 0.30 | 0.29 | 0.29 | | 0.37 | 0.35 | 0.33 | 0.33 | 0.56 | 0.51 | 0.44 | 0.42 |
| Total amino nitrogen (g%) | 2.12 | 2.12 | 2.18 | 2.14 | | 11.90 | 1.95 | 2.20 | 1.97 | 1.57 | 1.73 | 1.89 | 1.77 |
| Trimethylamine nitrogen (mg%) | 0.71 | 0.53 | 0.58 | 0.57 | | 0.82 | 0.63 | 0.64 | 0.75 | 0.19 | 0.93 | 0.98 | 1.01 |
| ATP-ase activity[a] | 829.2 | 850.3 | 881.4 | 856.4 | | 635.2 | 719.5 | 741.0 | 731.0 | 560.4 | 678.3 | 695.7 | 691.4 |
| TBA value | | | | | | | | | | | | | |

A, control; B, ascorbic acid glaze; C, citric acid glaze; D, sodium nitrite glaze.

[a] Expressed as μg of P that would have been liberated by the extract containing 100 mg of wet tissue in 15 min of incubation at 37°C.

Table 1 (continued)

| | Quick-frozen | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fresh | One month | | | | Four months | | | | Seven months | | | |
| | | A | B | C | D | A | B | C | D | A | B | C | D |
| Mackerel | | | | | | | | | | | | | |
| Moisture (g%) | 74.6 | 71.2 | 71.5 | 72.4 | 72.3 | 68.4 | 69.7 | 70.5 | 69.8 | 63.6 | 64.1 | 65.3 | 64.6 |
| Total crude proteins (N × 6.25, g%) | 14.8 | 16.5 | 16.3 | 15.8 | 15.8 | 17.4 | 16.8 | 16.4 | 16.5 | 20.3 | 20.1 | 19.2 | 19.8 |
| Total acid-soluble ribose (mg%) (R2) | 96.2 | 87.4 | 88.4 | 88.6 | 88.5 | 69.8 | 71.5 | 73.3 | 71.9 | 33.6 | 36.5 | 39.9 | 37.4 |
| Ba-acetate alcohol non-precipitable ribose (mg%) (R1) | 1.4 | 4.2 | 3.3 | 3.1 | 3.3 | 11.8 | 10.6 | 9.8 | 10.3 | 19.4 | 18.6 | 15.5 | 16.7 |
| $R = \frac{R1}{R2} \times 100$ | 1.5 | 4.8 | 3.7 | 3.5 | 3.7 | 16.9 | 14.8 | 13.4 | 14.4 | 57.8 | 50.9 | 38.9 | 44.7 |
| Total nonprotein nitrogen (g%) | 0.29 | 0.32 | 0.31 | 0.31 | 0.31 | 0.44 | 0.40 | 0.39 | 0.47 | 0.54 | 0.49 | 0.46 | 0.49 |
| Total amino nitrogen (g%) | 2.20 | 1.85 | 1.96 | 2.00 | 1.98 | 1.64 | 1.80 | 1.90 | 1.84 | 1.15 | 1.34 | 1.40 | 1.30 |
| Trimethylamine nitrogen (mg%) | 0.70 | 1.82 | 1.30 | 1.14 | 1.28 | 1.00 | 0.80 | 0.75 | 0.65 | 0.80 | 0.65 | 0.58 | 0.60 |
| TBA value | .009 | .095 | 0.078 | 0.065 | 0.070 | 0.160 | 0.126 | 0.120 | 0.127 | 0.255 | 0.199 | 0.170 | 0.204 |

| | Slow-frozen | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | One month | | | | Four months | | | | Seven months | | | |
| | A | B | C | D | A | B | C | D | A | B | C | D |
| Mackerel | | | | | | | | | | | | |
| Moisture (g%) | 70.8 | 71.3 | 72.1 | 72.1 | 67.5 | 68.9 | 69.9 | 68.7 | 61.8 | 63.6 | 64.4 | 63.7 |
| Total crude proteins (N × 6.25, g%) | 16.8 | 16.4 | 15.9 | 15.9 | 18.1 | 17.2 | 16.8 | 17.0 | 21.3 | 20.7 | 19.9 | 20.4 |
| Total acid-soluble ribose (mg%) (R2) | 85.8 | 88.1 | 89.5 | 88.9 | 64.6 | 68.3 | 72.0 | 69.8 | 31.3 | 36.5 | 40.0 | 37.9 |
| Ba-acetate alcohol non-precipitable ribose (mg%) (R1) | 4.4 | 3.4 | 3.5 | 3.5 | 12.0 | 10.5 | 9.9 | 10.6 | 20.9 | 18.6 | 15.6 | 16.7 |
| $R = \frac{R1}{R2} \times 100$ | 5.1 | 3.9 | 3.9 | 4.0 | 18.6 | 15.4 | 13.8 | 15.2 | 66.6 | 50.9 | 39.0 | 44.2 |
| Total nonprotein nitrogen (g%) | 0.33 | 0.32 | 0.31 | 0.31 | 0.46 | 0.43 | 0.41 | 0.42 | 0.59 | 0.51 | 0.49 | 0.51 |
| Total amino nitrogen (g%) | 1.78 | 1.90 | 1.95 | 1.90 | 1.60 | 1.54 | 1.70 | 1.62 | 0.88 | 0.92 | 1.02 | 0.96 |
| Trimethylamine nitrogen (mg%) | 1.82 | 1.43 | 1.0 | 1.43 | 1.30 | 1.12 | 0.90 | 1.05 | 1.0 | 0.93 | 0.86 | 0.84 |
| TBA value | 0.110 | 0.105 | 0.098 | 0.102 | 0.178 | 0.134 | 0.129 | 0.140 | 0.278 | 0.210 | 0.187 | 0.204 |

A, control; B, cold water glaze; C, sodium chloride glucose glaze; D, ascorbic acid glaze.

Table 2. Chemical changes occurring during frozen storage of sardines, at 6 storage periods (5, 8, 12, 16, 22 and 29 weeks).

| | | 5 weeks | | 8 weeks | | 12 weeks | | 16 weeks | | 22 weeks | | 29 weeks | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Fresh | C | B | C | B | C | B | C | B | C | B | C | B |
| Moisture (g%) | 78.5 | 74.9 | 77.2 | 74.2 | 76.5 | 73.3 | 76.0 | 72.5 | 75.1 | 71.3 | 74.4 | 70.1 | 73.7 |
| Total crude proteins (N × 6.25 g%) | 16.9 | 18.8 | 17.9 | 19.3 | 18.3 | 19.9 | 18.5 | 20.2 | 18.7 | 20.8 | 19.2 | 21.5 | 19.5 |
| Total acid-soluble ribose (mg%) (R2) | 129.4 | 105.8 | 114.0 | 94.7 | 110.5 | 89.1 | 104.0 | 81.2 | 94.7 | 72.4 | 86.4 | 78.5 | 61.9 |
| Ba-acetate alcohol nonprecipitable ribose (mg%) (R1) | 2.5 | 5.9 | 5.6 | 12.6 | 9.5 | 14.5 | 11.8 | 18.7 | 15.2 | 19.9 | 16.0 | 20.4 | 16.2 |
| $R = \frac{R1}{R2} \times 100$ | 1.9 | 5.6 | 4.9 | 13.4 | 8.6 | 16.3 | 11.3 | 23.0 | 16.1 | 27.5 | 18.5 | 33.0 | 20.6 |
| Total nonprotein nitrogen (g%) | 0.43 | 0.50 | 0.45 | 0.50 | 0.46 | 0.52 | 0.49 | 0.51 | 0.54 | 0.61 | 0.54 | 0.72 | 0.59 |
| Total amino nitrogen (g%) | 2.18 | 1.95 | 2.10 | 1.70 | 2.00 | 1.50 | 1.80 | 1.20 | 1.70 | 1.00 | 1.55 | 0.88 | 1.35 |
| Trimethyl amine nitrogen (mg%) | 0.6 | 2.2 | 1.8 | 3.0 | 2.6 | 0.85 | 0.54 | 0.81 | 0.60 | 1.0 | 0.9 | 1.6 | 1.0 |
| Free amino nitrogen (mg%) | 199 | 263 | 213 | 300 | 242 | 376 | 321 | 441 | 390 | 538 | 426 | 617 | 489 |
| Total volatile basic nitrogen (mg%) | 6.2 | 12.8 | 11.2 | 17.5 | 14.3 | 39.3 | 27.0 | 60.9 | 47.8 | — | — | 131.7 | 72.7 |

C, slow-frozen; B, quick-frozen.

Table 3. Some physical and chemical properties of sardine drip.

| Storage period (weeks) | Groups | Drip (cc) per 100 g of muscle | | Ash content (g%) | pH | Total nitrogen (g%) | NPN (g%) | TMA (mg%) | Ribose (mg%) |
|---|---|---|---|---|---|---|---|---|---|
| 5 | C | 3.4 | Reddish, transparent | 0.056 | 7.9 | 0.31 | 0.127 | 0.85 | 11.8 |
| | B | 2.5 | Reddish, transparent | 0.061 | 7.8 | 0.20 | 0.216 | 0.78 | 14.7 |
| 8 | C | 3.6 | Light-brown, transparent | 0.43 | 8.0 | 0.36 | 0.250 | ..... | 23.3 |
| | B | 2.2 | Light-brown, transparent | 1.0 | 7.9 | 0.27 | 0.228 | ..... | 24.9 |
| 12 | C | 5.8 | Dark-brown, turbid | 0.5 | 8.2 | 0.41 | 0.331 | 0.78 | 22.3 |
| | B | 2.9 | Greenish-brown, clear | 0.3 | 8.0 | 0.33 | 0.326 | 0.42 | 31.5 |
| 16 | C | 4.0 | Blackish-brown, turbid | 0.19 | 8.3 | 0.46 | 0.359 | 1.4 | 24.5 |
| | B | 2.1 | Dark-brown, opaque | 0.16 | 8.2 | 0.381 | 0.246 | 1.6 | 36.9 |
| 22 | C | 3.5 | Dirty-brown, turbid | 0.95 | 8.4 | 0.52 | 0.338 | 1.0 | 16.0 |
| | B | 2.3 | Dark-brown, slightly blackish | 0.74 | 8.3 | 0.41 | 0.256 | 1.2 | 15.0 |
| 29 | C | 3.9 | Blackish-green, opaque | 0.94 | 8.5 | 0.57 | 0.266 | 1.9 | 26.3 |
| | B | 2.8 | Dark-brown, opaque | 0.74 | 8.4 | 0.46 | 0.243 | 1.2 | 18.6 |

C, slow-frozen; B, quick-frozen.

cate that proteolysis is occuring during storage at −18°C. Gangal and Magar (1963) reported similar results in frozen crabmeat.

**Thiobarbituric acid value.** TBA values increased in all the fish studied. Pomphrets glazed with sodium nitrite showed a slower increase in TBA value than pomphrets with other glazes, whereas mackerel glazed with sodium chloride glucose glaze showed the lowest increase in TBA value. The higher TBA values of mackerel and sardines after storage may be a reflection of their higher fat content and thus susceptibility to oxidation as compared with pomphret.

**Drip.** Slow-frozen sardines exuded more drip than the quick-frozen. The pH of the drip increased steadily during storage and finally attained the values of 8.5 and 8.4 after 29 weeks for slow- and quick-frozen sardines, respectively. Large amounts of NPN, ribose, TMA, and total nitrogen were found in the drip.

**Organoleptic observations.** Both slow- and quick-frozen pomphrets were acceptable even after seven months of storage. A few yellowish patches were observed in slow-frozen pomphrets, but were rarely found in quick-frozen samples. Slow-frozen samples had a dull appearance with a dry and porous texture. The use of glazes helped to retain the appearance, taste, and quality of the samples over control groups in both quick- and slow-frozen batches. In the glazed pomphrets, ascorbic acid was very useful in protecting the appearance of the product. In both quick- and slow-frozen mackerel, yellowish patches under the skin and slight development of off-odor were noted after 7 months of storage but sodium chloride-glucose glaze was found most effective in maintaining palatability. In sardines, quick-frozen fish were comparatively superior in appearance to slow-frozen.

Over-all it was observed that the use of different glazes was distinctly advantageous in prolonging the storage life of fish by retarding desiccation.

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Thanks are due to the Council of Scientific and Industrial Research, New Delhi, for financing the work and Prof. A. F. D'Mallow, former Fishery Technologist, C.F.T.R.S. (Bombay Unit), for cold-storage facilities.

F. K. SHOUP, Y. POMERANZ, and C. W. DEYOE

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

# Amino Acid Composition of Wheat Varieties and Flours Varying Widely in Bread-Making Potentialities

## SUMMARY

**Eight composites of hard red winter wheat varieties and 5 hard red spring wheat varieties from several locations, and single samples of soft red winter, durum, and club (white) wheat were milled to flour on a semi-commercial mill, and their chemical composition, rheological properties, and bread-making potentialities were evaluated. Protein determined by the Kjeldahl procedure was highly correlated with protein estimates by the acid-orange-12 binding method. The histidine, arginine, threonine, glycine, and methionine concentrations in protein were negatively correlated, and the glutamic acid and proline concentrations were positively correlated, with the total protein contents of the wheats. The contents of almost all the amino acids increased as the protein of the wheat and flour samples increased. Only cystine and methionine in the wheat, and lysine, cystine, and methionine in the flour failed to follow this pattern. Milling wheat to flour lowered (in decreasing order) concentrations of lysine, arginine, aspartic acid, glycine, alanine, tyrosine, histidine, threonine, and valine. The concentrations of glutamic acid, proline, and phenylalanine were higher in flour than in wheat. The concentrations in protein of basic amino acids, glutamic acid, and threonine were significantly correlated with certain rheological properties or bread-making potentialities of the flours. Proteins of hard red spring wheats contained less lysine, arginine, and methionine, and more cystine, than hard red winter wheats.**

## INTRODUCTION

Studies on the amino acid composition of wheat concern differences in composition of the whole kernel and its structural parts or milled products; the effects of total protein levels on distribution of amino acids in the protein; and the relation of amino acid composition to bread-making potentialities. Studies on amino acid composition of cereals have provided useful information on the nutritional value and on the "chemical" formula of wheat proteins, but have thus far been rather disappointing in their failure to explain differences in the bread-making characteristics of wheat varieties. Investigations on the amino acid content of wheat flours milled from different varieties have been limited in scope; however, as pointed out by Miller *et al.* (1950), certain relationships may yet become apparent. The study reported here is part of an extensive program to correlate the composition of wheat flours with functional properties in bread-making.

## MATERIALS AND METHODS

**Wheat and flour samples.** Eight hard red winter and five hard red spring wheat samples were composited by variety from equal portions of wheat from the 1963 crop. Among the hard red winter wheats, 'Pawnee' (C.I. 11669), 'Comanche' (C.I. 11673), 'Quivira-Tenmarq × Marquillo-Oro' (C.I. 12995), 'Chiefkan × Tenmarq' (Ks. 501097), and 'Chiefkan × Tenmarq' (Ks. 501099), were from Clovis, N. Mex.; Lincoln and North Platte, Nebr.; Fort Collins, Colo.; Stillwater, Cherokee, Goodwell, and Woodard, Okla.; Bushland and Chillicothe, Tex.; and Garden City, Hays, Colby, and Manhattan, Kan. 'Yogo' (C.I. 8033) and 'Warrior' (C.I. 13190) were from Hymore and Brookings, So. Dak; St. Paul and Crookston, Minn.; and Huntley, Bozeman, Havre, and Moccasin, Mont. 'Karmont' (C.I. 6700) was from Huntley, Bozeman, Sidney, and Havre, Mont. Hard red spring wheats, 'Thatcher' (C.I. 10003), 'Selkirk' (C.I. 13100), 'Marquis' (C.I. 3641), 'Lee' (C.I. 12488), and 'Pilot' (C.I. 11428), were from Brookings, Eureka, Hymore, and Newell, So. Dak.; Dickenson, Fargo, Minot, and Williston, No. Dak.; Morris and Crookston, Minn.; and Havre, Huntley, and Bozeman, Mont. Single samples of soft red winter, 'Seneca' (C.I. 12529), durum, 'Wells' (C.I. 13333), and white club wheat, 'Omar' (C.I. 13072), were respectively obtained from agricultural experiment stations in Wooster, Ohio; Fargo, No. Dak.; and Pullman, Wash.

**Chemical analyses.** Chemical analyses were performed according to Cereal Laboratory Methods (AACC, 1962). Analytical determinations of wheat were made on samples ground on a Micro-Wiley mill to pass a 40-mesh sieve.

**Dye binding.** Dye absorption was determined with dye and equipment obtained from the Udy Analyzer Company. The reagent dye was acid

orange 12 in a phosphate-buffered system at pH 1.7. One-g samples of flour or 0.8-g samples of wheat were mixed for 3 min with 40 ml of the reagent dye solution in the reaction chamber. The mixture was then transferred to a squeeze-type polyethylene bottle fitted with a fiberglass filter disc in the dropper cap. Light transmittance through the filtered dye solution was determined colorimetrically as the filtrate was transferred dropwise into the flow-through cuvette. The colorimeter was previously calibrated to 32% transmittance with a reference dye solution.

**Amino acid analyses.** Acid hydrolysis was used in the preparation of samples for amino acid analyses. Test tubes were narrowed after the addition of 100 mg of sample, and 6*N* hydrochloric acid was added at the rate of approximately 1 ml per 10 mg protein. The tubes were placed in a dry-ice-alcohol bath and sealed under vacuum. Contents of tubes were hydrolyzed for 22 hr at 110°C and filtered through a fritted-disc funnel. The filtrate was evaporated to dryness 3 times under partial vacuum and diluted to 10 ml with 0.2*N* sodium citrate buffer, pH 2.2. All hydrolysates were stored at −20°C until analyzed. The hydrolysates were light tan, indicating little humin formation. Amino acid analyses were made by ion-exchange column chromatography technique (Moore *et al.*, 1958; Spackman *et al.*, 1958) on a Beckman amino acid analyzer, model 120B. Preliminary investigations on cereal grains with the sealed tube method, as described above, and various volumes of acid indicated no advantage of using large excess of acid (Waggle and Deyoe, 1965). Average Kjeldahl nitrogen accounted for by the amino acid analyses was 91.0% for whole wheat and 95.6% for wheat flour (Shoup, 1965). These amounts of nitrogen accounted for are in the same range as reported by Hepburn and Bradley (1965).

**Bread-making.** For bread-making evaluation, the wheat samples were milled on a Miag "Multimat." Baking experiments were on a laboratory scale. The bread was baked by a straight dough procedure by the following basic formula: 100 parts flour, water as needed, 2 parts yeast, 6.0 parts sucrose, 4.0 parts nonfat dry milk solids, 0.25 part 120°L malt syrup, 3.0 parts shortening, and 1.5 parts sodium chloride. An optimum mixing time with the straight dough procedure, and a 3-hr fermentation time at 30°C were employed. Punching and panning were performed mechanically. Baking time was 24 min at 218°C. Bakings were replicated at least twice, each at least at 3 levels of potassium bromate to determine the bromate response. Loaf volumes are reported as determined on freshly baked loaves from 100 g flour at bromate levels that gave the best bread judged by loaf volume, bread crumb, and grain. After the loaves had cooled, they were cut and their crumb grain evaluated by the following code: $S$ = satisfactory, $Q$ = questionable, and $U$ = unsatisfactory.

Table 1. Wheat characteristics.

| Code no. | Variety | Cereal investigation no. | Moisture (%) | Ash (%) | Sedimentation value | Wheat meal fermentation (min) |
|---|---|---|---|---|---|---|
| Hard red winter | | | | | | |
| 281 | Pawnee | 11669 | 11.1 | 1.5 | 38.5 | 124 |
| 282 | Comanche | 11673 | 11.6 | 1.7 | 62.4 | 239 |
| 283 | Qv-Tm × Mql-Oro | 12995 | 11.5 | 1.6 | 53.5 | 234 |
| 284 | Chiefkan × Tenmarq | 501097 | 11.5 | 1.6 | 33.6 | 97 |
| 285 | Chiefkan × Tenmarq | 501099 | 11.2 | 1.7 | 27.8 | 35 |
| 286 | Yogo | 8033 | 9.9 | 1.8 | 25.3 | 102 |
| 287 | Warrior | 13190 | 11.7 | 1.6 | 59.6 | 225 |
| 288 | Karmont | 6700 | 11.1 | 1.7 | 66.0 | 103 |
| Hard red spring | | | | | | |
| 289 | Thatcher | 10003 | 10.9 | 1.8 | 63.5 | 317 |
| 290 | Selkirk | 13100 | 10.9 | 1.8 | 64.0 | 185 |
| 291 | Marquis | 3641 | 10.4 | 2.0 | 59.3 | 312 |
| 292 | Lee | 12488 | 11.0 | 1.8 | 62.0 | 304 |
| 293 | Pilot | 11428 | 10.4 | 1.8 | 57.2 | 238 |
| Soft red winter | | | | | | |
| 294 | Seneca | 12529 | 9.9 | 1.5 | 40.0 | 99 |
| Durum | | | | | | |
| 295 | Wells | 13333 | 12.0 | 1.6 | 12.1 | 15 |
| Soft white wheat (club) | | | | | | |
| 296 | Omar | 13072 | 11.1 | 1.3 | 6.6 | 17 |

## RESULTS AND DISCUSSION

Certain chemical and bread-making parameters of the wheat samples and straight-grade flours are summarized in Tables 1 and 2. The amino acid composition of the 8 composites of hard red winter wheats and their straight-grade flours are summarized in Tables 3 and 4. The amino acid compositions of

Table 2. Flour characteristics.

| Code no. | Variety | Extraction (%) | Moisture (%) | Ash (%) | Absorption (%) | Mixing time (min) | Bromate requirement (mg %) | Loaf volume (cc) | Crumb grain[a] |
|---|---|---|---|---|---|---|---|---|---|
| Hard red winter | | | | | | | | | |
| 281 | Pawnee | 71.5 | 12.7 | 0.56 | 60.5 | 2¼ | 4.5 | 850 | Q–S |
| 282 | Comanche | 69.3 | 12.0 | 0.48 | 65.5 | 3¼ | 3.0 | 925 | S |
| 283 | Qv-Tm × Mql-Oro | 66.9 | 12.6 | 0.48 | 62.5 | 4⅞ | 2.0 | 863 | S |
| 284 | Chiefkan × Tenmarq | 73.9 | 12.1 | 0.58 | 66.8 | 1¾ | 5.0 | 740 | Q–U |
| 285 | Chiefkan × Tenmarq | 72.4 | 11.9 | 0.53 | 65.8 | 1¼ | 5.0 | 678 | U |
| 286 | Yogo | 72.7 | 12.2 | 0.45 | 59.0 | 2⅜ | 3.5 | 778 | Q–S |
| 287 | Warrior | 74.3 | 11.7 | 0.45 | 66.0 | 2⅝ | 2.5 | 865 | Q–S |
| 288 | Karmont | 71.7 | 10 8 | 0.49 | 65.5 | 1⅞ | 5.0 | 905 | Q–S |
| Hard red spring | | | | | | | | | |
| 289 | Thatcher | 69.9 | 13.0 | 0.46 | 65.0 | 3¼ | 2.0 | 1003 | S |
| 290 | Selkirk | 69.9 | 12.9 | 0.46 | 64.5 | 3⅝ | 3.0 | 973 | S |
| 291 | Marquis | 65.1 | 13.0 | 0.46 | 63.5 | 3⅜ | 2.0 | 915 | S |
| 292 | Lee | 67.7 | 12.6 | 0.45 | 66.0 | 3½ | 1.5 | 950 | S |
| 293 | Pilot | 65.0 | 12.9 | 0.44 | 63.5 | 3⅛ | 1.5 | 1003 | S |
| Soft red winter | | | | | | | | | |
| 294 | Seneca | 52.5 | 13.3 | 0.36 | 51.0 | 2⅛ | 2.0 | 835 | Q–S |
| Durum | | | | | | | | | |
| 295 | Wells | 71.2 | 12.2 | 0.74 | 67.5 | 1½ | 2.0 | 355 | U |
| Soft white (club) | | | | | | | | | |
| 296 | Omar | 71.5 | 12.2 | 0.40 | 49.0 | 2⅜ | 2.0 | 465 | U |

[a] S = satisfactory; Q = questionable; U = Unsatisfactory.

Table 3. Amino acid composition of hard red winter wheat (g amino acid per 100 g protein).[a]

| Code no. | 281 | 282 | 283 | 284 | 285 | 286 | 287 | 288 | Av. |
|---|---|---|---|---|---|---|---|---|---|
| Protein[b] | 14.7 | 16.0 | 14.8 | 15.0 | 16.1 | 12.7 | 14.8 | 16.9 | 15.1 |
| Lysine | 3.0 | 2.7 | 2.9 | 2.7 | 2.7 | 2.9 | 2.9 | 3.0 | 2.8 |
| Histidine | 2.5 | 2.4 | 2.3 | 2.4 | 2.4 | 2.4 | 2.4 | 2.3 | 2.4 |
| Ammonia | 3.3 | 3.4 | 3.6 | 3.6 | 3.7 | 3.5 | 3.8 | 3.8 | 3.6 |
| Arginine | 4.7 | 4.2 | 4.5 | 4.3 | 4.3 | 4.4 | 4.5 | 4.3 | 4.4 |
| Aspartic acid | 5.1 | 4.8 | 4.9 | 5.0 | 5.0 | 5.0 | 4.9 | 4.8 | 4.9 |
| Threonine | 2.9 | 2.7 | 2.8 | 2.7 | 2.7 | 3.0 | 2.8 | 2.6 | 2.8 |
| Serine | 4.6 | 4.5 | 4.6 | 4.5 | 4.5 | 4.7 | 4.5 | 4 5 | 4.5 |
| Glutamic acid | 31.4 | 33.1 | 32.5 | 32.9 | 32.6 | 30.3 | 32.5 | 33.0 | 32.3 |
| Proline | 10.1 | 10.5 | 10.3 | 10.7 | 10.6 | 10.4 | 10.7 | 11 2 | 10.6 |
| Glycine | 4.1 | 4.1 | 4.1 | 4.0 | 4.0 | 4.3 | 4.0 | 3 9 | 4.0 |
| Alanine | 3.6 | 3.4 | 3.4 | 3.5 | 3.9 | 3 8 | 3.4 | 3 3 | 3.5 |
| Half cystine | 2.7 | 2.2 | 2.5 | 2.4 | 2.2 | 2 8 | 2.4 | 2.1 | 2.4 |
| Valine | 4.4 | 4.2 | 4.2 | 4.1 | 4.2 | 4 5 | 4.2 | 4.1 | 4.2 |
| Methionine | 1.3 | 1.2 | 1.2 | 1.2 | 1.1 | 1.4 | 1.2 | 1.1 | 1.2 |
| Isoleucine | 3.5 | 3.4 | 3.3 | 3.3 | 3.4 | 3.5 | 3.4 | 3.4 | 3.4 |
| Leucine | 6.8 | 6.7 | 6.6 | 6.5 | 6.6 | 6.8 | 6.6 | 6.6 | 6.7 |
| Tyrosine | 1.7 | 1.6 | 1.8 | 1.7 | 1.6 | 1.7 | 1.6 | 1.5 | 1.7 |
| Phenylalanine | 4.5 | 4.8 | 4.6 | 4.7 | 4.6 | 4.6 | 4.4 | 4.7 | 4.6 |

[a] Sum of g amino acids and ammonia accounted for by the amino acid analyzer.
[b] %, Kjeldahl protein N × 5.70 (moisture-free basis).

the 5 hard red spring wheat composites, soft red winter, white club, and durum wheats are reported in Table 5; those of the straight-grade flours from those wheats are summarized in Table 6. The values for the amino acid composition of wheat and wheat flour agree satisfactorily with those reported in the literature (Barton-Wright and Moran,

Table 4. Amino acid composition of hard red winter wheat flour (g amino acid per 100 g protein[a]).

| Code no. | 281 | 282 | 283 | 284 | 285 | 286 | 287 | 288 | Av. |
|---|---|---|---|---|---|---|---|---|---|
| Protein[b] | 14.2 | 15.0 | 14.0 | 14.5 | 15.2 | 12.1 | 14.4 | 15.7 | 14.4 |
| Lysine | 2.0 | 1.9 | 2.0 | 1.9 | 2.2 | 2.1 | 2.1 | 2.0 | 2.0 |
| Histidine | 2.1 | 2.0 | 2.0 | 2.1 | 2.5 | 2.0 | 2.1 | 2.0 | 2.1 |
| Ammonia | 3.9 | 3.9 | 4.0 | 4.1 | 4.7 | 3.7 | 3.7 | 3.8 | 4.0 |
| Arginine | 3.4 | 3.0 | 3.3 | 3.1 | 3.6 | 3.2 | 3.2 | 3.1 | 3.2 |
| Aspartic acid | 3.9 | 3.7 | 3.8 | 3.8 | 3.8 | 3.9 | 3.9 | 3.7 | 3.8 |
| Threonine | 2.6 | 2.5 | 2.6 | 2.5 | 2.5 | 2.8 | 2.6 | 2.5 | 2.6 |
| Serine | 4.5 | 4.6 | 4.8 | 4.5 | 4.3 | 4.6 | 4.5 | 4.5 | 4.5 |
| Glutamic acid | 35.6 | 36.0 | 35.4 | 35.7 | 35.0 | 34.6 | 34.8 | 36.2 | 35.4 |
| Proline | 11.1 | 11.8 | 11.7 | 11.8 | 11.7 | 11.6 | 11.5 | 12.2 | 11.7 |
| Glycine | 3.4 | 3.4 | 3.6 | 3.3 | 3.3 | 3.4 | 3.5 | 3.3 | 3.4 |
| Alanine | 3.0 | 2.8 | 2.9 | 2.9 | 2.9 | 3.1 | 3.0 | 2.8 | 2.9 |
| Half cystine | 2.7 | 2.4 | 2.4 | 2.5 | 2.2 | 2.7 | 3.3 | 2.2 | 2.5 |
| Valine | 4.1 | 4.0 | 4.0 | 4.0 | 4.0 | 4.3 | 4.2 | 4.0 | 4.1 |
| Methionine | 1.3 | 1.2 | 1.2 | 1.3 | 1.1 | 1.4 | 1.2 | 1.2 | 1.2 |
| Isoleucine | 3.5 | 3.6 | 3.5 | 3.6 | 3.5 | 3.7 | 3.7 | 3.6 | 3.6 |
| Leucine | 6.8 | 6.7 | 6.7 | 6.8 | 6.6 | 6.9 | 6.8 | 6.7 | 6.7 |
| Tyrosine | 1.6 | 1.5 | 1.4 | 1.3 | 1.4 | 1.3 | 1.5 | 1.3 | 1.4 |
| Phenylalanine | 4.7 | 5.0 | 4.8 | 4.9 | 4.8 | 4.7 | 4.7 | 5.0 | 4.8 |

[a] Sum of g amino acids and ammonia accounted for by the amino acid analyzer.
[b] %, Kjeldahl protein N × 5.70 (moisture-free basis).

Table 5. Amino acid composition of hard red spring, soft red winter, durum, and white club wheats (g amino acid per 100 g protein[a]).

| | Hard red spring | | | | | Av. | SRW | Durum | Club |
|---|---|---|---|---|---|---|---|---|---|
| Code no. | 289 | 290 | 291 | 292 | 293 | | 294 | 295 | 296 |
| Protein[b] | 17.0 | 17.2 | 16.1 | 17.0 | 15.9 | 16.6 | 15.9 | 14.2 | 8.9 |
| Lysine | 2.6 | 2.8 | 2.6 | 2.5 | 2.7 | 2.6 | 2.7 | 2.6 | 3.2 |
| Histidine | 2.3 | 2.3 | 2.3 | 2.3 | 2.3 | 2.3 | 2.3 | 2.2 | 2.4 |
| Ammonia | 3.7 | 3.8 | 3.4 | 3.6 | 3.4 | 3.6 | 3.5 | 3.6 | 3.1 |
| Arginine | 4.1 | 4.1 | 4.3 | 4.3 | 4.3 | 4.2 | 4.2 | 4.3 | 4.7 |
| Aspartic acid | 4.9 | 4.8 | 5.0 | 5.0 | 5.2 | 5.0 | 4.9 | 5.1 | 5.9 |
| Threonine | 2.8 | 2.7 | 2.9 | 2.8 | 2.9 | 2.8 | 2.8 | 2.8 | 3.1 |
| Serine | 4.6 | 4.5 | 4.6 | 4.6 | 4.6 | 4.6 | 4.6 | 4.5 | 4.7 |
| Glutamic acid | 32.3 | 32.8 | 31.5 | 32.0 | 31.4 | 32.0 | 32.3 | 32.2 | 29.0 |
| Proline | 11.1 | 10.8 | 10.6 | 10.7 | 10.5 | 10.8 | 10.8 | 10.3 | 9.9 |
| Glycine | 4.1 | 3.8 | 4.1 | 3.9 | 4.1 | 4.0 | 3.9 | 3.7 | 4.4 |
| Alanine | 3.5 | 3.4 | 3.6 | 3.6 | 3.6 | 3.5 | 3.5 | 3.6 | 4.2 |
| Half cystine | 2.1 | 2.6 | 2.8 | 2.6 | 2.8 | 2.6 | 2.2 | 2.3 | 2.2 |
| Valine | 4.3 | 4.3 | 4.4 | 4.4 | 4.5 | 4.4 | 4.3 | 4.3 | 4.8 |
| Methionine | 1.0 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.1 | 1.3 | 1.3 |
| Isoleucine | 3.5 | 3.8 | 3.5 | 3.6 | 3.5 | 3.6 | 3.6 | 4.0 | 3.7 |
| Leucine | 6.7 | 6.7 | 6.7 | 6.8 | 6.8 | 6.7 | 6.8 | 6.8 | 7.1 |
| Tyrosine | 1.7 | 1.7 | 1.9 | 1.7 | 1.6 | 1.7 | 1.6 | 1.8 | 1.7 |
| Phenylalanine | 4.9 | 4.1 | 4.6 | 4.6 | 4.6 | 4.6 | 4.9 | 4.8 | 4.8 |

[a] Sum of g amino acids and ammonia accounted for by the amino acid analyzer.
[b] %, Kjeldahl protein N × 5.70 (moisture-free basis).

1946; Hepburn and Bradley, 1965; Hepburn *et al.*, 1957, 1960; Horn *et al.*, 1958; Lawrence *et al.*, 1958; McDermott and Pace, 1960; Pence *et al.*, 1950, 1964; Price, 1950; Simmons, 1962).

The data indicate a general uniformity in distribution of amino acids in proteins of wheats covering a wide range of breadmaking potentialities. The concentrations of lysine, arginine, and methionine in proteins of hard red winter wheats were somewhat higher, and in club wheat much higher, than in proteins of hard red spring wheats. There were no significant differences in glutamic acid or proline concentrations in the breadmaking wheats (hard red spring and hard red winter); but the proteins of the soft club wheat contained substantially less of the two amino acids. The average concentration of cystine was higher in hard red spring than in hard red winter wheats, and was much lower in durum and club wheats. These results must be interpreted with caution since amino acid composition is known to be affected by protein levels. Thus, valid comparisons between wheat varieties from different classes should be made on samples of comparable protein content. Alternatively, the effects of total protein levels on amino acid distribution for a certain wheat variety should be allowed for. The limited experimental material does not yet permit such corrections to be made.

Milling hard red winter wheat into flour resulted in an average decrease of 29% lysine, 12% histidine, 27% arginine, 22% aspartic acid, 7% threonine, 16% glycine, 18% alanine, 4% valine, and 16% tyrosine. Amino acids more concentrated in flour than in hard red winter wheat were: glutamic acid (10%), proline (10%), cystine (4%), isoleucine (5%), and phenylalanine (5%). Milling hard red spring wheat into flour lowered the concentration of lysine (33%), histidine (13%), arginine (29%), aspartic acid (24%), threonine (11%), glycine (19%), alanine (15%), valine (8%), and tyrosine (11%). Proteins of flour contained 10% more proline, 12% more glutamic acid, 12% more methionine, and 6% more phenylalanine than proteins of whole-grain spring wheat. Differences in amino acid composition of wheat and flour proteins are known to be affected by flour extraction, milling system, and probably also by milling properties of wheats from various classes

Table 6. Amino acid composition of hard red spring, soft red winter, durum, and white club wheat flours (g amino acid per 100 g protein[a]).

| | Hard red spring | | | | | Av. | SRW | Durum | Club |
|---|---|---|---|---|---|---|---|---|---|
| Code no. | 289 | 290 | 291 | 292 | 293 | | 294 | 295 | 296 |
| Protein[b] | 15.8 | 15.6 | 14.8 | 16.3 | 14.7 | 15.4 | 13.0 | 13.2 | 7.5 |
| Lysine | 1.9 | 1.7 | 1.9 | 1.7 | 1.7 | 1.8 | 2.4 | 2.0 | 2.7 |
| Histidine | 2.1 | 2.0 | 2.0 | 2.0 | 1.9 | 2.0 | 2.6 | 2.1 | 2.4 |
| Ammonia | 4.0 | 4.1 | 4.0 | 3.9 | 3.6 | 3.9 | 4.6 | 4.1 | 4.0 |
| Arginine | 3.1 | 3.0 | 3.1 | 3.0 | 2.9 | 3.0 | 3.7 | 3.4 | 3.7 |
| Aspartic acid | 3.8 | 3.6 | 3.8 | 3.7 | 3.8 | 3.8 | 3.7 | 4.3 | 4.4 |
| Threonine | 2.4 | 2.4 | 2.5 | 2.5 | 2.6 | 2.5 | 2.5 | 2.5 | 2.7 |
| Serine | 4.3 | 4.5 | 4.5 | 4.5 | 4.6 | 4.5 | 4.4 | 4.4 | 4.7 |
| Glutamic acid | 36.4 | 36.3 | 35.5 | 36.1 | 35.8 | 36.0 | 35.2 | 35.0 | 32.1 |
| Proline | 12.1 | 11.8 | 11.7 | 11.4 | 11.6 | 11.7 | 11.4 | 11.3 | 11.0 |
| Glycine | 3.3 | 3.1 | 3.4 | 3.2 | 3.3 | 3.3 | 3.2 | 3.1 | 3.5 |
| Alanine | 3.0 | 2.9 | 3.1 | 3.0 | 3.1 | 3.0 | 3.0 | 3.2 | 3.3 |
| Half cystine | 2.4 | 2.6 | 2.7 | 2.7 | 2.6 | 2.6 | 2.4 | 2.3 | 2.9 |
| Valine | 3.8 | 3.9 | 4.1 | 4.0 | 4.1 | 4.0 | 3.9 | 4.0 | 4.4 |
| Methionine | 1.2 | 1.2 | 1.3 | 1.3 | 1.4 | 1.3 | 1.2 | 1.3 | 1.5 |
| Isoleucine | 3.4 | 3.5 | 3.6 | 4.3 | 3.7 | 3.7 | 3.4 | 3.7 | 3.7 |
| Leucine | 6.5 | 6.6 | 6.8 | 6.7 | 6.9 | 6.7 | 6.5 | 6.7 | 7.0 |
| Tyrosine | 1.5 | 1.8 | 1.4 | 1.4 | 1.6 | 1.5 | 1.5 | 1.7 | 1.4 |
| Phenylalanine | 4.9 | 5.0 | 4.8 | 4.7 | 4.9 | 4.8 | 4.8 | 4.9 | 4.9 |

[a] Sum of g amino acids and ammonia accounted for by the amino acid analyzer.
[b] %, Kjeldahl protein N × 5.70 (moisture-free basis).

and varieties. The close similarity in amino acid distribution patterns of wheat and wheat flours in both classes seems to indicate that varietal effect is minor. Hepburn *et al.* (1957) showed similar trends when comparing wheat and flour of two commercial blends of hard red spring wheat and two blends of hard red winter wheat.

Tables 7 and 8 summarize correlation coefficients between amino acid composition and protein content or bread-making properties of flours. Table 7 shows correlations between bread-making properties and percent amino acid distribution in the proteins; the results in Table 8 compare the same bread-making properties with the amino acid content of the samples. Whereas, for example, an increase in protein content was accompanied by a reduction in the lysine concentration in the protein, the lysine content in the rich-protein sample was higher than in the low-protein sample. Consequently, a comparison between the two tables is useful in interpretation of the effects of protein contents on correlation coefficients. The statistical computations were made on 13 samples of bread-making wheats which included 8 hard red winter and 5 hard red spring wheats and flours milled from those wheats. It should be stressed again that, in addition to class and varietal effects, the results have been affected by protein levels, which ranged between 12.65 to 17.17% (in dry matter) for the 13 wheat samples, and between 12.07 and 16.25% for the 13 flour samples. Correlation coefficients of 0.55 were statistically significant at the 5% level, and those of 0.68 at the 1% level. Concentrations of histidine, arginine, threonine, glycine, and methionine in the protein decreased with increased protein content, and glutamic acid and proline increased. In the flours, concentrations of aspartic acid, threonine, glycine, valine, and leucine were correlated negatively with protein content, and only glutamic acid positively. However, when considered as percent of the sample, all the amino acids except cystine and methionine were positively corre-

Table 7. Correlations between protein content or bread-making properties and amino acid composition (g amino acid per 100 g protein).

| | Wheat protein | Flour protein | Lysine | Histidine | Ammonia | Arginine | Aspartic acid | Threonine | Serine | Glutamic acid |
|---|---|---|---|---|---|---|---|---|---|---|
| Wheat protein (Kjeldahl) | | | −.50 | −.57* | .36 | −.59* | −.38 | −.66* | −.54 | .59* |
| Flour protein (Kjeldahl) | | | −.43 | .04 | .32 | −.24 | −.61* | −.93** | −.49 | .77** |
| Wheat protein (Udy) | .96** | | −.62* | −.59* | .27 | −.48 | −.19 | −.52 | −.46 | .44 |
| Flour protein (Udy) | | .97** | −.20 | .17 | .32 | −.11 | −.52 | −.87** | −.55* | .65* |
| Loaf volume | | .62* | −.85** | −.65* | −.34 | −.74** | −.46 | −.43 | −.01 | .64* |
| Sedimentation value | | .65* | −.74** | −.59* | −.39 | −.77** | −.42 | −.41 | −.05 | .68** |
| Mixing time | | .07 | −.50 | −.57* | −.28 | −.40 | −.13 | −.02 | .49 | .24 |
| Flour absorption | | .74** | −.13 | .23 | .35 | −.19 | −.46 | −.75** | −.38 | .40 |
| Bromate requirement | | −.13 | .59* | .57* | .41 | .52 | −.06 | −.01 | −.20 | −.11 |

| | Proline | Glycine | Alanine | Half cystine | Valine | Methionine | Isoleucine | Leucine | Tyrosine | Phenylalanine |
|---|---|---|---|---|---|---|---|---|---|---|
| Wheat protein (Kjeldahl) | .68** | −.71** | −.35 | −.45 | −.36 | −.71** | .32 | −.13 | −.17 | −.01 |
| Flour protein (Kjeldahl) | .33 | −.68* | −.33 | −.35 | −.77** | −.41 | .19 | −.68** | .28 | .39 |
| Wheat protein (Udy) | .61* | −.63* | −.19 | −.34 | −.23 | −.62* | .21 | −.03 | −.11 | .12 |
| Flour protein (Udy) | .33 | −.59* | −.41 | −.31 | −.66* | −.53 | .20 | −.68** | .07 | .29 |
| Loaf volume | .12 | −.42 | .28 | .11 | −.31 | .23 | .32 | −.11 | .48 | .26 |
| Sedimentation value | .38 | −.34 | .11 | −.03 | −.36 | .08 | .18 | −.23 | .36 | .44 |
| Mixing time | −.05 | .21 | .17 | .13 | −.20 | .19 | .12 | −.04 | .29 | .10 |
| Flour absorption | .41 | −.40 | −.45 | −.18 | −.50 | −.52 | .12 | −.50 | −.07 | .40 |
| Bromate requirement | .16 | −.07 | −.57* | −.41 | −.03 | −.47 | −.37 | −.12 | −.29 | .14 |

* At 5% level.
** At 1% level.

Table 8. Correlation between protein content or bread-making properties and amino acid composition (g amino acid per 100 g sample).

| | Lysine | Histidine | Ammonia | Arginine | Aspartic acid | Threonine | Serine | Glutamic acid | Proline |
|---|---|---|---|---|---|---|---|---|---|
| Wheat protein (Kjeldahl) | .86** | .90** | .92** | .88** | .90** | .74** | .91** | .98** | .96** |
| Flour protein (Kjeldahl) | .53 | .76** | .82** | .74** | .96** | .62* | .78** | .90** | .89** |
| Wheat protein (Udy) | .74** | .89** | .88** | .92** | .92** | .77** | .90** | .94** | .92** |
| Flour protein (Udy) | .63* | .78** | .76** | .73** | .97** | .53 | .66* | .81** | .81** |
| Loaf volume | −.24 | .05 | .22 | .12 | .39 | .63* | .66* | .66* | .60* |
| Sedimentation value | −.12 | .12 | .19 | .07 | .40 | .62* | .64* | .67* | .66* |
| Mixing time | −.53 | −.41 | −.18 | −.30 | −.09 | .01 | .14 | .03 | −.02 |
| Flour absorption | .61* | .71** | .71** | .58* | .80** | .47 | .59* | .68** | .74** |
| Bromate requirement | .45 | .26 | .16 | .22 | .06 | −.26 | −.21 | −.15 | −.08 |

| | Glycine | Alanine | Half cystine | Valine | Methionine | Isoleucine | Leucine | Tyrosine | Phenylalanine |
|---|---|---|---|---|---|---|---|---|---|
| Wheat protein (Kjeldahl) | .87** | .81** | .42 | .84** | .40 | .90** | .94** | .72** | .76** |
| Flour protein (Kjeldahl) | .73** | .75** | .30 | .67* | .44 | .78** | .80** | .60* | .87** |
| Wheat protein (Udy) | .87** | .84** | .48 | .85** | .48 | .83** | .93** | .72** | .81** |
| Flour protein (Udy) | .66* | .65* | .29 | .63* | .32 | .72** | .70** | .45 | .76** |
| Loaf volume | .55* | .73** | .55* | .55* | .71** | .64* | .65* | .62* | .61* |
| Sedimentation value | .62* | .65* | .42 | .54 | .64* | .57* | .61* | .55* | .66* |
| Mixing time | .09 | .09 | .11 | −.12 | .13 | .04 | −.01 | .17 | .03 |
| Flour absorption | .63* | .54 | .35 | .59* | .26 | .59* | .63* | .34 | .70** |
| Bromate requirement | −.22 | −.36 | −.59* | −.21 | −.48 | −.30 | −.19 | −.27 | −.10 |

* At 5% level.
** At 1% level.

lated with wheat protein content. In the case of the flours the correlations were also positive with the exception of lysine, methionine, and cystine.

Orange G binds specifically under acidic conditions to free amino groups, the imidazole group of histidine, and the guanidyl group of arginine (Fraenkel-Conrat and Cooper, 1944). Orange G and acid orange 12 are used to determine the protein content of wheat and wheat flour (Udy, 1954, 1956, 1964). The amount of dye bound would be expected to depend on the protein content and amino acid composition of the tested samples. Correlation coefficients between protein determined by the Kjeldahl procedure, and by the acid orange 12 binding method, were highly significant for both the wheat and flour samples. However, the correlation between dye binding and the concentration in the protein of basic amino acids, lysine or histidine, was significant in the wheat samples at the 5% level only, and nonsignificant in the flour samples. The low correlations between the concentrations in the protein of the basic amino acids and dye binding, apparently resulted from decreases in concentrations of the basic amino acids with increase in total protein contents. This is borne out by the high positive correlations between dye binding and the weight of basic amino acids in the sample (Table 8).

Protein content was, as expected, correlated with loaf volume and sedimentation value. Significant negative correlations were observed between loaf volume and the concentration in protein of the three basic amino acids; the correlation between glutamic acid and loaf volume was positive. Similar trends were observed for correlations between sedimentation values and amino acid concentration in flour. It is difficult to evaluate whether the correlations indicate causative, or merely casual, relations between amino acid concentration and total protein content. Flour absorption was correlated negatively only with the concentration of threonine in the protein. The high correlations between

flour absorption and amino acid content (Table 8) would be expected since the amino acids contents were positively correlated with protein content. The protein content was found highly correlated with flour absorption. Bromate requirement increased as the concentration in the protein of lysine and histidine increased, and was correlated negatively with alanine concentration. It is interesting to note that cystine content was negatively correlated with oxidation requirements.

The results of this study, based on testing composites of wheat varieties from a number of locations, confirm the rather wide uniformity in amino acid composition of wheat and wheat flours reported by other workers. Changes in patterns of amino acid composition during milling of wheat into flour, certain correlations between dye binding and amino acid contents, and tentative correlations between amino acid composition and certain rheological and bread-making parameters indicate a need for additional research in this field.

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Cooperative investigations between Department of Flour and Feed Milling Industries, Kansas State University and Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture. Contribution No. 530, Kansas Agricultural Experiment Station, Manhattan.

Mention of firms or products does not imply endorsement.

The authors thank G. D. Miller for Kjeldahl nitrogen analyses, A. B. Ward for milling the wheats, K. F. Finney for baking tests, and D. H. Waggle for assistance in the statistical analysis of the data.

*Research Note*

**JONATHAN W. WHITE, JR.**

*Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division Agricultural Research Service, Philadelphia, Pennsylvania 19118*

# Methyl Anthranilate Content of Citrus Honey

## SUMMARY

**A photometric method for determination of methyl anthranilate (MA) was adapted for honey. Twenty-one citrus honeys from 3 states and 5 crops averaged 2.87 μg/g (range 0.84–4.37). For 12 non-citrus honeys, apparent MA content averaged 0.07 μg/g (range 0.00–0.28).**

## INTRODUCTION

Citrus honey is obtained predominantly from the orange, with lesser amounts from grapefruit and lemon. It is collectively handled in commerce as orange honey, originating principally from Florida and California. It is considered a premium honey and possesses a most distinctive and pleasant flavor. To Nelson (1930) its aroma was reminiscent of methyl anthranilate (MA), which is a component oil of orange flowers. He obtained a positive diazotization test for MA in an ether extract of orange honey. Lothrop (1932) later examined 17 honey samples qualitatively by applying the diazotization procedure to an ether extract of the steam distillate from 1 kg honey. Positive tests were obtained for only the three orange honey samples included. Mixtures of orange and tulip-tree honey gave positive tests for MA down to 10% of the former, and were negative at 5%.

More recently, Deshusses and Gabbai (1962) used thin-layer chromatography of a petroleum ether extract of honey to demonstrate the presence of methyl anthranilate in Spanish orange-blossom honey. By comparison of intensity of chromatographic spots, they estimated that 0.4–0.5 ppm of MA were originally present.

Several years ago we adapted a procedure (Anon., 1957) using diazotization and coupling with 1-naphthol-2-sulfonic acid (Sale and Wilson, 1926) for determination of MA in honey. Changes were primarily in reagent concentration and preparation, resulting in a much more stable, sensitive reagent with lower blank absorbance. Since recoveries of added MA in the range of 5–30 ppm were adequate, the procedure was applied to a number of citrus and non-citrus honey samples.

## MATERIALS AND METHODS

**Honey samples.** The 21 samples from the 1956-57 crops were from the collection made for an extensive analytical survey of U. S. honey (White *et al.*, 1962). They were kept in storage at −20°C between receipt and analysis. Other samples were obtained from producers. The cooperation of Wendell Shore and the Superior Honey Company, South Gate, California, is acknowledged for the 1965 samples.

**Method for methyl anthranilate in honey.** *Reagents: hydrochloric acid, 1N; sodium nitrite,* 1% in water; *hydrazine sulfate,* 3% in water; *1-naphthol-2-sulfonic acid, potassium salt,* dissolve 1.25 g, practical grade, in 50 ml hot water, add a little decolorizing charcoal and filter after a few minutes. Solution is stable in amber bottle at room temperature for at least 2 weeks; *sodium carbonate,* 25 g dissolved in 75 ml water.

*Equipment: distillation unit,* Parnas-Wagner all-glass micro-Kjeldahl distilling apparatus (Horwitz, 1955) with variable transformer for power input; *photoelectric photometer,* accepting 10-cm cuvettes, 500-mμ filter.

*Procedure.* Add 5 ml water to 10 g honey weighed to 1 mg, mix, and transfer into the distilling apparatus using 5 ml more water for washing. Distill, reducing voltage from 115 to 85 when foaming begins, and collect 50±1 ml. Condenser water should not exceed 15°C. It is not necessary to submerge the tip of the condenser as described in the original procedure for grape juice. Transfer the distillate to a 100-ml volumetric flask. Use 50 ml water for a blank. To each flask add 2.5 ml HCl and 0.4 ml $NaNO_2$, and let stand 2 min. Add 0.6 ml hydrazine solution, wash down the neck of the flasks with a few ml water, and let stand 1 min. Add 1 ml naphthol sulfonate solution, and immediately follow with 1.5 ml $Na_2CO_3$ solution. Mix, make to 100 ml, and determine absorbance in a 10-cm cuvette at 500 mμ in the photometer, using the prepared blank for reference. Calibrate against methyl anthranilate solution, 0–50 μg in the 50 ml analyzed.

## RESULTS AND DISCUSSION

**Effect of sample size.** Samples of a citrus honey weighing 5, 10, and 15 g were carried

through the procedure to determine if solids content during the distillation affected the result. As shown in Table 1, the 10-g sample

Table 1. Effect of sample size on methyl anthranilate (MA) value.

| Sample wt. | MA (μg/g) |
|---|---|
| 5.012 | 1.94 |
| 5.046 | 2.04 |
| 10.000 | 2.42 |
| 10.000 | 2.40 |
| 15.058 | 2.34 |
| 14.996 | 2.27 |

gives maximal yields of MA. All subsequent recovery work was done at this honey concentration.

**Recovery of added MA.** MA was added to 10-g honey samples in amounts shown in Table 2. These were subjected to the distillation and analysis as described. Recoveries ranged from 86 to 100%, averaging 94.6%. This recovery, in the microgram range, is comparable with that of Sale and Wilson (1926) in recovering MA added to grape juice, in the milligram range.

The analysis of 33 honey samples for MA is shown in Table 3. The analyses were made at different times, those of the 1956–57 crops after 6 years of storage at −20°C. It has been shown elsewhere (White *et al.*, 1961, 1964) that none of the characteristics measured (carbohydrates, acidity, invertase, diastase, color, and HMF content) change during such storage.

Table 3 shows separate averages for the 21 citrus honey samples and the 12 non-citrus honeys. The values are from single determinations. Standard deviation found for duplicate analyses of seven samples was 0.031 ppm. It is quite apparent that methyl anthranilate as measured by this procedure is characteristically present in citrus honeys and absent from non-citrus honey types. The traces shown in other honeys are not necessarily MA. An unexpectedly high value of 1.34 μg/g was obtained for a sample labeled "Chinquapin," from Florida, not shown in the table. This sample was excluded from the table because of the definite possibility that it was in fact mixed with some citrus. Further information on this point, with adequate sample control, is necessary.

Portions of three of the 1957 citrus samples included in Table 3 had also been stored for 6 years at room temperature. Table 4 shows the effect of such storage on MA content. A loss of about 15% of that present per year is evident.

The source of the considerable variation among samples from different areas and seasons is not known. It would not be advisable to set a value for MA content of citrus honey without further investigation of this variability with samples of honey gathered under controlled conditions. At this point, it can be concluded only that citrus honey may contain between 0.84 and 4.4 μg MA per gram honey, averaging 2.87 for 21 samples, and 12 samples of non-citrus honey gave an average of 0.07 μg/g.

Table 2. Recovery of methyl anthranilate added to honey.

| | Methyl anthranilate | | | |
|---|---|---|---|---|
| Honey type | Added[a] (μg) | Found[b] (μg) | Difference (μg) | Recovery (%) |
| Citrus | 6.95 | 6.10 | 0.85 | 87.8 |
| | 12.5 | 12.35 | 0.15 | 98.9 |
| | 13.9 | 12.0 | 1.9 | 86.2 |
| | 25 | 24.2 | 0.8 | 97.0 |
| Basswood | 5.70 | 5.70 | 0.0 | 100 |
| | 13.3 | 12.6 | 0.7 | 94.5 |
| | 27.8 | 26.0 | 1.8 | 93.5 |
| | 38.3 | 38.1 | 0.2 | 99.2 |
| Av. | | | | 94.6 |

[a] Indicated amount added to 10 g honey, then carried through procedure.
[b] Difference between result and amount naturally present.

Table 3. Methyl anthranilate (MA) in honey.

| Citrus | | | | Non-citrus | | | |
|---|---|---|---|---|---|---|---|
| Crop year | Origin | Type | MA[a] | Crop year | Origin | Type | MA |
| 1956 | Calif. | Orange | 0.85 | 1956 | Calif. | Vetch | 0.07 |
| 1957 | Fla. | Citrus | 3.16 | 1957 | Fla. | Saw Palmetto | 0.14 |
| | | | 3.10 | | | | |
| | | | 4.19 | | La. | Clover | 0.00 |
| | | | 3.23 | | Iowa | " | 0.28 |
| | | | 2.77 | | Col. | " | 0.03 |
| | | | 4.21 | | N.C. | Lespedeza | 0.05 |
| | | | 4.01 | | S.D. | Sweet-clover | 0.07 |
| | | | 3.95 | | | | |
| | | | 4.37 | | Md. | " | 0.02 |
| | | | 3.34 | | Mass. | Cranberry | 0.06 |
| 1963 | | | 1.86 | | Tenn. | Tulip | 0.02 |
| 1964 | | | 2.34 | 1958 | Fla. | Tupelo | 0.05 |
| | | | 2.19 | 1962 | Pa. | Basswood | 0.04 |
| | | | 1.42 | | | | |
| 1965 | Calif. | Orange | 3.41 | | Av. non-citrus | | 0.07 |
| | | | 3.32 | | | | |
| | | | 3.90 | | | | |
| | Arizona | Orange | 0.97 | | | | |
| | | | 0.84 | | | | |
| | | Orange-Mesquite | 2.86 | | | | |
| | Av. Fla. samples | | 3.15 | | | | |
| | Av. Calif. samples | | 2.87 | | | | |
| | Av. Arizona samples | | 1.56 | | | | |
| | Av. all citrus | | 2.87 | | | | |

[a] μg/g, fresh weight.

Table 4. Effect of storage (6 years) on methyl anthranilate content of citrus honey.

| | Method of storage | | | |
|---|---|---|---|---|
| | | | Loss | |
| | Frozen (ppm) | Room temp. (ppm) | ppm | % |
| 1 | 4.19 | 1.83 | 2.36 | 56.5 |
| 2 | 4.01 | 1.59 | 2.42 | 60.2 |
| 3 | 4.37 | 1.80 | 2.57 | 58.9 |

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Ms. rec'd 9/16/65.

PRASERT SAISITHI,[a] BUNG-ORN KASEMSARN,[b]
J. LISTON, and ALEXANDER M. DOLLAR
*University of Washington, College of Fisheries, Seattle, Washington 98105*

# Microbiology and Chemistry of Fermented Fish

## SUMMARY

**The microbiology and chemistry of Thai fish sauce was investigated in relation to the development of flavor and aroma. Total viable count decreased steadily as fermentation time advanced. The counts were higher when media containing 10% NaCl were used than when media containing 0.5% NaCl were used. Approximately 70% of the bacterial isolates from a nine-month-old fish sauce were halophiles of *Bacillus* types. These bacteria produced volatile acids from cultures in a medium prepared by hydrolyzing rockfish (*Sebastodes* sp.) with a mineral acid. A diethyl-ether-ethanol-soluble fraction contained the typical aroma of fish sauce. This extract could be separated further by column chromatography (AG1-X8, formate form) into a fraction containing amino compounds and a second fraction containing weakly acidic compounds. The amino acids, principally glutamic acid, histidine, proline, etc., and other amines were identified by thin-layer chromatography. The identification of acidic compounds has not been completed.**

## INTRODUCTION

Fermented fish products are commonly used as a food condiment in Southeast Asia. Most of the fermented products are strictly traditional and limited to local consumption. The variety of fermented fish products available has been reviewed by Amano (1961).

Fish sauce is known under different names in various countries of Southeast Asia (for example: Nuoc-mam in Cambodia and Viet-Nam; Nam-pla in Thailand and Laos; Patis in the Philippines; Ketjap-ikan in Indonesia; Ngapi in Burma). Approximately 250,000,000 people from the market area use the products. This figure does not include countries such as Hong Kong, Japan, and Malaysia, where only a minority of the population would utilize fish sauce. In Thailand, Viet-Nam, and Cambodia, fish sauce production is the biggest single fisheries industry (Subba Rao, 1961). In Thailand alone, the minimum production of sauce during 1961 was $8 \times 10^6$ gallons, requiring $66 \times 10^6$ pounds of fish (Sribhibhadh, 1965). The various grades of the liquefied fish products, Nuoc-man and Nam-pla, which contain salt (27.5–28.9 g/100 ml), organic nitrogen (0.6–2.0 g N/100 ml), and ammonia nitrogen (0.2–0.7 g N/100 ml), are a valuable supplement to cereal foods. As such, they supply as much as 7.5% of an individual's total nitrogen intake (Amano, 1961) and serve as a rich source of unidentified nutrients, vitamins, and minerals in the diet.

Traditionally, fish sauce is prepared from small pelagic fishes of the *Stolephorus* and *Sardinella* species. These fish, usually less than 6 inches long, are caught inshore by small fishing vessels using purse seines. As long as 24–48 hr may elapse before the fish reach the processing plant on shore, and since there is no refrigeration, spoilage will have begun.

The present studies were limited to a type of "fish sauce" produced commercially in Thailand. The normal procedure for making such a fish sauce begins with the mixing of uneviscerated fish with salt in the ratio of 1:3 (salt to fish) on a concrete floor. The mixture is transferred to fermentation tanks which are generally constructed of concrete and built into the ground, providing temperature control ($\sim 40°C$). After the tank is filled to the top, it is sealed off and remains sealed for at least 6 months. After the fish has liquefied, the product is drained off and filtered through sand filtering beds. The filtrate is transferred to earthenware containers and ripened in the sun for 1–3 months. The finished product is a clear dark-brown color and has a distinct aroma and flavor. The flavor and aroma determine its quality, and these characteristics develop progressively as the fermentation process advances.

The principal objectives of this study were: 1) to determine the types of bacteria present in fish sauce obtained from Thailand; 2) to study the action of the isolated bacteria upon

[a] Rockefeller Foundation Fellowship.

[b] Department of Fisheries, Technological Section, Bangkok, Thailand.

selected substrates; and 3) to determine the nature of flavor-active substances present in commercial fish sauces or in media inoculated with the bacteria isolated at different stages of fermentation.

## METHODS AND PROCEDURES

Fish sauce samples used in these experiments were obtained directly from Thailand in small sealed glass vials. They were air-shipped and refrigerated immediately upon arrival.

**Total viable count.** Serial decimal dilutions were made with phosphate buffer, pH 7.2, and plates were poured with brain-heart infusion (BHI) agar (Difco), to which either 0.5 or 10% NaCl was added (Series 1). In the second series (Series 2) the medium was nutrient agar (Difco) containing 0.5% yeast extract, to which either 10 or 20% NaCl was added. The plates were incubated aerobically and anaerobically (McIntosh Fildes jars) at 20°C for 4 days (Series 1) and at 37°C for 4 days (Series 2).

**Isolation of bacteria.** Samples were streaked in duplicate onto BHI agar containing 10% salt and incubated at room temperature under aerobic or anaerobic (McIntosh Fildes jars) conditions. After 4 days' incubation at 20°C, colonies were picked from the aerobic plates and transferred to BHI broth containing 10% salt. Colonies were picked from the anaerobic plates and transferred to cooked meat medium containing 10% salt.

The isolated cultures were purified, and the isolated organisms were classified according to Bergey's Manual (Breed *et al.*, 1957).

Rockfish (*Sebastodes* sp.) muscle (1,000 g) was blended for 3 min with concentrated HCl (555.5 ml). The acidified mixture was hydrolyzed by heating for 5 hr at 240°F and then neutralized with concentrated NaOH (453 ml, 50% w/v). The hydrolysate (final pH 6.2) was sterilized in screw-capped test tubes by autoclaving at 15 lb for 15 min.

The pure cultures of the isolates were streaked on BHI agar containing 10% salt and incubated at room temperature for 48–72 hr. The culture was picked up on a cotton swab and transferred to 1.5 ml of sterile 10% saline. The bacteria were suspended by agitation, and two drops of the suspension were inoculated into the fish muscle hydrolysate with a sterile Pasteur pipette. The cultures were incubated 31 days at 31°C to permit development of flavor and aroma. An untrained panel of 5 Thai students, who normally consume fish sauce, evaluated flavor and aroma.

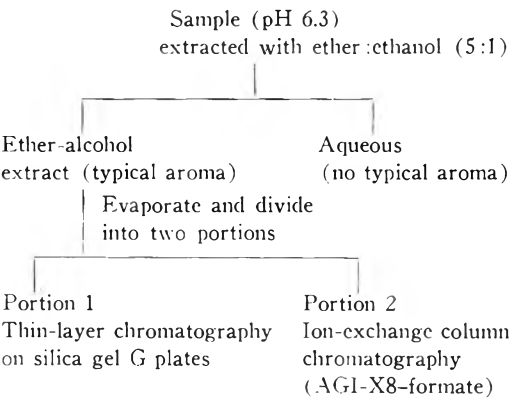
**Determination of volatile acids.** The samples of fish muscle hydrolysate, inoculated with a pure culture, were incubated for 3 days at 37°C. The volatile acids were determined in accordance with the standard method (Methods 18.11, 18.12, and 18.13; AOAC, 1950), except that the sample size was 5 ml instead of 50 g. Results are expressed as millimoles lactic acid per 100 ml sample.

**Chemical analysis of fish sauce samples.** The pH value of each sample was determined with a Beckman Zeromatic pH meter.

Total volatile base and total volatile acids were determined by the microdiffusion technique of Conway and Byrne (1933) as modified by Beatty and Gibbons (1937).

Titratable acidity was determined by the indicator method, as recommended in the Manual of Microbiological Methods (Anon., 1958). Since only a small amount of original sample was available, a 5-ml sample was used for titration instead of a 10-ml sample. Acidity was expressed as millimoles lactic acid per 100 ml sample. Salt was determined by the standard method (Method 18.14, 18.15; AOAC, 1950).

**Isolation and identification of groups of compounds having the typical aroma of fish sauce.** The substances responsible for the typical aroma of fish sauce could not be distilled. Therefore, the following scheme was developed to isolate them from fish sauce:




**Column chromatography.** Five ml of anion-exchange resin (formate form) was washed into a glass chromatographic tube (10 mm ID) with distilled water, and washing was continued until the pH of the eluate was about 5.2.

A 5-ml sample of the ether-soluble fraction was added to the top of the resin and eluted with approximately 75 ml of distilled water at the rate of 0.5 ml/min. The effluent was collected in a series of 2-ml samples, and the amino nitrogen was determined by the method of Moore and Stein (1954) against a leucine standard.

**Identification of amines and amino acids by thin-layer chromatography.** Each fraction (column chromatography) was spotted on silica gel G plates and chromatographed with a phenol-water (80:30) solvent system (Randerath, 1963). The

chromatograms were developed until the front had advanced 17 cm, and then amine groups were detected by heating for 10 min in a hot-air oven (110°C) after spraying with ninhydrin reagent (0.2% w/v in acetone).

**Aldehydes from Strecker degradation of amino acids.** Fourteen amino acids (arginine, aspartic acid, cystine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, tyrosine, and valine) were oxidized to aldehydes by treatment with ninhydrin by methods described by Hunter and Potter (1958).

**Determination of carbonyls in fish sauce.** The hydrazones were formed by allowing 1 ml of fish sauce to react with 15 cc of 2,-4-dinitrophenyl hydrazine (0.2% w/v in 2*N* HCl). The mixture was filtered through Whatman No. 1 filter paper. The residue was dissolved in 80% (w/v) ethanol and extracted with diethylether, and the ether extract was washed with 10% (w/v) $Na_2CO_3$ (Bush and Hockaday, 1962).

## RESULTS AND DISCUSSION

Total viable counts (Table 1) decreased steadily as fermentation time advanced. No data or samples are available which would reflect the total bacterial count on the fish before they were placed in the fermentation tanks. However, it is probable that the total viable count was higher at the time the fish were salted than after 15 days of fermentation. The present results are similar to those reported for the fish sauce of the Philippines (Hamm and Clague, 1950) and Viet-Nam (Truong, 1963). The fish begin to liquefy soon after being placed in the fermentation tanks, and this liquefaction must be due to endogenous enzymes originally present in the fish, or possibly to spoilage bacteria brought in with the fish. The species of fish generally used in the production of fish sauce are caught during the season when their plankton food is most abundant and when proteolytic enzyme activity is greatest (Kashiwada, 1952). Therefore, it is probable that these endogenous enzymes, rather than the bacteria, are responsible for the hydrolysis of the muscle in the first three months of fermentation (Shimidu, 1934).

The total viable counts were varied with the concentration of salt in the culture media. High counts obtained when the culture media contained 10% salt concentration (see Table 1) indicated the presence of halophiles. These bacteria could originate from the fish, the salt used in the fermentation process, or from contamination. Solar salt obtained from Thailand and other Southeast Asian countries had an average of 27,300 bacteria/g of salt when counted on media containing 10% or more NaCl incubated at 37°C (Bain *et al.*, 1957). The present results and those of Bain *et al.* (1957) confirmed that the principal species of organisms isolated were halophilic bacilli (about 78%). The optimum salt concentration for halophilic bacteria was found to be 20% (Gibbons, 1957), and the optimum pH between 6.5 and 7.5 (Linderberg, 1957). The bacteria seemed to grow better on a medium enclosed in a container where the relative humidity was high (75.5%) (Dussault, 1957). The growth characteristics of the halophilic bacteria, therefore, are similar to the conditions generally found in the fermentation process of fish sauce. Undoubtedly the bacteria obtain nutrients from the fish proteins and/or fish extractives. The aroma in fermented fish products has been claimed to be derived from the activity of various types of halophilic bacteria (Boez and Guillerm, 1930; van Veen, 1953; Amano, 1961; Visco and Fratoni, 1963). The bacteria isolated in these studies are capable of producing volatile acids: 10 of 17 *Bacillus*-type isolates produced measurable amounts of volatile acids.

Table 1. Fermented fish: Total viable counts (bacteria/ml).

| Fermentation time (months) | Brain-heart infusion (Difco) | | Nutrient agar (Difco) | |
|---|---|---|---|---|
| | 0.5% NaCl | 10% NaCl | 10% NaCl | 20% NaCl |
| 0.5 | | | $2.8\times10^{3}$ | $1.1\times10^{3}$ |
| 1 | $3.0\times10^{3}$ | $1.41\times10^{5}$ | $9.6\times10^{1}$ | $3.0\times10^{3}$ |
| 3 | $2.0\times10^{3}$ | $4.0\times10^{4}$ | $5.5\times10^{2}$ | $1.7\times10^{2}$ |
| 6 | $1.1\times10^{2}$ | $3.8\times10^{2}$ | $7.1\times10^{3}$ | $5.5\times10^{2}$ |
| 9 | $7.0\times10^{1}$ | $2.4\times10^{3}$ | $2.4\times10^{1}$ | $2.1\times10^{1}$ |
| 12 | $4.0\times10^{1}$ | $3.4\times10^{2}$ | $6.2\times10^{2}$ | $2.7\times10^{2}$ |

Table 2. Types of bacteria isolated from 9-month-old fish sauce (Nam-Pla) producing volatile acid when grown in pure culture (7 days' incubation at 31°C) in fish hydrolysate containing 19% salt.

| Species | No. of isolates | Identification numbers | Volatile acids (mmoles lactic acid per 100 ml) |
|---|---|---|---|
| *Bacillus* | 10 | 3 | 3.5 |
| | | 13 | 4.0 |
| | | 15 | 4.0 |
| | | 23 | 4.0 |
| | | 25 | 4.0 |
| | | 42 | 3.4 |
| | | 53 | 4.0 |
| | | 66 | 3.8 |
| | | 85 | 3.8 |
| | | 91a | 4.0 |
| "Coryneform" | 1 | 56 | 5.0 |
| *Streptococcus* | 2 | 74b | 3.5 |
| | | 81b | 4.0 |
| *Micrococcus* | 1 | 80 | 4.0 |
| *Staphylococcus* | 1 | 109 | 8.0 |

The remaining 7 failed to produce volatile acids, but may not have grown sufficiently during the period of incubation in the fish hydrolysate containing 19% NaCl. The largest amount of volatile acids was produced by *Staphylococcus* strain 109 (twice as great as the *Bacillus* spp.).

Low-molecular-weight volatile compounds such as methyl ketone (van Veen, 1953), organic acids (Nguyen and Vialard-Goudou, 1953; Truong, 1963; Yanagihara *et al.*, 1963) and carbonyl compounds (Yurkowski, 1965a,b) have often been stated to be the cause of the flavor and aroma of fermented fish products. Organic acids in particular were shown to be associated with the aroma of fish sauce (Nguyen and Vialard-Goudou, 1953; Truong, 1963). The activity of bacteria isolated from nine-month-old fish sauce would suggest that these organisms were capable of producing volatile acids and other products which contributed to the aroma and flavor of the sauce.

There was only a slight increase in volatile acids during the 12 months of fermentation, while the volatile base increased to a maximum of 14.7 meq/100 ml of sample by the 9th month and decreased sharply toward the 12th month to 3 meq/100 ml (Table 3). Ammonia nitrogen increased to a maximum of 14 millimoles/100 ml by 6 months of fermentation time, and total soluble nitrogen increased from 56 to 140 millimoles/100 ml during the 6th to 12th month of fermentation time. The analysis of fish sauce, therefore, would suggest that soluble nitrogen could be a good indicator of the events leading to the development of the desirable flavor in fish sauce.

The material responsible for the aroma of the fish sauce could be extracted with a diethyl-ether-alcohol mixture. Ion-exchange chromatography of this extract separated the compounds containing amino groups from volatile acids. None of the fractions containing amino groups had an odor which was typical of the aroma of fish sauce. The fractions eluted from the resin had an odor of trimethylamine or an odor similar to that of the Strecker degradation product of phenylalanine. The typical aroma substance of fish sauce was eluted from the ion-exchange resin with 4*N* formic acid, suggesting that it is most probably an acid.

To eliminate the possibility that the aroma originated through Strecker degradation of amino acids, 14 amino acids were treated with ninhydrin. Only 7 of the acids formed odorous products at room temperature. Glutamic acid yielded a product which was "meaty" and was only remotely similar in odor to the compounds eluted from the ion-

Table 3. Chemical analysis of Thai fish sauce.

| Fermentation period (mo). | pH | NaCl (%) | mmoles/100 ml | | | | |
|---|---|---|---|---|---|---|---|
| | | | Total N | Ammonia N | Titratable acid-lactic | Volatile acid-lactic | Volatile base (meq/100 ml) |
| 1 | 6.4 | 30.1 | 49 | 8 | 6.8 | 4.3 | 4.01 |
| 3 | 6.2 | 30.3 | 52 | 7 | 8.0 | 3.3 | 6.61 |
| 6 | 6.6 | 30.2 | 56 | 14 | 5.2 | 8.7 | 10.21 |
| 9 | 6.2 | 30.2 | 130 | 15 | 5.9 | 4.3 | 14.71 |
| 12 | 6.4 | 27.9 | 140 | 15 | 15.8 | 6.3 | 3.01 |

exchange resin with 4*N* formic acid. Phenylalanine yielded a product which was typical of the 8-to-12-ml fraction eluted from the resin. Methionine yielded a product which had a methyl-sulfide-like odor, while isoleucine, leucine, and valine had a sweet pelargonium-type odor not present in any fish sauce fraction. However, no aldehyde was found from Thai fish sauce by forming the hydrazone derivative and testing on thin-layer chromatography (Bush and Hockaday, 1962).

Identification of the compounds present in the aroma-containing diethylether fractions after further fractionation by ion-exchange chromatography is shown in Table 6. Thin-layer chromatography with silica gel G plates revealed the presence of amino acids, glutamic acid, histidine, alanine, leucine, phenylalanine, and proline, as well as amines—glucosamine, histamine, glutamine, and trimethylamine. Other compounds, which were present in the aroma-containing fraction when this fraction was chromatographed, were absent from the fraction obtained from fish sauce, included indole-acetic acid, indole-butyric acid, and beta-hydroxyphenyl pyruvic acid.

These studies support the conclusion of Jones (1961) that the flavor of Thai fish sauce can arise in part from glutamic acid, histidine, and proline. The aroma normally present in the fish sauce is probably a blend of amines and acids. Although further identification and quantitative analysis of these substances is necessary, the role of bacteria in the formation of volatile acids and amines was quite evident, since at least one organism

Table 4. Volatile aldehydes from "Strecker" degradation of α-amino acids.

| Amino acid | Type of aroma |
|---|---|
| arginine | none |
| aspartic acid | none |
| cystine | none |
| glutamic acid | meaty |
| histidine | none |
| isoleucine | sweet, pelargonium-like |
| leucine | sweet, pelargonium-like |
| lysine | none |
| methionine | methyl sulfide-like |
| phenylalanine | strong rose-like |
| threonine | none |
| tryptophane | none |
| tyrosine | rose-like |
| valine | weak pelargonium-like |

Table 5. Ion-exchange (AG1–X8) separation of the aromatic fraction soluble in ethyl ether extracted from 20 ml of fish sauce.

| Fraction (ml) | Amino nitrogen (μmole leucine/ml) | Aroma type |
|---|---|---|
| 0– 6 | 2.80 | Trimethylamine |
| 7–12 | 10.94 | Oxidized phenylalanine |
| 13–18 | 12.36 | none |
| 19–24 | 2.28 | none |
| 25–30 | 2.24 | none |
| 31–36 | 1.32 | none |
| 37–42 | 0.75 | none |
| 43–48 | 0.62 | none |
| 49–54 | 0.55 | none |
| 55–60 | 0.16 | none |

Column regenerated with 4*N* formic acid. Eluant was free of amino acids but contained the aroma which was typical of fish sauce.

Table 6. Chromatography of ion-exchange fractions (6, 8, 10 ml) of the diethylether-soluble fraction from Thai fish sauce (Nam-pla).

Thin layer: Silica gel G
Solvent system: Phenol-Water (80:30)
Detection: 0.2% ninhydrin in acetone
Reference: Randerath (1963)

| $R_f$ values sample | Corresponding to: | Note |
|---|---|---|
| 0.11 | Glutamic acid | |
| 0.18 | Glucosamine | |
| 0.20 | | Unidentified |
| 0.29 | Histidine | |
| 0.34 | dl-Alanine | |
| 0.37 | Glutamine | |
| 0.44 | | Unidentified |
| 0.53 | dl-Leucine | |
| 0.54 | l-Proline | |
| 0.61 | Phenylalanine | |

Table 7. Chromatography of ion-exchange fractions (6, 8, 10 ml) of the diethylether-soluble fraction from Thai fish sauce (Nam-pla).

Thin layer: Silica gel + 0.5% oxalic acid
Solvent system: Phenol-Water (80:30)
Detection: 0.2% ninhydrin in acetone
Reference: Randerath (1963)

| $R_f$ values sample | Corresponding to: | Note |
|---|---|---|
| 0.12 | Glucosamine | |
| 0.15 | Trimethylamine | |
| 0.22 | Histamine | |
| 0.28 | | Unidentified |
| 0.32 | Glutamine | |
| 0.39 | | Unidentified |
| 0.49 | | Unidentified |
| 0.56 | | Unidentified |

produced a typical aroma and flavor when incubated with acid-hydrolyzed rockfish muscle

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Ms. rec'd 6/23/65.

Contribution 210, College of Fisheries, University of Washington.

**ALLEN A. KRAFT and JOHN C. AYRES**
*Department of Dairy and Food Industry, Iowa State University, Ames, Iowa 50010*

# Competitive Growth of Microorganisms and Fluorescence Development on Inoculated Chicken

## SUMMARY

**Studies were conducted to determine the effects of competitive growth of microorganisms and development of fluorescence on inoculated poultry during refrigerated storage. *Pseudomonas fluorescens* rapidly outgrew *Staphylococcus aureus* in broth at 15°C when the initial concentration of staphylococci was about ten times as great as that of the *Pseudomonas*. On stored chicken previously chilled in water containing various levels of pseudomonads, *S. aureus*, *Echerichia coli*, and *Streptococcus faecalis*, growth of the fluorescing pseudomonads greatly surpassed that of the other organisms. Fluorescing bacteria attained large populations on chicken even when *S. aureus* initially outnumbered the fluorescing spoilage organisms by 100:1. Little change occurred in the populations of *S. aureus* recovered from inoculated poultry during storage for one week at 5°C.**

Information concerning the associative effects of growth of bacteria on processed poultry is desirable for evaluating potential health hazard or for control of spoilage. Many studies have been reported on the inhibitive effect that other organisms have on the growth of *Staphylococcus aureus* in foods. These investigations were reviewed by Graves and Frazier (1963), who found that, of 870 cultures isolated from food products, more than 25% (248) were inhibitory to *S. aureus;* the remainder either stimulated growth or had no effect. Their results were in agreement with those of Oberhofer and Frazier (1961) in that streptococci were among the most consistently inhibitory genera of various organisms isolated from foods. The latter workers reported that a "typical" culture of *Escherichia coli* markedly inhibited the growth of two strains of *S. aureus* in broth; but, in general, the effect of *E. coli* varied with the strain used. *S. aureus* did not influence growth of *E. coli*. Peterson *et al.* (1962) observed that *S. aureus* was repressed by mixed populations of microorganisms obtained from chicken pot pies. Retardation of growth was enhanced as the proportion of staphylococci was decreased and as incubation temperature was lowered from 37° to 20°C. Troller and Frazier (1963a) reported similar results for the ratio of staphylococci to inhibiting organisms. Those workers (1963b) also noted that a species of *Pseudomonas* caused inhibition of *S. aureus* by competition for vitamins and amino acids. Antagonism of *Pseudomonas fluorescens* and other pseudomonads toward sporeforming bacteria, staphylococci, and micrococci was shown by Lewis (1929) to be caused by a thermostable bactericidal agent. Zyskind *et al.* (1965) believed that the pyocyanine of *P. aeruginosa* was not similar to the staphylolytic substance produced by the strains of *P. aeruginosa* and *P. fluorescens* used in their study.

Reeves (1965) recently reviewed the action of the antibiotics known as bacteriocins. Bacteriocins produced by various bacteria act only on strains of the same or closely related species. Inhibitory action of the bacteriocins pyocin and fluocin, respectively produced by *P. aeruginosa* and *P. fluorescens*, has been described by Hamon (1956) and Hamon *et al.* (1961).

According to Oberhofer and Frazier (1961), cultures of *Pseudomonas* from chicken and other meat did not influence the growth of *S. aureus*. The present investigation was undertaken to evaluate the effects of *Pseudomonas* on the growth of other organisms, particularly *S. aureus*, and, conversely, the influence of other bacteria on *Pseudomonas* when mixed cultures were employed in different proportions as inocula on chicken during chilling.

## MATERIALS AND METHODS

Used as test organisms were cultures of *Pseudomonas aeruginosa* (2F41, Department of Bacteriology, Iowa State University, Ames), *P. fluorescens* (strain K, isolated in this laboratory from spoiled chicken, and strain 15G, supplied by Dr. W. L. Gaby, Department of Microbiology, Hahnemann Medical College, Philadelphia, Pa.), *Staphylococcus aureus* (S-EY), *Escherichia coli* (EC-1) from chicken, and *Streptococcus faecalis* (M-18) from turkey meat. These bacteria were grown

for 24–48 hr at 30°C in brain heart infusion (BHI, Difco) broth and mixed in various proportions for use as inocula. When competitive growth in broth was evaluated, one-third ml of each of the cultures of *S. aureus, E. coli,* and *S. faecalis* was added to a suspension of 0.1 ml *P. fluorescens* (15G) in 25 ml of BHI broth, or one-third ml of the *Pseudomondas* culture was added to a mixed inoculum of 0.1 ml of the other organisms. Broth cultures were incubated at 5 and 15°C and sampled at intervals for numbers of bacteria.

Freshly cut chicken wings were purchased from a retail store on the day that the poultry was delivered by the wholesaler. The chicken was chilled for 16–20 hr at about 5°C in demineralized water containing the following inocula: 1) equal volumes of all of the test organisms; 2) *P. fluorescens* (K or 15G) and *P. aeruginosa* only; 3) equal volumes of *E. coli, S. aureus,* and *S. faecalis*; 4) *S. aureus* only; and 5) different combinations of *S. aureus* with *Pseudomonas* in the chill water. Control samples were not inoculated. Bacterial counts were taken of chill waters after introduction of inocula but before the chicken was added.

After chilling overnight, the wings were allowed to drain and were then packaged in 195 LSAD cellophane. Packages were stored in a display case at approximately 5°C. During defrosting twice daily, the temperature in the case increased to 10°C but did not remain above 5°C for more than 1 hr.

Surfaces of wings were sampled with a swab technique. Media and incubation procedures for enumeration of organisms were as follows: trypticase soy agar (BBL) for total aerobes (3–4 days at 15°C), violet red bile agar (BBL) for coliforms (24 hr at 37°C), KF streptococcus agar (Difco) for enterococci (48 hr at 37°C), Medium B of King *et al.* (1954) for fluorescing bacteria (4–5 days at 15°C), and staphylococcus medium no. 110 (Difco) fortified with egg yolk (Herman and Morelli, 1960). A surface plating procedure (Silliker *et al.*, 1958) was employed for fluorescing bacteria and for staphylococci; plates for the latter organisms were incubated for 72 hr at 30°C. Colonies showing zones of precipitation and clearing of the yolk on the staphylococcus medium were considered to give a positive reaction. Cultures of these bacteria were tested for coagulase production by a tube method (Difco Manual, 1953).

Fluorescence measurements were made of asparagine broth (Georgia and Poe, 1931) inoculated with a swab from the poultry. Flasks containing the inoculated medium were shaken 18–20 hr at 15 or 30°C, and incubated without shaking for an additional 18–20 hr. The broths were then centrifuged, fluorescence of the supernatant liquid was determined with a Coleman Model C photofluorometer equipped with $B_1$ and $PC_2$ filters and with 6 ppm fluorescein as standard.

The numbers of aerobic organisms recovered were greater with incubation at 15°C than at 5°C. Counts of total aerobic bacteria in broth incubated at 5°C were approximately 6% greater when pseudomonads composed a higher proportion of the initial inoculum than when the lower ratio of pseudomonads to other bacteria was added.

## RESULTS AND DISCUSSION

For testing competitive growth in broth, all organisms were grown together at 5 and 15°C. However, for the sake of clarity in presentation of graphs, growth curves are given in three different figures. Fig. 1 shows growth curves for *P. fluorescens* and *S. aureus* in BHI broth at 5 and 15°C. *P. fluorescens,* at an initial level of about one million cells per ml, rapidly outgrew *S. aureus* in combination with *E. coli* and *S. faecalis* at 15°C, although the initial concentration of *S. aureus* was nine times that of *P. fluorescens*. *P. fluorescens,* at 5°C, increased in numbers to a greater population than that of *S. aureus* at either 5 or 15°C during the first day of incubation. However, during the remainder of the storage period, both types of

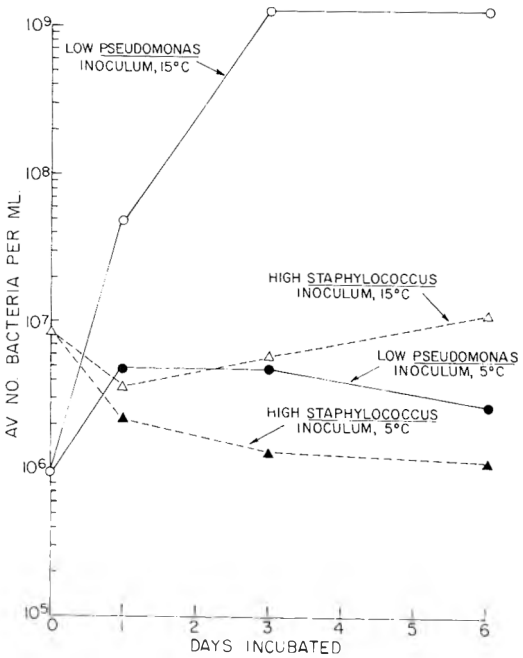


Fig. 1. Growth of mixed cultures of *P. fluorescens* and *S. aureus* in broth at 5 and 15°C.

bacteria slowly declined in numbers at the lower temperature, although populations of *P. fluorescens* remained greater than those of *S. aureus* throughout the 6-day holding period. Curves for a higher level of inoculum of *P. fluorescens* ($3 \times 10^7$ cells per ml) and lower initial concentration of *S. aureus* ($2 \times 10^5$ cells per ml) are not presented, but at 5°C the pseudomonads demonstrated slight increases in numbers and the staphylococci decreased at about the same rate as they did in the curve shown in Fig. 1. In a detailed study of growth of staphylococci in competition with other organisms, Peterson *et al.* (1962) demonstrated that *S. aureus* failed to grow in mixed cultures with psychrophiles at temperatures below 20°C, but the psychrophiles grew normally. Two strains of *S. aureus* tested by Walker and Harmon (1965) also decreased in populations in broth and in milk incubated at 5°C.

*S. faecalis* and *E. coli* differed appreciably in growth with incubation at either 5 or 15°C (Fig. 2). The level of inoculum did not influence the rate of development of *S. faecalis* in broth at 15°C, although this organism reached higher numbers when the initial concentration was almost $4 \times 10^6$ cells per ml than it did when the original concentration of cells was only one tenth as great. In BHI broth the growth of *S. faecalis* was similar to that of *P. fluorescens*. Both types of bacteria grew rapidly to produce large populations. By contrast, *E. coli* at 15°C grew more slowly than *S. faecalis* or *P. fluorescens*, regardless of initial numbers of cells. At 5°C, *E. coli* decreased in numbers throughout the 6-day incubation period whereas populations of *S. faecalis* remained almost constant (Fig. 3).

These observations made with broth cultures indicated that development of *P. fluorescens* did not appear to be influenced by the strains of *S. aureus*, *E. coli*, or *S. faecalis* used. According to Fredericq (1957), the colicins, a group of bacteriocins formed by species of *Escherichia*, *Shigella*, and *Salmonella*, show antibiotic activity against species closely related to the colicinogenic strain. Certain strains of *E. coli* were reported to be effective in inhibiting *Salmonella* in egg white (Flippin and Mickelson, 1960; Mickelson and Flippin, 1960). The strain of *E. coli* tested in this work, however, apparently did not produce colicins, or if it did they were not active against the *Pseudomonas*.

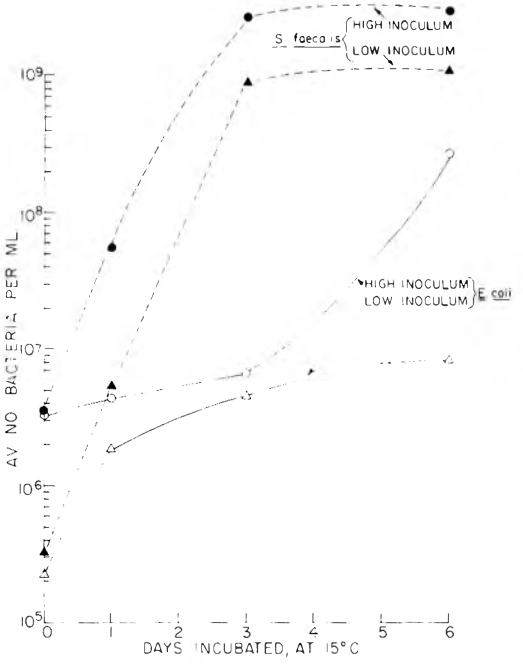


Fig. 2. Growth of mixed cultures of *S. faecalis* and *E. coli* in broth at 15°C.

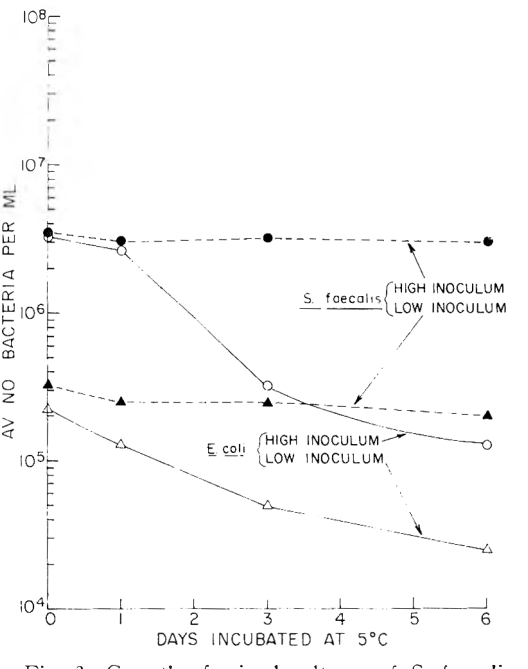


Fig. 3. Growth of mixed cultures of *S. faecalis* and *E. coli* in broth at 5°C.

Minimum generation times for the test bacteria at high and low initial concentrations are presented in Table 1. Of the test organisms grown in association in broth, *S. faecalis* most closely resembled *P. fluorescens* in rate of growth at 15°C. Lower levels of inocula of *S. faecalis* and *E. coli* resulted in shorter generation times than did higher levels. Possibly, the greater total population density with larger inocula caused greater competition for nutrients in the medium, producing a slower growth rate. The low inoculum of *S. aureus* ($1.8 \times 10^5$ cells per ml) resulted in decreased numbers of cells during incubation for 6 days, and no generation time is given.

Chill waters containing different combinations of contaminants were sampled before the chicken was added. Counts of various types of organisms recovered are presented in Table 2. Only the kinds of bacteria added as inocula were recovered from the water; numbers of organisms ranged from about one to ten million cells per ml.

At different intervals during storage, determinations were made of fluorescence of asparagine broth cultures inoculated with organisms from the chicken. The chicken initially was chilled in water containing either pseudomonads and *S. aureus* alone or in combination, or in water with *E. coli, S. faecalis*, and *S. aureus* with and without pseudomonads added.

Fig. 4 relates fluorescence of asparagine broth cultured from chicken during storage to the various combinations of organisms used as inocula in the chill waters. With incubation at 30°C, little difference was noted between fluorescence development when only *P. fluorescens* and *P. aeruginosa* were added to the chill medium and amount of fluorescence when *E. coli, S. faecalis* and *S. aureus* were included with the pseudomonads. Since photofluorometer values were similar for the two combinations of organisms tested at 30°C, the data were combined to form one curve. This procedure was also followed when photofluorometer readings for other groups of inocula were alike (e.g., *Pseudomonas* alone, *Pseudomonas* with other organisms, or *E. coli, S. faecalis,* and *S. aureus* with no *Pseudomonas,* incubated at 15°C). In broth incubated at 30°C, fluorescence was greatest when the chicken was previously inoculated with *Pseudomonas,* regardless of whether other organisms were initially present as contaminants in the chill water. Production of the green fluorescent pigment complex, pyoverdine, by the pseudomonads, was not retarded. When the broth was incubated at 15°C, pyoverdine formation was not as profuse as it was with incubation at 30°C, but at 15°C there was greater fluorescence development when no pseudomonads were added to the original inoculum containing *E. coli, S. faecalis*, and *S. aureus*. Naturally occurring fluorescent pigment producing organisms recovered from the poultry meat were apparently able to develop and form pyoverdine in the broth

Table 2. Numbers of bacteria per ml recovered from inoculated chill waters.

| | No. bacteria per ml × 1000 in Water containing | | |
|---|---|---|---|
| | *E. coli* *S. faecalis* *S. aureus* | *P fluorescens* *P. aeruginosa* | All organisms test |
| Total aerobes | 1,200 | 13,000 | 4,300 |
| Fluorescing *Pseudomonas* | $<10^1$ | 12,000 | 3,900 |
| Coliforms | 970 | $<10^1$ | 470 |
| Enterococci | 1,100 | $<10^1$ | 520 |
| Staphylococci | 1,500 | $<10^1$ | 1,100 |

Table 1. Minimum generation times of organisms grown in BHI broth at 15°C.

| Culture | No. bact./ml × 1,000 Initially | At time *t* | *t* (hr) | Generation time (hr) |
|---|---|---|---|---|
| *P. fluorescens* (15G) | 27,000 | 2,000,000 | 24 | 3.9 |
| | 950 | 49,000 | 24 | 4.2 |
| *S. faecalis* (M-18) | 330 | 910,000 | 24 | 5.9 |
| | 3,700 | 56,000 | 24 | 6.1 |
| *E. coli* (EC-1) | 220 | 1,900 | 24 | 7.7 |
| | 3,400 | 280,000 | 144 | 22.7 |
| *S. aureus* (S-FY) | 8,200 | 11,000 | 144 | 347 |

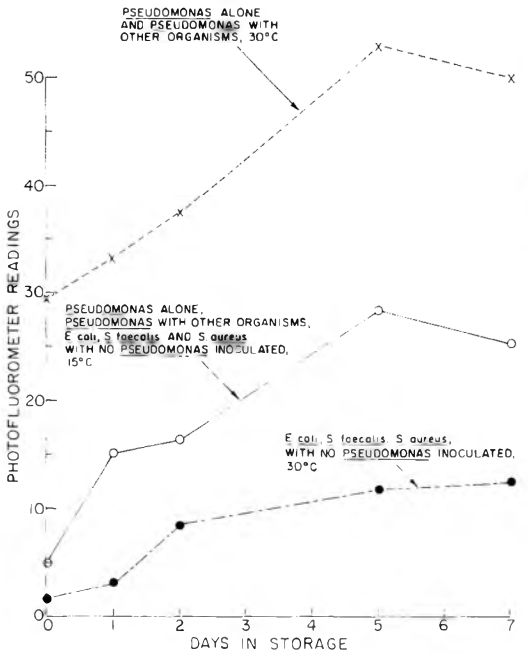


Fig. 4. Relation of different contaminants to fluorescence of broth with organisms from chicken stored for one week at 5°C.

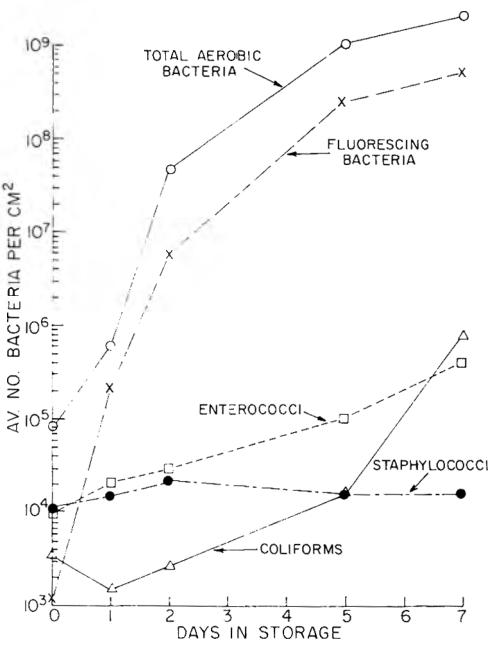


Fig. 5. Numbers of different bacteria recovered from chicken inoculated with *E. coli, S. faecalis,* and *S. aureus,* and stored at 5°C.

to a greater extent at 15°C than at 30°C.

The numbers of various kinds of organisms found on chicken stored at 5°C and originally inoculated with *E. coli, S. faecalis,* and *S. aureus* are shown in Fig. 5. Fluorescent pigment-producing pseumonads developed rapidly and were present in large numbers after 5 and 7 days of storage, even though these organisms were not added to the original inoculum. Of the types of bacteria enumerated, fluorescing organisms composed the greatest portion of the total aerobes. When *P. fluorescens* and *P. aeruginosa* were included with other contaminants in the chill water, the growth curves in Fig. 6 were obtained. Enterococci and coliforms demonstrated gradual increases in numbers on chicken chilled in water with or without *Pseudomonas* added, although coliform counts declined during the first day or two of storage. Staphylococci maintained fairly constant populations; the numbers of these bacteria decreased slightly when the pseudomonads were included in the contaminated chill water.

Further information concerning the associative growth of staphylococci and pseudomonads on poultry was gained by chilling the chicken in waters containing different proportions of these organisms. Ratios, in

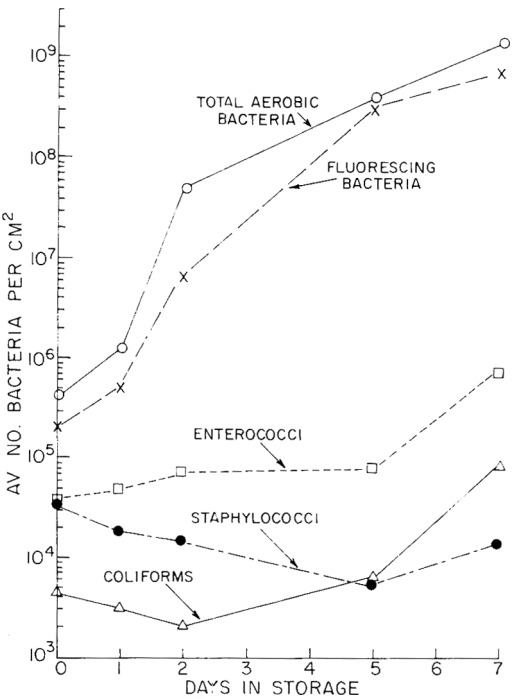


Fig. 6. Numbers of various organisms on chicken inoculated with *E. coli, S. faecalis, S. aureus, P. fluorescens,* and *P. aeruginosa,* and stored at 5°C.

ml, of *S. aureus* to a mixed culture of *P. fluorescens* and *P. aeruginosa* were 1:1, 4:1, 9:1, 10:0.1, and 5:0. Counts of staphylococci in relation to fluorescing organisms in the chill medium were proportional to the volume of inoculum of each of these types of bacteria. For example, 9 ml of a suspension of *S. aureus* in combination with 1 ml of an inoculum of pseudomonads resulted in recoveries of approximately 9,000,000 staphylococci to 1,000,000 fluorescing pseudomonads from the chill water. Control samples had no inoculum added. Little difference was observed in the growth of fluorescing bacteria on chicken inoculated with any of the combinations of *S. aureus* and *Pseudomonas* (Fig. 7). Bacterial counts from chicken inoculated with all combinations except the inoculum of staphylococci and pseudomonads in a ratio of 10:0.1 were averaged together to form one curve. No significant suppression of growth of fluorescing pseudomonads resulted when the initial population of *S. aureus* was about one hundred times that of the *Pseudomonas*.

Numbers of staphylococci remained fairly constant throughout the storage period (Fig. 8). Counts of staphylococci were greatest when the proportion of these organisms to pseudomonads also was greatest. However, the inoculum of *S. aureus* without *Pseudomonas* resulted in no higher loads of staphylococci on chicken than those found when *Pseudomonas* were included. Similar to results discussed previously, fluorescing organisms naturally present on the poultry developed at a rapid rate. No attempt was made in this work to determine if *P. fluorescens* produced substances inhibitory to *S. aureus*, or if populations of staphylococci remained at low levels because of depletion of nutrients. Further investigations are needed to determine the mechanisms of inhibition of staphylococci on poultry meat.

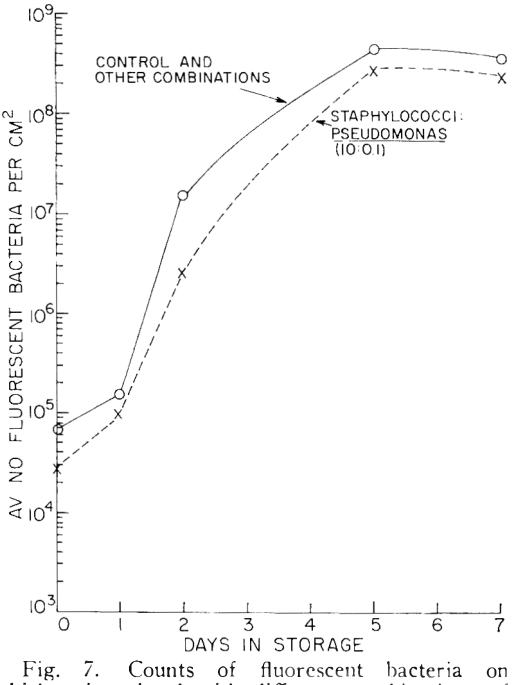


Fig. 7. Counts of fluorescent bacteria on chicken inoculated with different combinations of *Pseudomonas* and *S. aureus* and stored for one week at 5°C.

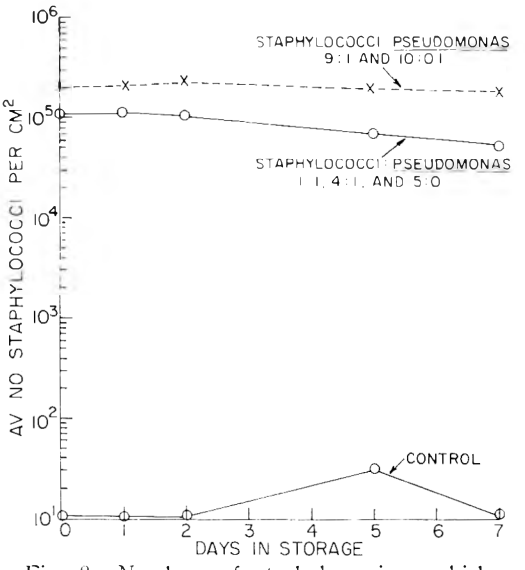


Fig. 8. Numbers of staphylococci on chicken inoculated with different combinations of *Pseudomonas* and *S. aureus* and stored for one week at 5°C.

From these studies, it is apparent that the growth of *S. aureus*, *E. coli*, and *S. faecalis* on chicken stored at low temperature is greatly exceeded by that of *Pseudomonas*, even when the latter forms only a small proportion of the initial flora. Growth curves presented in Figs. 5 and 7 indicate that spoilage of poultry by *Pseudomonas* will still occur when initial numbers of staphylococci are greater than those of fluorescing spoilage organisms. Conditions favorable for growth of psychrophiles on poultry meat appear to limit reproduction of *S. aureus* so

that it does not attain numbers in the order of several million per gram required to produce illness (Dack *et al.*, 1960).

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Journal Paper No. J-5186 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa. Project No. 1392, Center for Agricultural and Economic Development cooperating.

The investigation was supported in part by PHS research grant EF-00113-11 from the Division of Environmental Engineering and Food Protection, Public Health Service.

The authors thank Mrs. Carmen Rey for able technical assistance in the laboratory.

JOHN E. AMOORE and DELPHA VENSTROM

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

# Sensory Analysis of Odor Qualities in Terms of the Stereochemical Theory

## SUMMARY

**In correlating chemical constitution with odor character, the matching-standards method of Schutz was modified for use with the seven primary odors of the stereochemical theory. Information is given regarding suitable standard chemicals, threshold concentrations, and matching odor intensities. This method of odor analysis provides, for any unknown odor, a set of seven numerical values representing the similiarities between that odor and each of the seven standards. Odor analyses are reported for the seven standards themselves, for mixtures of two or three standards, and for 21 compounds closely related in odor to one or another of the standards. The analyses by the panel agreed well with literature descriptions of the odors. The method was further applied to analyzing a series of 18 compounds with complex odors involving two or more primes, compounds with odors disputed in the literature, and compounds with no description at all. Examples are mentioned of the similar odors of isosteric compounds, and the divergent odors of isomers. The experimental findings are generally in keeping with the stereochemical theory.**

The aroma of a food is due to numerous volatile constituents. Powerful analytical techniques have identified many of the compounds responsible. However, the relationship between each structural formula and the corresponding odor is as yet only partially understood. Currently under investigation, as a means of bridging this gap in our knowledge, is the "stereochemical theory of olfaction" (Amoore, 1962a,b). The theory postulates that the odor quality of a chemical compound can be correlated with the physical fit of its molecules into certain "receptor sites" at the olfactory nerve endings. This concept is being tested experimentally. The site-fitting potential of a compound may be measured by the displacement that occurs when its molecular model is placed in a water-filled model of the receptor site (Amoore, 1964). However, the odor quality of the chemical is difficult to estimate quantitatively because odor is a subjective impression and liable to human variability.

Schutz (1964) recently proposed a panel method which appears acceptable for describing odors. It is called the "matching-standards method for characterizing odor qualities." Indeed, Schutz suggested that his method could be used for testing the stereochemical theory of odor. Accordingly, we modified the matching-standards method for use with the stereochemical theory. The method yields for any substance its "odor dimension analysis," a numerical description of its odor, by a panel of judges, in terms of the seven primary odors of the stereochemical theory. This paper presents sensory scores for 46 compounds.

Stereochemical analyses of these same compounds have now been made, and the corresponding sensory and stereochemical assessments have been compared (Amoore, 1965a). The measurements, which have been applied to 107 compounds (Amoore and Venstrom, 1965), prove that there is an extremely significant correlation ($P < 10^{-9}$) between the sensory and the stereochemical determinations of olfactory similarity. The steric theory of odor has recently been reviewed in the context of flavor research (Amoore, 1965b).

## MATERIALS AND METHODS

**Sources of odorants.** Nearly all the chemicals examined were used as received from the supplier (reagent-grade whenever available). The compounds were from Eastman Organic Chemicals, apart from the following: Formic acid, acetic acid, and benzene were from Allied Chemical; chloroform and diethyl ether from Mallinckrodt; potassium cyanide from J. T. Baker Chemical Co.; naphthalene and 1,1,2,2-tetrabromoethane from Fisher Scientific Co.; hexamethylethane, cyclooctanone, and cyclopentadecanone from Aldrich Chemical Co.; *l*-menthol from Fluka A. G.; and

*d,l*-menthone, geraniol, and musk xylol (2,4,6-trinitro-3,5-dimethyl *tert.*-butylbenzene) from K. & K. Laboratories, Inc. The following were gifts from Givaudan-Delawanna, Inc.: Thibetolide® (15-hydroxypentadecanoic acid lactone), Versalide® (1,1,4,4-tetramethyl-6-ethyl-7-acetyl-1,2,3,4-tetrahydronapthalene), cedrol, santalol, benzyl acetate, and phenylethylmethylethyl carbinol.

The *n*-amyl acetate, *tert*-amyl acetate, and cyclopentyl acetate were highly purified samples furnished by Dr. Roy Teranishi of this laboratory.

**Odor panel.** The subjects (13 men and 16 women) were staff members ranging widely in familiarity with chemicals and sensory procedures. No preliminary testing or selection of panelists was undertaken. Attendance averaged about 85%. The testing room has 5 individual booths with separate serving hatches, weak green illumination (7.5-watt lamps), and a charcoal-filtered air-ventilation system.

**Standard primary odorants.** The standards employed in this paper are the outcome of extensive testing and discussion, in this laboratory and in that of Dr. J. W. Johnston, Jr., of Georgetown University. The final selection (Fig. 1) should be regarded as a working set of standards, open to further refinement but quite practical as they are. The standards were diluted with odorless triple-distilled water. The solutions (20 ml) were presented to the judges in 125-ml Pyrex glass Erlenmeyer flasks with ground-glass stoppers, standard taper 22.

**Threshold measurements.** The primary standard odorants (not further purified) were submitted to odor threshold measurements by the panel. Apart from the vessels, the procedure was that of Guadagni *et al.* (1963). The "operational threshold" defined by those workers is the concentration of odorant that yielded just 70% correct responses (significant at the 1% level) in a paired comparison test against pure water as a control.

**Intensity matching.** A solution (1 ppm) of the musk, Thibetolide®, was adopted as the standard of intensity. Solutions of the other 6 standards were prepared at concentrations that appeared to the authors to approximate the intensity of the musk standard. Then all seven solutions were ranked by the panel of judges in order of intensity of odor. Keeping the musk concentration the same, the concentrations of the other standards were altered on successive days until none of the standards fell outside the limits of rank sums prescribed by Kramer (1963) for significance at the 5% level. The "matched concentrations" for the primary odor standards listed in Table 1 were arrived at by this procedure. These solutions deteriorate, so standards *A, B, D,* and *E* were made up freshly 3 times a week, *F* and *G* twice a week, and *C* once a week. The odorants and solutions were stored at room temperature. Five sets of 7 flasks containing the standard odors were prepared. The flasks were emptied and refilled with stock solution for each day's testing and, in the case of exceptionally volatile odorants (ether and chloroform), several times during the testing.

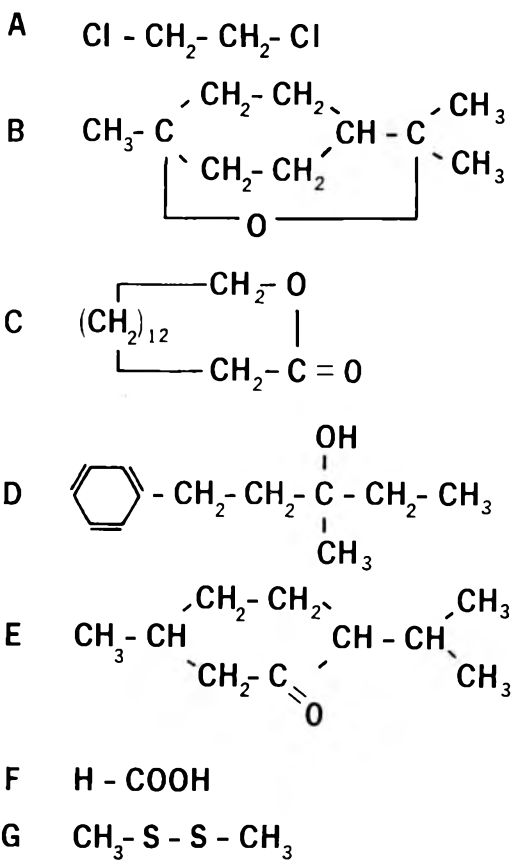


Fig. 1. Structural formulae of the seven primary standard odorants. See Table 1 for the chemical names and other data.

**Odor dimension analysis.** The unknown solutions (5 flasks of each) were freshly prepared for each session. Whenever possible, water was the diluent. With three compounds the odor of the aqueous solution was not intense enough, so they were dissolved in odorless mineral oil (Chevron white oil No. 3, N.F., Standard Oil Co., San Francisco). The concentrations of odorants intended for presentation as unknowns were first matched by the panel against two of the standards (*A* and *E*) to determine the equivalent intensity.

The judge was handed an enamel tray holding the primary odor standards, a flask containing the

Table 1. Primary odor standards.

| Code | Primary odor | Standard compound | Solubility in water[a] (ppm[b]) | Odor threshold (ppm[b]) | Matched concentration (ppm[b]) | Ratio: matched/ threshold |
|---|---|---|---|---|---|---|
| A | Ethereal | 1,2-Dichlorocthane | 6,900 | 29 | 800 | 28 |
| B | Camphoraceous | 1,8-Cineole | 3,800 | 0.012 | 10 | 830 |
| C | Musky | 15-Hydroxypentadecanoic acid lactone | 1.1[c] | 0.0007 | 1[d] | 1430 |
| D | Floral | *d,l*-β-Phenylethyl-methylethyl carbinol | 1,200[c] | 6.4 | 300 | 47 |
| E | Minty | *d,l*-Menthone | 780 | 0.17 | 6 | 35 |
| F | Pungent | Formic acid (90%) | ∞ | 1,500 | 50,000 | 33 |
| G | Putrid | Dimethyl disulfide | 2,800[c] | 0.0012 | 0.1 | 83 |

[a] At 20–25°C; literature values except where indicated.
[b] Parts per million of water; concentrations are v/v, except for the musk, which is w/v.
[c] Observed approximately in this work.
[d] Warm the water to about 40°C to melt the musk, then shake vigorously to dissolve.

unknown solution, and a score sheet (Fig. 2). He was expected to sniff the unknown and compare its odor with that of the first standard. The scale of similarity is based on that of Schutz (1964); a rating of 8 means extreme similarity, and a rating of 0 means no similarity. Then the judge compared the unknown odor with those of the other standards, examining them in the order indicated in Fig. 2. When the subject finished judging one unknown, the sample and score sheet were withdrawn, and replaced by a different sample and a fresh score sheet. The second unknown was judged in the same way as the first. Only two unknown samples were presented per session, which took 3–5 min of the judge's time.

Each unknown compound was presented to the panel twice (in nonconsecutive sessions). After each session the judges' ratings of the unknown's similarity to each standard were totaled up and averaged. Finally, the average scores for the two separate presentations of a given unknown were averaged. This routine yielded seven numerical values (rounded off to one decimal) which represent an analytical description of the unknown odor by a panel of observers in terms of the primary odors of the stereochemical theory. This procedure is herein called "odor dimension analysis."

**Reproducibility of the results.** We experienced difficulty with the large variation between the ratings ascribed by different judges to the same comparison of an unknown and a standard odor. A single column of raw data may contain all ratings from 0 to 8 for the same comparison. Nevertheless, the panel average score, struck at the foot of the column, was reasonably reproducible, even when the presentations were months

**ODOR DIMENSION ANALYSIS**

Select the standards in the order indicated. Compare the unknown with each standard in turn, and rate the degree of similarity on the following scale:

Extremely similar ..... 8
7
Very similar ..... 6
5
Moderately similar ..... 4
3
Slightly similar ..... 2
1
Not similar ..... 0

Ignore any differences in intensity, and concentrate on odor quality.

UNKNOWN ODOR #_____

| STANDARD ODOR | DEGREE OF SIMILARITY |
|---|---|
| D | |
| F | |
| B | |
| C | |
| E | |
| G | |
| A | |
| | |

Fig. 2. Score sheet for odor dimension analysis.

Table 2. Odor dimension analyses of the primary odor standards.

| Primary odor[a] | Degrees of similarity to standards[b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ether | Camphor | Musky | Floral | Minty | Pungent | Putrid |
| Ethereal | **6.3** | 1.2 | 0.6 | 1.1 | 1.1 | 0.4 | 0.1 |
| Camphoraceous | 1.0 | **6.2** | 1.2 | 1.3 | 2.1 | 0.2 | 0.1 |
| Musky | 0.5 | 0.5 | **5.9** | 1.9 | 1.2 | 0.3 | 0.0 |
| Floral | 0.7 | 1.3 | 1.8 | **6.3** | 1.7 | 0.1 | 0.0 |
| Minty | 0.6 | 1.7 | 0.6 | 2.4 | **6.7** | 0.2 | 0.0 |
| Pungent | 0.6 | 0.3 | 0.5 | 0.6 | 0.4 | **7.1** | 0.7 |
| Putrid | 0.3 | 0.2 | 0.3 | 0.1 | 0.1 | 1.5 | **7.0** |

[a] Compounds and matched concentrations as in Table 1.
[b] Means of two presentations to about 25 judges. For scale of similarity, see Fig. 2.

apart. We obtained standard deviations (SD) of panel average scores for 15 compounds (the seven standards and eight amyl acetate isomers) which were analyzed four times (instead of the usual twice). The SD appeared to average about ±0.5, with the panel average score in the range of 2.0–6.0. At either end of the similarity scale the SD decreased appreciably.

Schutz (1964) measured the reproducibility between two separate presentations of an unknown with the rank-order correlation for his 9 standards. For 10 unknowns, Schutz reported $\rho = 0.93$. For our 7 standards and 8 unknowns (the amyl acetate isomers) we found $\rho = 0.88$, which closely approaches the reproducibility Schutz found, and is significant at the 5% probability level.

## RESULTS AND DISCUSSION

**Odor thresholds.** The thresholds observed with the whole panel are shown in Table 1. The strongest standard odorant, Thibetolide®, can be detected at less than one millionth the concentration needed for the weakest. Hence, equal dilutions of odorants would not be an acceptable basis for comparing odor qualities. With two of the primary standards (1,8-cineole and dimethyl disulfide), enough tests were made to obtain approximate personal thresholds for all the individual judges. For both odorants the range of individual thresholds was remarkably wide, 300-fold. Yet none of the judges was obviously anosmic in everyday experience.

Group thresholds were computed for men, women, smokers, nonsmokers, old (over 40), and young. However, for none of these categories of judges, and for none of the standard odorants, have we observed any convincing differences in threshold. The data also provided a test of Le Magnen's (1948) remarkable finding that Exaltolide® (Thibetolide®) had little or no odor for the majority of men, but that women perceived an intense odor. With this compound, we observed no difference in the thresholds of the male and female subdivisions of our panel. We are unable to explain this discrepancy.

**Matching concentrations.** The concentrations of the primary odorants that appeared to match the musk standard in intensity are shown in Table 1 as matched concentrations. The last column of Table 1 shows that the matched concentration does not bear a constant ratio to the threshold. The ratio ranges from 28, for the ethereal standard, to 1430, for the musky standard, which shows that no fixed multiple of the threshold concentration could provide a satisfactory choice of concentration for presentation to the panel.

**Odor analyses of the primary standards.** The recognizability and mutual distinctiveness of the standards were checked by submitting them (as "unknowns") to the panel for the odor dimension analysis procedure. The results are shown in Table 2. Ideally, this matrix would consist of a diagonal row of 8's, representing extreme similarity of the primary odorants to themselves, surrounded by 0's indicating no similarity between different standards. The actual scores however, are not so perfect. The self-comparisons of the standards (heavy type in Table 2) register about 6–7, with an average of 6.5 (6 means very similar), and the cross

Table 3. Index of similarity values for the primary odor standards.

| Primary odor | Index of similarity ($D$) values[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Ether | Camphor | Musky | Floral | Minty | Pungent | Putrid |
| Ethereal | 0.6 | 7.3 | 8.1 | 7.7 | 8.0 | 8.9 | 9.3 |
| Camphoraceous | | 2.0 | 7.6 | 7.0 | 6.5 | 9.3 | 9.6 |
| Musky | | | 1.0 | 6.0 | 7.7 | 8.8 | 9.3 |
| Floral | | | | 1.0 | 6.5 | 9.3 | 9.7 |
| Minty | | | | | 1.4 | 9.6 | 10.1 |
| Pungent | | | | | | 0.9 | 8.4 |
| Putrid | | | | | | | 0.9 |

[a] The index of similarity, $D = \sqrt{\Sigma d^2}$, is the square root of the sum of the squared algebraic differences between the seven corresponding means for a given pair of standards (Schutz, 1964), e.g. for camphor/musky, $D = \sqrt{(1.0-0.5)^2 + (6.2-0.5)^2 + \ldots + (0.1-0.0)^2} = 7.6$. The cross-comparisons are from the data of Table 2. The self-comparisons (heavy type) are from Table 2 compared with a complete duplicate set of measurements.

comparisons range from 0 to 2, average 0.7 (2 is slightly similar).

Schutz (1964) suggested computing an "index of similarity" ($D$), to express quantitatively the overall resemblance between a given pair of unknown odors, taking all the degrees of similarity into consideration. The definition of the $D$ index is a footnote to Table 3. A low $D$ index means similarity, and a high $D$ index means dissimilarity. Indexes of similarity were calculated for the seven primary standard odorants (Table 3). The cross comparisons for pairs of primary odorants all show high $D$ indexes, in the range of 6–10. Although, in theory, $D$ could rise as high as 21, two very different primes ($D$ index about 10) represent as great a contrast in odor as we have encountered.

At the other end of the $D$ scale are self-comparisons of the standard odorants with themselves (heavy type in Table 3). Theoretically, the $D$ index would be zero for complete identity, but the actual values are greater, in the range of 0.6–2.0. This represents the inherent variability of the method.

**Odor analyses of mixtures of primary standards.** Interspersed among other unknowns, all 21 possible binary mixtures of the 7 standards were presented (once only) to the panel. Each standard odorant was present in the mixture at half its matched concentration. The panel average scores correctly identified both constituents of the mixture for all but one of the 21 pairs. The average scores for the primes actually present were 3.9, which contrasts well with the low average score of 0.9 against primes not included.

Of the 35 possible ternary mixtures among the primary standards, 7 were tried on the panel. The average score for primes included in the mixture (each at one-third of its matched concentration) was 2.3, and the scores against primes not present averaged 0.9. Hence, in going from single primes through two-prime to three-prime mixtures, the scores for primes present fell, roughly proportionately to their concentration, from 6.5 to 3.9 to 2.3, whereas the scores against primes not present remained around 0.8.

**Odor analyses of some primary odorants.** The procedure was next tested on 21 compounds which, according to the descriptions of their odors in standard reference works (Beilstein's Handbuch, 1918; Givaudan Index, 1961) appeared to fall into one or other of the 7 primary odor classes of the stereochemical theory. The degrees of similarity to the standards are shown in Table 4. The scores registered for each compound against its nominal primary odor are set in heavy type.

For instance, the group of three ethereal odorants all came out with higher degrees of similarity to the ethereal than to any of the other standards. The first two, diethyl ether and chloroform, had scores comparable with those of the standard, 1,2-dichloro-

Table 4. Odor dimension analyses of 21 compounds representing primary odors.

| Nominal primary odor | Compound | Matched concn.[a] (ppm) | Degrees of similarity to standards | | | | | | | *D* index[d] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ether | Camphor | Musky | Floral | Minty | Pungent | Putrid | |
| Ethereal | Diethyl ether | 200 | **5.1** | 1.9 | 0.7 | 1.4 | 1.8 | 0.3 | 0.0 | 1.5 |
| | Chloroform | 1,300 | **6.7** | 0.6 | 0.2 | 0.6 | 0.9 | 0.4 | 0.0 | 1.0 |
| | *n*-Propanol | 8,000 | **2.3** | 1.3 | 1.0 | 2.0 | 1.5 | 0.4 | 0.2 | 4.1 |
| Camphor-aceous | *d,l*-Camphor | 40,w/v | 1.6 | **5.4** | 1.3 | 0.9 | 2.3 | 0.3 | 0.2 | 1.1 |
| | Hexachloroethane | 10,w/v | 1.1 | **2.1** | 0.6 | 0.9 | 0.7 | 0.5 | 1.9 | 4.8 |
| | *tert.*-Amyl alcohol | 300 | 2.2 | **1.8** | 0.7 | 1.2 | 1.7 | 0.8 | 0.8 | 4.7 |
| Musky | Musk xylol | 14,000,w/v[c] | 0.4 | 0.7 | **5.1** | 2.8 | 1.0 | 0.1 | 0.2 | 1.2 |
| | Versalide® | 2,w/v | 0.5 | 0.7 | **5.3** | 2.3 | 0.8 | 0.1 | 0.0 | 0.9 |
| | Cyclopentadecanone | 2.5,w/v[b] | 0.3 | 0.4 | **5.5** | 2.5 | 1.0 | 0.1 | 0.1 | 0.6 |
| Floral | Geraniol | 40[b] | 0.9 | 1.8 | 2.2 | **4.4** | 2.1 | 0.3 | 0.0 | 2.0 |
| | β-Phenylethyl alcohol | 400 | 0.5 | 0.8 | 2.0 | **3.4** | 1.9 | 0.5 | 0.3 | 3.0 |
| | Benzyl acetate | 100 | 1.0 | 1.4 | 1.2 | **3.9** | 1.8 | 0.2 | 0.0 | 2.5 |
| Minty | *l*-Menthol | 75,w/v | 0.6 | 3.8 | 0.6 | 1.7 | **3.4** | 0.0 | 0.0 | 3.6 |
| | Cyclohexanone | 150 | 1.9 | 2.1 | 1.2 | 1.8 | **1.9** | 0.3 | 0.3 | 5.0 |
| | Diethyl sulfate | 800 | 1.9 | 1.9 | 1.3 | 2.4 | **2.1** | 0.2 | 0.2 | 4.8 |
| Pungent | Acetic acid | 7,000 | 0.5 | 0.4 | 0.1 | 0.3 | 0.3 | **4.6** | 0.9 | 2.5 |
| | Acetaldehyde | 150 | 1.6 | 1.0 | 0.4 | 1.4 | 1.1 | **1.0** | 0.9 | 6.3 |
| | Allyl alcohol | 500 | 2.3 | 1.1 | 0.6 | 0.9 | 0.8 | **1.3** | 1.8 | 6.2 |
| Putrid | Dimethyl sulfide | 0.05 | 0.7 | 0.3 | 0.9 | 0.5 | 0.4 | 0.9 | **3.7** | 3.5 |
| | *n*-Butyl mercaptan | 0.1 | 0.8 | 0.6 | 0.5 | 0.5 | 0.2 | 0.9 | **2.6** | 4.5 |
| | *n*-Butylamine | 500 | 0.8 | 0.7 | 0.7 | 0.5 | 0.6 | 0.7 | **2.6** | 4.6 |

[a] Dilutions are v/v in water, except where indicated.
[b] Super-saturated aqueous "solution;" matched concentration represents a fairly stable colloidal suspension.
[c] Solution in mineral oil; matched concentration unattainable in aqueous dispersion.
[d] Index of similarity between each nominal primary odorant and the corresponding standard primary odorant in Table 2. For definition of *D*, see footnote to Table 3.

ethane, itself. This is reflected by the low *D* indexes of 1.5 and 1.0, which indicate near identity between the odors of these unknowns and the ethereal standard. The *n*-propanol, however, should barely be included in the same class, because its *D* index of 4.1 approaches the level for different primary odors (6 and above).

The next section of Table 4 gives data for three nominally camphoraceous odorants. Camphor itself scored highly similar to the standard, 1,8-cineole. Hexachloroethane, however, was rated as being almost as putrid as camphoraceous. The *tert*-amyl alcohol undobutedly has a considerable camphoraceous component in its odor, but the panel average scores show a greater resemblance to the ethereal standard. Hence, by this method of odor analysis, *tert*-amyl alcohol should perhaps be transferred to the ethereal list, or at least be regarded as a complex odorant, exhibiting both camphoraceous and ethereal components in its odor, as well as some minty character.

The three musky odorants were taken from quite different chemical families, yet all three gave almost identical scores, and registered great similarity to the musky standard, as shown by the *D* indexes. This result agrees with the evidence obtained by Johnston and Sandoval (1962), through a different olfactometric procedure, that the various chemical families of musky odorants belong to a single primary odor category.

The floral odorants all showed a satisfactory degree of similarity to the prototype floral primary. However, for each of the nominal minty odorants the mint score came out only second-highest among the standards. This result shows that the three chemicals do have in common a substantial minty character although, quantitatively, the minty property is not the strongest component of the odor. Mintiness may represent a distinct cooling effect, registered by the trigeminal nerve, not the olfactory. Anosmic persons may still detect the presence of minty substances.

The pungent primary probably does not represent a true olfactory response either, but a mild pain reaction mediated through the trigeminal nerve endings. Anosmic subjects usually respond to pungent compounds. Acetic acid showed good similarity to the primary standard (formic acid), but the other two examples registered very low pungent scores. When the pungent character is present, particularly in the undiluted chemical, it may attract attention even though it is not quantitatively the strongest component of the odor.

The evil-smelling trio at the foot of Table 4 all gave substantially higher scores against putrid than for any other standard, but the *D* index showed that they fell considerably short of close similarity to the putrid standard itself, dimethyl disulfide.

Taken as a whole, the data of Table 4 are encouraging since they show fair to good agreement between the literature descriptions of the odors of these chemicals, and the scores obtained from our panel of judges working with a common set of standard primary odorants for comparison.

**Analyses of some complex odors.** Compounds with complex odors composed of several primes can be studied semiquantitatively by this method of odor dimension analysis. In Table 5 the column on the left shows the literature description of the odor. The first example, tetrabromoethane, was described as possessing an odor reminiscent of camphor and chloroform (Anschütz, 1883). Chloroform itself is ethereal (Beilstein, 1918; and see Table 4). The odor dimension analysis showed a score of 3.5 for ethereal and 1.3 for camphor character. Hence, for our panel of observers the ethereal note was much more apparent than was the camphoraceous. In fact, they placed the secondary note in the minty class (score 1.9). Interestingly enough, Johnston (1965) considered this compound to have a minty nuance, and used it to contribute mintiness in his formulations of certain complex odors.

Two compounds which typify the almond odor are benzaldehyde and nitrobenzene. Their patterns of scoring were closely similar: in fact, the *D* index for this pair was 1.3, which is within the observed range for identical odors (up to 2.0). The individual scores for benzaldehyde were greatest for the camphoraceous, floral, and minty components; ethereal and musky were weaker;

Table 5. Odor dimension analyses of 18 compounds representing complex odors.

| Nominal complex odor | Compound | Matched concn.[a] (ppm) | Degrees of similarity to standards | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Ether | Camphor | Musky | Floral | Minty | Pungent | Putrid |
| Ether-camphor | 1,1,2,2-Tetrabromoethane | 25 | 3.5 | 1.3 | 0.6 | 1.3 | 1.9 | 0.5 | 0.0 |
| Almond | Benzaldehyde | 20 | 1.3 | 1.7 | 1.1 | 2.7 | 2.1 | 0.1 | 0.1 |
| Almond | Nitrobenzene | 5 | 1.3 | 1.9 | 1.6 | 1.5 | 1.9 | 0.2 | 0.3 |
| Mint/almond | Cyclooctanone | 450,w/v | 1.5 | 3.3 | 0.3 | 0.9 | 2.4 | 0.3 | 0.6 |
| Almond/pungent | Hydrogen cyanide | 270,w/v[d] | 0.6 | 0.6 | 0.2 | 0.4 | 0.4 | 2.6 | 2.4 |
| Aromatic | Chlorobenzene | 5 | 2.2 | 1.5 | 1.1 | 1.2 | 1.1 | 0.3 | 0.4 |
| Aniseed | Isoquinoline | 20 | 0.9 | 1.9 | 2.0 | 2.1 | 1.8 | 0.2 | 0.3 |
| Lemon | *d*-Limonene | 5,000[c] | 1.0 | 1.3 | 0.9 | 3.2 | 1.9 | 0.2 | 0.0 |
| Cedar | Cedrol | (400 mg)[c] | 0.3 | 1.4 | 3.3 | 2.8 | 1.3 | 0.1 | 0.0 |
| Garlic | Diallyl sulfide | 0.25 | 0.9 | 0.4 | 0.3 | 0.4 | 0.4 | 1.2 | 3.7 |
| Rancid | *n*-Valeric acid | 25 | 0.3 | 0.7 | 0.6 | 0.4 | 0.4 | 1.3 | 3.9 |
| Sandalwood | Santalol | 35[b] | 0.8 | 2.0 | 2.0 | 1.4 | 1.2 | 0.5 | 0.5 |
| Pungent | Hexamethylethane | 20,000,w/v[c] | 1.3 | 2.8 | 0.9 | 0.7 | 0.9 | 0.8 | 1.7 |
| Characteristic | Benzene | 200 | 3.6 | 1.1 | 0.7 | 0.8 | 0.9 | 0.3 | 0.3 |
| Characteristic | Naphthalene | 1 | 1.8 | 2.8 | 0.7 | 0.9 | 1.2 | 0.6 | 0.6 |
| — | *n*-Amyl acetate | 40 | 1.4 | 2.0 | 1.8 | 3.4 | 2.7 | 0.4 | 0.2 |
| — | *tert.*-Amyl acetate | 100 | 2.0 | 2.6 | 1.3 | 1.9 | 2.9 | 0.5 | 0.4 |
| Pleasant | Cyclopentyl acetate | 52 | 0.9 | 1.6 | 1.5 | 4.2 | 1.4 | 0.1 | 0.0 |

[a,b,c] See footnotes to Table 4.

[d] Potassium cyanide (651 mg/L), neutralized with an equivalent quantity (10 ml/L) or N-sulfuric acid.

[e] Dry powder; matched concentration unattainable in water and impracticably high in mineral oil.

pungent and putrid made virtually no contribution. The theoretical analysis of odorants into classes according to molecular shapes earlier indicated that the almond odor is complex, made up of camphoraceous, floral, and minty primary odors (Amoore, 1962b).

Cyclooctanone has been variously described as smelling like almonds (Wallach, 1907) or mint (Mager, 1893). To our panel the strongest component of the odor was camphoraceous, followed by minty, with the other primary odors of much less importance. However, the $D$ index of 2.6 showed kinship with benzaldehyde, whereas the $D$ index against the camphoraceous standard was 3.2 and against the minty standard was 5.0. This illustrates the value of the $D$ index in showing up resemblances between the total effects of two odorants which may not be apparent in the individual scores for the primary odor components.

The odor of hydrogen cyanide is recorded, in almost any book that mentions it (e.g. Beilstein), as almond-like. Its odor, if indeed it has this quality, is an embarrassment to the stereochemical theory of olfaction on account of the very small size of the HCN molecule compared with the molecules of other almond-smelling compounds (Amoore, 1963). However, the conventional description of the odor of HCN has been authoritatively disputed by Meyer (1935); he asserted that the odor is penetrating and pungent, quite different from that of bitter almond oil.

The panel scores for hydrogen cyanide show a pattern of response very different from that to benzaldehyde. The first five primes were very low, whereas pungent and putrid, almost totally absent in benzaldehyde, respectively scored 2.6 and 2.4. The $D$ index between HCN and benzaldehyde was 4.7, which approaches the 6.0 or more found for two different primaries. Nitrobenzene, it will be recalled, had a low $D$ index of 1.3 compared with benzaldehyde. Hence we conclude, in agreement with Meyer, that there is practically no resemblance between the odors of HCN and benzaldehyde, and that the dominant primary odor in HCN is pungency. This evidence indicates that the stereochemical theory has no case to answer with respect to the alleged almond odor of HCN.

The almond odor was the first of seven complex odor classes considered earlier (Amoore, 1962b) and broken down according to stereochemical principles into their probable primary odor components. One example of each of these complex odors was offered to the panel for analysis. The results are included in Table 5. The aromatic odor of chlorobenzene, the aniseed odor of isoquinoline, and the lemon odor of limonene all gave patterns of scoring rather similar to that of the almond odor.

Commercial cedrol crystals exhibit much of the odorous character of cedarwood (although purified cedrol is odorless; H. C. Saunders, personal communication). The panel analysis scores show that muskiness is the most important constituent, followed closely by floral, and with some camphoraceous and minty aspects, but with little contribution from the remaining three primes. This result accords with stereochemical expectations based on other cedar-like compounds (Amoore, 1962b). Johnston (1965) recently reported the converse of the above theoretical and experimental analysis of a complex odor; he has been attempting synthesis of the cedarwood odor. In an olfactometer he mixes four air streams bearing vapors of chemicals representing these four primary odors. When the correct proportions of the components were found, an approximation of a nonresinous woody odor resulted. Although the exact character of cedarwood or sandalwood continues to be elusive, the approximate synthesis of a complex odor from its theoretically and practically analyzed primes must be an important achievement.

The garlic odor is represented by diallyl sulfide. In agreement with expectations (Amoore, 1962b) the major primary components were ethereal, pungent, and putrid. However, predictions were not well fulfilled with the rancid odor of $n$-valeric acid. It was expected that the major components would be ethereal, minty, and pungent, but the panel placed the compound strongly in the putrid class, with pungency a poor sec-

ond and no other important contributions.

Sandalwood has not yet been submitted to stereochemical analysis. It is of interest in connection with Johnston's (1965) experiments on the synthesis of woody odors from their primes. The scoring pattern for santalol was rather like that of cedrol, but with less emphasis on musk and floral, and more on camphor and ethereal (*D* index 2.2 for santalol compared with cedrol).

Hexamethylethane is another compound whose Beilstein odor description appeared to constitute an exception to the stereochemical theory of odor. It was described as "Geruch stechend, sehr durchdringend." As pointed out in an earlier paper (Amoore, 1962b) its molecular shape indicates that it should smell camphoraceous, and it contains no electrophilic groupings to contribute any pungency. When hexamethylethane was presented to the odor dimension analysis panel (Table 5) the results disagreed with the Beilstein description and, instead, strongly supported the stereochemical prediction. The camphoraceous score was 2.8, and the pungent score only 0.8. In fact the compound very closely resembled its isosteric relative hexachloroethane in odor (see Table 4; the *D* index is 0.9). This contradiction was resolved when the original literature was consulted. It appears that Henry (1906) first announced that hexamethylethane had "une odeur piquante, très pénétrante," but two weeks later (1907a) he revised this to "son odeur camphrée est très pénétrante." Unfortunately the misleading earlier description was the one that found its way into Beilstein (1918, vol. 1, p. 165). Two weeks after the revision, Henry (1907b) himself pointed out the close similarity in odor between hexamethylethane and hexachloroethane.

The odor dimension analysis method can also be applied to odorous compounds with either no descriptions in the literature, or only uninformative remarks. In the latter category come the familiar benzene and naphthalene, whose odors are described unhelpfully in Beilstein as "characteristic." The judges placed these firmly in the ethereal and camphoraceous classes, respectively.

To close the list of odorants chosen to illustrate the usefulness of the matching-standards method, we have taken three members from the isomeric family of amyl acetates, which is being studied in considerable detail in this laboratory. The straight-chain parent compound, *n*-amyl acetate, has no literature odor description known to us. Nor has its highly branched-chain isomer, *tert*-amyl acetate. The latter has a globular molecule, more able to fit the camphoraceous receptor site of the stereochemical theory than its straight-chain isomer. In agreement with this concept, the *tert*-amyl ester did register a higher camphoraceous score than the *n*-amyl acetate. Cyclization of the 5-carbon amyl radical yields cyclopentyl acetate, which has the kite-shaped molecule typical of floral odorants (Amoore, 1962b). The Beilstein description is simply "pleasant," and indeed it did show a higher floral score than did its straight-chain relative.

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Ms. rec'd 5/7/65.

We are very grateful to the members of the odor panel for their extended cooperation and tolerance. We thank Dr. D. Althausen and Mr. D. Guadagni for continued interest and advice.

Reference to a company or product does not imply endorsement.

**HERBERT STONE and SHIRLEY OLIVER**
*Department of Food Sciences and Nutrition*
*Stanford Research Institute, Menlo, Park, California 94025*

# Effect of Viscosity on the Detection of Relative Sweetness Intensity of Sucrose Solutions

## SUMMARY

**Data are presented to demonstrate the relationship between the subjective mouthfeel of selected gums, cornstarch, gum tragacanth, carboxymethyl cellulose and methyl cellulose, and their rheological properties as measured by a viscosimeter. The mouthfeel of the gum solutions was evaluated subjectively on a 7-point intensity scale of degree of sliminess. The viscous solutions containing sucrose were ranked more sweet by most of the panel; however, rankings between pairs of viscous and non-viscous solutions were not significantly different. Sucrose detection thresholds for the panel also changed as a function of viscosity. Sensitivity for sucrose was highest in water, lowest in the presence of carboxymethyl cellulose, and intermediate with cornstarch. The results of these tests and their application to foods is discussed.**

## INTRODUCTION

In the sensory evaluation of foods, it is generally acknowledged that the primary determinants of acceptance or rejection are color, taste, and odor. Recent research has drawn attention also to the importance of mouthfeel, a textural property (Szczesniak and Farkas, 1962; Wick, 1963). The Sensory Testing Guide (1964) defines mouthfeel as "the mingled experience deriving from the sensations of the skin in the mouth during and/or after ingestion of a food or beverage. It relates to density, viscosity, surface tension and other physical properties of the material being sampled."

Szczesniak and Farkas (1962) demonstrated a correlation between mouthfeel and rheological properties of a large number of gum solutions. Their data indicated that "slimy mouthfeel" was related to a basic characteristic of the gum and, coupled with data on viscosity vs. rate of shear, established three gum classes (nonslimy, somewhat slimy, and very slimy). Thus it should be possible to predict, with some degree of confidence, the mouthfeel characteristics of a particular gum from objective measurements. However, all foods that utilize gums are not tasteless, and it would be important to know if any changes occur in the presence of the gum. Mackey and Valassi (1956) and Mackey (1958) reported that thresholds for the four basic tastes were lower in water solutions than in food products and that taste substances were more difficult to detect in foams or in gels. Taste sensitivity to quinine, caffeine, or saccharine in oil or water plus gel indicated that sensitivity was greatest when water was the solvent, and least when oil was the solvent.

Thus, it should be possible to decrease sucrose levels in certain types of foods, i.e., fruit syrups, while retaining the same sweetness levels through selection of appropriate gums. If this assumption is correct, the relative sweetness intensity of gum solutions should increase as a function of the increased viscosity of the experimental solutions. To test this hypothesis, a series of investigations was undertaken to determine the relative sweetness intensity of sucrose solutions in aqueous solutions and in the presence of various gums.

## METHODS

**Materials.** Five gums (encompassing the viscosity range of nonslimy to extremely slimy properties) described by Szczesniak and Farkas (1962) were obtained commercially. These included cornstarch, 2% (National Starch and Chemical Corp., CWS-50-5850, No. 1215), gum tragacanth, 1% (Morningstar-Paisley Inc., No. 1), carboxymethyl cellulose, 1.0% (Hercules Powder Co., 7HSXP), methyl cellulose, 2.6% (Dow Chemical Co. No. 65HGSH), and pectin (Sunkist Pectin, N. Y., No. 3442, pure citrus). The listed gum concentrations were the same as those used by Szczesniak and Farkas. Because the methyl cellulose was reported to be too unpalatable and rather difficult to work with, the carboxymethyl cellulose was substituted. The tests with pectin were discontinued because of "citrus-like" off-flavors which tended to interfere with normal perception.

**Viscosity measurements.** Viscosity measure-

ments were made at room temperature (22±1°C) with a Brookfield viscosimeter, model HAT, at spindle speeds 1 and 3, and the values were converted to centipoises. A plot of viscosity vs. rpm data (Fig. 1) for the test gums was in agreement with results of Szczesniak and Farkas (1962).

**Sensory evaluations.** Standardized test techniques and procedures were employed in all experiments. Facilities included air-conditioned partitioned booths with controlled lighting and sinks for expectoration. Solutions were prepared according to Pfaffmann *et al.* (1954).

Four different experiments, including intensity and hedonic ratings and threshold and viscosity measurements, were conducted with a trained five-man panel.

Experiment 1 consisted of intensity ratings, on a 100-point scale, of sucrose solutions at 1, 2, 5, 10, and 15% (.029, .058, .146, .292, and .438*M*). Zero equalled no noticeable taste; 50, moderate sweet taste; 100, most intense sweet taste. Daily for 18 days, subjects received an identified reference sample of intensity 50 (6%), followed by five test solutions in random sequence. Subjects also rated their hedonic impression of each solution on a 9-point hedonic (like-dislike) scale (Jones *et al.*, 1955). These data were converted to digits—1, dislike extremely; 9, like extremely—and mean scores were plotted.

In Experiment 2, the mouthfeel of four representative gums was evaluated on a 7-point intensity scale with descriptive words based on that of Szczesniak and Farkas. The major differences were that a profile-type panel was not used, and each subject evaluated the gums separately. In pretest sessions, the panel received instructions on mouthfeel properties and samples representing the viscosity range being tested. The test gums were constarch (nonslimy), gum tragacanth (moderately slimy), carboxymethyl cellulose gum (slimy), and methyl cellulose gum (extremely slimy). Each gum was evaluated 15 times by each subject, but only the last 10 replications were included in the analysis.

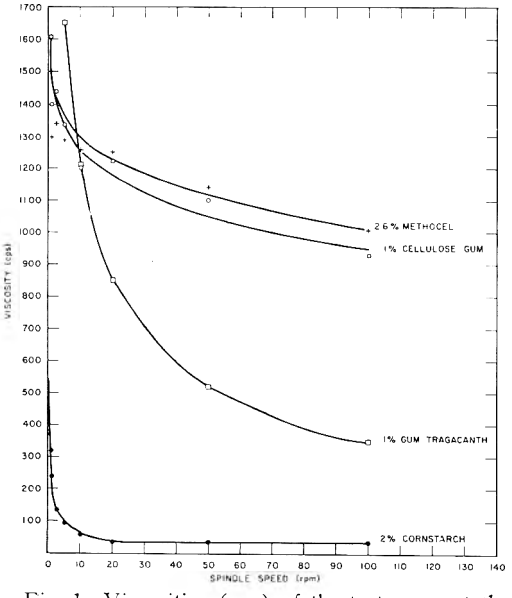


Fig. 1. Viscosities (cps) of the test gums at the different spindle speeds (rpm).

Experiment 3 involved ranking the sweetness of 1, 2, 5, and 10% sucrose solutions against solutions containing cornstarch (nonslimy) or carboxymethyl cellulose based on increasing sweetness. Each pair was tested 20 times, and the final 15 responses were included in the analysis.

Experiment 4 was a threshold experiment for sucrose in aqueous solutions with and without added cornstarch and carboxymethyl cellulose gum. A randomized single sample presentation was used. Subjects received 12 solutions daily, of which 2–4 were blanks, and were instructed to indicate the samples containing sucrose. The initial test concentrations were selected to include the reported median detection threshold for sucrose (.01*M*), .035, .0175, .008, and .004*M*. For most of the panel, the initial test concentrations were the higher levels, and the lower intensities were introduced only in the later stages of testing. Therefore, no subject received more than three intensity levels on any test day. This permitted easy adjustment to higher or lower intensities according to individual sensitivity. When thresholds were completed in the aqueous system, the experiments were repeated in the presence of cornstarch and carboxymethyl cellulose.

**Analysis of data.** Whenever possible, each subject's scores were treated separately because significant differences among individuals were evident in all the experiments.

Mean scores for individuals and for the panel were used to characterize the hedonic scaling data and the mouthfeel properties of the gums. The rankings of the sucrose solutions with and without added gum were analyzed by the Wilcoxon matched-pairs signed-ranks test and the Friedman two-way analysis of variance (Siegel, 1956). The method of least squares was used to determine the sucrose detection thresholds in the aqueous and gum solutions (Goulden, 1952).

## RESULTS

The data from the preliminary experiments (Fig. 2) show that the panel had an optimum sucrose preference at approximately 5%. The data also revealed marked individual differences in preference for sucrose. The lower part of Fig. 2 shows

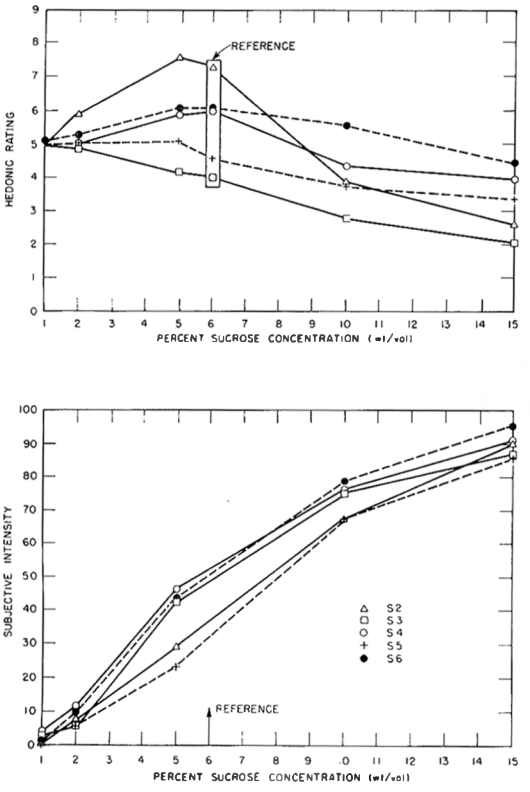


Fig. 2. Hedonic ratings of the sucrose solutions by the individual panelists (upper figure). The subjective intensities of these sucrose solutions are shown in the lower figure.

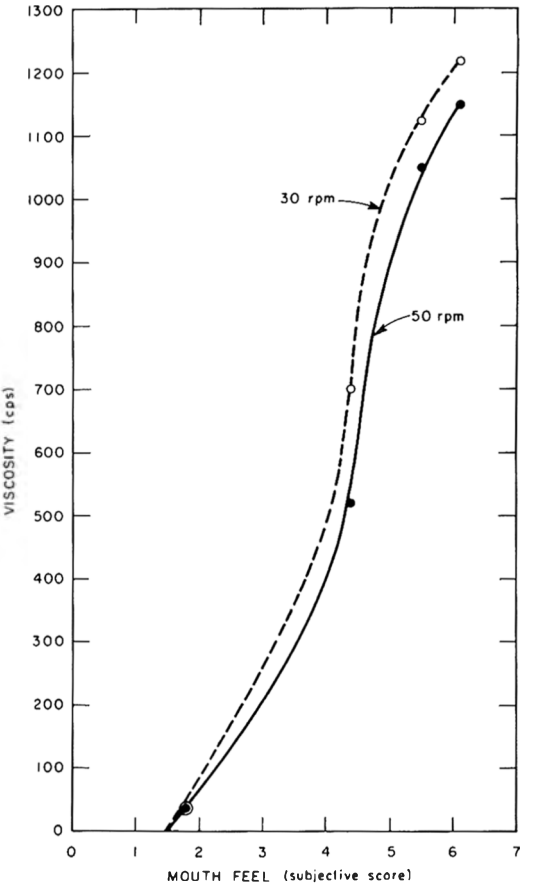


Fig. 3. Relationship between subjective mouthfeel and the viscosity of the test gums at 30 and 50 rpm. These data were derived from the subjective ratings in Table 1 and the viscosity measurements shown in Fig. 1. The curves at 30 and 50 rpm are not significantly different.

that the panel rated the intensity of the sucrose solutions on a linear basis, and that differences between the individual panelists were less obvious than on the basis of hedonic ratings.

The results of subjective evaluation of the mouthfeel of the selected gums are shown in Table 1 and Fig. 3. Fig. 3 illustrates the validity of the relationship between subjective mouthfeel and the rheological properties of the test gums. Thirty. and 50 rpm were selected as typical of tongue movement during mastication, as suggested by Szczesniak and Farkas (1962).

Table 1. Mean ratings for subjective mouthfeel of test gums.[a]

| Subject | Percent concentration of added gum | | | |
|---|---|---|---|---|
| | Starch 2% | Gum tragacanth 1% | Carboxymethyl cellulose 1% | Methyl cellulose 2.6% |
| II | 1.2[b] | 5.4 | 4.9 | 5.6 |
| III | 2.0 | 3.6 | 6.3 | 6.6 |
| IV | 2.1 | 4.0 | 6.0 | 6.8 |
| V | 1.0 | 4.8 | 5.1 | 5.6 |
| VI | 2.0 | 4.1 | 5.3 | 6.2 |
| $\bar{X}$[c] | 1.7 | 4.4 | 5.5 | 6.1 |

[a] The panel used a 7-point scale: 1, nonslimy, to 7, extremely slimy.
[b] Each subject evaluated each gum 10 times.
[c] Mean score for the panel.

Ranking the sucrose solutions based on increasing sweetness intensity with or without added cornstarch or gum yielded the data in Tables 2 and 3. In general, the more viscous solution was listed more sweet when compared to the same sucrose solution without added gum. However, there were individual exceptions. Analysis of the individual rankings by the Wilcoxon matched pairs signed-rank test showed significant differences for some of the rankings but not in any consistent manner (Siegel, 1956). A Friedman two-way analysis of variance of the average ranks yielded no significant differences between the pairs at any test concentration (Siegel, 1956).

The results of the threshold experiments (Table 4) indicated the panel was most sentitive to su-

Table 2. Average rankings of sweetness for aqueous solutions with and without added cornstarch.[a]

| | Percent sucrose concentration | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 5 | | 10 | |
| Subject | None | Cornstarch | None | Cornstarch | None | Cornstarch | None | Cornstarch |
| II | 1.07[b] | 1.07 | 3.33 | 3.26 | 5.26 | 5.46 | 7.15 | 7.34 |
| III | 1.66 | 1.60 | 3.33 | 2.53 | 5.33 | 5.0 | 7.34 | 7.15 |
| IV | 1.0 | 1.0 | 3.06 | 3.13 | 5.13 | 5.13 | 7.26 | 7.07 |
| V | 1.13 | 1.20 | 2.53 | 2.13 | 5.33 | 5.40 | 6.86 | 7.46 |
| VI | 1.20 | 1.27 | 3.33 | 3.20 | 5.20 | 5.46 | 7.15 | 7.20 |
| $\bar{X}$[c] | 1.212 | 1.228 | 3.116 | 2.850 | 5.250 | 5.290 | 7.152 | 7.244 |

[a] 2% cornstarch (CWS-50-5850, No. 1215 National Starch Co.).
[b] Mean of 15 replications per subject.
[c] Mean score for the panel.

Table 3. Average rankings of sweetness for aqueous solutions with and without added cellulose gum.[a]

| | Percent sucrose | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 5 | | 10 | |
| Subject | None | Gum | None | Gum | None | Gum | None | Gum |
| II | 1.47[b] | 1.53 | 3.33 | 3.67 | 5.10 | 5.90 | 7.30 | 7.70 |
| III | 1.36 | 1.70 | 3.60 | 3.40 | 5.16 | 5.50 | 7.46 | 7.53 |
| IV | 1.46 | 1.53 | 3.13 | 3.86 | 5.06 | 5.93 | 7.83 | 7.16 |
| V | 1.60 | 1.67 | 3.10 | 3.96 | 5.53 | 5.43 | 7.40 | 7.50 |
| VI | 1.73 | 1.46 | 3.43 | 3.43 | 5.93 | 5.07 | 7.70 | 7.30 |
| $\bar{X}$[c] | 1.52 | 1.58 | 3.31 | 3.67 | 5.35 | 5.56 | 7.53 | 7.43 |

[a] 1% carboxymethyl-cellulose (Hercules Powder Co., 7HSXP).
[b] Mean of 15 replications per subject.
[c] Mean score for the panel.

Table 4. Detection thresholds for sucrose in solutions of varying viscosity.[a]

| | Molar concentration | | |
|---|---|---|---|
| Subject | Aqueous | With cornstarch | With cellulose gum |
| II | .0099 | .0113 | .0181 |
| III | .0125 | .0128 | .....[b] |
| IV | .0075 | .0111 | .0149 |
| V | .0125 | .0122 | .0173 |
| VI | .0125 | .0160 | .0150 |
| $\bar{X}$[c] | .0105 | .0128 | .0163 |

[a] 50% detection threshold.
[b] Subject was not available for testing.
[c] Mean score for the panel; reported value for sucrose in an aqueous system is .01*M* (.005–.016), according to Pfaffmann (1959).

crose in water, and least sensitive in the presence of cellulose gum. Cornstarch decreased sensitivity to sucrose only slightly. The sucrose thresholds in cornstarch increased for all subjects except one, but still fell within the normal range for aqueous sucrose thresholds. Most important is the decreased sensitivity with increasing viscosity, a relationship exhibited by all panelists. These data are in agreement with data of Mackey and Valassi (1956) and Mackey (1958) indicating that discrimination was decreased in the presence of a viscous material.

## DISCUSSION

These experiments provide additional evidence for the effect of viscosity on the relative sweetness intensity of sucrose solutions.

Although rankings of the pairs of viscous and nonviscous solutions were not significantly different, the data suggest that more viscous material possesses greater relative sweetness. Use of the restricted scale probably reduced some of the differences; however, in preliminary tests this type of scale was understood more readily by the panel. Another explanation may be that sucrose concentrations were too low, and that much greater differences are present at higher concentrations. These experiments remain to be undertaken.

Several subjects reported the more viscous solutions as being smoother and possessing

a more lingering sweetness. But, as would be expected, response to the aqueous samples was much faster. One problem encountered with many of the gums was the presence of undesirable off-flavors. The cornstarch possessed a typical "raw starch" taste which was just perceivable at the sucrose concentrations used in these tests. In addition, the more viscous materials possessed slimy characteristics which may have been sufficiently disagreeable to mask any increase in relative sweetness. As demonstrated by Pangborn and Hansen (1963) and others, food associations are important in affecting food acceptance. If the effect of contrast existed, it may have been sufficient to eliminate any potentially large difference in relative sweetness (Kamenetzky, 1959).

Although these explanations are speculative, the contrast (and convergence) effects known to occur in foods were presumably operating in the test situations. While additional testing may have yielded significant differences, the objectives of the experiment did not permit testing for a significant difference *per se*. Considerably more work is needed on the sensory properties of viscous solutions before definitive conclusions can be reached.

Individuals showed considerable variation in their rankings of the same sucrose concentration with different gums. Mean rankings of relative sweetness were greater with cellulose gums than with cornstarch. Apparently, the panel shifted its frame of reference for relative sweetness for both aqueous and gum solutions. In contrast to this shift in relative sweetness with increased viscosity, the sucrose detection thresholds showed the opposite; a decrease in sensitivity with increasing viscosity. The decrease represented a change in the molar concentration from 50 to 17%. For the four subjects who completed all tests, the average decrease in sensitivity was 18% in the presence of cornstarch and 36% in the presence of cellulose gum. Similar changes in sensitivity might be anticipated for gums possessing intermediate viscosities. It should be emphasized, however, that the percentages reported are intended only to show the extent of change possible, and should not be misconstrued as final quantitative values. It would be interesting to establish why sensitivity decreased and relative sweetness intensity increased with increasing viscosity. Either the viscous material interfered with the perception process by coating the tongue, or it retarded the rate of release of the sucrose into the mucous bathing the receptor sites.

These experiments provide a better insight into the sensory properties of viscous materials and their role in food acceptance. The problem seems complex, however, and it appears that investigators should consider the mouthfeel properties of foods more carefully.

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Ms. rec'd 7/23/65.

The authors thank the sensory panel for its cooperation and assistance in evaluating the various gum solutions. Thanks also to Mr. A. H. Knechtel, of Hercules Powder Company (San Francisco), for the samples of sodium carboxylmethyl cellulose gum, and to Mr. Beaumar of National Starch and Chemical Corporation (New York) for the samples of corn starch (CWS).

This research was supported by a grant from the Institute Research and Development program of Stanford Research Institute.