QUANTITATIVE DETERMINATION OF CERTAIN SHORT-CHAIN ACIDS IN FROZEN WHOLE EGGS BY GAS-LIQUID CHROMATOGRAPHY

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John Edward Steinhauer

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THESIS

This is to certify that the

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QUANTITATIVE DETERMINATION OF CERTAIN
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John Edward Steinhauer

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ABSTRACT

QUANTITATIVE DETERMINATION OF CERTAIN SHORT-CHAIN ACIDS IN FROZEN WHOLE EGGS BY GAS-LIQUID CHROMATOGRAPHY

By John Edward Steinhauer

Organic acids in whole egg samples were evaluated using gas-liquid chromatography (GLC) and Association of Official Agriculture Chemists (AOAC, 1960) procedures. The recovery of acids from whole egg samples by GLC procedures were as accurate, and generally less variable, than those recovered by AOAC (1960) procedures. Lactic and succinic acids were recovered from the whole egg samples and chromatographed as their butyl ester derivatives with an internal standard. Acetic, propionic, and butyric acids were recovered from whole egg samples and chromatographed as the acids per se with an internal standard. Formic and acetic acids were recovered from whole egg samples and chromatographed as their butyl ester derivatives without an internal standard. Quantitation of butyl formate and butyl acetate was accomplished by comparing the chromatogram peak heights of the butyl esters

recovered from the egg samples with the chromatogram peak heights of a known concentration of butyl ester standards.

Whole eggs which had undergone natural bacterial decomposition were analyzed by GLC to determine the quantity of formic, acetic, propionic, and butyric (C_1-C_4 acids) and lactic and succinic acids present. Microbiological examinations of the decomposed eggs were also made to determine the total, salmonella, staphylococcus, and coliform counts.

The recovery of lactic acid from whole egg samples by GLC procedures ranged from 98 percent at the low concentration (0.43 mg/100 g egg) to 99 percent at the high concentration (47.70 mg/100 g egg). The recovery of succinic acid from whole egg samples by GLC procedures ranged from 103 percent at the low concentration (1.22 mg/100 g egg) to 98 percent at the high concentration (73.20 mg/100 g egg).

The recovery of acetic, propionic, and butyric acids from whole egg samples by GLC methods were, respectively, 114 percent at the low concentration (1.17 mg/100 g egg) to 100 percent at the high concentration (12.67 mg/100 g egg); 100 percent at the low concentration (1.38 mg/100 g egg) to 101 percent at the high concentration (14.87 mg/100 g egg); and 103 percent at the low concentration (1.70 mg/100 g egg) to 99 percent at the high concentration (18.37 mg/100 g egg).

The recovery of formic acid as its butyl ester from whole egg samples by GLC procedures was 104 percent at the low concentration (1.25 mg/100 g egg) to 96 percent at the high concentration (24.76 mg/100 g egg). The recovery of acetic acid as its butyl ester from whole egg samples by GLC procedures ranged from 102 percent at the low concentration (1.15 mg/100 g egg) to 98 percent at the high concentration (33.32 mg/100 g egg).

Analysis of whole eggs which had undergone natural bacterial decomposition showed that lactic acid was present in the highest concentration (214.9 mg/100 g egg), followed by acetic acid (34.8 mg/100 g egg) recovered as butyl acetate, acetic acid (35.6 mg/100 g egg), recovered as acetic acid, succinic acid (29.8 mg/100 g egg), formic acid (17.8 mg/100 g egg), and propionic acid (4.6 mg/100 g egg). No butyric acid was detected. The high total, coliform, and salmonella counts indicated that a relationship exists between the types and numbers of microorganisms present and the quantity and kind of acids produced in decomposed eggs.

QUANTITATIVE DETERMINATION OF CERTAIN SHORT-CHAIN ACIDS IN FROZEN WHOLE EGGS BY GAS-LIQUID CHROMATOGRAPHY

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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 salmonella 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.

Egg products are presently analyzed for both viable and nonviable microbial count, the presence or absence of salmonella and coliforms, the odor of the product, and the presence of formic, acetic, lactic, or succinic acids to determine the product's acceptability based on wholesomeness.

Current AOAC laboratory procedures for the determination of formic, acetic, lactic, and/or succinic acids as indices of bacterial decomposition in frozen whole eggs are laborious and time consuming. In the meantime, valuable lots of frozen eggs may be held under seizure awaiting results. At the present time, formic acid is measured gravimetrically by its reaction with mecuric chloride;

acetic acid and succinic acids are determined by titration following elution from a silicic acid column; and lactic acid is determined colorimetrically through its reaction with ferric chloride.

Since gas-liquid chromatography offers the advantages of simplicity, rapidity and sensitivity, an investigation to improve the speed, accuracy, and/or precision in the determination of formic, acetic, lactic, and succinic acids in frozen whole eggs was initiated.

LITERATURE REVIEW

Examination of Frozen Eggs

Bacterial and physical changes occur in eggs of good and poor quality during preparation, freezing, commercial storage, and prolonged storage. Schneiter, et al., (1943) reported that (1) frozen eggs of good quality are able to withstand at least two complete thawings and refreezings without significant change in bacterial content or without acquiring abnormal appearance or odor; (2) eggs of poor quality, including cracks, leakers, and dirty eggs usually have high bacterial counts and this condition leads to progressive decomposition of the product unless it is rapidly frozen and maintained in the frozen condition; (3) prolonged storage of frozen eggs over a six year period resulted in a considerable reduction of bacterial count, but the total count still served 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/g of liquid or frozen egg with determinable amounts of either formic or acetic acid, or lactic acid in excess of 7 mg/100 g of liquid egg, demonstrated the presence of decomposed eggs.

Further, certain types of decomposed eggs can be present in liquid and frozen eggs without being detected by these bacteriological and chemical methods.

Winter and Wilkin (1947) reported that more bacteria were destroyed in slowly frozen egg products than in rapidly frozen products in 30 pound containers.

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

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

Steinhauer, et al., (1967) reported that the number of bacteria in eggs stored up to eight days at 16 C increased from 8.0×10^2 to 3.6×10^8 g 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.

These authors also reported that total viable bacteria in six commercial frozen egg samples varied from 12×10^5 to 61×10^7 microorganisms/g of egg. Acetic acid in these samples was relatively high, varying from 0 to 56.2 mg/100 g egg and succinic acid varied from 0.2 to 4.5 mg/100 g egg. Sixteen additional samples of liquid or frozen eggs contained no formic acid; only one sample contained acetic acid; three samples contained succinic acid; and 15 samples contained lactic acid. A general relationship was found between numbers of bacteria and concentrations of lactic and succinic acids.

Microbiology of Frozen Eggs

The bacterial flora of frozen egg products is important, because it influences the wholesomeness of the products, their keeping quality, and their functional properties in the preparation of food. Haines (1939), Gibbons, et al., (1944) and Winter, et al., (1946) reported that more than 80 percent of freshly laid eggs are bacteriologically sterile. However, nearly all commercial liquid, frozen and dried egg products contain several hundred to several million bacteria/g, according to reports by Redfield (1920), Johns (1944), McFarlane, et al., (1947) and Winter and Wrinkle (1949).

The dirt on egg shells and the practices used in egg-breaking plants account for most of the bacteria found in liquid, frozen, and dried egg products (Haines, 1939; Rosser, 1942; Stuart and McNally, 1943; Zagaevsky and Lutikova, 1944; McFarlane, et al., 1945; Johns and Berard, 1945; Gunderson and Gunderson, 1945; Solowey, et al., 1946; Penniston and Hedrick, 1947 and Winter and Wrinkle, 1949).

Wrinkle, et al., (1950) reported that a very heterogeneous and variable flora may be expected in frozen egg products, since the dirt on the shell consists of fecal material, soil, nest and feather dust; and microorganisms are present in the air, on equipment and on workers in the breaking plants.

Haines (1939) showed that the percentage distribution of microflora in washings from shell eggs was as follows: nonspore forming rods, 38 percent; sporing rods, 30 percent; cocci, 25 percent; yeasts, 4 percent; and molds, 3 percent. More recently, Zagaevsky and Lutikova (1944) and Gunderson and Gunderson (1945) reported somewhat similar flora on shell eggs.

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 predominantly 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, and 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 catabolism.

Thimann (1955) reported that three groups of bacteria produce lactic acid from sugar in 80-98 percent yield, with only traces of by-products. These homofermentative forms include 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 appearing as CO_2, hydrogen, alcohol, formic or acetic acid. These groups are 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, and all members of the genera Streptococcus,

Pediococcus, Microbacterium, and a large number of lactobacilli and certain bacilli ferment glucose predominantly 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 pH greatly influenced the yields of metabolic products in fermentations conducted in phosphate buffer but not in those in bicarbonate buffer. As the pH 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 a condensation of a 3-carbon compound with formic acid.

Braak (1928) showed that glycerol is readily fermented by Escherichia and Aerobacter. With the former,

the products are mainly alcohol and lactic and acetic acids; with the latter, they are mainly alcohol and acetic and formic acids.

Doudoroff (1942) reported that certain facultatively 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 species of E. coli reported that, depending on the pH 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 pH. Mickelson and Werkman (1938) reported that pH has a marked effect upon the 2, 3-butanediol fermentation of Aerobacter. Above pH 6.3, acetic and formic acids accumulate and below pH 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 carbohydrate 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 13 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 pH 5, 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 formate to CO_2 and hydrogen, and as a consequence, formic acid 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; Friedemann, 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 eggs become sour on 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 accompanied by rapid bacterial increase.

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 formic acid in biological materials. Fincke (1913) developed a gravimetric procedure for the determination of formic acid based on its reaction with mecuric chloride, and Hanak and Kurschner (1930) reported the development of a permanganate colorimetric method for the determination of formic acid. A relatively simple mecuric chloride volumetric method for the estimation of formic and acetic acids was developed by Fuchs (1929). More recently Grant (1948) proposed a colorimetric method

for the microdetermination of formic acid based on the reduction of the acid to formaldehyde.

Dyer (1917) proposed the original procedure for the separation, identification and determination of volatile fatty acids by steam distillation. This procedure is based on the fact that under 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 (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 GLC, James and Martin (1952) developed new procedures whereby formic acid and its

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 derivative to determine volatile fatty acids by GLC 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 analyses.

Shelly, et al., (1963) described a procedure whereby the volatile short-chain fatty acids (C_1-C_4) present in frozen eggs were quantitatively determined using GLC. A similar method was also used by Grey and Stevens (1966) for the quantitative determination of formic, acetic and propionic acids from microbial fermentations.

Lactic and Succinic Acids

Friedemann and Graeser (1933) proposed a titrimetric method for the determination of lactic acid through its reaction with sodium bisulfite. Hillig (1937 a, 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 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 chromatography to isolate both lactic and succinic acids. The identification of lactic acid was accomplished by microscopic comparison of the isolated zinc lactate with a known solution of pure zinc lactate. 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 the use of filter paper chromatography (Magasanik and Umbarger, 1950).

Gehrke and Georlitz (1963) reported the development of 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 chromatography and quantitated the amounts by titration with KOH.

Gas-Liquid Chromatography

Historical

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 inert material, the method is Gas-Liquid Chromatography (GLC).

Early in the 14th century, Brunschwig (1512) employed the principle of gas-liquid chromatography in the purification of ethyl alcohol by use of a bath sponge and olive oil.

After the theoretical suggestion of Martin and Synge (1941) gas-liquid chromatography was first introduced in 1952 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.

Description of Operation

Gas-liquid chromatography employs columns charged with a liquid phase which is applied in a thin layer to an inert carrier or supporting material and the support should not exert any absorption action on the materials to be separated (Bayer, 1961). The materials are separated or fractionated on these columns by the elution process. The gaseous substances to be separated are placed on the column by means of suitable dosing devices and driven through the column by a carrier gas. This author reported that mixtures are separated as they pass through the column and the separated fractions emerge at the end of the column along with the carrier gas. The substances in the flowing carrier gas stream are then detected or characterized by measuring a chemical or physical property. This measurement is continuously recorded either by direct reading or by means of a device which automatically delivers a permanent record of the data in question. Thus, there results a diagram in which the observed figures are plotted against time, and the measured values are now in simple relation to the concentration of the substance, the values depending on the type of measuring instrument employed.

Apparatus

1. The mobile phase.--Hardy and Pollard (1960) reported that the gases most frequently used for the mobile phase are nitrogen, helium, hydrogen and carbon dioxide and the choice of mobile phase is intimately related to the type of detector employed. The flow rate can be controlled with either a simple manometer or needle valve and measured by moving-bubble flowmeters or rotameters.
2. The solid support.--The general requirements for the solid support material used in gas-liquid partition chromatography are: (1) must have a high surface area; (2) must not break up under reasonable compaction or under the handling procedure required to prepare the material and fill the chromatographic column; (3) must pack uniformly in the column; (4) must be chemically inert towards the samples which are to be put through the column; and (5) must not be appreciably absorptive (Johns, 1958).

Burchfield and Storrs (1962) and Szymanski (1964) reported that a number of important supports are made from diatomaceous earth. The material consists of the skeletons of diatoms, and chemically, it is primarily silica with trace impurities. Various treatments of the diatomaceous earth yield

different types of supports; the more commonly known are Celite, Firebrick, Sterchamol and Chromosorb.

Absorption of solutes on the solid support may result in asymmetrical peaks due to tailing; this is particularly noticeable in the chromatography of polar compounds, or when the stationary liquid is nonpolar. The deactivation of supports by treatment with chemicals which form films over the solid particles have been reported. These include dimethyldichlorosilane (Horning, et al., 1959) hexamethyldisilazane (Bohemen, et al., 1960) and metallic silver (Ormerod and Scott, 1959).

Highly absorptive materials, other than diatomaceous earths, have been used as solid supports. Decora and Dinneen (1960) prepared a finely divided inorganic material by exhaustive extraction of Tide, a synthetic detergent.

3. The stationary liquid.--The selection of a stationary liquid is the most important part in developing a gas chromatographic method, since it determines whether any given pair of solutes can be separated (Ober, 1958). The liquid selected as the stationary phase should be nonvolatile and thermally stable at the operating temperature of the chromatograph.

Burchfield and Storrs (1962) stated that when compounds which have the same polarity and different boiling points are to be separated, a nonpolar liquid phase is the most satisfactory. The most common liquids used in the nonpolar class include squalane, Apiezons, silicones and esters of high-molecular weight alcohols and dibasic acids. However, if the compounds under study are to be separated according to polarity, a polar liquid such as polyethylene glycol or one of the polyesters of the short-chain dibasic acids or alcohols should be used.

Integral Detectors

1. Measurement of volume or pressure.--Janak (1954) used carbon dioxide as the mobile gas to carry the separated components of mixtures from the column into the nitrometer containing a concentrated solution of potassium hydroxide. The carbon dioxide was absorbed and the residual gases measured in a burette.
2. Direct titration.--James and Martin (1952) titrated volatile fatty acids and bases by passing the gas stream from the column through a titration cell.

Differential Detectors

1. Thermal conductivity.--The measurement of thermal conductivity has been used for many years for GLC analysis and the design and operation of thermal conductivity cells was given by Minter and Burdy (1951). The principle of the detector is based on the fact that when a constant flow of gas passes over a fine wire heated by a constant electric current the rate of heat loss by the wire is constant, and any change in composition of the gas stream will cause a change in heat loss and thus a change in resistance. The wires are connected in a Wheatstone bridge circuit and any change of bridge output, due to a change of wire resistance, is amplified and recorded.
2. Gas density balance.--Martin and James (1956) developed a detector called a gas-density balance which consisted of a metal block bored with a series of tubes connected together in a manner analogous to a Wheatstone bridge to compensate for pressure differences due to the flow of gas. Any difference in density caused a flow of gas in a cross-channel in the metal block. This cross-channel contained a flow-detector consisting of an electrically heated filament close to two

connected thermojunctions and a flow of gas altered the temperature of these thermojunctions. This thermoelectric output was subsequently amplified and recorded.

3. Flame ionization.--This detector was first used by Scott (1957) to measure the gases from a separatory column. The gas mixture was burned in a micro burner and the temperature of the flame was determined and recorded.

   A more recent and widely used variation of the Scott detector is the flame ionization detector proposed by Harley, et al., (1958). This detector measures the current which develops in a flame because of the ionization of organic compounds, and there is a linear relationship between the detector response and the molar concentration of the substance.

4. Argon detector.--This detector developed by Lovelock (1958) utilizes argon as the carrier gas and its atoms are excited to a metastable state by ionizing radiations as they enter the detector. The excited argon atoms are stable until they collide with organic molecules, whereupon secondary electrons are produced, giving rise to an ionization current which is proportional to the concentration of solute in the carrier gas.

5. Electron capture.--Compounds which contain halogen atoms and polar functional groups can be identified by electron capture (Burchfield and Storrs, 1962). Neutral molecules which pass through an ionization chamber can capture free electrons to form negative molecule-ions. The energy change accompanying the reaction is the electron affinity of the compound.

EXPERIMENTAL PROCEDURES

This investigation was divided into four parts. Part I involved the development of a quantitative procedure for the determination of lactic and succinic acids by GLC. Part II involved the determination of acetic, propionic and butyric as the straight chain acids in eggs using a modified procedure previously developed by Shelley, et al., (1963).

Since formic acid is also an index of wholesomeness of egg products and cannot be detected per se using flame ionization, Part III of this study was the development of a procedure whereby formic and acetic acids were quantitatively determined by GLC using their butyl ester derivatives.

Part IV involved the determination of formic, acetic, propionic, butyric, lactic and succinic acids in frozen whole eggs that had been allowed to decompose by the microorganisms naturally present.

General

Apparatus

1. Gas-Liquid Chromatograph.--An F and M Scientific Co. Model 810 dual column flame ionization instrument equipped with a Model 9294 N Honeywell recorder was used for all GLC analyses in this study.
2. Liquid-Liquid Extractor.--This apparatus was identical in design with that used in AOAC (1960) procedure 15.012.
3. Steam Distillation Assembly.--This assembly consisted of a 2000 ml round bottom flask which served as a steam generator and was connected to a two neck 1000 ml round bottom distillation flask by means of a suitable arrangement of glass and rubber tubing and neoprene stoppers. The steam entered the distillation flask through the center neck and the volatile products in the sample were allowed to escape through a Vigreux fractioning column connected to the other neck. The volatile products, after passage through the Vigreux column, were condensed in a Graham coil condenser and subsequently collected in a 2000 ml graduated cylinder. Heat for both the steam generator and the distillation flask was supplied by means of individual

heating mantles connected to powerstats. Figure 1 shows the details of this apparatus.

Reagents

Reagent grade chemicals and deionized water were used throughout this study. Wherever possible internal standards, acids and reagents used in this study were checked for purity using GLC, and redistilled when necessary.

Blanks

All titration and steam distillation operations were preceded by determination of blanks using distilled water and appropriate reagents.

Part I. Lactic and Succinic Acids

A. Preparation of Standard Acid Solutions

1. Lactic acid: N/10.--Approximately 4.25 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 distilled water and mixed.
2. Succinic acid: N/10.--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,

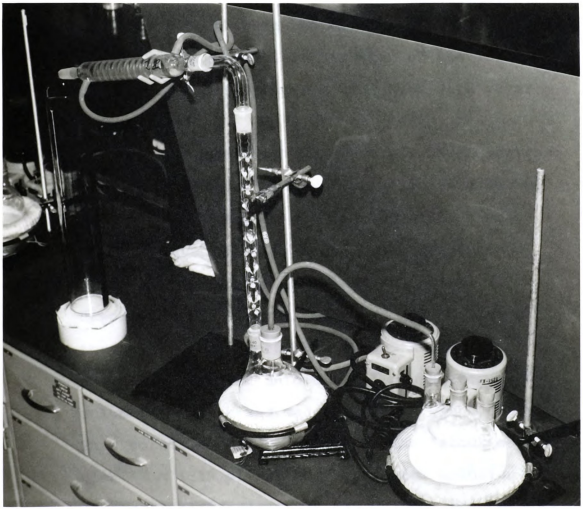

Fig. 1 - Photograph of steam distillation assembly.

approximately 2.95 g were weighed, transferred to a 400 ml beaker and dissolved in 150-200 ml water. The dissolved solution was transferred to a 500 ml volumetric flask; the beaker was rinsed several times to insure complete transfer of the acid. The solution was diluted to the mark with water and mixed.

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 N/10 solution of sodium hydroxide to a phenolphthalein end point.

3. Preparation of Internal Standard.--Approximately three g of butyl decanoate (K and K Labs) were accurately weighed into a 25 ml glass stoppered volumetric flask and made to volume with acetone. This solution was designated as the "stock solution." A 1:10 dilution of the stock solution was prepared using acetone as the diluent and designated as the "diluted stock solution."

B. Preparation of the Columns

Twenty g of high temperature stabilized ethylene glycol adipate (Analabs) were weighed into a 500 ml round bottom flask and dissolved in 175-200 ml acetone. Eighty 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 use of a rotating evaporator at reduced pressure in a water bath at 45 C. The packing material was further dried in an air oven for 12 hr at 100 C.

The columns were packed using a vacuum and Vibra-graver tool to evenly distribute the packing material within the column. The packed columns were installed in the Model 810 chromatograph and conditioned for 24 hr at 175 C. Nitrogen was used as the conditioning gas at a flow rate of 60 ml/min.

C. Operating Parameters for the Gas-Liquid Chromatograph

Column	72" X 1/4" copper tubing	
Carrier gas	Helium	
Pressure	40 psi	
Flow rates	A column - 60 ml/min	
	B column - 62 ml/min	
Hydrogen	20 psi	
Air	640 ml/min @ 35 psi	
Temperatures	Detectors	250 C
	Injection port	250 C
	Column	160 C
Chart speed	30 in/hr	
Sample size	1 µl	

Range	10
Attenuation	4-32

D. Calibration of the Gas-Liquid Chromatograph

Approximately one g each of butyl lactate (Eastman Chemical) butyl decanoate, and dibutyl succinate (K and K Labs) were accurately weighed into the same 25 ml volumetric flask, diluted to the mark with acetone and designated as the stock solution. A 1:10 dilution of the stock solution was prepared by pipetting 2.5 ml of the stock mixture into a 25 ml volumetric flask and diluting to the mark. Various volumes of the 1:10 dilution mixture of the standard butyl esters were pipetted into a five ml volumetric flask to provide a range of concentration for each butyl ester at a given sensitivity. 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. Using the calculation outlined by Shelley, et al., (1963) the detector response value, R, of butyl lactate and dibutyl succinate relative to butyl decanoate was calculated using the formula: $R_{BE} = (h_{BE})(c_{IS})/(c_{BE})(h_{IS})$, where h_{BE} and h_{IS} are the peak heights of the butyl ester and butyl decanoate, respectively, and c_{BE} and c_{IS} are the corresponding concentrations (mg/five ml acetone solution).

E. Determination of Percent Acid Recovery Using Liquid-Liquid Extractor

To determine the optimum time necessary for the extraction of lactic and succinic acids from the egg filtrate using liquid-liquid extraction, standard N/10 lactic and succinic acid solutions were prepared. Ten ml of each of the standard acid solutions were pipetted into a 50 ml beaker and titrated with N/10 sodium hydroxide to a phenolphthalein end point. The contents of the beaker were quantitatively transferred into the liquid-liquid extractor and acidified with four drops of concentrated sulfuric acid. Ten ml of water and 40 ml of ether were placed in a 50 ml round bottom flask with a 19/38 ground glass joint; the flask was connected to the 24/40 joint of the side arm of the extractor with an adapter and placed in a preheated 50 ml heating mantle. Temperature was regulated with a powerstat at a setting of 90. The extraction was timed from the fall of the first drop of ether. Following extraction, the flask was removed from the heat source, disconnected from the extractor and the ether portion evaporated on a steam bath under a stream of dry air. After cooling, the remaining acid-water mixture was titrated with N/10 sodium hydroxide to a phenolphthalein end point. The percent recovery was calculated by use of the formula:

$$\frac{\text{ml N/10 NaOH required after extraction}}{\text{ml N/10 NaOH required before extraction}} \times 100$$

F. Determination of Butyl Esterification Efficiency for Lactic and Succinic Acids

Ten replicate, five and 15 ml aliquots of accurately standardized N/10 lactic and succinic acid solutions, respectively, were pipetted into individual 50 ml 19/38 standard taper round bottom flasks. The water was evaporated using a steam bath and a stream of dry air. One ml of 1-butanol, two drops of concentrated hydrochloric acid and approximately 500 mg of anhydrous sodium sulfate were added to the flask containing the dried material. The flask was connected to a condenser equipped with a drying tube filled with soda lime and the mixture was gently refluxed for two hr with a 50 ml heating mantle connected to a powerstat set at 40.

After butyl esterification was completed, the sides and joint of the condenser were rinsed with one 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 through a microfunnel. An appropriate quantity of internal standard was pipetted into the flask and the contents were made to volume with acetone. Triplicate one µl aliquots were injected into the chromatograph and the lactic and

succinic acids were recovered as butyl lactate and dibutyl succinate.

G. Preparation of Frozen Whole Eggs for Analysis

Two cases of Grade A fresh medium (or larger) eggs were obtained from the Michigan State University Poultry farm. The eggs were broken and the liquid contents placed in a 1.5 gallon stainless steel Waring blendor jar and blended for 15 sec at low speed. The blended eggs were transferred to a 30 gallon stainless steel, multiple paddle mixer and mixed for an additional 15 min.

Five 5000 g lots of the blended whole eggs were prepared for subsequent analysis by adding increasing aliquots of each of the previously prepared standard lactic and succinic acid solutions to each lot. This procedure was carried out by pipetting the desired quantities of the standard acid solutions into a tared 1.5 gallon stainless steel Waring blendor jar, and adding a quantity of the blended eggs sufficient to make a total weight of 5000 g (acid solution plus eggs). The total mixture was then blended for 15 sec at low speed. From the 5000 g acid-egg mixture, 20 sub-samples of 225 g each were placed into one pint plastic containers with snap-on lids and immediately frozen and held at -10 C until analyzed.

The mg concentrations/100 g of egg of both lactic and succinic acids were as follows:

Sample No.	Lactic acid (mg/100 g egg)	Succinic acid (mg/100 g egg)
1	0.43	1.22
2	3.40	7.30
3	6.80	12.20
4	13.65	24.30
5	34.10	48.70
6	47.70	73.20

A sixth 5000 g lot of the blended eggs was removed from the mixer and treated the same as the five previous lots, with the exception that no acids were added; these samples were used as controls.

H. Preparation of Egg Samples for GLC Analysis

1. Acid Extraction.--The frozen egg samples were thawed under running cold tap water and a 200 g aliquot was accurately weighed into a tared 1000 ml wide-mouth erlenmeyer flask, and 500 ml of water were added. Seventy-five ml of N/1 sulfuric acid and 125 ml of 20 percent phosphotungstic acid were added, and the contents of the flask diluted to 1000 g with water, stoppered and shaken vigorously for one min. The sample was divided and filtered through two 24 cm folded filter papers

(Whatman No. 12) into 600 ml beakers. Filtration proceeded for 60 min. The filtrates were then combined and 450 ml of the filtrate were transferred by pipette to a 600 ml beaker and reduced to approximately 30 ml using a hot plate and a steam bath.

The material was quantitatively transferred to the inner tube of a liquid-liquid extractor by washing through a small funnel with water. The beaker was rinsed several times with water and the total volume of liquid in the extractor was made to approximately 60 ml. Four drops of concentrated sulfuric acid were added and washed with a small quantity of water into the extractor. 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. Fifty ml of ether and several boiling chips were placed in a 50 ml 19/38 standard taper round bottom flask, the flask and 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 90 on the powerstat was found to satisfactorily maintain a steady flow of ether through the liquid material. The heating mantle was preheated for several

minutes prior to initiation of the extraction to prevent superheating of the ether. The samples were extracted for 24 hr, starting with the fall of the first drop of ether from the condenser.

2. Esterification.--After acid extraction was completed, the flask containing the ether extract was disconnected from the apparatus and the ether evaporated on a steam bath. Esterification was accomplished using the procedure outlined under Part I-F.

3. Determination.--After butylation was completed, the sides and joint of the condenser were washed with one ml of acetone and the washings were added to the contents of the flask. The contents of the flask were quantitatively transferred to an 8-10 ml volumetric flask through a micro-funnel. The 8 ml graduation mark was made with a marking pen after pipetting 8 ml of acetone into the 10 ml volumetric flask.

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 micro-funnel was rinsed with one ml of acetone which was also added to the contents of the volumetric flask. Using acetone, the butyl ester-acetone mixture was diluted to the

8 ml mark, stoppered and mixed well. A one µl aliquot was injected into the chromatograph and the peak heights of butyl lactate and dibutyl succinate were measured. The R value data previously determined gave an approximation (based on peak height, detector sensitivity and attenuation) of the concentrations of butyl lactate and dibutyl succinate in the 8 ml sample. Using this information, the necessary quantity of butyl decanoate which should be added to provide a peak height commensurate with the peak heights of the butyl lactate and dibutyl succinate 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.

I. Calculations

From the chromatograms of the samples, the peak heights of butyl lactate, butyl decanoate, and dibutyl succinate were measured. Rearrangement of the R value equation gives the following:

$$c_{BE} = ({}^{h}BE)\ \ ({}^{c}IS)/({}^{R}BE)\ \ ({}^{h}IS)$$

Since R values for each butyl ester were previously determined, unknown concentrations were calculated by substituting peak heights and the appropriate R values in the above equation. After determining the mg concentration of butyl lactate and dibutyl succinate present in the injected aliquot, the concentration value for butyl lactate and dibutyl succinate was multiplied by 8050 and 6775, respectively. These factors convert the quantity of butyl lactate and dibutyl succinate present in the injected aliquot to mg of lactic and succinic acids/100 g egg. The formulas for determining these conversion factors were derived as follows:

	Column a	Column b	Column c	Column d	Column e
Lactic	$\left[0.6162\right]$	$\left[\frac{10,000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{84.8}\right]$	$\left[\frac{100}{200}\right]$
Succinic	$\left[0.5134\right]$	$\left[\frac{10,000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{82.9}\right]$	$\left[\frac{100}{200}\right]$

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 ml (g) of the original 1000 g of

egg-water-phosphotungstic-sulfuric acid mixture was used.

Column d is the factor which accounts for the 84.8 and 82.9 percent efficiency of butylation for lactic and succinic acids, respectively.

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

J. Preparation of Egg Samples for AOAC Analysis

Triplicate egg samples containing lactic acid from the same lot of eggs analyzed by GLC were analyzed according to AOAC (1960) procedures 15.012-15.013 and 16.039-16.040. The lactic acid content of the egg samples was quantitated colorimetrically using a Spectronic 20 Spectrophotometer (Bausch and Lomb) and matched tubes at 370 millimicrons.

Triplicate egg samples containing succinic acid from each lot of eggs analyzed by GLC were analyzed according to AOAC (1960) procedures 16.039-16.042. The succinic acid content of the eggs was quantitated by titrating the acid with sodium hydroxide following its elution from a silicic acid column.

Part II. Acetic, Propionic, and Butyric Acids

A. Preparation of Standard Acid Solutions

Approximately N/10 acetic, propionic, and butyric acid solutions were prepared by pipetting the calculated volume of each acid into separate 500 ml volumetric flasks (6.0 ml acetic, 7.4 ml propionic, and 8.8 ml butyric).

To determine the normality and the exact mg concentration of each acid/ml of solution, 20 ml aliquots were removed by pipette and titrated with an accurately standardized N/10 solution of sodium hydroxide to a phenolphthalein end point.

B. Preparation of Internal Standard

Approximately three g of butyl octanoate (K and K Labs) were accurately weighed into a 25 ml glass-stoppered volumetric flask and made to volume with acetone. This solution was designated as the "stock solution." A 1:10 dilution of the stock solution was prepared using acetone as the diluent and designated as the "diluted stock solution."

C. 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 the mark with acetone, and tightly stoppered.

D. Preparation of the Columns

These procedures were identical to those outlined in Part I-B and were discussed previously.

E. Operating Parameters for the Gas-Liquid Chromatograph

Column	72" X 1/4" copper tubing	
Carrier gas	Helium	
Pressure	40 psi	
Flow rates	A column - 50 ml/min	
	B column - 51 ml/min	
Hydrogen	20 psi	
Air	640 ml/min @ 35 psi	
Temperatures	Detector	250 C
	Injection port	250 C
	Column	120 C
Chart speed	30 in/hr	
Sample size	1 µl	
Range	10	
Attenuation	4-32	

F. Calibration of the Gas-Liquid Chromatograph

This procedure was identical to that outlined in Part I-D, except that one g each of acetic, propionic,

butyric, and butyl octanoate were substituted for butyl lactate, dibutyl succinate, and butyl decanoate.

G. Determination of Acid Recoveries by Steam Distillation

One hundred ml each of the standard formic, acetic, propionic, and butyric acid solutions were pipetted into individual 200 ml volumetric flasks and diluted to the mark with water. Three 10 ml aliquots of each acid were removed by pipette, placed in a 125 ml erlenmeyer flask with 10 ml of water, and titrated with N/100 sodium hydroxide to a phenolphthalein end point.

Ten ml aliquots of each acid were pipetted into the distillation flask of the steam distillation assembly. Four hundred and thirty five ml of water and five ml of concentrated sulfuric acid were added to the flask providing a total volume of 450 ml in the distillation flask. Heating mantles controlled by rheostats were used to adjust the distillation apparatus to (1) provide a distillation rate of 1200 ml/90 ± 5 min and (2) maintain the volume in the distillation flask at 450 ± 10 ml throughout the 90 min distillation period. After distillation, the Graham condenser was disconnected from the Vigreux fractionating column, the condenser was washed several times with water, and the washings were added to the contents of the graduated cylinder. The 1200 + ml of distillate were quantitatively transferred to a 1500 ml beaker and quantitatively

neutralized to a phenophthalein end point with N/100 sodium hydroxide. Ten replicate 10 ml aliquots of each acid were distilled using the above procedure.

H. Preparation of Frozen Whole Eggs for Analysis

Frozen whole egg samples containing known amounts of acetic, propionic, and butyric acids were prepared for subsequent analysis using the procedures outlined in Part I-G.

The concentrations in mg/100 g egg of acetic, propionic, and butyric acids were as follows:

Sample No.	Acetic (mg/100 g egg)	Propionic (mg/100 g egg)	Butyric (mg/100 g egg)
1	1.17	1.38	1.70
2	3.17	3.72	4.59
3	6.34	7.44	9.29
4	12.67	14.87	18.37

I. Preparation of Egg Samples for GLC Analysis

1. Acid Extraction.--The frozen egg samples were thawed under cold running tap water, and a 200 g aliquot was accurately weighed into a tared 1000 ml wide-mouth erlenmeyer flask. Five hundred ml of water were added, followed by 75 ml of N/1 sulfuric acid. The contents of the flask were gently swirled until thoroughly mixed.

Seventy five ml of 20 percent phosphotungstic acid solution were added, and the contents of the flask made to 1000 g with water. The flask was stoppered and vigorously shaken for one min. The sample was divided and filtered through two 24 cm folded filter papers (Whatman No. 12) into 600 ml beakers. Filtration proceeded for 60 min. The filtrates were then combined and 450 ml of the filtrate were transferred by pipette to the distillation flask of the steam distillation assembly and the acids were distilled and collected using the procedure outlined in Part II-H.

2. Determination.--The distillate was quantitatively neutralized with N/100 sodium hydroxide using phenolphthalein as the indicator. An excess of base (usually 1-1.5 ml of N/10 sodium hydroxide) was added to the neutralized distillate and the volume was reduced using a hot plate and steam bath. When the color became discharged, more base was added.

When the volume reached approximately 25 ml, the distillate was transferred to a 50 ml beaker and evaporated to approximately one to two ml on a steam bath. The sodium salts of the acids were quantitatively transferred to a 12 ml graduated centrifuge tube using a 1:1 (v/v) mixture

of acetone and water. The acetone and water were evaporated under a stream of dry air on a steam bath. The dried salts were loosened with a microspatula and one ml of the dichloroacetic mixture was added to the centrifuge tube.

The contents of the tube were mixed for one min on a Vortex Jr. mixing apparatus to insure complete dispersal of the salts and liberation of the volatile acids. A volume of the butyl octanoate internal standard (as specified for the acid content of the sample in Table 1) was pipetted into the tube. The sample was diluted to two ml, stoppered and mixed again with the Vortex mixer. The precipitated sodium dichloracetate was allowed to settle out and one µl of the clear solution was injected into the chromatograph.

J. Calculations

The peak heights of acetic, propionic, and butyric acids and butyl octanoate were measured from the chromatograms of the samples. Rearrangement of the R value equation gave the following formula:

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

Since R values for each acid were previously determined, unknown acid concentrations were calculated by substituting the appropriate peak heights and R values in the

Table 1. Volume of internal standard added per millileter of base required to neutralize steam distillate.

N/100 NaOH for Neutralization of Steam Distillate (Before Addition of Excess)	Volume of Internal Standard Added		Total Volume After Dilution
ml	ml	concentration	ml
1.0 - 10.0	0.10	1:10 dilution of stock	2.0
10.1 - 20.0	0.20	1:10 dilution of stock	2.0
20.1 - 30.0	0.50	1:10 dilution of stock	2.0
30.1 - 50.0	0.10	stock solution	2.0

above equation. After determining the concentration of acetic, propionic, and butyric acids present in the injected aliquot, the concentration value for acetic, propionic, and butyric acids was multiplied by 5355, 4631, and 4444, respectively. These factors converted the quantity of acetic, propionic and butyric acids present in the injected aliquot to mg of acetic, propionic, and butyric acids/100 g of egg. These conversion factors were derived as follows:

	Column a	Column b	Column c	Column d
Acetic	$\left[\frac{2000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{90.4}\right]$	$\left[\frac{100}{200}\right]$
Propionic	$\left[\frac{2000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{96.8}\right]$	$\left[\frac{100}{200}\right]$
Butyric	$\left[\frac{2000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{100}\right]$	$\left[\frac{100}{200}\right]$

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

Column b is a factor which corrects for the fact that only 450 ml (g) of the original 1000 g of egg-water-phosphotungstic acid-sulfuric acid mixture was used.

Column c is a factor which accounts for the quantity of acetic, propionic, and butyric acids recovered by steam distillation.

Column d is a factor which converts mg acid in 200 g egg to mg acid in 100 g egg.

K. Preparation of Egg Samples for AOAC Analysis

Triplicate samples of the lots of eggs analyzed by GLC were analyzed for their acetic, propionic, and butyric acid content using AOAC (1960) procedures 16.035-16.036 and 18.019-18.021. The C_2-C_4 acid content of the eggs was quantitated by titrating the acid with sodium hydroxide following the elution of each acid from a silicic acid column.

Part III. Formic and Acetic Acids

A. Preparation of Standard Acid Solutions

Standard N/10 solutions of formic and acetic acids were prepared as outlined in Part II-A.

B. Preparation of the Column

The columns were prepared and packed as outlined in Part I-B.

C. Operating Parameters for the Gas-Liquid Chromatograph

Column	72" X 1/4" copper tubing
Carrier gas	Helium
Pressure	40 psi
Flow rates	A column - 76 ml/min
	B column - 78 ml/min

Hydrogen	20 psi	
Air	640 ml/min @ 35 psi	
Temperatures	Detectors	250 C
	Injection port	250 C
	Column	60 C
Chart speed	30 in/hr	
Sample size	1 µl	
Range	10	
Attenuation	4-32	

D. Calibration of the Gas-Liquid Chromatograph

Approximately one-half g each of butyl formate and butyl acetate (K and K Labs) were accurately weighed into the same 10 ml volumetric flask, diluted to the mark with acetone, and designated as the stock solution.

Two dilutions, 1:10 and 1:50, of the stock solution were prepared and designated as "dilute stock solution A" and "dilute stock solution B", respectively. Various volumes of the diluted stock solutions of the standard butyl esters were injected into the chromatograph to provide a range of concentration for each butyl ester at a given sensitivity. Triplicate injections at each sensitivity were made and the peak heights corresponding to each concentration at a given sensitivity were measured. The peak heights and concentrations were used in the calculation of the acid concentrations in the unknowns.

E. Determination of Butyl Esterification Efficiency for Formic and Acetic Acids

Ten ml aliquots of accurately standardized N/20 formic and acetic acid solutions were pipetted into a 50 ml 19/38 standard taper round bottom flask. The water was evaporated on a steam bath under a stream of dry air. The acids were esterified by the procedure outlined in Part I-F.

After esterification, the contents of the flask were quantitatively transferred to a five ml volumetric flask and diluted to the mark with acetone. One µl aliquots were injected into the chromatograph and the formic and acetic acids were recovered as butyl formate and butyl acetate.

Using the peak heights and the concentration at a given sensitivity of the standards chromatographed as described in Part III-D, the following formula was applied to calculate the concentration of the butyl formate and butyl acetate recovered:

$$\text{Concentration of unknown butyl ester} = \frac{(\text{Peak height of unknown butyl ester}) \times (\text{Concentration of standard butyl ester})}{\text{Peak height of standard butyl ester}}$$

F. Preparation of Frozen Whole Egg Samples

Whole egg samples containing known concentrations of formic and acetic acids were prepared according to the procedures outlined in Part I-G.

The concentrations in mg/100 g of egg of both formic and acetic acids were as follows:

Sample No.	Formic (mg/100 g egg)	Acetic (mg/100 g egg)
1	1.25	1.15
2	2.47	3.30
3	4.94	6.60
4	9.88	13.20
5	24.76	33.32

G. Preparation of Egg Samples for GLC Analysis

1. Acid Extraction.--The formic and acetic acids were extracted from the frozen whole eggs as outlined in Part II-I.
2. Determination.--The steam distillate was reduced to approximately 10 ml in volume on a hot plate and steam bath, and quantitatively transferred to a 50 ml round bottom flask. The distillate was evaporated to dryness on a steam bath under a stream of dry air.

   The dried sodium salts of formic and acetic acid were converted to their butyl esters using the procedures outlined in Part I-F, beginning with "One ml of 1-butanol. . . ."

   After butylation was complete, the sides and joint of the condenser were rinsed with one ml of acetone and the washings added to the contents

of the round bottom flask. The contents of the flask were quantitatively transferred through a microfunnel to a five ml volumetric flask. The round bottom flask was rinsed five times with one-half ml quantities of acetone, and the washings added to the contents of the volumetric flask. The contents of the flask were made to volume with acetone and one µl aliquots were injected into the chromatograph.

H. Calculations

The peak heights of butyl formate and butyl acetate were measured from the chromatograms of the samples. Using the formula previously given in Part III-E, the mg concentration of butyl formate and butyl acetate recovered, was calculated.

After determining the mg concentration of butyl formate and butyl acetate present in the injected aliquot, the concentration value for butyl formate and butyl acetate was multiplied by 3205 and 3575, respectively. These factors convert the quantity of butyl formate and butyl acetate present in the injected aliquot to mg of formic and acetic acids/100 g of egg. The formulas for determining these conversion factors were derived as follows:

Column

	a	b	c	d	e	f
Formic	$\left[0.447\right]$	$\left[\frac{5000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{106}\right]$	$\left[\frac{100}{77.4}\right]$	$\left[\frac{100}{200}\right]$
Acetic	$\left[0.517\right]$	$\left[\frac{5000}{1}\right]$	$\left[\frac{1000}{450}\right]$	$\left[\frac{100}{95}\right]$	$\left[\frac{100}{90.4}\right]$	$\left[\frac{100}{200}\right]$

where: Column a is a factor which converts butyl formate and butyl acetate to formic and acetic acid.

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

Column c is a factor which corrects for the fact that only 450 ml (g) of the original 1000 g of egg-water-phosphotungstic-sulfuric acid mixture was used.

Column d is a factor which accounts for the 106 and 95 percent efficiency of butylation for formic and acetic acids, respectively.

Column e is a factor which accounts for the 77.4 and 90.4 percent acid recovery from steam distillation for formic and acetic acids, respectively.

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

I. Preparation of Egg Samples for AOAC Analysis

Triplicate formic and acetic acid samples from the same group of eggs analyzed by GLC were analyzed according to AOAC (1960) procedures 16.035-16.036 and 18.019-18.022, respectively. The formic acid content of the egg samples was quantitated gravimetrically by its reaction with mercuric chloride. The method of acetic acid quantitation was previously explained in Part II-K.

Part IV. Determination of Formic, Acetic Propionic, Butyric, Lactic, and Succinic Acids in Decomposed Liquid Whole Eggs by GLC

A. Preparation of Decomposed Frozen Whole Eggs for Analysis

Fifteen dozen Grade A large eggs were purchased from the Michigan State University Food Stores Department. The eggs were broken, and the liquid contents placed in a 1.5 gallon stainless steel Waring blendor jar (previously sanitized) and blended for 15 sec at low speed. The blended eggs were transferred to a 30 gallon stainless steel, multiple paddle mixer (previously sanitized) and mixed for an additional 15 min.

The top of the mixer was covered with aluminum foil and the eggs were allowed to decompose by natural microbial contamination for a 56 hr period at 27 ± 2 C. After further mixing for 10 min, 20 sub-samples of 225 g

each were placed in one pint plastic containers with snap-on lids and immediately frozen and held at -10 C until analyzed.

B. Microbiological Examination

Prior to sampling and freezing, an 11 ml aliquot of the decomposed whole eggs was removed by pipette and placed in 99 ml of sterile saline. Serial dilutions were made and plated on tryptone glucose extract (TGE) agar, brilliant green agar, eosin methylene blue (EMB) agar, and S-110 agar. The total count was determined from the TGE agar following a 48 hr incubation at 32 C. The salmonella and coliform counts were determined from the brilliant green and EMB agars following a 48 hr incubation at 37 C. The staphlococcal contamination was determined from the S-110 agar following a 48 hr incubation at 37 C.

C. Preparation of the Egg Samples for GLC Analysis

1. Formic and Acetic Acids.--Formic and acetic acids were extracted from the egg samples and quantitatively determined by GLC using the procedures outlined in Part III.
2. Acetic, Propionic, and Butyric Acids.--These acids were extracted from the egg samples and quantitatively determined by GLC using the procedures outlined in Part II.

3. Lactic and Succinic Acids.--These acids were extracted from the egg samples and quantitatively determined by GLC using the procedures outlined in Part I.

RESULTS AND DISCUSSION

Columns

Four different types of liquid phases of various percentages were evaluated in the course of this study. The liquid phases used were Carbowax 1540 at 10, 15, and 25 percent levels; Carbowax 20 M at 10, 15, and 20 percent levels; Apiezon L at 5, 7.5, and 10 percent levels; and ethylene glycol adipate (EGA) at 15 and 20 percent levels. Commercially prepared standards of the butyl esters and acids were chromatographed on these columns under various temperature conditions and carrier gas flow rates and the columns were evaluated for their effect on peak symmetry and resolution of the standard esters and acids. The two Carbowaxes gave good separation of the C_2-C_4 acids, however, the peaks of the acids and the butyl esters on these columns and the Apiezon L columns were not symmetrical. Poor resolution between the solvent and butyl lactate and the solvent and butyl formate occurred on the Apiezon L columns. Except for differences in retention times, necessitating changes in the temperature conditions of the experimental runs, the ethylene glycol adipate at either the 15 or 20 percent level was quite satisfactory

in regard to peak symmetry and resolution for the C_2-C_4 acids and butyl lactate and dibutyl succinate. At the 20 percent level, however, slightly greater resolving power between the butyl acetate and butanol were observed, hence, the 20 percent EGA column was adopted for use in these studies.

Esterification

Since neither lactic nor succinic acids are volatile under ambient conditions an initial attempt was made to prepare and chromatograph the methyl esters of these acids in order to determine if the methyl esters would provide a suitable method for quantitation of the lactic and succinic acids present in frozen whole eggs. Initially, standard solutions of lactic and succinic acids were prepared, and aliquots of these solutions were dried and refluxed with methanol using the same methodology as previously described under Part I-F. The methyl esters were chromatographed, along with an internal standard, to determine the esterification efficiency. The results obtained showed highly inconsistent esterification and/or losses in transferring the esters to the chromatograph (as high as 20-30 percent variation) in the 10 replicates tested. These results using methyl esters are similar to those reported by Jensen, et al., (1967), Oette and Ahrens (1961) and Vorbeck, et al., (1961). When chromatographing

these methyl esters, it was also noticed that there were nearly similar retention times for the excess methanol peak and the methyl lactate peak. Further, when the oven temperature of the chromatograph was reduced in order to achieve a greater separation between the methanol and the methyl lactate, the time required for the emergence of the dimethyl succinate peak was greatly increased, and the peak itself was unsymmetrical and not sharply defined.

Butyl ester derivatives of lactic and succinic acids for determining these acids in eggs was attempted to increase the esterification yields and/or reduce the loss of the esters during transfer to the GLC unit. The percentage yields and precision of both butyl lactate and dibutyl succinate, as shown in Tables 2 and 3, indicated that butyl derivatives were suitable for quantitation of the lactic and succinic acids in eggs.

Higher molecular weight esters were not considered in this study since these lead to the development of polymeric products and side reactions (Shelley, et al., 1963).

The quantitative determination of formic acid, per se, present in eggs could not be accomplished using the chromatograph available since formic acid readily decomposes to CO_2 and water on contact with metal. In an attempt to overcome this problem, an all-glass column and injection system was installed in the chromatograph. However, no response for formic acid at any sensitivity could

Table 2. Determination of esterification efficiency of lactic acid.

Sample	Lactic Acid (mg)	Equivalent as Butyl Lactate (mg)	Recovery from GLC (mg)	Recovery (%)
1	47.88	77.71	65.00	83.6
2	47.88	77.71	64.25	82.6
3	47.88	77.71	65.25	83.9
4	47.88	77.71	65.25	83.9
5	47.88	77.71	65.00	83.6
6	47.88	77.71	64.25	82.6
7	47.88	77.71	66.25	85.2
8	47.88	77.71	67.75	87.1
9	47.88	77.71	69.00	88.7
10	47.88	77.71	67.75	87.1
			Average	84.8

Range: 82.6 - 88.7 percent

Table 3. Determination of esterification efficiency of succinic acid.

Sample	Succinic Acid (mg)	Equivalent as Dibutyl Succinate (mg)	Recovery from GLC (mg)	Recovery (%)
1	69.20	134.94	114.25	84.6
2	69.20	134.94	112.50	83.3
3	69.20	134.94	114.25	84.6
4	69.20	134.94	110.00	81.5
5	69.20	134.94	108.00	80.0
6	69.20	134.94	111.00	82.2
7	69.20	134.94	112.00	83.0
8	69.20	134.94	108.00	80.0
9	69.20	134.94	114.25	84.6
10	69.20	134.94	115.50	85.6
			Average	82.9

Range: 80.0 - 85.6 percent

be detected. It was theorized that the formic acid decomposed on contact with the metal of the detectors after leaving the glass column and prior to reaching the flame. Since the use of butyl derivatives for lactic and succinic acids had proven successful, butyl derivatives of formic and acetic acids were prepared and chromatographed. The esterification yields, shown in Tables 4 and 5, indicated that formic and acetic acids could be quantitatively recovered. However, due to short retention times of butyl formate, butyl acetate, and the excess butanol, no suitable internal standard could be used without an overlapping of the peaks and incomplete resolution. Higher percentage liquid phases (up to 30 percent) and longer columns (up to 10 feet) did not sufficiently improve resolution to allow the use of an internal standard. Therefore, quantitation was achieved by injecting known concentrations of commercially available butyl formate and butyl acetate. The peak heights of the standards were compared with the peak heights of the unknowns, and the concentrations of the unknowns were calculated as previously described under Part III-E. The elution positions of butyl formate, butyl acetate, and butanol are shown in Figure 2. Butyl acetate was esterified with good precision (92-97 percent) as was butyl formate (105-107 percent), however, the reason for the 105 percent recovery of butyl formate could not be ascertained. All of the esterification efficiencies were

Table 4. Determination of esterification efficiency of formic acid.

Sample	Formic Acid (mg)	Equivalent as Butyl Formate (mg)	Recovery from GLC (mg)	Recovery (%)
1	20.8	46.6	49.3	106
2	20.8	46.6	49.6	106
3	20.8	46.6	49.1	105
4	20.8	46.6	49.1	105
5	20.8	46.6	49.1	105
6	20.8	46.6	49.2	106
7	20.8	46.6	48.8	105
8	20.8	46.6	49.7	107
9	20.8	46.6	50.0	107
10	20.8	46.6	49.1	105
			Average	106

Range: 105 - 107 percent

Table 5. Determination of esterification efficiency of acetic acid.

Sample	Acetic Acid (mg)	Equivalent as Butyl Acetate (mg)	Recovery from GLC (mg)	Recovery (%)
1	20.9	40.3	38.1	95
2	20.9	40.3	38.0	94
3	20.9	40.3	38.5	96
4	20.9	40.3	38.3	95
5	20.9	40.3	38.5	96
6	20.9	40.3	38.0	94
7	20.9	40.3	39.0	97
8	20.9	40.3	38.6	96
9	20.9	40.3	38.3	95
10	20.9	40.3	38.2	95
			Average	95

Range: 94 - 97 percent

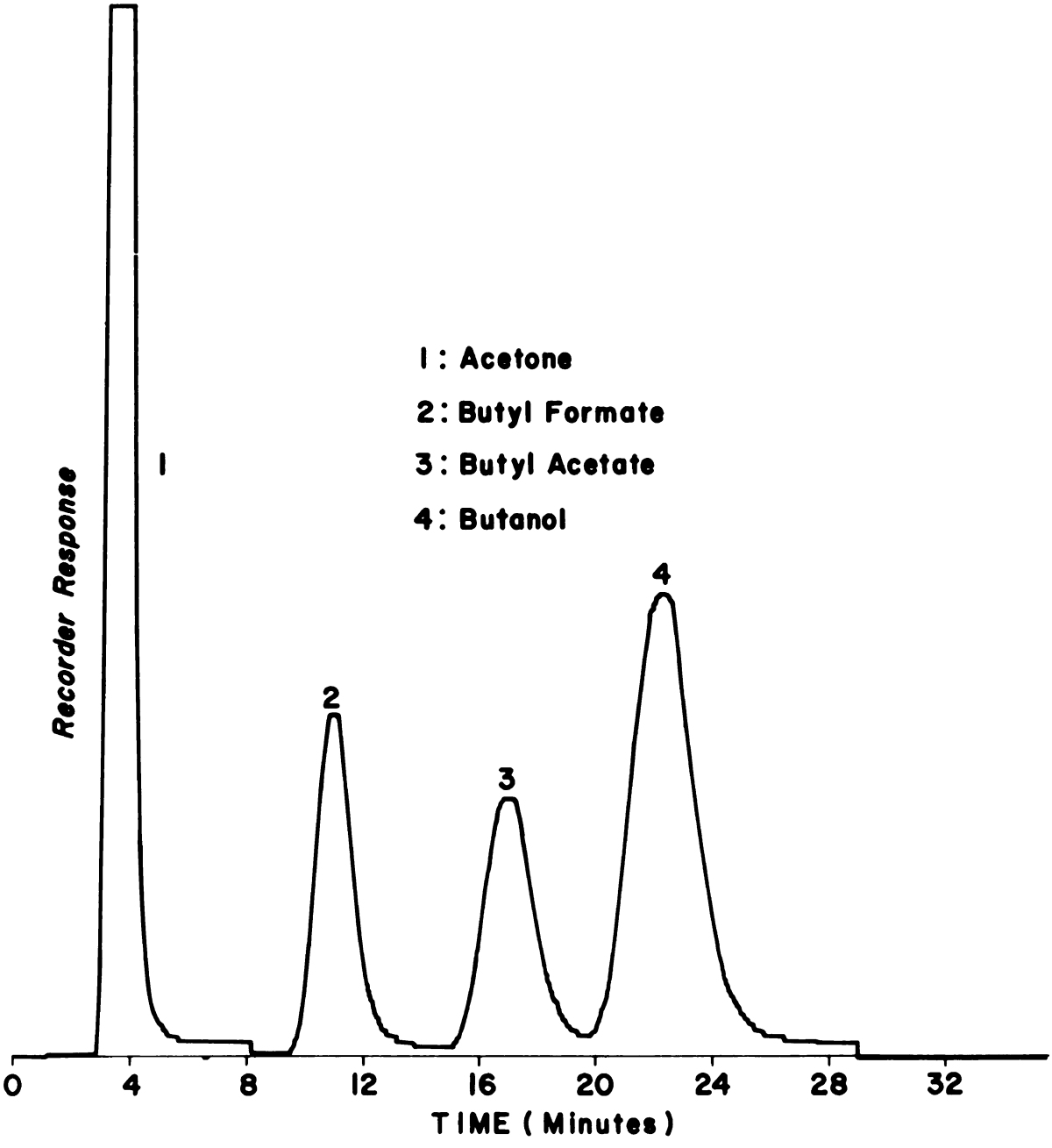

Fig. 2 - A gas chromatogram of butyl formate and butyl acetate showing their order of elution and retention times.

relatively precise (± three percent maximum) and the recoveries of butyl formate, butyl acetate, butyl lactate, and dibutyl succinate from the egg samples were within the error allowed.

Internal Standard

Since detector response varied with each compound injected, internal standards were used, when possible, in the quantitation procedures. The elution positions of the internal standards, butyl octanoate relative to the C_2-C_4 acids, and butyl decanoate relative to the butyl esters of lactic and succinic acids, are shown in Figures 3 and 4, respectively. In this laboratory, methyl enanthate as the internal standard for the C_2-C_4 acids as described by Shelley, et al., (1963) was unsuccessful since its retention time was the same as that of acetic acid. Butyl octanoate was selected because its retention time was greater, yet sufficiently close to the C_2-C_4 acids to permit rapid analysis.

The response values of the C_2-C_4 acids relative to butyl octanoate, and butyl lactate and dibutyl succinate relative to butyl decanoate, were calculated according to the procedure previously described by Shelley, et al., (1963). The R values over a wide range of acid and butyl ester concentrations relative to these internal standards are presented in Tables 6 and 7. Shelley, et al., (1963)

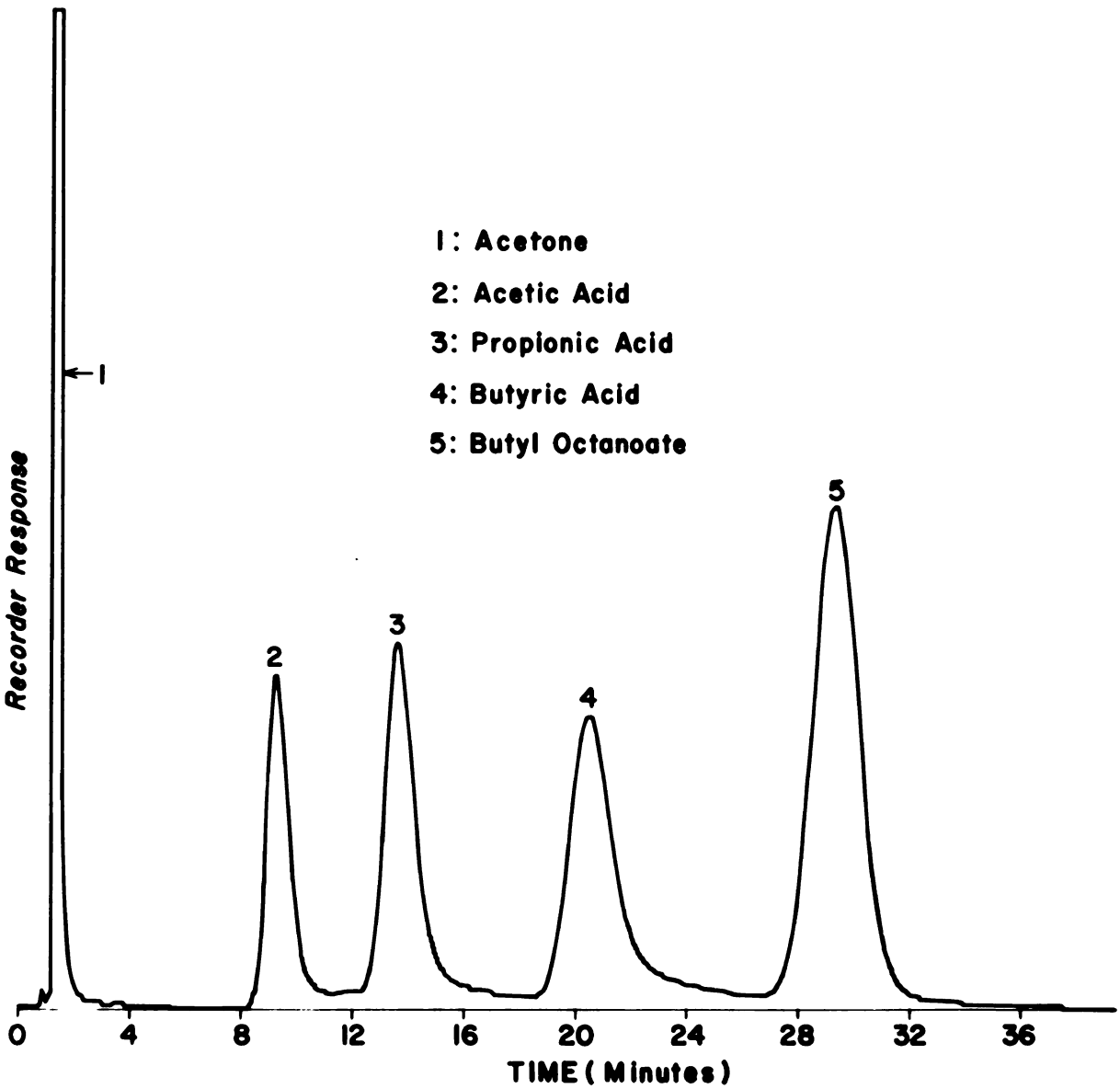

Fig. 3 - A gas chromatogram of acetic, propionic, and butyric acid and butyl octanoate showing their order of elution and retention times.

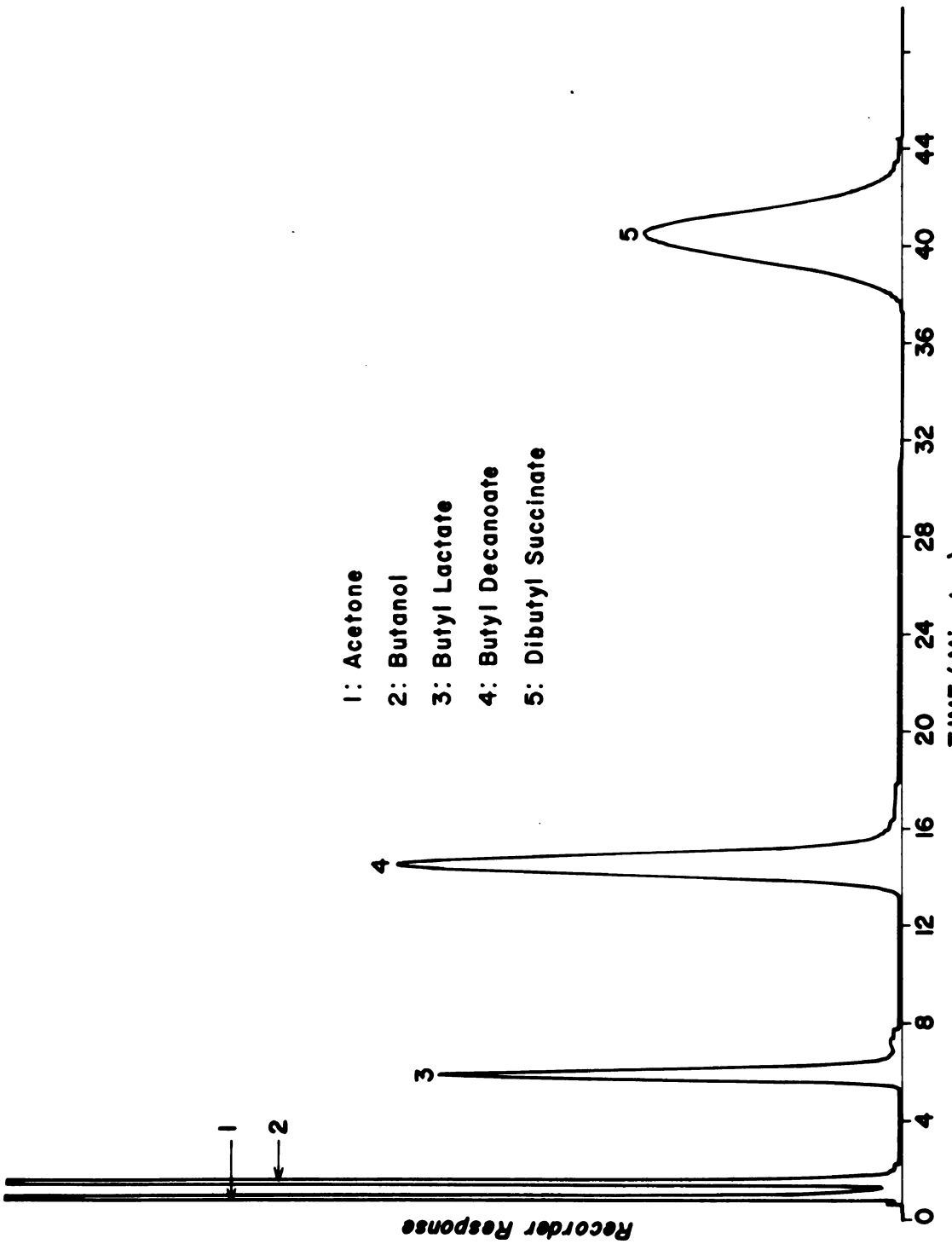

Fig. 4 - A gas chromatogram of butyl lactate, butyl decanoate, and dibutyl succinate showing their order of elution and retention times.

Table 6. Response values, R, of acetic, propionic and butyric acids relative to butyl octanoate.

Range of Acid Concn. in Sample (mg)	Chromatograph Range	Chromatograph Attenuation	R Value Low	R Value High	R Value Range	Ave. R Value
			Acetic Acid			
0.10 - 0.50	10	4	0.660	0.758	0.098	0.720
0.51 - 1.00	10	8	0.716	0.765	0.049	0.739
1.01 - 2.50	10	16	0.571	0.642	0.071	0.609
2.51 - 7.50	10	32	0.705	0.757	0.052	0.733
			Propionic Acid			
0.10 - 0.50	10	4	0.764	0.858	0.094	0.825
0.51 - 1.00	10	8	0.860	0.910	0.070	0.888
1.01 - 2.50	10	16	0.635	0.742	0.107	0.685
2.51 - 7.50	10	32	0.838	0.891	0.053	0.864
			Butyric Acid			
0.10 - 0.50	10	4	0.600	0.672	0.072	0.645
0.51 - 1.00	10	8	0.686	0.728	0.042	0.708
1.01 - 2.50	10	16	0.558	0.643	0.085	0.597
2.51 - 7.50	10	32	0.711	0.751	0.040	0.730

Table 7. Response values, R, of butyl lactate and dibutyl succinate relative to butyl decanoate.

Range of Ester Concn. in Sample (mg)	Chromatograph Range	Chromatograph Attenuation	R Value Low	R Value High	R Value Range	Ave. R Value
		Butyl Lactate				
0.34 - 1.50	10	4	1.441	1.519	0.078	1.448
1.51 - 3.50	10	8	1.424	1.465	0.040	1.442
3.51 - 7.50	10	16	1.275	1.314	0.039	1.322
7.51 -10.00	10	32	1.339	1.390	0.051	1.371
		Dibutyl Succinate				
0.50 - 2.50	10	4	0.301	0.315	0.014	0.310
2.51 - 5.00	10	8	0.303	0.316	0.012	0.308
5.01 - 7.50	10	16	0.309	0.330	0.021	0.323
7.51 -10.00	10	32	0.301	0.309	0.008	0.306

reported that variations in R values decreased as the concentration of the internal standards and the compounds under study increased. As shown in Tables 6 and 7, the R value variations generally decreased with increasing concentration of internal standards, acids, and butyl esters.

GLC Analysis of C_2-C_4 Acids

A modified procedure of Shelley, et al., (1963) was adopted for the quantitative analysis of the short-chain acids. These authors used an exact amount of dichloroacetic acid to acidify the sodium salts of the acids and to improve the stability of the final solution prior to GLC analysis. In this study, an excess of dichloroacetic acid was used to release the acids from their sodium component with no apparent loss of solution stability or changes in retention times. The excess dichloroacetic acid had the same retention time as acetone and consequently, did not interfere with the resolution of the acids.

The use of phosphoric acid on the columns was also omitted in this study since the material tended to bleed off the column during prolonged runs. This caused fouling of the detector and resulted in recorder noise and pen spiking.

Acid Extraction

According to AOAC (1960) procedures lactic and succinic acids are completely extracted by ether in a liquid-liquid extractor in three hr. In this study, a 24 hr extraction time was necessary for complete extraction (Table 8). It is possible that a larger extraction apparatus would enable a larger volume of ether to pass through the sample and may reduce the time required for complete extraction.

The steam distillation apparatus used in these studies was a modification of the apparatus used in standard AOAC (1960) determinations. The apparatus adopted gave increased yields of the C_1-C_4 acids compared to the AOAC (1960) procedure, as shown in Tables 9, 10, 11 and 12. The apparatus used in these studies (see Figure 1) gave recoveries averaging 77.4 percent for formic acid; 90.4 percent for acetic acid; 96.8 percent for propionic acid; and 100 percent for butyric acid.

The use of larger distillation flasks and steam generators as described under Experimental Procedures-General, improved the precision of the rate of distillation since the larger volumes were not affected by temperature and air movement fluctuations as much as the standard AOAC apparatus. The Vigreux fractionating column was inserted between the distillation flask and the condenser to prevent entrainment of the sulfuric acid and

Table 8. Extraction times and percent acid recovery for lactic and succinic acids using liquid-liquid extraction.

Replication	Extraction Time (hr)	ml NaOH Required Before Extraction	ml NaOH Required After Extraction	Percent Recovery
1	3	9.255	0.500	0.05
2	3	9.300	0.500	0.05
3	3	9.255	0.500	0.05
4	3	9.300	0.500	0.05
1	8	9.325	8.675	93.03
2	8	9.320	7.250	77.79
3	8	9.350	7.600	81.28
4	8	9.325	7.925	84.99
1	18	9.550	9.025	94.50
2	18	9.400	8.850	94.15
3	18	9.325	8.750	93.83
4	18	9.325	9.125	97.86
1	24	9.250	9.250	100.00
2	24	9.255	9.250	99.94
3	24	9.300	9.300	100.00
4	24	9.255	9.255	100.00

Table 9. Recovery of formic acid by steam distillation[1/].

Distillations	N/100 NaOH required/ aliquot after distillation (ml)	Recovery (%)
1	28.95	77.4
2	30.95	79.5
3	28.90	74.3
4	29.90	76.8
5	30.50	78.4
6	30.80	79.1
7	30.10	77.3
8	30.50	78.4
9	30.00	77.1
10	30.80	79.1
	Average	77.4

1/ Stock solution of formic acid required an average of 38.92 ml of N/100 NaOH per aliquot before distillation.

Table 10. Recovery of acetic acid by steam distillation[1/].

Distillations	N/100 NaOH required/ aliquot after distillation (ml)	Recovery (%)
1	40.85	90.0
2	40.60	89.4
3	40.70	90.0
4	41.40	91.0
5	40.80	89.9
6	41.80	92.0
7	41.80	92.0
8	41.90	92.2
9	40.00	88.1
10	40.60	89.4
	Average	90.4

1/ Stock solution of acetic acid required an average of 45.40 ml of N/100 NaOH per aliquot before distillation.

Table 11. Recovery of propionic acid by steam distillation[1/].

Distillations	N/100 NaOH required/ aliquot after distillation (ml)	Recovery (%)
1	36.00	94.0
2	37.50	97.4
3	36.00	94.0
4	36.50	95.3
5	37.20	97.1
6	38.00	99.2
7	37.10	96.9
8	37.50	97.9
9	38.00	99.2
10	37.10	96.9
	Average	96.8

1/ Stock solution of propionic acid required an average of 38.30 ml of N/100 NaOH per aliquot before distillation.

Table 12. Recovery of butyric acid by steam distillation[1/].

Distillations	N/100 NaOH required/ aliquot after distillation (ml)	Recovery (%)
1	36.30	100.1
2	36.25	100.0
3	36.25	100.0
4	36.20	99.9
5	36.25	100.0
6	36.60	101.0
7	36.20	99.9
8	36.20	99.9
9	36.25	100.0
10	36.25	100.0
	Average	100.0

1/ Stock solution of butyric acid required an average of 36.25 ml of N/100 NaOH per aliquot before distillation.

other compounds which would affect the quantitative neutralization of the distillate.

At the initiation of these studies an attempt was made to extract all the C_1-C_4 acids, and lactic and succinic acids using a liquid-liquid extraction procedure as suggested by Ramsey (1963). The acids were trapped in 25 ml of N/10 KOH solution in the side-arm flask of the extractor. Following evaporation to dryness, the potassium salts of the acids were esterified with butanol, made to volume, and chromatographed using temperature programming. Butyl formate, butyl acetate and dibutyl succinate were successfully recovered by this procedure, but the excess butanol, butyl propionate, butyl butyrate, and butyl lactate exhibited identical retention times.

Egg Samples

The initial quantity of eggs used for the GLC analysis was increased from 80 g (recommended in the AOAC, 1960 procedure) to 200 g to insure an accurate determination of the acids present and to provide a sufficiently high acid concentration for detection by the chromatographic unit. For this study an attempt was made to secure frozen whole eggs containing acetic, formic, lactic and succinic acids from the Michigan Department of Agriculture, Laboratory Division, for developing the GLC procedures. However, the quantity of eggs available that

contained all of the acids being studied was not sufficient to permit more than a single analysis for any one group of acids. For this reason, egg samples containing known amounts of the acids under study were prepared in the laboratory to insure a sufficient quantity of egg samples containing an accurately known concentration of acids for each experiment.

In the preparation of the egg samples for analysis of lactic and succinic acids, the quantity of succinic acid added was adjusted so the ratio of succinic acid to lactic acid was approximately 2:1 (mg/mg). This adjustment was made so complete peaks of both butyl lactate and dibutyl succinate could be obtained at a given sensitivity, thus eliminating duplicate runs and providing more accurate information for the evaluation of this procedure.

Volumetric flasks, graduated at the 8 and 10 ml volumes were used to determine the quantity of butyl decanoate necessary for quantitation of butyl lactate and dibutyl succinate present in the unknown sample. This provided a rapid and accurate means of establishing the necessary level of sensitivity of the chromatographic instrument so that the peak heights of the unknowns and the internal standard remained within the boundaries of the chart paper. The loss of a small portion of the esters on the first concentration seeking run did not materially affect the recoveries of the esters.

Recovery of Acids

Tables 13 and 14 show the percentage recoveries of lactic and succinic acids, respectively, using GLC and AOAC (1960) procedures. In nearly all the samples tested the standard deviation from the mean was greater using AOAC (1960) procedures as compared to the standard deviation using GLC procedures, indicating that GLC gave more precise measurements. The accuracy of the GLC method was also better as compared to the AOAC (1960) methods in the quantitative recovery of lactic and succinic acids. As was expected the accuracy and precision of both methods improved with increasing concentrations of acids.

A comparison of the recoveries of acetic, propionic, and butyric acids using GLC and AOAC (1960) procedures is presented in Table 15, and the results show that the standard deviation of the mean for both procedures was similar. This indicates that both procedures were about equal in precision. Again, the accuracy of both methods improved with increasing concentrations of acids. Here the main advantages of the GLC procedure were reduced analysis time and detection of lower quantities of the C_2-C_4 acids as compared to the standard AOAC (1960) procedure.

The recoveries of formic and acetic acids by GLC and AOAC (1960) procedures are presented in Table 16. Considering that no internal standard was employed, the

Table 13. Recovery of lactic acid from frozen whole eggs using GLC and AOAC (1960) procedures.

Sample	mg/100 g Egg (Added)	mg/100 g Egg (Recovered)		Average Percent Recovery	
		GLC 1/	AOAC 2/	GLC	AOAC
1	0.43	0.42±0.07	0.44±0.07	98	102
2	3.40	3.60±0.17	3.95±0.16	106	116
3	6.80	6.90±0.20	7.07±0.31	101	104
4	13.65	13.15±0.38	12.90±0.46	96	95
5	34.10	33.33±0.44	35.40±2.29	98	104
6	47.70	47.22±0.51	49.87±0.54	99	105

1/ Average of four replications ± standard deviation of the mean.

2/ Average of three replications ± standard deviation of the mean.

Table 14. Recovery of succinic acid from frozen whole eggs using GLC and AOAC (1960) procedures.

Sample	mg/100 g Egg (Added)	mg/100 g Egg (Recovered)		Average Percent Recovery	
		GLC 1/	AOAC 2/	GLC	AOAC
1	1.22	1.26±0.05	1.27±0.05	103	104
2	7.30	7.48±0.22	7.36±0.14	102	101
3	12.20	12.33±0.00	13.08±1.25	101	107
4	24.30	24.35±0.44	24.30±1.99	100	100
5	48.70	48.65±0.48	44.90±0.62	100	92
6	73.20	71.83±0.18	67.40±2.65	98	92

1/ Average of four replications ± standard deviation of the mean.

2/ Average of three replications ± standard deviation of the mean.

Table 15. Recovery of acetic, propionic, and butyric acids from frozen whole eggs using GLC and AOAC (1960) procedures.

Sample	mg/100 g Egg (Added)	mg/100 g Egg (Recovered)		Average Percent Recovery	
		GLC 1/	AOAC 2/	GLC	AOAC
		Acetic			
1	1.17	1.33±0.05	Trace	114	-
2	3.17	3.13±0.13	3.53±0.07	99	113
3	6.34	6.45±0.06	6.20±0.08	102	98
4	12.67	12.70±0.08	12.77±0.07	100	101
		Propionic			
1	1.38	1.38±0.03	Trace	100	-
2	3.72	3.55±0.38	3.73±0.12	98	100
3	7.44	7.43±0.03	7.43±0.21	100	100
4	14.87	14.80±0.14	14.87±0.16	101	100
		Butyric			
1	1.70	1.75±0.13	Trace	103	-
2	4.59	4.70±0.18	4.43±0.16	102	97
3	9.29	9.08±0.15	9.35±0.24	98	101
4	18.37	18.18±0.03	18.23±0.07	99	99

1/ Average of four replications ± standard deviation of the mean.

2/ Average of three replications ± standard deviation of the mean.

Table 16. Recovery of formic and acetic acids from frozen whole eggs using GLC and AOAC (1960) procedures.

Sample	mg/100 g Egg (Added)	mg/100 g Egg (Recovered)		Average Percent Recovery	
		GLC 1/	AOAC 2/	GLC	AOAC
		Formic			
1	1.25	1.30±0.07	1.33±0.05	104	106
2	2.47	2.68±0.07	2.48±0.06	109	100
3	4.94	5.01±0.04	4.91±0.05	101	99
4	9.88	9.72±0.07	9.93±0.01	98	101
5	24.76	23.68±0.11	23.64±0.07	96	95
		Acetic			
1	1.15	1.17±0.10	Trace	102	-
2	3.30	3.15±0.08	3.34±0.00	95	101
3	6.60	6.61±0.10	6.45±0.05	100	98
4	13.20	13.13±0.10	13.03±0.17	99	99
5	33.32	32.75±0.57	32.98±0.02	98	99

1/ Average of four replications ± standard deviation of the mean.

2/ Average of three replications ± standard deviation of the mean.

precision of the GLC method, as shown by the relatively small standard deviations of the means, compared favorably with the precision of the AOAC (1960) methods, and there were no significant differences in accuracy between the two methods.

The quantity of acids recovered from the decomposed liquid whole eggs by GLC is summarized in Table 17.

The viable counts obtained from the brilliant green and EMB agars show that the highest percentage of the organisms present were coliforms and Salmonella (Table 18). The high content of lactic acid and the presence of formic, acetic, and succinic acids indicate that the microorganisms present were heterofermentative (Doudoroff, 1942 and Thimann, 1955). The absence of butyric acid indicates that no members of the clostridia or bacillus genera fermenting glucose to butyric acid were present.

Table 17. Quantity of C_1-C_4 and lactic and succinic acids recovered from decomposed whole eggs.

Acid	mg/100 g Egg
Formic	17.8 ± 0.07
Acetic (recovered as acetic acid)	34.8 ± 0.00
Acetic (recovered as butyl acetate)	35.6 ± 0.01
Propionic	4.6 ± 0.05
Butyric	None detected
Lactic	214.9 ± 2.10
Succinic	29.8 ± 0.00

Table 18. Numbers of viable bacteria recovered from decomposed whole eggs.

Medium	Selected Bacteria	Number of Viable Bacteria (per g of Egg)
Tryptone Glucose Extract	Total	1.7×10^9
Brilliant Green	salmonella	5.1×10^8
Eosin Methylene Blue	coliforms	3.5×10^8
S-110	staphlococcus	1.0×10^5

SUMMARY

Using columns packed with 20 percent (weight percent) high temperature stabilized ethylene glycol adipate on 120-130 mesh solid support, the recoveries of formic, acetic, propionic, butyric, lactic, and succinic acids from whole egg samples were evaluated by gas-liquid chromatography procedures and compared with recoveries obtained by standard Association of Official Agriculture Chemists (AOAC, 1960) procedures.

Lactic and succinic acids were recovered from the whole egg samples, and chromatographed, as their butyl ester derivatives along with an internal standard. Acetic, propionic, and butyric acids were recovered from whole egg samples and chromatographed, as the acids, *per se* along with an internal standard. Formic and acetic acids were recovered from whole egg samples and chromatographed, as their butyl ester derivatives without an internal standard. Quantitation of butyl formate and butyl acetate was accomplished by comparing the chromatogram peak heights of the butyl esters recovered from the egg samples with the chromatogram peak heights of a known concentration of butyl ester standards. The GLC procedures offered, in

addition to reduced analysis time, the advantages of greater precision compared to standard AOAC methods, and an accuracy comparable to AOAC determinations.

The recovery of lactic acid from whole egg samples by GLC procedures ranged from 93 percent at the low concentration (0.43 mg/100 g egg) to 99 percent at the high concentration (47.70 mg/100 g egg). The recovery of succinic acid from whole egg samples by GLC procedures ranged from 103 percent at the low concentration (0.43 mg/100 g egg) to 99 percent at the high concentration (47.70 mg/100 g egg). The recovery of succinic acid from whole egg samples by GLC procedures ranged from 103 percent at the low concentration (1.22 mg/100 g egg) to 98 percent at the high concentration (73.20 mg/100 g egg).

The recovery of acetic, propionic, and butyric acids from whole egg samples by GLC procedures were, respectively, 114 percent at the low concentration (1.17 mg/100 g egg) to 100 percent at the high concentration (12.67 mg/100 g egg); 100 percent at the low concentration (1.33 mg/100 g egg) to 101 percent at the high concentration (14.87 mg/100 g egg); and 103 percent at the low concentration (1.70 mg/100 g egg) to 99 percent at the high concentration (18.37 mg/100 g egg).

The recovery of formic acid as its butyl ester from whole egg samples by GLC procedures ranged from 104 percent at the low concentration (1.25 mg/100 g egg) to

96 percent at the high concentration (24.76 mg/100 g egg). The recovery of acetic acid as its butyl ester from whole egg samples by GLC procedures ranged from 102 percent at the low concentration (1.15 mg/100 g egg) to 98 percent at the high concentration (33.32 mg/100 g egg).

The GLC evaluation of the organic acid content of liquid whole eggs allowed to decompose by natural microbial contamination revealed the development of 214.9 mg of lactic acid, 35.6 mg of acetic acid (recovered as acetic acid), 34.8 mg of acetic acid (recovered as butyl acetate), 29.8 mg of succinic acid, 17.8 mg of formic acid, and 4.6 mg of propionic acid/100 g of egg. No butyric acid was detected. The microbiological examination of these same eggs showed a total count of 1.7×10^9 bacteria, and the presence of 5.1×10^8 viable salmonella 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.

LITERATURE CITED

A.O.A.C. 1960. Official Methods of Analysis. 9th Ed., Assoc. Off. Agr. Chemists., Washington, D.C.

Barker, S. B. and W. H. Summerson 1941. The colorimetric determination of lactic acid in biological materials. J. Biol. Chem., 138, 535-554.

Bayer, E. 1961. Gas Chromatography. Elsevier Publishing Co., New York and London.

Blackwood, A. C., A. C. Neish and G. A. Ledingham 1956. Dissimilation of glucose at controlled pH values by pigmented and nonpigmented strains of Escherichia coli. J. Bact. 72, 497-499.

Bohemen, J., S. H. Langer, R. H. Perrett and J. H. Purnell 1960. A study of the adsorptive properties of firebrick in relation to its use as a solid support in gas-liquid chromatography. J. Chem. Soc., 2444-2451.

Braak, H. R. 1928. Onderzueking over vergisting von Glycerine. Dissertation, Delft.

Brunschwig, H. 1512. Liberde arte distillandi. Quoted in Bayer, E. 1961. Gas Chromatography. Elsevier Publishing Co., New York and London.

Burchfield, H. P. and E. E. Storrs 1962. Biochemical Applications of Gas Chromatography. Academic Press, New York and London.

Claborn, H. V. and W. I. Patterson 1948. The determination and identification of lactic and succinic acids in foods. J. Assoc. of Off. Agr. Chemists, 31, 134-139.

Craig, B. M., A. P. Tullock and N. L. Murty 1963. Quantitative analysis of short chain fatty acids using gas-liquid chromatography. J. Am. Oil Chemists' Soc., 40, 61-63.

Crawford, I. P. 1954. A new fermentative pseudomonad, Pseudomonas formicans, n. sp. J. Bact., 68, 734-738.

Decora, A. W. and G. V. Dinneen 1960. Gas-liquid chromatography of pyridines using a new solid support. Anal. Chem., 32, 164-169.

DeMoss, R. D., R. C. Bard and I. C. Gunsalus 1951. The mechanism of the heterolactic fermentation: a new route of ethanol formation. J. Bact., 62, 499-511.

Doudoroff, M. 1942. Studies on the luminous bacteria. II. Some observations on facultatively anaerobic species. J. Bact., 44, 461-467.

Dyer, D. C. 1917. A new method of steam distillation for the determination of the volatile fatty acids, including a series of colorimetric qualitative reactions for their identification. J. Biol. Chem., 28, 445-472.

Fincke, H. 1913. Nachweis und bestimmung der ameisen saure. Biochem. Z., 51, 253-287.

Foster, L. F. 1921. The biochemistry of Streptococcus hemolyticus. J. Bact., 6, 211-237.

Friedemann, T. E. and J. B. Graeser 1933. The determination of lactic acid. J. Biol. Chem., 100, 291-308.

Friedemann, T. E. 1939. The carbohydrate metabolism of streptococci. J. Biol. Chem., 130, 757-761.

Fuchs, P. 1929. Mabanalytische schnell methode zur bestimmung von ameisensaure und essignsaure nebencinander. Z. anal. Chem., 78, 125-127.

Gallagher, F. H. and R. W. Stone 1939. Dissimilation of glucose by members of the genus bacillus. J. Bact., 38, 235.

Gehrke, C. W. and D. F. Goerlitz 1963. Quantiative preparation of methyl esters of fatty acids for gas chromatography. Anal. Chem., 35, 76-80.

Gehrke, C. W. and W. M. Lamkin 1961. Quantitative determination of steam-volatile fatty acids by gas-liquid chromatography. J. Agr. Food Chem., 9, 85-88.

Gibbons, N. E., R. L. Moore and C. O. Fulton 1944. Dried whole egg powder. XV. The growth of Salmonella and other organisms in liquid and reconstituted egg. Can. J. Research, 22, 169-173.

Gibbs, M., R. Dumrose, F. A. Bennett and M. R. Bubeck 1950. On the mechanism of bacterial fermentation of glucose to lactic acid studied with C^{14}-glucose. J. Biol. Chem., 184, 545-549.

Grant, W. M. 1948. Colorimetric microdetermination of formic acid based on reduction to formaldehyde. Anal. Chem., 20, 267-269.

Grey, T. C. and B. J. Stevens 1966. Analysis of the lower free fatty acids from microbial fermentations. Anal. Chem., 38, 724-726.

Gunderson, M. F. and S. D. Gunderson 1945. Eggs can be washed clean. U.S. Egg Poultry Mag., 51, 533, 560-562.

Gunsalus, I. C. 1944. Butylene glycol production by the so-called Ford strain of Bacillus subtilis. J. Bact., 48, 261-262.

Gunsalus, I. C. and C. F. Niven, Jr. 1942. The effect of pH on the lactic acid fermentation. J. Biol. Chem., 145, 131-136.

Gunsalus, I. C. and W. W. Umbreit 1945. The oxidation of glycerol by Streptococcus faecalis. J. Bact., 49, 347-357.

Haines, R. B. 1939. Microbiology in the preservation of the hen's egg. Dept. Sci. Ind. Research, Food Invest. Spec. Rpt. No. 37, Low Temp. Research Sta., Cambridge, England.

Hammer, B. W. 1920. Volatile acid production of S. lacticus and the organisms associated with it in starters. Iowa State Coll. Agr. Expt. Sta. Bull. No. 63.

Hanak, A. and K. Kurschner 1930. Neues bestimmungsverfahren der ameisensaure in fruchtsaften. Z. Untersuch. Lebensm., 60, 278-290.

Hankinson, C. L., W. J. Harper and E. M. Mikolajeik 1958. A gas-liquid chromatographic method for volatile fatty acids in milk. J. Dairy Sci., 41, 1502-1509.

Hardy, C. J. and F. H. Pollard 1960. Review of Gas-Liquid Chromatography. Chromatographic Reviews, Vol. 2, Elsevier Publishing Co., New York and London.

Harley, J., W. Nel and V. Pretorius 1958. A new detector for vapour phase chromatography. Nature, 178, 1244-1246.

Hawke, J. C. 1957. The fatty acids of butterfat and the volatile acids formed on oxidation. J. Dairy Res., 24, 366-371.

Hillig, F. 1937a. Colorimetric determination of lactic acid in milk and milk products. J. Assoc. Off. Agr. Chemists, 20, 130-140.

Hillig, F. 1937b. Colorimetric determination of lactic acid in tomato products. J. Assoc. Off. Agr. Chemists, 20, 303-307.

Hillig, F. 1937c. Colorimetric determination of lactic acid in fruits and fruit products. J. Assoc. Off. Agr. Chemists, 20, 605-610.

Hillig, F. and L. Knudsen 1942. Determination of volatile fatty acids. J. Assoc. Off. Agr. Chemists, 25, 176-195.

Hillig, F., A. P. Dunnigan, W. Horwitz and L. L. Warden 1960. Authentic packs of edible and inedible frozen eggs and their organoleptic, bacteriological, and chemical examination. J. Assoc. Off. Agr. Chemists, 43, 108-113.

Horning, E. C., E. A. Moscatelli and C. C. Sweeley 1959. Polyester liquid phases in gas-liquid chromatography. Chem. and Ind. (London), 751-752.

Horrocks, L. A., D. G. Cornwell and J. B. Brown 1961. Quantitative gas-liquid chromatography of fatty acid methyl esters with the thermal conductivity detector. J. Lipid Res., 2, 92-94.

Isherwood, F. A. and C. S. Hanes 1953. Separation and estimation of organic acids on paper chromatograms. Biochem. J., 55, 824-830.

James, A. T. and A. J. P. Martin 1952. Gas-liquid chromatography: the separation and microestimation of volatile fatty acids from formic acid to dodecanoic acid. Biochem. J., 50, 679-690.

James, A. T. and A. J. P. Martin 1956. Gas-liquid chromatography: the separation and identification of the methyl esters of saturated and unsaturated acids from formic acid to N-octadecanoic acid. Biochem. J., 63, 144-152.

Janak, J. 1954. Collection Czech Chem. Commons., 19, 684. In Chromatographic Reviews, Vol. 2, 1960. Elsevier Publishing Co., London and New York.

Jensen, R. G., J. G. Quinn, D. L. Carpenter and J. Sampugna 1967. Gas-liquid chromatographic analysis of milk fatty acids; a review. J. Dairy Sci., 50, 119-126.

Johns, C. K. 1944. Microbiological aspects of egg powder. Sci. Agr., 24, 373-382.

Johns, C. K. and H. L. Berard 1945. Further bacteriological studies related to egg drying. Sci. Agr., 25, 551-565.

Johns, T. 1958. The behavior of the solid support in gas-liquid partition chromatography. Gas Chromatography, Academic Press, Inc., New York.

Kennedy, E. P. and H. A. Barker 1951. Paper chromatography of volatile acids. Anal. Chem., 23, 1033-1034.

Knaysi, G. and I. C. Gunsalus 1944. A study of the so-called Marburg and the Lawrence and Ford strains of Bacillus subtilis. J. Bact., 47, 381-389.

Langwill, B. 1924. The character of acids produced by hemolytic and non-hemolytic streptococci from pathogenic sources and from milk. J. Bact., 9, 79-94.

Ledingham, G. A. and A. C. Neish 1954. Industrial Fermentations., Vol. 2, Chem. Publ. Co. New York Edited by L. A. Underkofler and R. J. Hickey.

Lepper, H. A., M. T. Bartram and F. Hillig 1944. Detection of decomposition in liquid, frozen and dried eggs. J. Assoc. Off. Agr. Chemists, 27, 204-223.

Lepper, H. A. and F. Hillig 1948. Succinic acid in decomposed egg products. J. Assoc. Off. Agr. Chemists, 31, 734-739.

Long, H. F. and B. W. Hammer 1936. Classification of the organisms important in dairy products. I. Streptococcus liquefaciens. Iowa State Coll. Agr. Expt. Sta. Bull. No. 206.

Lovelock, J. E. 1958. A sensitive detector for gas chromatography. J. Chromotog., 1, 35-46.

Magasanik, B. and H. E. Umbarger 1950. The separation and identification of keto acids by filter paper chromatography. J. Am. Chem. Soc., 72, 2308-2309.

Martin, A. J. P. and R. L. M. Synge 1941. A new form of chromatogram employing two liquid phases. I. A theory of chromatography. II. Application to the microdetermination of the higher monoaminoacids in proteins. Biochem. J., 35, 1358-1369.

Martin, A. J. P. and A. T. James 1956. Gas-liquid chromatography: The gas-density meter, a new apparatus for the detection of vapours in flowing gas streams. Biochem. J., 63, 138-143.

McFarlane, V. H., A. J. Watson and H. E. Goresline 1945. Microbiological control in the production of spray-dried whole egg powder. U.S. Egg Poultry Mag., 51, 250-257, 270-273, 275-277, 279-286, 364-366, 368.

McFarlane, V. H., A. J. Watson, R. R. Sutton and W. Kurman 1947. Microbiology of spray-dried whole egg. I. Plate and direct counts. Food Res., 12, 311-327.

Mickelson, M. and C. H. Werkman 1938. Effect of aldehydes and fatty acids as added hydrogen acceptors on the fermentation of glucose by Aerobacter indologenes. J. Bact., 37, 619-628.

Minter, G. C. and L. M. J. Burdy 1951. Thermal conductivity bridge for gas analysis. Anal. Chem., 23, 143-147.

Nelson, M. E. and C. H. Werkman 1935. Dissimilation of glucose by heterofermentative lactic acid bacteria. J. Bact., 30, 547-557.

Ober, S. S. 1958. The interrelationship of column efficiency and resolving power in gas chromatography. In "Gas Chromatography" (V. J. Coates, H. J. Noebels, and I. S. Fagerson, eds.) New York, Academic Press. 41-50.

Oette, K. and E. H. Ahrens, Jr. 1961. Quantitative gas-liquid chromatography of short-chain fatty acids as 2-chloroethanol esters. Anal. Chem., 33, 1847-1851.

Ormerod, E. C. and R. P. W. Scott 1959. Gas chromatography of polar solutions with a non-polar liquid base. J. Chromatog., 2, 65-68.

Osburn, O. L., H. G. Wood and C. H. Werkman 1936. Determination of volatile fatty acids by the partition method. Ind. Eng. Chem., Anal. Ed., 8, 270-275.

Pederson, C. S. and R. S. Breed 1928. The fermentation of glucose by organisms of the genus Serratia. J. Bact., 16, 163-185.

Penniston, V. and L. R. Hedrick 1947. The reduction of bacterial count in egg pulp by use of germicides in washing dirty eggs. Food Tech., 1, 240-244.

Platt, T. B. and E. M. Foster 1958. Products of glucose metabolism by homofermentative streptococci under anaerobic conditions. J. Bact., 75, 453-459.

Prescott, S. C. and C. G. Dunn 1940. Industrial Microbiology, McGraw-Hill, New York.

Pucher, G. W. and H. B. Vickery 1941. Determination of succinic acid in plant tissue. Ind. Eng. Chem., Anal. Ed., 13, 412-415.

Puziss, M. and S. C. Rittenberg 1957. Studies on the anaerobic metabolism of Bacillus anthracis and Bacillus cereus. J. Bact., 73, 48-51.

Ramsey, H. A. 1963. Separation of organic acids in blood by partition chromatography. J. Dairy Sci., 46, 480-483.

Ramsey, L. L. and W. I. Patterson 1945. Separation and identification of the volatile saturated fatty acids (C_1-C_4). J. Assoc. Off. Agr. Chemists, 28, 644-656.

Reid, R. L. and M. Lederer 1952. Separation and estimation of saturated C_2-C_7 fatty acids by paper partition chromatography. J. Biol. Chem., 50, 60-67.

Redfield, H. W. 1920. Examination of frozen eggs and interpretation of results. USDA Bull. 846.

Rosser, F. T. 1942. Preservation of eggs. II. Surface contamination of egg shell in relation to spoilage. Can. J. Research D, 20, 291-296.

Schneiter, R., M. T. Burtram and H. L. Lepper 1943. Bacteriological and physical changes occurring in frozen eggs. J. Assoc. Off. Agr. Chemists, 26, 172-182.

Scott, R. P. W. 1957. In Vapour Phase Chromatography, Butterworths Science Publication, London.

Shelley, R. N., H. Salwin and W. Horwitz 1963. Quantitative determination of formic, acetic, propionic, and butyric acids by gas chromatography. J. Assoc. Off. Agr. Chemists, 46, 486-493.

Solowey, M., E. H. Spaulding and H. E. Goresline 1946. An investigation of a source and mode of entry of Salmonella organisms in spray-dried whole egg powder. U.S. Egg Poultry Mag., 49, 28-31, 45-47.

Stanier, R. Y. 1947. Acetic acid production from ethanol by fluorescent pseudomonads. J. Bact., 54, 191-194.

Stark, J. B., A. E. Goodban and H. S. Owens 1951. Paper chromatography of organic acids. Anal. Chem., 23, 413-415.

Steinhauer, J. E., L. E. Dawson, W. L. Mallmann and R. J. Wilkinson 1967. Microbial counts and certain organic acids in liquid and frozen whole eggs. Food Tech., 21, 161-162.

Stokes, J. L. 1949. Fermentation of glucose by suspensions of Escherichia coli. J. Bact., 57, 147-158.

Stokes, J. L. 1956. Enzymatic aspects of gas formation by Salmonella. J. Bact., 72, 269-275.

Stuart, L. S. and E. H. McNally 1943. Bacteriological studies on egg shell. U.S. Egg Poultry Mag., 49, 28-31, 45-47.

Szymanski, H. A. 1964. Biomedical Applications of Gas Chromatography. Plenum Press, New York.

Thimann, K. V. 1955. The Life of the Bacteria. The MacMillan Co., New York.

Vorbeck, M. L., L. R. Mattick, F. A. Lee and C. S. Pederson 1961. Preparation of methyl esters of fatty acids for gas-liquid chromatography. Anal. Chem., 33, 1512-1514.

Werkman, C. H. 1930. Determination of organic acids in mixtures. Ind. Eng. Chem., Anal. Ed., 2, 302-304.

White, A. G. C., R. H. Steele and W. A. Pierce, Jr. 1955. The effect of pH on the fermentation of glucose and galactose by Streptococcus pyogenes. J. Bact., 70, 82-86.

Winter, A. R., G. F. Stewart, V. H. McFarlane and M. Solowey 1946. Pasteurization of liquid egg products. III. Destruction of Salmonella in liquid whole egg. Am. J. Pub. Health, 36, 451-460.

Winter, A. R. and M. Wilkin 1947. Holding and storage of egg products to control bacteria. American Egg and Poultry Review, 8, 74.

Winter, A. R. and C. Wrinkle 1949. The control of bacteria in frozen egg products. U.S. Egg Poultry Mag., 55, No. 1, 7-9, 18-19; No. 2, 20-23, 30-32; No. 3, 28-31, 44.

Wolfe, J. B., D. Ivler and S. C. Rittenberg 1954. Malonate decarboxylation by Pseudomonas fluorescens. II. Magnesium dependency and trapping of active intermediates. J. Biol. Chem., 209, 875-883.

Wood, W. A. 1961. Fermentation of carbohydrates and related compounds. In the Bacteria, Vol. II, Ed. by Gunsalus and Stanier, Academic Press, London.

Wrinkle, C., H. H. Weiser and A. R. Winter 1950. Bacterial flora of frozen egg products. Food Res., 15, 91-98.

Zagaevsky, J. S. and P. O. Lutikova 1944. Sanitary measures in the egg-breaking plant. U.S. Egg Poultry Mag., 51, 17-20, 43-46, 75-77, 88-90, 121-123.