IMPROVED METHODS FOR DETERMINATION OF CERTAIN ORGANIC ACIDS IN LIQUID WHOLE EGG AND THE INFLUENCE OF PASTEURIZATION ON THEIR CONCENTRATION

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John G. Reagan

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ABSTRACT

IMPROVED METHODS FOR DETERMINATION OF CERTAIN ORGANIC ACIDS IN LIQUID WHOLE EGG AND THE INFLUENCE OF PASTEURIZATION ON THEIR CONCENTRATION

By

John G. Reagan

Liquid whole egg to which acetic, butyric, lactic, propionic and succinic acids had been added and liquid whole egg which had previously been incubated at room temperature to different microbial populations were pasteurized at 60.5 C for 3.5 min. The pasteurizer was a continuous flow system, developed by using two temperature controlled water baths, through which egg products were pumped in 7 mm o.d. glass tubing. This treatment reduced bacterial numbers a minimum of 99. percent.

A liquid-liquid extraction method was developed for the extraction of all five acids instead of the liquid-liquid and steam distillation extraction methods currently employed in AOAC (1965) procedures. This extraction procedure involved the centrifugation of the denatured egg to obtain the supernatant and a modification of the

extractor by increasing the 100 ml capacity sample holding section to 500 ml.

The concentration of each acid before and after pasteurization was evaluated in liquid whole egg samples after freezing and thawing. The recovery of the five acids was evaluated by gas-liquid chromatography. Twenty percent diethylene glycol succinate on 120-130 mesh Anakrom ABS was used for the column packing material.

Lactic and succinic acids were recovered from the liquid whole egg samples and chromatographed as their butyl ester derivatives along with an internal standard, butyl decanoate. Acetic, propionic and butyric acids were recovered from liquid whole egg samples and chromatographed as the acids per se along with the same internal standard used for the butyl ester derivative determinations.

By programming the column temperature, the dibutyl succinate peak became sharper when compared to the peak obtained under isothermal conditions which are currently advocated for detecting the ester derivatives of acids in eggs by use of GLC procedures (Steinhauer, 1968 and Salwin and Bond, 1969).

The pasteurization process did not affect the concentration of the short-chained organic acids evaluated. There was no consistent difference in the organic acid concentration between pasteurized and nonpasteurized egg products. Thus, if the acids are present in the liquid

whole egg product before pasteurization, they will be present in the pasteurized product at about the same level. An open batch type pasteurization system could conceivably result in a small loss of some of the volatile organic acids such as acetic, butyric and propionic acids.

The fresh egg samples contained acetic and lactic acid concentrations of 3.91-4.31 mg/100 g egg and 2.55-2.96 mg/100 g egg, respectively. These results and the results of Landes and Dawson (1969) do not agree with the court decision (Anon., 1959) that when acetic and/or formic acids are present, the egg product contains decomposed substances.

Liquid whole egg product incubated until a microbial population of 4.5×10^6 was obtained, contained an increase in only acetic and lactic acids. A relatively high percentage of these two acids were already present in the fresh egg sample.

Sensory evaluations showed significant differences in flavor between fresh egg and egg with short-chain organic acids added. Even at the lowest level of addition, 5 ppm of each acid, an unnatural flavor was detected. A significant difference in odor between control and treated egg was observed only in the egg to which 200 ppm of each acid was added. No significant differences in odor were observed in the incubated egg samples.

A modified procedure was developed and evaluated for evaluating short-chain organic acids in liquid whole egg. Accurate and valid analyses of the short-chain organic acids in pasteurized whole egg products can also be made since the pasteurization procedure does not result in a loss of the acids used to determine the wholesomeness of the whole egg products.

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INTRODUCTION

The measurement of egg product quality, which includes wholesomeness, may consist of physical, chemical and biological tests. Wholesomeness of a food product can be measured by the presence or absence of disease-producing bacteria, such as salmonellae and staphylococcus; by bacteria closely associated with disease-producing microorganisms, such as coliforms and enterococci; and/or by total microbial population, which is a measurement of the degree of contamination from all sources. Chemical tests are also conducted to determine the presence or absence of toxic materials or chemical compounds which may indicate decomposed substances.

In an effort to improve egg product quality by reducing the microbial population and destroying all food poisoning bacteria, such as salmonellae, current federal regulations require that all liquid egg products, which may enter interstate commerce, be pasteurized.

Egg products are presently analyzed for both viable and non-viable microbes, the presence or absence of salmonellae and coliforms, the odor of the product and the

presence of acetic and lactic acids to determine the product's wholesomeness (U.S.D.A., 1967). Chemical tests for the presence of formic and succinic acids are also conducted on a routine basis. Since whole eggs are analyzed for the presence of these organic acids by regulatory agencies as a part of their inspection program, it is important to know if the required pasteurization process affects the acid content of the eggs. Gas-liquid chromatography was used to determine the presence and amount of acetic, butyric, lactic, propionic and succinic acids since it offers the advantage of being accurate, rapid and sensitive.

The primary objective of this study was to determine what effect pasteurization had on the concentration of the five organic acids in liquid whole egg. Other objectives of no less importance were the improvement of the methods used to determine the five acids in liquid whole egg and to determine if the acids in the egg enabled taste panel members to detect a difference in flavor or odor.

REVIEW OF LITERATURE

Examination of Frozen Egg

Bacterial and physical changes occur in eggs of high and low quality during preparation, freezing and storage. Schneiter, et al., (1943) reported that (1) frozen egg of good quality is able to withstand at least two complete thawings and refreezings without significant changes in bacterial content or without acquiring an abnormal appearance or odor; (2) shell eggs of low quality, including cracks, leakers and dirty eggs usually have high bacterial counts and this condition leads to progressive decomposition of the liquid egg product unless it is rapidly frozen and maintained in that state; (3) prolonged storage of frozen egg for a six year period results in a considerable reduction in viable bacteria count, but the total count (direct microscopic) serves as a reliable index of the original quality of the product.

Lepper, et al., (1944) reported that a direct microscopic count of over 5,000,000 bacterial cells/g of liquid or frozen egg with determinable amounts of either formic acid or acetic acid, or lactic acid in excess of

7 mg/100 g of liquid egg, demonstrated the presence of decomposed egg. Further, certain types of decomposed egg could be present in liquid and frozen eggs without being detected by these bacteriological and chemical methods.

According to Lepper and Hillig (1948), succinic acid was not found in shell eggs of acceptable edible quality or in frozen egg prepared from such shell eggs. It was formed during some process of egg decomposition, either in the shell or after separation from the shell.

Decomposition, either chemical or bacteriological, does not develop in sound, edible and wholesome liquid egg which is promptly frozen after breaking and mixing (Hillig, et al., 1960). Their studies confirmed that decomposed egg is present when frozen egg contains a direct microscopic count of over 5,000,000/g of egg with determinable amounts of either formic and/or acetic acids or lactic acid present in excess of 7 mg/100 g of egg. Succinic acid was also demonstrated as an additional chemical index of decomposition.

Steinhauer, et al., (1967) reported that the number of bacteria in liquid whole eggs stored up to eight days at 16 C increased from 8.0×10^2 to 3.6×10^8/g of liquid egg. Lactic acid increased from 0.48 mg/100 g egg to 18.15 mg/100 g egg and succinic acid was detected in small amounts in all samples of egg evaluated. Very little acetic or formic acid was found.

The organic acid content of liquid whole egg allowed to decompose from natural microbial contamination was found to be 214.9 mg of lactic acid, 35.6 mg of acetic acid, 29.8 mg of succinic acid, 17.8 mg of formic acid and 4.6 mg of propionic acid per 100 g of liquid whole egg (Steinhauer, 1968). No butyric acid was detected. The microbiological examination of this same egg product showed a total plate count of 1.7×10^9 bacteria/g of egg, with the presence of 5.1×10^8 viable salmonellae and 3.5×10^8 coliforms, indicating that the bulk of the acids found in the eggs may have originated from enteric and/or lactic acid microorganisms.

Landes and Dawson (1969) found that a number of different acids were present in fresh whole egg products. Those present in the highest quantities were formic, acetic and lactic acids. After microbial decomposition these three acids were still the most predominant acids in the egg.

Salwin (1968) reported the presence of β-hydroxybutyric acid in incubated chicken egg (incubator rejects). The acid was not observed in fresh eggs, in nonincubated decomposed eggs nor in incubated turkey eggs. This is important to regulatory agencies since these eggs are not permitted in foods produced for human consumption (U.S.D.A., 1967).

The results of a court case during 1959 involving the shipment of frozen whole eggs from Tennessee to Illinois established a precedent for the validity of organoleptic tests, microbial population and the presence of certain organic acids in frozen whole eggs suspected of being decomposed or of containing decomposed eggs (Anon., 1959). The following conclusions of law applicable to this study were made:

1. "Organoleptic tests by the use of the sense of smell are determinative of the presence of decomposed substances in frozen eggs when the odor of decomposition is present. Decomposition can exist, however, even when no odor is obtained but this decomposition will be detected by bacteriological and chemical analyses."
2. "The presence of bacteria in frozen whole egg in an amount in excess of 5,000,000 per gram of egg by direct microscopic count is determinative of the presence of decomposed substance in eggs."
3. "The presence of acetic, formic or succinic acid in any measurable quantity in frozen whole egg is determinative of the presence of decomposed substances in the eggs."

4. "The presence of lactic acid in excess of seven milligrams per gram of egg in combination with a direct microscopic bacteria count of 5,000,000 or more is determinative of the presence of decomposed substances in frozen eggs."

History of Heat Pasteurization

The preservation of foods by application of heat was known as early as 1766 when Spallanzani preserved meat extract in sealed flasks by heating at 100 C for one hr. The limited application of heat which we know today as pasteurization was introduced by Pasteur in 1864 to prevent wine spoilage. In 1871, Pasteur studied fermentations used in the brewing industry and in 1872 bottle beer was heated at 50-55 C to prevent it from becoming sour or putrid. It was in this connection that the term "pasteurized" or "pasteurization" was first used (Sommer, 1946).

Milk is one product which most Americans now take for granted as being pasteurized, and rightly so because of state and federal regulations. The pasteurization of milk was first employed for infant feeding. Soxhelt in Germany in 1886 proposed heated milk for infants, and in the United States, Jacobi by 1889 had been using heated milk for some time in his pediatric practice. In spite of the favorable attention which pasteurization was commanding from physicans and public health authorities, milk

distributors of that time were inclined to practice pasteurization secretly (Sommer, 1946). However, when Russell and Hastings (1900) and Rosenau (1912) showed that a heat treatment of 20 min at 60 C is adequate to destroy Mycobacterium tuberculosis and other pathogenic organisms, the foundation was laid for the modern pasteurization treatment of milk.

Pasteurization of Egg Products

General

Pasteurization, as a means of controlling the bacterial content of liquid, frozen and dried egg products, has been advocated for the last 25-30 years. At first it was generally believed by some workers that it was impossible to heat liquid egg products at a high enough temperature or for a long enough time to kill bacteria without causing coagulation of the proteins or otherwise impairing the culinary properties of the egg. However, it is now recognized that pasteurization of liquid egg products serves several functions. It reduces the viable bacteria count, destroys possible pathogenic bacteria such as salmonellae and improves the keeping quality of the liquid whole egg without affecting the functional qualities of the egg product. Garibaldi, et al., (1969b) reported that 95 percent of the samples in commercially broken eggs contained less than one salmonellae per gram. However, one sample contained over 100 salmonellae per

gram and this is why pasteurization has become necessary in the liquid egg products industry.

Henningsen Brothers first attempted to pasteurize egg products on a commercial scale in 1938 to reduce bacterial counts by utilizing a batch process (Goresline, et al., 1951). It was also about the time of World War II, 1940-46, that the egg drying industry began to flourish in this country. One of the accepted practices was to preheat the liquid egg before drying in order to reduce viscosity, dryer temperature and to increase plant production. One of the plant operators observed that the preheating process lowered the standard plate counts and especially the coliform bacterial counts (Winter, 1952). Schneider (1946, 1951) showed that the commercial practice of preheating liquid whole egg (140 F and above) prior to spray-drying effectively reduced the incidence of salmonellae organisms in whole egg powder. He also demonstrated that preheating and short-time pasteurizing high quality liquid whole egg at 60.5 C for two to five min entirely eliminated salmonellae organisms from processed egg powder.

Winter, et al., (1946a, 1948) and Steele, et al., (1967) pasteurized liquid whole egg in a laboratory pasteurizer and in plate and tubular heaters. They were able to kill more than 99 percent of the viable bacteria and all of the coliforms in liquid whole egg without

coagulating the product. During this same time period, researchers recognized that salmonellae could be killed using the principle of pasteurization (Winter, et al., 1946b; Gibbons, et al., 1946; Goresline, et al., 1951; Kraft, et al., 1966; Murdock, et al., 1960; Schneider, 1946, 1951 and Speck and Tarver, 1967). Goresline, et al., (1951) advocated pasteurization of whole egg at 60 C for three min for total destruction of salmonellae. There are many published reports concerning the time-temperature relationships that must be used to reduce or eliminate salmonellae from liquid whole egg. Among the more important papers published during the past twenty to twenty-five years are: Winter, et al., (1946a,b), Solowey, et al., (1948), Stewart (1949), Goresline, et al., (1951), Schneider (1946, 1951), Winter (1952), Annellis, et al., (1954), Osborne, et al., (1954), Heller, et al., (1962), Brooks (1962) and Sugihara, et al., (1966). Most of the research was performed using plate type pasteurizers or laboratory equipment. Based on this information, two processes have been adopted by official agencies. In the United States, liquid whole egg must be held at a minimum of 60 C for a minimum of 3.5 min, whereas in the United Kingdom and Denmark the requirements include heating at 64 C for 2.5 min (Brant, et al., 1968). Scalzo, et al., (1969) suggested that holding times should be based on the time required for the fastest particle, rather than the

average particle, to traverse the holding tube. The latest available U.S.D.A. regulations on grading and inspection of egg products (U.S.D.A., 1967) include holding times for the fastest particle as well as the holding time for the average particle passing through the system. Table 1 shows some approved time-temperature requirements for pasteurizing egg products. Note that the times are uniform with the temperature varying to achieve the desired destruction of salmonellae using the two uniform times for the fastest and average particle. Garibaldi, et al., (1969c) reported D values for S. typhimurium TM-1 in many types of egg products.

At the present time only continuous flow processes of the HTST (High-Temperature Short-Time) type are approved. Work continues, however, to develop an approved "batch-type" process which might allow small processors to pasteurize liquid egg products without the large capital outlay required for most HTST equipment (Brant, et al., 1968; Walters, et al., 1968; Maurer, 1966; Gibbons, et al., 1946 and Patterson, et al., 1966). However, according to one commercial equipment manufacturer (Kentco) (Anon., 1967), a continuous DeLaval pasteurizer can be purchased for $10,000 and a complete batch pasteurization system would cost about the same, including the equipment and operating expense for heating and refrigerating the product, since a batch type process does not have a regeneration capability.

TABLE 1.--Typical approved pasteurization requirements of liquid egg products in the United States (U.S.D.A., 1967).

Liquid egg product	Minimum temperature requirements	Minimum holding time requirements	
		Fastest particle	Average particle
	^{0}F	minutes	
Albumen (without use of chemicals)	134 132	1.75 3.10	3.50 6.20
Whole egg	140	1.75	3.50
Salt whole egg (added salt content $\geq$ 2 percent)	146 144	1.75 3.10	3.50 6.20
Sugar whole egg (2-12 percent sugar added)	142 140	1.75 3.10	3.50 6.20
Plain yolk	142 140	1.75 3.10	3.50 6.20
Sugar yolk (added sugar content $\geq$ 2 percent)	146 144	1.75 3.10	3.50 6.20
Salt yolk (2-12 percent salt added)	146 144	1.75 3.10	3.50 6.20

As shown in Table 1, egg yolk, egg albumen, whole egg and other egg products require different times and temperatures for pasteurization. Wilkin and Winter (1947) found that egg albumen should be pasteurized at a temperature not to exceed 57.8 C and preferably not greater than 56.7 C to avoid troublesome film formation on the sides of the pasteurizer. Bacteria were killed more easily in egg albumen than in egg yolk or whole egg. In order to reduce albumen coagulation, two other methods have been discovered in which certain chemicals are necessary for effective pasteurization. The method developed by Rogers, et al., (1966) incorporates hydrogen peroxide and a catalase, at 51.7 C, and the method developed by Cunningham and Lineweaver (1965) incorporates the use of lactic acid and aluminum sulfate to prevent the proteins from denaturing at the heating temperature of 60 C.

Another egg pasteurization method has been tested by Ballas Egg Products Co., and equipment for this process is marketed by Kentco (Anon., 1967). This process involves pasteurizing egg albumen at 56.7 C with a 3.5 min holding time. A unique feature of this process is the vacuum chamber which is maintained at 17-20 inches of vacuum. This chamber is usually positioned in the line after the egg has passed through the regenerator. According to the inventor, the vacuum chamber reduces the degree of "cooking" of the egg albumen on the plates during heating

and yields an albumen product with a higher beating rate and an albumen which has a greater sugar-carrying capacity. The reason for and the degree of the beneficial effects of the vacuum chamber have not been verified (U.S.D.A., 1969).

The adjustment of $_{p}H$ in the liquid whole egg before pasteurization and its effect on efficiency of the heat treatment to destroy certain bacteria has been studied by several workers. Cotterill, et al., (1964, 1965) indicated that liquid whole egg can be more efficiently pasteurized at $_{p}H$ 8.5 to 8.7 so long as physical and functional characteristics permit. Some organisms, such as staphylococci and salmonellae, have maximum heat resistance near $_{p}H$ 5.0. When a $_{p}H$ above 8.0 is not suitable they suggest using a $_{p}H$ below 4.0. They also found that the minimum temperature to kill 99.99 percent of the salmonellae in inoculated egg albumen is 2 C higher at $_{p}H$ 7.0 than at $_{p}H$ 8.6, using the same holding time. Garibaldi (1968) reported that the use of acetic acid in the pasteurization of liquid egg yolk reduced the heat resistance of salmonellae in these products. Garibaldi, et al., (1969a) reported a synergistic effect between EDTA and lactic acid. The D values also decreased with an increase in $_{p}H$ above 7.0, and when chelating agents were used.

Sebring, et al., (1963) reported the greatest increase in viscosity of liquid whole egg during heat

pasteurization at $_{p}H$ 7.5 while the least increase was at $_{p}H$ 8.5. They studied the range of $_{p}H$ from 7.5 to 11.0.

Protein Changes During Pasteurization

Parkinson (1967, 1968a,b) studied the changes in the protein composition of liquid whole egg after pasteurization (no time-temperature relationships were mentioned, but assume they were the conditions used in the United Kingdom or at least the temperature). He showed that protein changes do occur, with heating time a more important factor than temperature in influencing changes. A heating time of five min caused significant changes in the fractionation patterns of both soluble and insoluble proteins. His studies support the view that heat treatment of whole egg results in the aggregation of some of the proteins. He also reported that unfrozen new-laid egg, stale egg and frozen new-laid egg all showed an increase in insoluble material after pasteurization, and frozen egg contained more insoluble materials than unfrozen egg. Gel filtration results showed that the proportion of compounds of high molecular weight in the insoluble proteins was markedly increased by pasteurization and even more markedly by freezing.

Chemical Method for Determining Adequate Pasteurization

Recent research has attempted to provide chemical methods which would provide positive tests to indicate

when minimum pasteurization had been met. Brooks (1962) suggested the use of α-amylase to evaluate the adequacy of pasteurization. It is inactivated at a unique time and temperature, 64.4 C for 2.5 min, which also destroys Salmonella senftenberg (Shrimpton, et al., 1962). Reinke and Baker (1966) suggested that the α-amylase test could be used at temperatures below 64.4 C (down to 61.1 C) to evaluate the pasteurized product. Cunningham (1966) found the phosphatase test to be unsatisfactory since the phosphatase retained some of its activity after 5 min at 70 C. Brooks (1962) concluded that catalase activity reduction would only indicate that some heat treatment had been applied rather than the minimum required for pasteurization.

Effect of Heat on Organic Acid Formation

In the literature reviewed no mention was made relating the effects of pasteurization temperature on the content of the organic acids in liquid whole egg or any other egg product. Fluid milk subjected to time-temperature relationships, which greatly exceed those used in commercial fluid milk pasteurization, resulted in an increase in acid formation as the temperature and time of heating increased (Duncombe, 1924 and Gould, 1945b). Whittier and Benton (1927) and Gould (1945a) also stated that the increased acid formation was due to the breakdown

of lactose. However, Crowe and Deane (1940) reported that pasteurization lowered product $_{p}H$ but decreased the titratable acidity, whereas, Whittier and Benton (1926, 1927) and Gould (1945b) reported increased titratable acidity when milk was held at 110-120 C for several hours. Morr, et al., (1957) indicated that the addition of phosphate to milk resulted in an increase in rate of production of each of the acids studied. Formic and lactic acids were the predominant acids before and after heat treatment with or without the addition of phosphate. Withycombe and Lindsay (1969) reported a loss of free fatty acids in milk subjected to five different heat treatments.

El Miladi, et al., (1969) studied seven organic acids in canned tomato juice subjected to a retort temperature of 220 F for 20 min. They found an increase in total acidity and an increase in five of the acids studied. A decrease was noted in two of the acids, succinate and α-ketoglutarate.

Kirchner, et al., (1953) reported the effects of canning and prolonged storage time on the formation of certain volatile water-soluble constituents in grapefruit juice. Acetic acid was the primary volatile acid found, but was not detected in the fresh juice. A relatively small amount of acetic acid was found in the freshly canned juice (1.9 mg/kg) with a 12 fold increase (to 23.3 mg/kg) after four years storage at 30 C. Small amounts of

two other unidentified acids, not present in the fresh juice, were detected in the freshly canned juice, but the amount present decreased during the storage period. Pasteurization was accomplished at 88 C for an unspecified length of time.

Pasteurization of Egg Products by Non-Heat Methods

The following work gives some indication of the potential of other treatments for the destruction of microorganisms in egg products and in particular, the destruction of salmonellae. It should be noted that the methods referred to in this section are not approved for official use at this time. Due to the heat sensitivity of egg products, numerous workers have attempted to develop "cold" pasteurization methods or treatments (U.S.D.A., 1969).

Ionizing radiation treatment offers the unique feature of being able to pasteurize a frozen product, such as a 30-lb package of eggs, without thawing or without opening the package. This type of treatment is especially attractive since it permits in-package pasteurization, which avoids recontamination and heat damage. Ionizing radiation pasteurization of food products, which will destroy salmonellae, can be accomplished at about one-tenth the dose required for sterilization, or 0.2 to 0.5 Mrad (Brogle, et al., 1957; Brooks, et al., 1959; Comer,

et al., 1963; Ingram, et al., 1961; Ley, et al., 1963 and Mossel, 1960).

Beta and gamma energy are equally effective in killing salmonellae. However, the use of gamma rays would be needed to penetrate a thick container such as a 30-lb container of liquid eggs. The mechanism of death by irradiation is apparently different from destruction by heat as shown by studies using S. senftenberg 775 W. This organism is very heat resistant yet it is slightly less resistant to radiation destruction than are other salmonellae isolates (Brogle, et al., 1957; Mossel, 1960 and Nickerson, et al., 1957).

Yolk-containing products show flavor changes at pasteurizing doses of radiation. Flavor changes appear to be less if the product is irradiated in the frozen state. The change is slight enough that pasteurized products would be satisfactory for sponge cakes. The flavor change is readily detected and is objectionable in scrambled egg and custard (Brogle, et al., 1957 and Proctor, et al., 1953). Brogle, et al., (1957) showed that the off-flavor components are largely volatilized during spray drying. Mossel (1960) observed somewhat more serious damage to liquid whole egg and concluded that the process was not promising. Since the heat treatment, as used in the United States, causes little or no damage to functional properties and no flavor change, heat pasteurization is the preferred method at the present time.

Egg albumen products are only slightly damaged by pasteurizing doses of radiation. Flavor, volume and texture of angel cakes made from egg white solids irradiated at 0.7 Mrad did not differ from those cakes made with commercially prepared albumen (Nickerson, et al., 1957).

Ethylene and propylene oxides (typical epoxides) are gases that have been used to sterilize spices, gums and other materials. Epoxides can be used to destroy salmonellae and other microorganisms at room temperature (Sair, 1966). These same oxides were effective in a simple fumigation process on spray-dried powders, but not on pan-dried egg white. Their effectiveness is influenced by the moisture content of the powder. The epoxides may also react, in some cases, to yield undesirable chlorohydrins. Approval to use epoxides to "pasteurize" egg products does not appear likely in the near future. However, the applicability of these gases to powders makes them attractive.

Microorganisms as Sources of Certain Short-Chain Acids

All members of the genera Streptococcus, Pediococcus, Microbacterium, a large number of lactobacilli, certain bacilli and certain molds ferment glucose mainly to lactic acid with the formation of trace amounts of formic and acetic acids (Prescott and Dunn, 1940).

Ledingham and Neish (1954) compared the end products of glucose and pyruvate fermentations of Enterobacteriaceae, Bacillaceae and Pseudomonadaceae grown on the same culture medium. They found that formic and lactic acids are among the main products of glucose fermentation, while acetic and lactic acids may be derived from glucose and pyruvate metabolic pathways.

Thimann (1955) reported that three groups of bacteria produce lactic acid from sugar with only traces of by-products. These homofermentative forms included Thermobacterium, Streptobacterium and Streptococcus. Three other groups are heterofermentative, that is, they produce a mixed fermentation in which only about half of the sugar is converted to lactic acid. The remainder appears as CO_2, hydrogen, alcohol, formic or acetic acid. These groups include Betabacterium, Leuconostoc and Bacterium bifidum.

The fermentation displayed by facultative organisms, including the Enterobacteriaceae, bacilli and others, yields a wide variety of products among which organic acids are the major components (Wood, 1961). A characteristic of this fermentation is the prominence of the phosphorlytic cleavage of pyruvate to formate and acetate. All the members of the genera Streptococcus, Pediococcus, Microbacterium and a large number of lactobacilli and certain bacilli ferment glucose predominantely to lactic acid with formation of trace

amounts of volatile acids. Nelson and Werkman (1935) and DeMoss, et al., (1951) showed that several species of Lactobacillus and Leuconostoc exhibit heterolactic fermentations and among the main end products are lactic and acetic acids. These results agree with those of Gibbs, et al., (1950) who showed that Lactobacillus casei, Lactobacillus plantarum and Streptococcus faecalis ferment glucose to lactate.

Thimann (1955) reported that the formic-fermenting bacteria, often termed the coli-typhosum or colon-typhoid-dysentery group, contain species of Escherichia, Aerobacter, Proteus, Salmonella and Shigella. Stokes (1949) reported that aerobically grown cells of E. coli ferment glucose to ethanol, acetic, formic, lactic and succinic acids. The $_{p}H$ greatly influenced the yields of metabolic products in fermentations conducted in phosphate buffer but not those in bicarbonate buffer. As the $_{p}H$ increased from 5.62 to 7.96, the quantity of lactic acid produced decreased while the quantity of volatile and succinic acids increased. Also the quantitative data strongly suggested that succinic acid originated by the condensation of a 3-carbon compound with formic acid.

Doudoroff (1942) reported that certain facultative anaerobic bacteria of the genera Achromobacter possessed a mixed acid type of fermentation similar to that of E. coli. Blackwood, et al., (1956) using several strains of

E. coli, reported that, depending on the $_{p}H$ of the medium, ethanol, formic, acetic and lactic acids were the major glucose fermentation products, with succinic acid present in lower amounts. They also noted that the production of succinic acid is affected very little by changes in $_{p}H$. Mickelson and Werkman (1938) reported that $_{p}H$ had a marked effect upon the 2,3-butanediol fermentation of Aerobacter. Above $_{p}H$ 6.3, acetic and formic acids accumulate and below $_{p}H$ 6.3, acetic acid is converted to other products.

According to Gallagher and Stone (1939), Knaysi and Gunsalus (1944) and Puziss and Rittenberg (1957), many members of the genus Bacilli produce formic, acetic, butyric, lactic and succinic acids from the dissimilation of glucose. Wood (1961) reported that among the clostridia and certain bacilli, butyric acid is a characteristic product of carbohyrate fermentation. Gunsalus (1944) reported that in Bacillus subtilis fermentation, thiamine promotes the formation of CO_2 and other products of pyruvate metabolism, whereas in the absence of thiamine, lactate becomes the major product.

Stanier (1947) studied thirteen strains of fluorescent pseudomonads and reported that some of them have the ability to convert alcohol to acetic acid. The author also noted that the pseudomonads cannot tolerate media more acid than $_{p}H$ 5.0, and that they vigorously

attack peptones and proteins. More recently, Wolfe, et al., (1954) and Crawford (1954) showed that members of the Pseudomonas genera ferment glucose to formic, acetic, lactic and succinic acids.

Stokes (1956) demonstrated that Salmonella do not cleave formic acid to CO_2 and hydrogen, and as a consequence, formate accumulates in glucose fermentations.

Pederson and Breed (1928) and Wood (1961) reported that some members of the genus Serratia produce a mixture of organic acids which include formic, acetic, lactic and succinic acids.

The by-products of glucose fermentation by species of the Streptococcus genera have been extensively studied (Hammer, 1920; Foster, 1921; Langwill, 1924; Long and Hammer, 1936; Friedmann, 1939; Gunsalus and Umbreit, 1945 and White, et al., 1955). According to Gunsalus and Niven (1942), the homofermentative streptococci primarily metabolize glucose to lactic acid. However, these authors showed that a change in $_pH$ caused the production of lactic acid to decrease and the production of formic and acetic acids to increase. Platt and Foster (1958) studied the glucose fermentation balances for seven typical homofermentative lactic acid streptococci grown anaerobically with and without $_pH$ controls. The products they found included lactic, acetic and formic acids.

According to Lepper, et al., (1944) when liquid egg becomes sour after standing at a temperature above that of refrigeration, formic and acetic acids are formed, lactic acid content is substantially increased and this development of acids is a result of an increase in bacterial population.

Methods of Determining Volatile Short-Chain Fatty Acids and Organic Acids

Volatile Short-Chain Fatty Acids

Several methods have been proposed for the determination of the volatile acids. Dyer (1917) proposed the original procedure for the separation of volatile fatty acids by steam distillation. This procedure is based on the fact that under certain specified conditions the volatile acids distill at a constant rate, and in a mixture of volatile acids, each acid distills as if it were present singly. This procedure has been modified somewhat by several workers including Werkman (1930), Osburn, et al., (1936) and Hillig and Knudsen (1942).

Ramsey and Patterson (1945) and Ramsey (1963) proposed methods for the separation and identification of micro-amounts of formic, acetic, propionic and butyric acids (C_1-C_4). The volatile acids were separated on a silicic acid chromatographic partition column, and the C_1-C_4 acids were positively identified by microscopic

examination of their characteristic crystalline mercurous salts.

Attempts have also been made to separate and identify the short-chain volatile acids using paper chromatography (Stark, et al., 1951; Kennedy and Barker, 1951; Reid and Lederer, 1952 and Isherwood and Hanes, 1953).

With the advent of gas-liquid chromatography (GLC), James and Martin (1952) developed new procedures whereby formic acid and the higher homologs were quantitatively separated. Hawke (1957) chromatographed the volatile C_1-C_6 acids recovered from oxidized butterfat, and Hankinson, et al., (1958) developed a GLC procedure for the quantitative determination of C_1-C_6 and C_8 acids from milk.

The use of methyl ester derivatives to determine volatile fatty acids by GLC analysis was proposed by Horrocks, et al., (1961), Gehrke and Lamkin (1961) and Gehrke and Goerlitz (1963). Other ester derivatives such as the 2-chloroethanol esters were proposed by Oette and Ahrens (1961) and butyl, phenacyl and decyl esters were used by Craig, et al., (1963) for GLC analysis.

Shelly, et al., (1963) described a procedure whereby the volatile short-chain fatty acids (C_1-C_4) present in frozen egg were quantitatively determined using the GLC method. A similar method was also used by Grey

and Stevens (1966) for the quantitative determination of formic, acetic and propionic acids from microbial fermentations.

Steinhauer and Dawson (1969b) developed GLC procedures for formic, acetic, propionic and butyric acids in liquid whole egg, using butyl esters for the formic and acetic acids. They also injected acetic, propionic and butyric acids directly on the column. Landes and Dawson (1969) used a procedure similar to that outlined by Ramsey (1963), to determine the volatile and nonvolatile acids in liquid whole egg by silicic acid column procedures.

Lactic and Succinic Acids

Friedmann and Graeser (1933) proposed a titrimetric method for the determination of lactic acid based on its reaction with sodium bisulfite. Hillig (1937a,b,c) based a colorimetric procedure for the determination of lactic acid on its reaction with ferric chloride. Barker and Summerson (1941) described a method for the colorimetric determination of lactic acid in biological materials. Lactic acid was converted into acetaldehyde by treatment with sulfuric acid and the acetaldehyde was then determined by its color reaction with p-hydroxydiphenyl in the presence of cupric ions.

Pucher and Vickery (1941) developed a toluidine gravimetric method for succinic acid determination whereby

the acid was quantitatively determined by the formation of a succinyl-p-toluide derivative.

Claborn and Patterson (1948) developed a method for the determination and identification of both lactic and succinic acids in foods. These authors used silicic acid column chromatography to isolate both lactic and succinic acids. Lactic acid was identified by microscopic comparison of the isolated zinc lactate with pure zinc lactate crystals. Succinic acid was similarly identified by comparing the unknown with pure crystals of barium succinate.

The separation and identification of lactic and succinic acids has also been accomplished by filter paper chromatography (Magasanik and Umbarger, 1950).

Gehrke and Goerlitz (1963) developed a macro and micro gas chromatographic method for the determination of lactic and succinic acids as their methyl esters. The esterification involved the reaction of iodomethane with the silver salts of the acids. These authors reported no loss of the more volatile methyl esters.

Ramsey (1963) separated lactic and succinic acids from blood using silicic acid column chromatography and quantitated the amounts by titration with KOH. Landes and Dawson (1969), using a very similar technique, separated several nonvolatile acids including lactic and succinic acids from liquid whole egg.

Steinhauer and Dawson (1969a) separated and identified lactic and succinic acids, as butyl ester derivatives, from liquid whole egg by GLC procedures. Salwin and Bond (1969) made propyl ester derivatives of the lactic and succinic acids from egg and also separated them using GLC methods. The propyl ester derivatives were made using a 10 percent boron trifluoride-n-propyl alcohol solution. They used the same procedure to determine the lactic and succinic acids in beef, shrimp and cottage cheese whey as well as in liquid whole egg.

Gas-Liquid Chromatography

The term "gas chromatography" is used for all those chromatographic techniques in which a mobile gas phase carries the substance to be separated through a stationary phase packed into a suitable container (Hardy and Pollard, 1960). When the stationary phase is an absorbent liquid supported by an inert material, the method is Gas-Liquid Chromatography (GLC).

After the theoretical suggestion of Martin and Synge (1941), gas-liquid chromatography was first introduced by James and Martin (1952). This method made possible the separation and estimation of small amounts of volatile substances with a very wide range of boiling points and gave great impetus to many fields of research.

As reported in the previous section, the use of gas-liquid chromatography is increasing for determining

the short-chain organic acids in biological materials. Steinhauer (1968) reviewed the basic description and the components used in a gas-liquid chromatograph.

EXPERIMENTAL PROCEDURES

This investigation was divided into five parts. Part I involved the construction of a laboratory pasteurizer for liquid whole egg and egg products. Part II involved the improvement in a method to extract and recover acetic, propionic, butyric, lactic and succinic acids from liquid whole egg using one extraction procedure instead of two different procedures as reported by Steinhauer and Dawson (1969a,b) and current AOAC procedures (AOAC, 1965).

In Part III, known amounts of the above mentioned acids were added to liquid whole egg. The egg was then pasteurized, frozen and finally, the acid content of the egg was determined before and after pasteurization, utilizing GLC procedures for detection of the acids.

In Part IV, fresh liquid whole egg was incubated at room temperature until different levels of microbial population were reached, then the products were pasteurized and, as in Part III, the amounts of the above mentioned acids were determined in the incubated egg before and after pasteurization.

In Part V the acceptability of partially decomposed egg was determined by odor evaluation while odor and flavor evaluations were used to determine acceptability of the eggs to which known amounts of all five acids had been added.

Formic acid content was not examined in this study.

Equipment and Reagents

Pasteurizer

This equipment consisted of three units as shown in Figure 1. The first unit was a water bath maintained at a temperature of 55 C (Precision-Freas water bath model P2). The second unit was a Magniwhirl constant temperature water bath, model 1140S, in which temperature was maintained at 60.5 $\pm$ 0.5 C. A stirrer was used to help circulate the water. Both water baths were covered to minimize evaporation losses. The third section consisted of coiled glass chromatographic tubing in a slush-ice and salt bath at 0 C. Glass tubing, 5 mm i.d., 7 mm o.d., was used to conduct the egg product through the preheater and main heater sections. The tubing in the heating sections was U-shaped with a ball and socket joint on respective ends (a ball joint was on one end and a socket joint on the other) and when put together the joints were held by suitable pinch clamps.

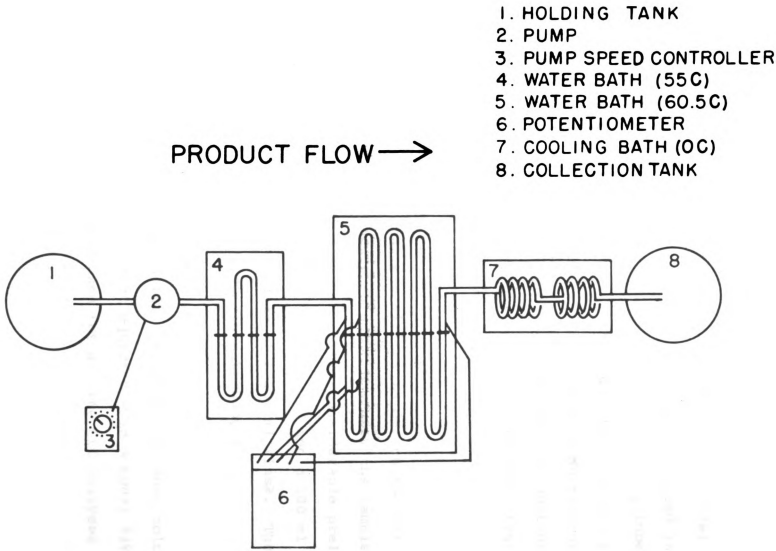

Figure 1.--Diagram of laboratory egg pasteurizer.

A Masterflex pump (Cole-Palmer) model WZlRO3l and Masterflex SCR Controller model 7020C were used to pump the egg thru the system at a constant rate. A model 7014-F pump head was used. Thermocouple wires were glued into place through holes punched in the glass holding tubes located in the main heater at appropriate intervals and were attached to a Honeywell Electronik 16 Multipoint Strip Chart Recorder. The glass tubing between the heating and cooling baths was connected by appropriate sized tygon tubing.

Liquid-Liquid Extractor

This apparatus was similar in design to that used in AOAC (1965) procedure 15.012. However, the sample holding portion was modified, after considerable preliminary research, to hold 500 ml of liquid and a 100 ml round bottom flask was used instead of a 250 ml flask. The total length of the extractor remained unchanged.

Gas-Liquid Chromatograph

An F and M Scientific Co. model 810 dual column flame ionization instrument equipped with a model 9294N Honeywell recorder was used for all the GLC analyses in this study.

Reagents

Reagent grade chemicals and distilled-deionized water were used throughout this study, except when

distilled water is mentioned. Whenever possible, internal standards, acids and reagents were checked for purity using the GLC and if necessary were redistilled.

Preparation of Standard Reagent Solutions

1. Acetic acid: 0.1N. Three ml glacial acetic acid (Mallinckrodt Chemical Co., analytical reagent grade) were pipetted into a 500 ml volumetric flask and made to volume with deionized water and mixed.
2. Butyric acid: 0.1N. Three and one-half ml butyric acid (Fisher Scientific Co., certified reagent grade) were pipetted into a 500 ml volumetric flask and made to volume with deionized water and mixed.
3. Lactic acid: 0.1N. Five ml of 85 percent lactic acid (Fisher Scientific Co., certified reagent grade) were pipetted into a 500 ml volumetric flask and made to volume with deionized water and mixed.
4. Propionic acid: 0.1N. Four and one-half ml of propionic acid (Fisher Scientific Co., certified reagent grade) were pipetted into a 500 ml volumetric flask and made to volume with deionized water and mixed.

5. Succinic acid: 0.1N. Five g of succinic acid (Fisher Scientific Co., certified reagent grade) were placed in an aluminum weighing pan and dried in an air oven at 105 C for 24 hr. After cooling, approximately 2.95 g were weighed and transferred to a 500 ml flask and dissolved in deionized water and made to volume.

   The normality and the exact mg concentration of each acid per ml of solution were determined by titrating 20 ml aliquots with an accurately standardized 0.1N solution of sodium hydroxide to a phenolphthalein end point.

6. Preparation of internal standard. Approximately 2.5 and 1.1 g of butyl decanoate (K and K Labs) were accurately weighed into separate 100 ml glass stoppered volumetric flasks and made to volume with acetone. These solutions were designated as stock solutions. As needed, various dilutions of the two stock solutions could easily be made to satisfy the need for a desired concentration of internal standard for a particular sample.

7. Preparation of dichloroacetic acid solution. Twenty five g of redistilled dichloroacetic acid (Fisher Scientific Co., certified reagent

grade) were accurately weighed into a 200 ml volumetric flask, diluted to volume with acetone, and tightly stoppered.

Separation and Identification of Acids

Egg Preparation and Pasteurization Procedures

A random sample of twenty eggs from a total sample of sixty dozen eggs was used to determine quality of the eggs. Both candling and Haugh unit methods (U.S.D.A., 1960) were used for quality determinations. The remaining eggs were broken and the liquid contents were placed in a 1.5 gal stainless steel Waring blendor jar and blended for 15 sec at low speed. The blended egg was then strained through a U.S. No. 20 mesh sieve, to remove egg shell and extraneous material, into a 30 gal stainless steel multiple paddle mixer and the eggs were mixed for 15 min until the first sample was taken. The multiple paddle mixer was located in a walk-in cooler maintained at 3 C. Total bacterial plate counts of the egg product were determined using tryptone glucose extract agar from samples taken before removing the first and last samples for other treatments. The plates were incubated 48 hr at 32 C and then counted.

The egg was pumped through the pasteurizer at a setting of 73/100 on the speed control unit attached to the

pump unit (flow rate approximately 100 ml/min). This provided the necessary holding time of 3.5 min after the egg reached a temperature of 60 C in the main heating unit. An air bubble of several inches in length preceded the egg product traveling in the glass tube. Since some water remained in the tubes following a clean-up procedure between samples, the first 175 ml of pasteurized product were discarded. After 2000 ml of pasteurized egg were collected, eight sub-samples of 225 g each were placed in one pint plastic containers with snap-on lids and held at -20 C until analyzed.

After each sample was pumped through the pasteurizer, the following clean-up procedure was performed:

1. The model 7014-F pump head was replaced by the model 7015-F pump head. This allowed a greater volume of cleaning solution to be pumped in a shorter length of time.
2. A cleaning solution of water and Score, a highly chlorinated dish washing compound (Economics Lab., Inc.), was used to remove the slight amount of egg which coagulated during each sample run.
3. After one-half gal of the cleaning solution was pumped through, the system was rinsed with one gal of distilled water.

4. The model 7014-F pump head was put back on the pump motor to be used for the next sample run.

Acid Extraction

The frozen egg samples were thawed under running cold tap water and shaken approximately every 15-20 min to hasten thawing. Total thawing time required was approximately 2.5 hr. A 200 g aliquot was accurately weighed into a tared 1000 ml wide-mouth erlenmeyer flask. It should be noted that separate 200 g aliquots were used for the acids determined as butyl ester derivatives (lactic and succinic) and for the acids determined as acids per se (acetic, butyric and propionic). Three hundred g of deionized water were added and the egg-water mixture swirled to aid mixing. Seventy-five g of 1N sulfuric acid were added after the addition of 125 g of 20 percent (w/w) phosphotungstic acid-water solution. The contents were stoppered and shaken vigorously for one min. The sample was placed into four 250 ml polyethylene centrifuge bottles (all bottles and contents weighed to within 0.1 g) and placed in a Sorvall RC-2B centrifuge and centrifuged for 13 min at 10,000 rpm. The supernatant from the four centrifuge bottles for each sample was then combined and 450 g of the supernatant were placed in the liquid-liquid extractor. The $_{p}H$ of the supernatant was 1.60. The inner tube was placed in the extractor and a sufficient quantity of diethyl ether was added to the extractor to raise the

total volume of liquid in the extractor to the level of the side arm. Eighty ml of diethyl ether and three glass boiling beads were placed in a 100 ml 24/40 standard taper round bottom flask, the flask and the extraction assembly were connected and continuous ether vaporization in the extraction flask was accomplished by means of a heating mantle connected to a powerstat. A setting of 97/100 on the powerstat was found to satisfactorily maintain a steady flow of ether through the liquid material and recover a high level of acid after a 24 hr extraction period. The heating mantle was preheated for several minutes prior to initiation of the extraction to prevent superheating of the ether. The extraction time began with the fall of the first drop of ether from the condenser.

Determination of the Acid Recovery Using Liquid-Liquid Extraction

To determine the optimum time and the amount of acetic, butyric, lactic, propionic and succinic acids extracted from the egg filtrate using liquid-liquid extraction, standard 0.1N solutions of the above acids were prepared as outlined previously under preparation of standard solutions. The percent recovery for each acid was determined separately. Ten ml aliquots of the standard acid solution to be run were pipetted into three 125 ml erlenmeyer flasks and stoppered. Then additional 10 ml aliquots were pipetted into separate extractors.

The erlenmeyer flasks and contents were stoppered and set aside until the extractions were completed. Deionized water (440 ml) and ten drops concentrated sulfuric acid were added to each extractor except for a blank which contained 450 ml deionized water and the ten drops of sulfuric acid. The remaining steps thru completion of the extraction was completed in the same manner and sequence as reported in the preceding section from "The inner tube was placed in the extractor . . ."

Following extraction, the flask was removed from the heat source, cooled so the ether no longer went into the condenser, disconnected from the extraction unit and then titrated with 0.05N alcoholic potassium hydroxide to a phenolphthalein end point. The three flasks containing 10 ml aliquots of the respective acids were also titrated at this time. The percent recovery was calculated by the use of the formula:

$$\frac{\text{ml 0.05N KOH required after extraction - blank}}{\text{ml 0.05N KOH required for 10 ml acid}} \times 100$$

Determination of Butyl Esterification Efficiency for Lactic and Succinic Acids

Ten replicate, five ml aliquots of accurately standardized 0.1N lactic and succinic acid solutions, respectively, were pipetted together into individual 100 ml 24/40 standard taper round bottom flasks. The water was evaporated using a steam bath and a stream of dry air.

After drying, approximately 1.3 ml of 1.25N HCl in n-butanol (Regis Chemical Co.) and approximately 300 mg of anhydrous sodium sulfate were added to the flask containing the dried material. The flask was connected to a condenser by a 19/38-24/40 adapter. The condenser was equipped with a drying tube filled with soda lime and the mixture was gently refluxed for 2 hr with a 100 ml heating mantle connected to a powerstat with a setting of 50/140.

After butyl esterification was completed, the sides and joint of the condenser were rinsed with 1 ml of acetone and the washings were added to the contents of the flask. The contents of the flask were quantitatively transferred to a 10 ml volumetric flask by use of a microfunnel. An appropriate quantity of internal standard (butyl decanoate) was pipetted into the flask (always a volume of 1 ml) and the contents were made to volume with acetone from the rinsings of the esterification flask. Triplicate one µl aliquots were injected into the chromatograph and the lactic and succinic acids were recovered as butyl and dibutyl esters of lactic and succinic acid, respectively. The percent esterification was calculated by the use of the formula:

$$\frac{\text{Amount of butyl ester recovered}}{\text{Theoretical recovery of butyl ester if 100 percent esterification}} \times 100$$

Preparation of the Extracted Sample for GLC Injection

For the samples in which the volatile acids were determined as the acids per se, enough ammonium hydroxide, usually less than one-half ml, was added to make the medium basic, after removal of the boiling flask from the extraction unit. Cresol red was used as the indicator. When the medium was not made basic, the concentration of the volatile acids decreased 40-50 percent during ether removal. Ether was removed from all samples by placing the flask over a 65-70 C hot water bath. All samples were immediately removed from over the water bath after the ether was evaporated. When higher temperatures were used, the ether had a tendency to superheat, thus losing the sample.

After the ether was evaporated from the volatile acid samples, salts formed from the addition of ammonium hydroxide were liberated by the addition of 1 ml of the dichloroacetic acid solution to the flask. After ether was removed from the samples in which lactic and succinic acids were to be determined, the samples were esterified using the procedure outlined in the previous section, beginning with addition of 1.3 ml of 1.25N HCl in n-butanol.

After the above steps were completed, the contents of each flask were quantitatively transferred to a 10 ml volumetric flask by the use of a microfunnel. The round

bottom flask was rinsed five times with one-half ml aliquots of acetone and the washings added to the contents of the volumetric flask. The microfunnel was rinsed with 1 ml of acetone which was also added to the contents of the volumetric flask. Using, acetone, the acid-acetone or butyl ester-acetone mixture was diluted to approximately 8-8.5 ml, stoppered and mixed well. A one µl aliquot was injected into the chromatograph and the peak heights of the acids or of the butyl esters were measured. The previously determined R value data gave an approximation (based on peak height, detector range and attenuation) of the concentration of the acids or butyl esters in the 8-8.5 ml sample. Using this information, the necessary quantity of butyl decanoate which should be added to provide a peak height commensurate with the peak height of the acids or butyl esters in the unknown was determined. Following the addition of this amount of internal standard, the contents of the volumetric flask were diluted to the 10 ml mark, and one µl aliquots were injected into the chromatograph.

Preparation of the GLC Columns

Ten g of high temperature stabilized diethylene glycol succinate (DEGS) (Analabs) were weighed into a 500 ml round bottom flask with a 24/40 standard taper neck and dissolved in approximately 100 ml acetone. Forty g of

120-130 mesh Anakrom ABS (Analabs) were added to the dissolved solution and mixed with the aid of a magnetic stirrer.

The acetone was removed by the use of a Buchi rotating evaporator at reduced pressure and with the aid of a 45 C water bath, as needed. The flask containing the slurry was removed several times during the acetone evaporation step and swirled to prevent the mixture from sticking to the sides of the flask. After the material had dried to the point of not sticking to the sides of the flask and was rolling as the flask turned, it was removed from the flask and further dried in an air oven for 12 hr at 100 C.

The columns were packed using a vacuum and a Burgess Vibro-Tool to evenly distribute the packing material within the column. The packed columns were installed in the chromatograph and conditioned for 24 hr at 185 C. Nitrogen (water pumped) was used as the conditioning gas at a flow rate of 60 ml/min.

GLC Operating Parameters

Table 2 gives the operating parameters for the GLC. As shown, the column temperature for the determination of the butyl ester derivatives was programmed. A temperature of 130 C was used until the butyl lactate and the internal standard were eluted (approximately 18 min after injection) and then the column temperature was

TABLE 2.--Operating parameters for the gas-liquid chromatograph.

Parameter	Butyl Esters	Straight Acids
Column	72" x 1/4" copper tubing	same
Carrier gas	Helium	same
Pressure	40 psi	same
Flow rates	A column: 60 ml/min	same
	B column: 50 ml/min	61 ml/min
Hydrogen	62 ml/min @ 20 psi	same
Air	625 ml/min @ 40 psi	same
Temperatures	Detectors: 250 C	same
	Injection port: 250 C	same
	Column: 130-170 C	120 C
Chart speed	15 in/hr	same
Sample size	1 µl	same
Range	10	same
Attenuation	2-16	1-8

increased at the rate of 6 C/min until a column temperature of 170 C was obtained and the 170 C temperature was maintained until the dibutyl succinate was eluted (approximately 36 min after injection).

Calibration of the Gas-Liquid Chromatograph

1. Volatile acids.--Approximately one g each of acetic acid (Mallinckrodt), butyric acid (Fisher), propionic acid (Fisher) and one-half g butyl decanoate (K and K Labs) were accurately weighed into the same 25 ml volumetric flask. The mixture was diluted to volume with acetone and designated as the volatile acid stock solution. A 1:10 dilution of this stock solution was prepared by pipetting 2.5 ml of the stock solution into a 25 ml volumetric flask and diluting to volume with acetone. This diluted mixture was designated as the diluted volatile acid stock solution. Various volumes of the diluted stock mixture were pipetted into separate 5 ml volumetric flasks to provide a range of concentration for each acid at a given sensitivity. Following five replicate one µl injections of each concentration into the GLC, the peak heights of the acetic, butyric and

propionic acids and of the butyl decanoate were measured. Using the calculation procedure outlined by Shelley, et al., (1963) the detector response values, R, of acetic, butyric and propionic acids relative to butyl decanoate were calculated using the formula:

$$R_A = (h_A)\ (c_{IS})/(h_{IS})\ (c_A)$$

where h_A and h_{IS} are the peak heights of the acid and butyl decanoate, respectively, and c_A and c_{IS} designate the corresponding concentrations (mg/ml acetone solution) of the acid and the butyl decanoate, respectively.

2. Butyl esters.--Approximately one g each of butyl lactate (Eastman Chemical), butyl decanoate (K and K Labs) and dibutyl succinate (K and K Labs) were accurately weighed into the same 25 ml volumetric flask, diluted to volume with acetone and designated as the butyl ester stock solution. A 1:10 dilution of this stock solution was prepared by pipetting 2.5 ml of the stock solution into a 25 ml volumetric flask and diluting to volume with acetone. This diluted mixture was then designated as the diluted butyl ester

stock solution. Various volumes of the diluted butyl ester stock solution were pipetted into separate 5 ml volumetric flasks to provide a range of concentration for each butyl ester at a given sensitivity on the GLC. Following five replicate one µl injections of each concentration into the GLC, the peak heights of the butyl lactate, butyl decanoate and dibutyl succinate were measured. Again using the calculation procedure proposed by Shelley, et al., (1963) the detector response values, R, of butyl lactate and dibutyl succinate relative to butyl decanoate were calculated using the formula:

$$R_{BE} = (h_{BE})\ (c_{IS})/(h_{IS})\ (c_{BE})$$

where h_{BE} and h_{IS} are the peak heights of the butyl ester and butyl decanoate, respectively. The designations c_{BE} and c_{IS} are the corresponding concentrations (mg/ml acetone solution) of the butyl ester and butyl decanoate, respectively.

Calculations

From the chromatograms of the samples, the peak heights of the acids and the butyl esters, including the

internal standard, were measured. Rearrangement of the R value equation gives the following:

1. For the acids:

$$c_A = (h_A)\ (c_{IS})/(h_{IS})\ (R_A)$$

2. For the butyl esters:

$$c_{BE} = (h_{BE})\ (c_{IS})/(h_{IS})\ (R_{BE})$$

Since the R values for each acid and butyl ester were previously determined, unknown concentrations were calculated by substituting peak heights and the appropriate R value in the above equations. After determining the mg concentration of the acid or the butyl ester present in the injected aliquot, the concentration value for each acid or butyl ester was multiplied by a conversion factor which converts the quantity of acid or butyl ester present in the injected aliquot to mg of the respective acid/100 g egg. The formula for determining these conversion factors was derived as follows:

Column

Acid	a	b	c	d	e	f	g
Acetic		$\frac{10,000}{1}$	$\frac{700}{450}$	$\frac{100}{92.1}$		$\frac{100}{200}$	84449
Butyric		$\frac{10,000}{1}$	$\frac{700}{450}$	$\frac{100}{99.7}$		$\frac{100}{200}$	78012
Lactic	0.6162	$\frac{10,000}{1}$	$\frac{700}{450}$	$\frac{100}{90.1}$	$\frac{100}{89.0}$	$\frac{100}{200}$	59767
Propionic		$\frac{10,000}{1}$	$\frac{700}{450}$	$\frac{100}{98.5}$		$\frac{100}{200}$	78962
Succinic	0.5134	$\frac{10,000}{1}$	$\frac{700}{450}$	$\frac{100}{96.8}$	$\frac{100}{89.0}$	$\frac{100}{200}$	46349

Where: Column a is the factor which converts butyl lactate or dibutyl succinate to lactic and succinic acid.

Column b is the dilution factor where a one µl (0.001 ml) aliquot for each injection was taken from a 10 ml sample.

Column c is the factor which corrects for the fact that only 450 g of the original 700 g of the egg-water-phosphotungstic-sulfuric acid mixture was used.

Column d is the factor which accounts for the quantity of the acids recovered by liquid-liquid extraction.

Column e is the factor which accounts for the esterification efficiency of butylation for lactic and succinic acids.

Column f is the factor which converts mg acid/200 g of egg to mg acid/100 g of egg.

Column g lists the conversion factor for each acid used in this study.

Preparation of Whole Egg for Analysis

Acids Added to the Egg

Two cases (60 dozen) of U.S. Grade AA large eggs were obtained from the Hamilton Farm Bureau through the Michigan State University Food Stores.

Three 4000 g lots of the blended whole egg were prepared for subsequent pasteurizing and analysis by adding increasing aliquots of each of the previously prepared acetic, butyric, lactic, propionic and succinic acids to each lot. This procedure was carried out by pipetting the desired quantities of the standard acid solutions into a tared 1.5 gal stainless steel Waring blendor jar, and adding a quantity of the blended egg sufficient to make a total weight of 4000 g (acid plus egg). The total mixture was then blended for 10 sec at low speed. From the 4000 g acid-egg mixture, six sub-samples of 225 g each were placed into one pint plastic containers with snap-on lids and immediately frozen and held at -20 C until analyzed.

The remaining acid-egg mixture was then pasteurized in the laboratory pasteurizer previously described. The sub-samples were also handled in the same manner as

described in the pasteurization section. A fourth 4000 g lot of the blended egg was handled and treated in the same manner as the three previous lots, with the exception that no acids were added; this lot was used to provide the control samples for this section.

The mg concentrations/100 g of egg for all the acids used were as follows:

Sample No.	Acetic Acid	Butyric Acid	Lactic Acid	Propionic Acid	Succinic Acid
1	0.510	0.500	0.465	0.506	0.543
2	5.098	4.998	4.651	5.058	5.430
3	20.390	19.990	18.604	20.230	20.634

Eggs Incubated at Room Temperature

Eggs were obtained from the same source and treated in the same manner as described previously up through the sampling step.

Three 4000 g lots of the blended whole egg were weighed into tared six liter erlenmeyer flasks. The egg remained at room temperature for 11, 14 and 17 hr, respectively. The egg samples were not inoculated as preliminary results showed sufficient contamination to yield the desired bacterial growth at the times indicated. From each 4000 g lot of incubated egg, six sub-samples of 225 g each were placed into one pint plastic containers

with snap-on lids and immediately frozen and held at -20 C until analyzed.

The remaining incubated egg samples for each time period were then pasteurized using the procedure as outlined in the egg preparation and pasteurization procedure section. Before and after pasteurization, a total plate count of organisms in each egg sample was determined in duplicate at several dilutions. The plate counts were made on tryptone glucose extract agar and incubated 48 hr at 32 C and counted.

A fourth 4000 g lot of the blended egg was handled and treated in the same manner as the three incubated samples except that it was not incubated, and was used as the control.

Sensory Evaluation

Odor Evaluation

A sub-sample from each treatment was removed from the freezer and thawed under cold running tap water. After thawing and mixing, approximately 100 g of each sample were poured into brown colored jars and allowed to warm up to room temperature. The panelists were asked to evaluate the odor of each sample and compare it with a reference sample, which was a freshly broken, frozen and then thawed whole egg sample. Table 3 shows the instructions and evaluation form provided each panelist.

TABLE 3.-- Egg odor evaluation form.

EGG ODOR EVALUATION

Name:_____ Date:_____

Instructions:

Compare the odor of the numbered samples with the reference sample provided. Rank each sample using the rating scale below. Assume the reference sample has a rating of 8. If there is an off-odor please try to identify.

RATING SCALE:

8	No detectable off-odor, comparable to high quality fresh eggs
7 1/2	Very slight off-odor
7	Slight but not unpleasant off-odor
6 1/2	Definite but not unpleasant off-odor
6	Pronounced off-odor (slightly unpleasant)
5	Unpleasant off-odor
4	Definite unpleasant off-odor
3	Pronounced off-odor
2	Repulsive odor
1	Definite repulsive odor
0	Pronounced repulsive odor

SAMPLE NO.	RATING	OFF-ODOR IF IDENTIFIABLE
_____	_____	_____
_____	_____	_____
_____	_____	_____

A reference sample was also included in the set of unknowns.

Flavor Evaluation

A sub-sample of each treatment to which the acids were added, was removed from the freezer and thawed under cold running tap water. After thawing, a 200 g sample was blended with 70 g skim milk and 15 g vegetable cooking oil in a Waring blendor for 10 sec. After blending, the egg-milk-oil mixture was placed in separate 600 ml beakers, the beakers were placed in a pan of boiling water and the egg-milk-oil mixture was cooked to the same degree of doneness. The egg-milk-oil mixture was stirred constantly throughout the cooking process. Control samples, including duplicate samples, consisted of U.S. A quality eggs which had been blended with similar amounts of milk and oil, and cooked in the same manner. After cooking, the scrambled egg samples were placed in small individual plastic cups for tasting by the panelists.

A triangle test was used to determine if panel members could distinguish a difference between treated and reference samples. All samples were offered to panel members under red lights to mask color differences.

Table 4 shows the score card used in this evaluation of egg flavor.

TABLE 4.--Score card used for flavor evaluation of scrambled egg products.

EGG FLAVOR EVALUATION

Name:_____ Plate No._____ Date:_____

Two of these samples are identical and one is different. Disregarding textural differences due to cooking technique, please check the different sample.

SAMPLE NO.		WHICH DO YOU PREFER?	
_____	( )	Different Sample	( )
_____	( )	Identical Samples	( )
_____	( )		

RESULTS AND DISCUSSION

Pasteurization

Table 5 shows the total plate counts of viable bacteria in egg products before and after pasteurization. The liquid egg was pumped through the tubes in the preheater maintained at 55 C and reached a temperature of 52 C. It continued through the tubes in the main water bath at 60 C. A product temperature of 60 C was reached in 30 sec and a temperature of 60-61 C was maintained for 3.5 min. The product then passed through the cooling tank and out into an appropriate container. A flow rate of 100 ml/min was maintained.

A slower flow rate, which would be necessary for a system without the preheater, resulted in excessive coagulation of egg in the tubes.

The bacterial count of egg held at 3 C up to 5 hr reached 4.5×10^4 bacteria / g with the product having an initial bacterial count of 4.1×10^4/g. After pasteurization, the counts were reduced a minimum of 2 log numbers and the maximum number found was 1.4×10^3/g. This is a reduction during pasteurization of 99.00 to 99.97 percent.

TABLE 5.--Influence of heat treatment on numbers of bacteria in whole egg products, either incubated or with acids added.

Sample	Treatment		Number of viable organisms/g[a]	
	Time	Temp	Nonpasteurized	Pasteurized
	hr	°C		
			Acids Added	
1	0.5	3	4.1×10^4	4.0×10^2
2	5.0	3	4.5×10^4	4.3×10^2
			Incubated	
3	0.5	3	4.0×10^4	4.0×10^2
4	11.0	24	1.3×10^5	6.3×10^2
5	14.0	24	5.4×10^5	8.1×10^2
6	17.0	24	4.5×10^6	1.4×10^3

[a]Each figure represents an average of two replicate plate counts per sample.

Acid Extraction

One objective of this study was to develop a common extraction procedure for all the acids studied. The two motivating reasons for this were to shorten or dispense with (1) the filtration step and (2) the condensing step. Elimination of the filtration step resulted in larger percentage recoveries of the acids (this method recovered 450 of 700 g, whereas, the AOAC (1965) procedures recover only 450 of 1000 g). Elimination of the condensing step saved time as well as avoided the formation of some undesirable reaction products which were frequently formed when the filtrate was heated to concentrate it. These reaction products fouled up the chromatographic column and necessitated several hours of clean up time after the sample was injected.

Steinhauer (1968) used a modification of the standard steam distillation apparatus outlined by AOAC (1965) to extract the C_1-C_4 volatile acids. He also used the AOAC (1965) liquid-liquid extraction procedure for the extraction of lactic and succinic acids except he used a 50 ml boiling flask instead of a 250 ml flask as used in the AOAC procedures.

Continuous extraction for 24 hr was required to recover sufficient acids when using a 100 ml boiling flask. This size flask was used since it could be used to esterify the lactic and succinic acids and eliminated the

need for product transfer from a larger flask. A 250 ml flask (or larger) can be used in this procedure and this flask size reduces the 24 hr extraction time to 3-5 hr. When the larger flask size was used the cooling condensers had to be connected to the water source individually, instead of in series, due to the increased amount of ether being evaporated. A 50 ml boiling flask was too small to allow sufficient quantities of ether to pass through this system in 24 hr to collect 90 percent or more of each acid. About 85 percent of the formic acid present can be recovered in a 24 hr extraction period.

The AOAC (1965) procedures and Steinhauer (1968) suggested that 5 to 10 ml of water be added to the boiling flask either during extraction or evaporation of the ether. In this study the elimination of water did not appear to affect the recovery of the acids and eliminated the time necessary to evaporate the water. However, as mentioned previously, when ether was evaporated from the samples used to determine the volatile acids, an alkaline solution is necessary to prevent loss of these acids.

The recovery of acetic, butyric, lactic, propionic and succinic acids by these modified liquid-liquid extraction procedures are shown in Tables 6, 7, 8, 9 and 10. More than 90 percent of each acid was recovered by these procedures. This varies from 90 percent recovery for lactic acid (Table 8) to 99.7 percent recovery of

TABLE 6.--Recovery of acetic acid by liquid-liquid extraction.

Extraction	0.05N alcoholic KOH required/aliquot after extraction[a] (ml)	Recovery (%)
1	19.15	93.64
2	19.05	93.17
3	19.18	93.80
4	18.47	90.30
5	18.60	90.90
6	18.80	92.00
7	18.78	91.83
8	18.85	92.15
9	18.78	91.83
10	18.78	91.83
	Average	92.14±1.15[b]

[a]Stock solution of acetic acid required an average of 20.45 ml of 0.05N KOH per 10 ml aliquot before extraction.

[b]The standard deviation of the mean.

TABLE 7.--Recovery of butyric acid by liquid-liquid extraction.

Extraction	0.05N alcoholic KOH required/aliquot after extraction[a] (ml)	Recovery (%)
1	19.70	98.01
2	19.40	96.50
3	19.95	99.22
4	20.02	99.61
5	19.25	95.74
6	19.80	98.45
7	20.00	99.50
8	19.87	98.87
9	20.05	99.75
10	19.93	99.15
	Average	98.47±1.32[b]

[a]Stock solution of butyric acid required an average of 20.10 ml of 0.05N KOH per 10 ml aliquot before extraction.

[b]The standard deviation of the mean.

TABLE 8.--Recovery of lactic acid by liquid-liquid extraction.

Extraction	0.05N alcoholic KOH required/aliquot after extraction[a] (ml)	Recovery (%)
1	17.75	89.44
2	17.98	90.56
3	18.30	92.20
4	18.20	91.67
5	17.98	90.56
6	17.48	88.07
7	17.82	89.77
8	18.05	90.91
9	17.70	89.20
10	17.60	88.66
	Average	90.10±1.31[b]

[a]Stock solution of lactic acid required an average of 19.85 ml of 0.05N KOH per 10 ml aliquot before extraction.

[b]The standard deviation of the mean.

TABLE 9.--Recovery of propionic acid by liquid-liquid extraction.

Extraction	0.05N alcoholic KOH required/aliquot after extraction[a] (ml)	Recovery (%)
1	20.65	97.64
2	20.60	97.40
3	21.00	99.29
4	20.95	99.05
5	21.10	99.76
6	21.20	100.24
7	21.08	99.68
8	21.40	101.18
9	21.20	100.25
10	21.65	102.40
	Average	99.69±1.50[b]

[a]Stock solution of propionic acid required an average of 21.15 ml of 0.05N KOH per 10 ml aliquot before extraction.

[b]The standard deviation of the mean.

TABLE 10.--Recovery of succinic acid by liquid-liquid extraction.

Extraction	0.05N alcoholic KOH required/aliquot after extraction[a] (ml)	Recovery (%)
1	20.45	98.79
2	20.17	97.44
3	20.03	96.76
4	19.38	93.60
5	20.17	97.44
6	19.90	96.15
7	20.05	96.86
8	20.17	97.44
9	20.03	96.76
10	20.03	96.76
	Average	96.80±1.34[b]

[a]Stock solution of succinic acid required an average of 20.70 ml of 0.05N KOH per 10 ml aliquot before extraction.

[b]The standard deviation of the mean.

propionic acid (Table 9). These are satisfactory recoveries, although the butyric, lactic and succinic acid recoveries are slightly lower than those reported by Steinhauer (1968). He reported recoveries of 100 percent for lactic and succinic acids using a liquid-liquid extraction procedure and 90.4, 96.8 and 100 percent for acetic, propionic and butyric acids, respectively, using steam distillation extraction.

Esterification

The esterification efficiency of lactic and succinic acids are shown in Tables 11 and 12. Similar recoveries and similar variability were obtained from each acid. The esterification recovery rate was 89.0 percent for both lactic and succinic acids. Steinhauer (1968) reported butyl esterification efficiency of 84.8 and 82.9 percent for lactic and succinic acids, respectively.

In an attempt to reduce the esterification time to less than 2 hr, a short time-high temperature method was tried. This involved placing the sample in an acylation tube, adding 1 ml of 1.25N HCl in n-butanol and 250 mg sodium sulfate, tightening the lid and then placing the tube with contents in a paraffin oil bath (150 C) for 5-15 min. This procedure produced the desired esterification but the results were inconsistent due possibly to the product being trapped in the sodium sulfate at the bottom of the acylation tube. Another problem was the transfer

TABLE 11.--Determination of esterification efficiency of lactic acid.

Sample	Lactic Acid (mg)	Equivalent as Butyl Lactate (mg)	Recovery from GLC (mg)	Recovery (%)
1	9.674	15.700	14.381	91.6
2	9.674	15.700	14.177	90.3
3	9.674	15.700	13.989	89.1
4	9.674	15.700	13.785	87.8
5	9.674	15.700	13.785	87.8
6	9.674	15.700	14.300	91.1
7	9.674	15.700	13.596	86.6
8	9.674	15.700	13.722	87.4
9	9.674	15.700	13.847	88.3
10	9.674	15.700	14.146	90.1
			Average	89.0±1.6[a]

[a]The standard deviation of the mean.

TABLE 12.--Determination of esterification efficiency of succinic acid.

Sample	Succinic Acid (mg)	Equivalent as Dibutyl Succinate (mg)	Recovery from GLC (mg)	Recovery (%)
1	10.102	19.700	18.026	91.5
2	10.102	19.700	17.888	90.8
3	10.102	19.700	17.691	89.8
4	10.102	19.700	17.080	86.7
5	10.102	19.700	17.691	89.8
6	10.102	19.700	17.730	90.0
7	10.102	19.700	17.001	86.3
8	10.102	19.700	17.159	87.1
9	10.102	19.700	17.257	87.6
10	10.102	19.700	17.829	90.5
			Average	89.0 ± 1.9[a]

[a]The standard deviation of the mean.

of the lactic and succinic acids from the 100 ml boiling flask to the acylation tube and evaporating the acetone and ether without superheating and blowing the contents out of the acylation tube. Because of these problems the 2 hr esterification method was used.

Since neither lactic nor succinic acids are volatile under ambient conditions, the normal GLC procedure is to make ester derivatives from these two acids.

Although the methyl ester is possibly the most commonly used ester derivative for gas chromatography of organic acids, Salwin and Bond (1969) experienced great difficulty in extracting the methyl esters of lactic and succinic acids from the reaction mixture quantitatively because of their great solubility in water. These workers also tried using chloroform, ether, petroleum ether, mixtures of the solvents and salting-out procedures, but as many as eight extractions were required and the results were not satisfactory. Steinhauer (1968) found inconsistent esterification and/or losses in transferring the methyl esters to be chromatographed (as high as 20-30 percent variation). When chromatographing the methyl esters, it was also noted that there were nearly similar retention times for the excess methanol peak and the methyl lactate peak. When the column temperature was lowered to achieve greater separation, the time required for the emergence of the dimethyl succinate peak was

greatly increased, and the peak itself was unsymmetrical and not sharply defined. These results using methyl ester derivatives are similar to those reported by Jensen, et al., (1967), Oette and Ahrens (1961) and Vorbeck, et al., (1961).

Steinhauer and Dawson (1969a) found the use of butyl ester derivatives suitable for quantitation of the lactic and succinic acids in liquid whole egg. Salwin and Bond (1969) reported butyl ester derivatives sufficiently water insoluble to permit their recovery by a single extraction with chloroform or ether. However, they were not used because gas chromatographic resolution was poorer than with propyl lactate and at a column temperature of 130 C, the peaks were not well separated for butyl lactate and acetophenone (the internal standard).

Propyl ester derivatives, prepared with BF_3-n-propyl alcohol reagent, were used by Salwin and Bond (1969) and Staruszkiewicz (1969). These authors prefer this ester derivative for similar studies.

Kuksis and Vishwakarma (1963) indicated that the trimethylsilyl derivatives of lactic and of a number of other organic acids could be separated by the use of gas-liquid chromatography. Salwin and Bond (1969) found these ester derivatives to be less satisfactory than the propyl ester derivatives when applied to GLC analysis.

Shelly, et al., (1963) reported that the use of higher molecular weight ester derivatives led to the development of polymeric products and side reactions.

Since the use of butyl ester derivatives had been used successfully in this laboratory and the work of Salwin and Bond (1969) and Staruszkiewicz (1969) was published after work was under way, the butyl ester derivatives of lactic and succinic acids were used. However, the use of propyl ester derivatives, or at least the use of the BF_3-alcohol mixture, could be more satisfactory since the esterification time is much faster (10 min vs 120 min). The use of the propyl ester derivative and the BF_3-alcohol esterification method are being used in the development of a method for GLC analysis to be used in AOAC procedures (Salwin and Bond, 1969 and Staruszkiewicz, 1969).

Steinhauer (1968) attempted to collect and make butyl ester derivatives of all the C_1-C_4 acids studied and to chromatograph them using a temperature programmed column. He successfully recovered the butyl ester derivatives of formic, acetic and succinic acids, but the excess butanol and the butyl ester derivatives of propionic, butyric and lactic acids all exhibited an identical retention time.

Columns

The liquid phase, diethylene glycol succinate (DEGS), was compared with the ethylene glycol adipate (EGA) liquid phase, both at the 20 percent level. Commercially prepared standards of the butyl esters and the acids were chromatographed on these two columns and

were evaluated for peak symmetry, resolution of the standard butyl esters and acids and retention time. The retention time was less for the DEGS column, peak symmetry was satisfactory for both columns and resolution was slightly better using the DEGS column on all the butyl esters and acids except acetic acid. Because of the shorter retention times for the DEGS column, it was adopted for use in these studies.

Earlier work in this laboratory (Steinhauer, 1968) showed 20 percent ethylene glycol adipate (EGA) to be best of several liquid phases studied. At the present time, the AOAC procedures for their GLC trials recommend the use of 10 percent stabilized DEGS on 100-120 mesh Gas Chrom Z (Salwin and Bond, 1969 and Staruszkiewicz, 1969). Another very promising liquid phase for this type of work is FFAP. In some recent preliminary studies in this laboratory, this liquid phase effectively separated the acids and butyl ester derivatives used in this study, and it has the advantage of complete separation of the acids or butyl ester derivatives (including the use of butyl decanoate as the internal standard) in as little as 8-10 min depending on the column size, percent liquid phase on the solid support and the temperature program used for the butyl ester derivatives.

To increase the sharpness of the dibutyl succinate peak and to increase the R value, a temperature program was developed. The butyl lactate and butyl decanoate

were eluted at a temperature of 130 C, then the column temperature was increased six degrees C per min until a temperature of 170 C was reached. The 170 C column temperature was maintained until the dibutyl succinate was eluted. This procedure increased the calculated R value to a range of 0.90-0.95. Steinhauer (1968) reported a R value of approximately 0.30 using an isothermal column temperature of 130 C. Using the isothermal column condition the dibutyl succinate was not as sharp as the butyl lactate or the butyl decanoate peaks. Salwin and Bond (1969) and Staruszkiewicz (1969) also used an isothermal column temperature of 130 C for the separation of propyl ester derivatives.

It is thus possible to extract a sample, inject it in the GLC and have the results within a normal 8-9 hr workday for either the acids recovered as the propyl esters or as the acids per se. This would require the use of a 250 ml boiling flask to reduce the extraction time to 3 to 4 hr, the use of the BF_3-alcohol reagent mixture for esterification of the lactic and succinic acids in 10-15 min, the acid extraction procedure as outlined in this study and the use of the FFAP liquid phase.

Internal Standard and R Values

Since the detector response varied with each compound injected, an internal standard was used in the

quantitation procedures. In this study, butyl decanoate was found to be satisfactory for use in both the volatile acid and butyl ester derivative determinations. The response values of the C_2-C_4 acids, butyl lactate and dibutyl succinate to butyl decanoate were calculated according to the procedure reported by Shelley, et al., (1963) and are presented in Tables 13 and 14.

The R values for all the acids and butyl ester derivatives had a tendency to increase as the concentration increased. Variability remained fairly constant throughout the various concentration ranges. Acetic acid had the lowest R values, approximately 0.50, while dibutyl succinate had the highest R values, approximately 0.93.

Steinhauer and Dawson (1969a,b) used two different internal standards. Butyl octanoate was used for the acids determined as the acids per se and butyl decanoate was used for the acids determined as their butyl ester derivatives. Salwin and Bond (1969) and Staruszkiewicz (1969) used acetophenone as the internal standard for the propyl ester derivatives of lactic and succinic acids.

Shelley, et al., (1963) reported that variations in R values decreased as the concentration of the internal standards and the compounds under study increased. Steinhauer (1968) reported that R value variations generally decreased with increasing concentration of internal standards, acids and butyl ester derivatives.

TABLE 13.--Response values, R, of acetic, propionic and butyric acids relative to butyl decanoate.

Range of Acid Conc. in Sample (mg/ml)	Chromatograph		R Value			Ave. R Value
	Range	Attenuation	High	Low	Range	
			Acetic Acid			
0.15-0.50	10	1	0.481	0.442	0.039	0.464
0.51-1.00	10	2	0.513	0.460	0.053	0.488
1.01-2.00	10	4	0.519	0.478	0.041	0.509
2.01-4.00	10	8	0.522	0.485	0.037	0.507
4.01-8.00	10	16	0.550	0.505	0.045	0.537
			Propionic Acid			
0.15-0.50	10	1	0.655	0.596	0.059	0.637
0.51-1.00	10	2	0.708	0.648	0.060	0.676
1.01-2.00	10	4	0.697	0.649	0.048	0.682
2.01-4.00	10	8	0.708	0.668	0.040	0.683
4.01-8.00	10	16	0.740	0.678	0.062	0.717
			Butyric Acid			
0.15-0.50	10	1	0.639	0.621	0.018	0.630
0.51-1.00	10	2	0.674	0.623	0.051	0.649
1.01-2.00	10	4	0.699	0.640	0.059	0.664
2.01-4.00	10	8	0.706	0.659	0.047	0.679
4.01-8.00	10	16	0.736	0.683	0.053	0.716

TABLE 14.--Response values, R, of butyl lactate and dibutyl succinate relative to butyl decanoate.

Range of Ester Conc. in Sample (mg/ml)	Chromatograph		R Value			Ave. R Value
	Range	Attenuation	High	Low	Range	
			Butyl Lactate			
0.15-0.50	10	2	0.893	0.868	0.025	0.883
0.51-1.00	10	4	0.926	0.878	0.048	0.901
1.01-2.00	10	8	0.910	0.899	0.011	0.907
2.01-4.00	10	16	0.948	0.911	0.037	0.928
4.01-8.00	10	32	0.950	0.907	0.043	0.931
			Dibutyl Succinate			
0.15-0.51	10	2	0.956	0.888	0.068	0.923
0.51-1.00	10	4	0.959	0.877	0.082	0.929
1.01-2.00	10	8	0.930	0.895	0.035	0.910
2.01-4.00	10	16	0.981	0.932	0.049	0.958
4.01-8.00	10	32	1.043	0.952	0.051	0.975

The elution positions of the internal standard, butyl decanoate, relative to the C_2-C_4 acids and to the butyl ester derivatives of lactic and succinic acids are shown in Figures 2 and 3, respectively.

Recovery of Acids

Tables 15 and 16 show the amount of acids recovered from both pasteurized and nonpasteurized liquid whole egg which had been frozen and thawed, to which known amounts of acid were added before the pasteurization and/or freezing step. Pasteurization of the liquid whole egg did not affect the recovery of the five acids studied. Of the five acids studied, only lactic and acetic acids were found in detectable amounts in the fresh egg. The lactic acid concentration was 2.78-2.96 mg/100 g egg and the acetic acid concentration was 4.27-4.31 mg/100 g fresh whole egg. The amount of these two acids found in fresh liquid whole egg agreed with the unpublished work of Landes and Dawson (1969) who used a silicic acid column. The amount of variation in the samples was comparable to that reported by Steinhauer (1968).

Table 17 shows the amount of acid recovered from egg samples incubated at room temperature to different bacterial population levels. Even at a population of 5×10^6 viable cells/g of egg only acetic and lactic acids were detectable of the five acids studied. The initial concentration of acetic acid was approximately

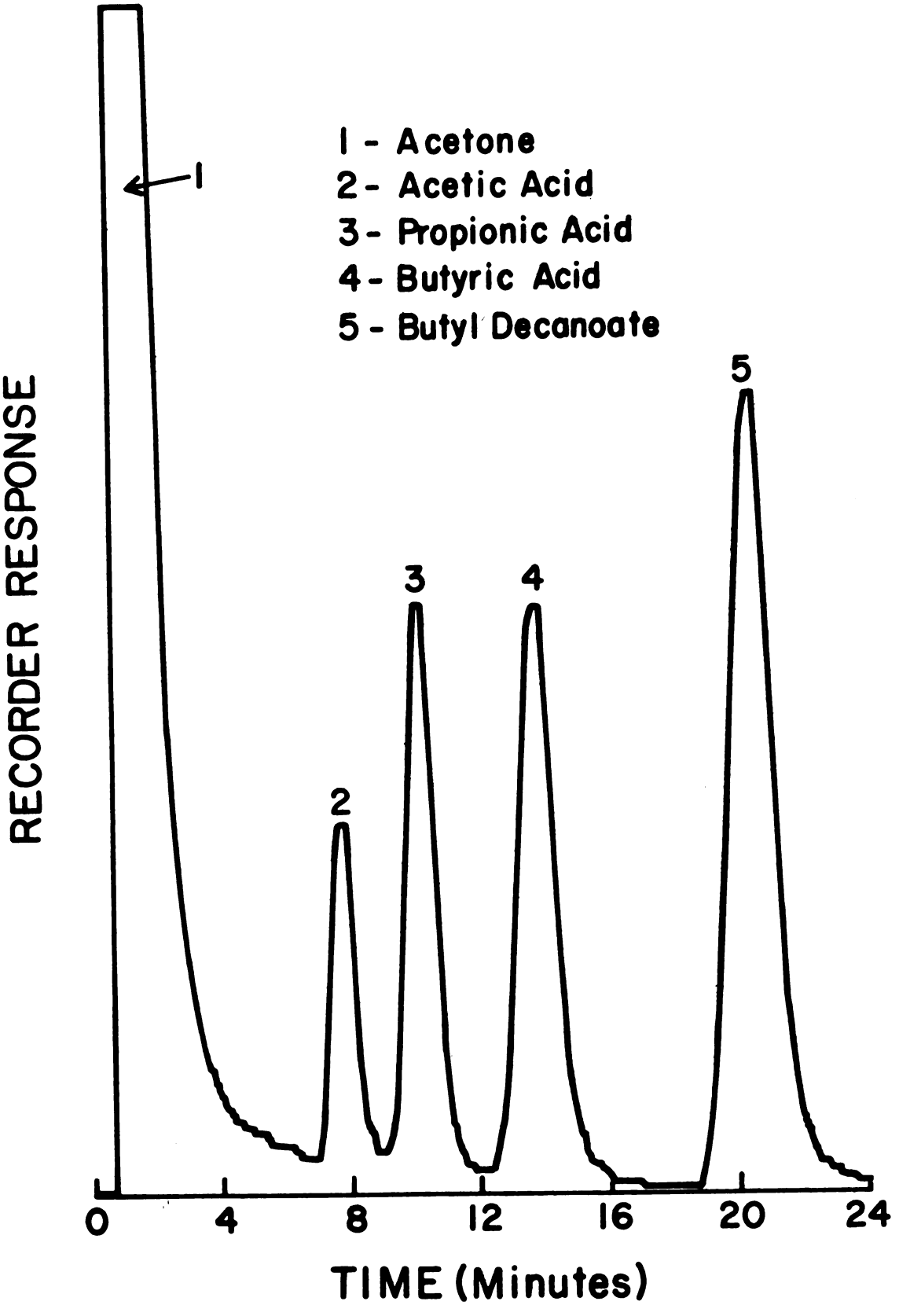

Figure 2.--A gas chromatogram of acetic, propionic and butyric acids and butyl decanoate showing their order of elution and retention times.

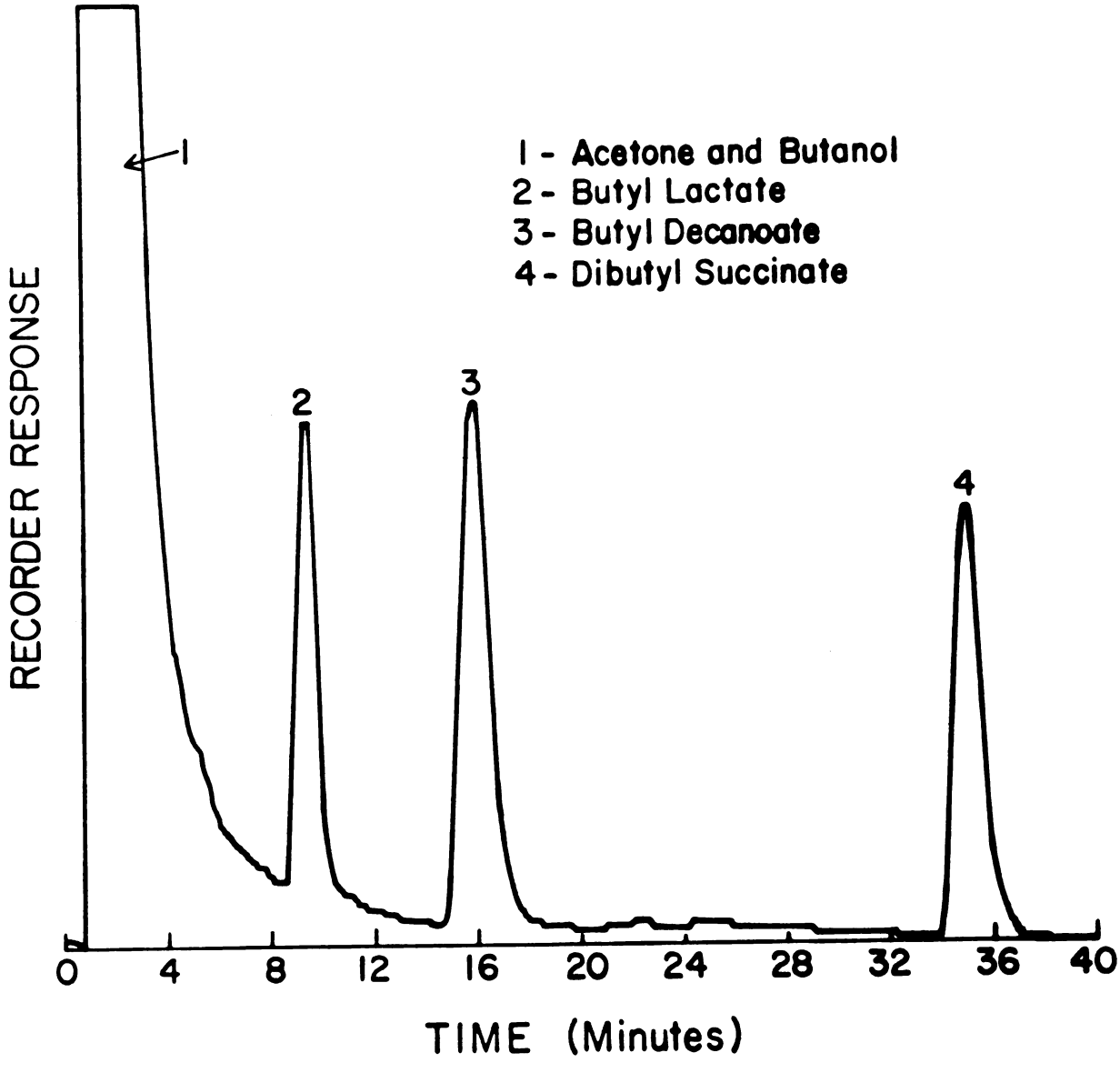

Figure 3.--A gas chromatogram of butyl lactate, butyl decanoate and dibutyl succinate showing their order of elution and retention times.

TABLE 15.--Recovery of acetic, propionic and butyric acids from liquid frozen whole egg using GLC procedures.

Sample	mg/100 g egg (added)	mg/100 g egg[a] (recovered)	
		nonpasteurized	pasteurized
		Acetic	
1	none	4.27 ± 0.23	4.31 ± 0.25
2	0.51	4.75 ± 0.18	4.82 ± 0.21
3	5.10	9.30 ± 0.29	9.31 ± 0.30
4	20.39	24.40 ± 0.45	24.38 ± 0.39
		Propionic	
1	none	none detected	
2	0.51	0.52 ± 0.05	0.49 ± 0.04
3	5.10	5.00 ± 0.19	5.08 ± 0.10
4	20.23	20.25 ± 0.31	20.33 ± 0.37
		Butyric	
1	none	none detected	
2	0.50	0.55 ± 0.07	0.50 ± 0.03
3	5.00	4.90 ± 0.23	5.11 ± 0.17
4	19.99	19.87 ± 0.38	19.93 ± 0.22

[a]Average of three samples and three injections of each sample ± standard deviation of the mean.

TABLE 16.--Recovery of lactic and succinic acids from liquid frozen whole egg using GLC procedures.

Sample	mg/100 g egg (added)	mg/100 g egg[a] (recovered)	
		nonpasteurized	pasteurized
		Lactic	
1	none	2.96 ± 0.22	2.78 ± 0.18
2	0.47	3.48 ± 0.19	3.45 ± 0.10
3	4.65	7.65 ± 0.31	7.54 ± 0.23
4	18.60	21.25 ± 0.47	21.74 ± 0.52
		Succinic	
1	none	none detected	
2	0.54	0.49 ± 0.04	0.55 ± 0.06
3	5.43	5.50 ± 0.18	5.38 ± 0.14
4	20.63	20.37 ± 0.54	20.48 ± 0.47

[a]Average of three samples and three injections of each sample ± standard deviation of the mean.

TABLE 17.--Recovery of acetic, butyric, lactic, propionic and succinic acids from frozen liquid whole egg incubated at room temperature and then frozen, using GLC procedures.[a]

Sample	Total plate count before pasteurization	mg/100 g egg[b] (recovered)	
		nonpasteurized	pasteurized
		Acetic	
1	4.0×10^4	4.02 ± 0.27	3.91 ± 0.19
2	1.3×10^5	4.15 ± 0.15	4.20 ± 0.07
3	5.4×10^5	4.33 ± 0.23	4.31 ± 0.20
4	4.5×10^6	5.14 ± 0.31	5.29 ± 0.27
		Lactic	
1	4.0×10^4	2.55 ± 0.31	2.65 ± 0.23
2	1.3×10^5	2.69 ± 0.44	2.72 ± 0.18
3	5.4×10^5	3.55 ± 0.44	3.47 ± 0.14
4	4.5×10^6	4.01 ± 0.30	3.92 ± 0.34

[a]No quantities, even in trace amounts, of butyric, propionic or succinic acids were detected.

[b]Average of three samples and three injections of each sample ± standard deviation of the mean.

4.0 mg/100 g of egg and after incubation to a bacterial population of 4.5×10^6 the concentration had only increased to 5.2 mg/100 g egg. Lactic acid was similar with an initial concentration of 2.6 mg/100 g egg and then increasing to 4.0 mg/100 g of egg with a microbial population of 4.5×10^6.

Tables 15, 16 and 17 also reveal the lack of a consistent pattern as to which product, pasteurized or nonpasteurized, had the higher acid concentration.

Steinhauer (1968) reported finding formic, acetic, propionic, lactic and succinic acids extracted from decomposed liquid whole egg which had a microbial population of 1.7×10^9/g of egg. He used GLC methods for the acid determinations. Steinhauer, et al., (1967) reported finding small amounts of succinic acid in all samples of egg evaluated. In this study a small peak was observed on the chromatogram in the vicinity of the location of the dibutyl succinate peak, but when dibutyl succinate was added the retention time was about 2 min after this unknown peak. The reason for no trace amounts of propionic and succinic acids in the eggs with a microbial population of 4.5×10^6 is not known, except that it may be due to the presence of different microbial species. The identification of bacteria was not made in this study. It is conceivable, as pointed out in the literature review, that the presence of different bacterial species could result in different

acids in varying amounts. The diet of the laying hen may also play an important role in the composition of the acids found in the fresh egg.

Acetic acid was found in measurable quantities and Landes and Dawson (1969) reported measurable quantities of both acetic and formic acids in the fresh egg. Therefore, it may be necessary to reevaluate the criteria now used to determine wholesomeness since acetic and formic acids may be found in fresh eggs.

Sensory Evaluation

Egg products were evaluated for odor and flavor differences to determine the detectable level caused by an increase in the acids added, or by the increase in acids or other by-products resulting from an increase in microbial growth.

Table 18 shows the flavor scores of eggs evaluated by the taste panel. A combination of the five acids used in this study, added from 5 to 200 ppm, resulted in significant differences in flavor. Even the lower level of acid addition (5 ppm) resulted in detection by a significant number of panelists.

A majority of the panelists who correctly identified the different sample preferred the reference samples over the sample to which the acids had been added. However, there were one or two panel members who preferred the

TABLE 18.--Flavor evaluation of scrambled whole egg to which acetic, butyric, lactic, propionic and succinic acids were added at varying levels of concentration, using the triangle test.

Sample	Amount of each acid added	No. panelists	No. correctly identifying different sample	No. preferring different sample[a]
1	none[b]	15	6	3
2	5 ppm	14	11**	1
3	50 ppm	15	9**	2
4	200 ppm	15	10**	1

**Significant at 1 percent level.

[a]Includes only panelists who correctly identified the different sample.

[b]This control sample was from the same lot of egg as samples 2, 3 and 4. The reference samples were from a different lot of egg.

sample with the acids added at each level of addition. The reference samples were always the identical samples.

Table 19 shows the results from the odor evaluation of raw egg products. No significant differences were found among the eggs which had been incubated at room temperature and which had a microbial population up to 4.5 million cells per gram before pasteurization. The panel results, after smelling the eggs to which varying amounts of acids had been added, showed a significant difference in odor perception in only those eggs to which 200 ppm of each acid was added. This difference was significant at the 1 percent level. The off-odor was described by some panelists as sour, musty, acid or rotten-H_2S, but most panelists were unable to describe the off-odor present. The lack of a definite and consistent characteristic odor development in these egg products may limit the real value of the panel evaluation data, since odor was not closely related to one acid or one bacteria.

TABLE 19.--Odor evaluation of liquid whole egg to which acids had been added at varying concentrations or had been incubated at room temperature to obtain different microbial populations.

Sample	Treatment	No. panelists	Average odor score
	Incubated[a]		
	No. organisms		
1	4.0×10^4	13	7.23
2	1.3×10^5	13	7.50
3	5.4×10^5	13	7.54
4	4.5×10^6	13	7.23
	Acids Added[b]		
1	none	16	7.50
2	5 ppm	16	7.38
3	50 ppm	16	7.50
4	200 ppm	16	5.72**

**Significant at the 1 percent level: studentized t-test.

[a]Reference sample had a score of 7.30.

[b]Reference sample had a score of 7.57.

SUMMARY

A laboratory procedure was developed for the pasteurization of liquid whole egg. This process included two accurately controlled temperature water baths, specific lengths of 7 mm o.d. glass tubing immersed in each bath, a cold water bath and a positive pressure pump to pump an accurately controlled rate of product through the system. Whole egg products were satisfactorily pasteurized by maintaining product temperature of 60-61 C for 3.5 min in the main water bath. The pasteurization process was a closed, continuous flow system.

Liquid whole egg samples incubated at 24 C for up to 17 hr, and liquid whole egg to which acetic, butyric, lactic, propionic and succinic acids were added at rates of 5, 50 and 200 ppm were pasteurized and the acid recovery percentages calculated. This heat treatment reduced bacterial populations a minimum of 2 log numbers or 99 percent.

A liquid-liquid extraction procedure was developed to extract all five of the above mentioned acids from liquid whole egg instead of the liquid-liquid and steam

distillation extraction methods currently employed in AOAC (1965) procedures. This modified liquid-liquid extraction procedure eliminates the filtration and condensing steps now used in the AOAC (1965) procedures. Recoveries of from 90 to 99 percent were achieved using this modified liquid-liquid extraction procedure.

The esterification efficiency for the formation of the butyl ester derivatives of lactic and succinic acids increased to 89 percent.

Improved GLC procedures were developed for the separation and quantitation of these five organic acids. A column temperature program resulted in a sharper dibutyl succinate peak, when compared to the peak obtained under the isothermal condition currently advocated for detecting the ester derivatives by GLC procedures.

Lactic and succinic acids were recovered from whole egg samples and chromatographed as their butyl ester derivatives along with an internal standard, butyl decanoate. Acetic, propionic and butyric acids were recovered from whole egg samples and chromatographed as the acids per se along with the same internal standard used for the butyl ester derivatives.

Twenty percent diethylene glycol succinate on 120-130 mesh Anakrom ABS was used for the column packing material.

The pasteurization process did not affect the concentration of the short-chained organic acids studied. No consistent or significant difference was found in amounts of acids recovered from pasteurized or nonpasteurized eggs. Thus, when the acids are present in the liquid whole egg before pasteurization, they will be present in the pasteurized product in the same concentration range as before pasteurization. An open batch type pasteurization system could conceivably reduce slightly the concentration of some of the volatile organic acids such as acetic, butyric and propionic acids.

The fresh egg samples studied had acetic and lactic acid concentrations of 3.91-4.31 mg/100 g egg and 2.55-2.96 mg/100 g egg, respectively. This study and the work of Landes and Dawson (1969) would seemingly be in opposition with the position taken by some researchers and the courts, who maintain that when acetic and/or formic acids are present, the egg product contains decomposed egg.

An evaluation of the content of the five organic acids studied in liquid whole egg allowed to reach a microbial population of 4.5 million, revealed the development of only acetic and lactic acids, with the major portion of both acids already present in the fresh egg sample.

Odor and flavor evaluations of egg samples either incubated or to which acids were added and which were pasteurized, were obtained to verify this procedure as an appropriate method for the evaluation of wholesomeness of liquid or frozen egg. Taste panel evaluations showed significant differences in flavor, detectable in eggs to which 5 ppm or more of all acids were added. A significant difference in odor of egg was observed in those products to which 200 ppm of all acids were added. No significant differences in odor were observed among the incubated egg samples.

The combination of all improvements in procedures used or referred to in this study, that is, esterification, extraction and GLC procedures, can result in accurate analyses of liquid egg products in about 8 hr. Accurate and valid analyses of pasteurized egg can also be made since the pasteurization procedure does not result in a loss of the acids used to determine wholesomeness of egg products.

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