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QUANTITATIVE GAS CHROMATOGRAPHIC DETERMINATION OF β-HYDROXYBUTYRIC ACID WITH APPLICATION TO EGGS*

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SUMMARY

A quantitative method for the analysis of β-hydroxybutyric acid has been developed through the gas chromatography of its propyl ester. The ester was formed by reaction with BF_3–propanol and an internal standard, acetophenone, was added to the reaction mixture. The propyl ester and internal standard were extracted with chloroform and chromatographed isothermally on a diethylene glycol succinate column. Inasmuch as β-hydroxybutyrate may enter into polymerization reactions, calibration procedures were developed for the simultaneous determination of lactic, succinic, and β-hydroxybutyric acids as well as for β-hydroxybutyric acid alone. A discussion of the problems which arise and of the techniques needed for reliable results is given. The retention time of propyl β-hydroxybutyrate differs from those of the propyl esters of the α- and γ-isomers and of the propyl esters of a number of other acids. When the method was applied to the analysis of eggs, recoveries were greater than 90% with good precision.

INTRODUCTION

The need for a reliable quantitative method for the analysis of β-hydroxybutyric acid exists in the fields of biochemistry and medicine. The available methods generally lack specificity and have been described as cumbersome and unsatisfactory[1].

Several papers have been published on the gas chromatographic (GLC) determination of β-hydroxybutyric acid as its methyl ester. However, none of them reported quantitative data on the recovery of the acid from natural materials[2–4].

Interest in developing a quantitative method arose from the identification in this laboratory of β-hydroxybutyric acid in incubator-reject eggs[5]. The acid was observed as a prominent component when such eggs were analyzed for lactic and succinic acids by a GLC procedure[6]. β-Hydroxybutyric acid may also have been responsible for one of the unidentified GLC peaks observed earlier by BETHEA AND WONG in incubator-reject eggs[7]. The GLC method for lactic and succinic acids[6] has

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now been modified to include β-hydroxybutyric acid. Acetophenone is used as an internal standard and the acids are chromatographed as their propyl esters under isothermal conditions. Inasmuch as β-hydroxybutyrate may enter into polymerization reactions, calibration procedures were developed for β-hydroxybutyric acid alone and also in combinations with lactic and succinic acids. The procedure for the analysis of eggs is described but it should be readily applicable to other foods and biological materials through the use of appropriate sample extraction procedures.

EXPERIMENTAL

Apparatus

Gas chromatograph. A Barber-Colman Series 5000 gas chromatograph with flame ionization detector was used for measurements. The operating temperatures were: detector bath, 200°; injector block, 200°; and column temperature, 115 to 150°, depending upon the liquid phase. Gas flow rates were adjusted for optimum detector response with the carrier gas (nitrogen or helium) set at 75 ml/min. An 8 ft. column containing 10 % diethylene glycol succinate was operated at 130° and an electrometer setting of 9×10^{-10} A full scale deflection on a 5 mV recorder. Up to 15 μg (equivalent to 25 mg/100 g sample) of β-hydroxybutyric acid was chromatographed according to the procedure, without altering the electrometer controls.

GLC columns. Various columns were used with the choice dependent on the purposes intended. For the routine analysis of acids in eggs, an 8 ft. by 4 mm I.D. glass U-column of 100–120 mesh Gas Chrom Z (Applied Science Laboratories, Inc.), coated with 10 % DEGS (diethylene glycol succinate, stabilized, Analabs, Inc.) and conditioned 24 h at 200°, has proven very reliable. The propyl lactate peak separates from the solvent front and there is baseline resolution of acetophenone and propyl β-hydroxybutyrate. A typical chromatogram is shown in Fig. 1. The previously described slurry method was used to prepare the packing[6]. Reoplex 400 (Supelco, Inc.), 15 % on 60–80 mesh Gas Chrom Z, was also used with equivalent results.

FFAP (Supelco, Inc.), 5–10 % on 100–110 mesh Anakrom ABS (Analabs, Inc.),

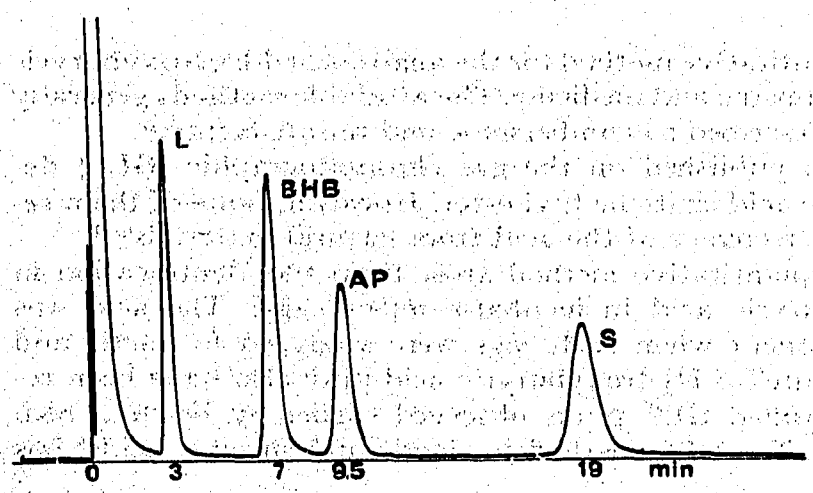


Fig. 1. Chromatogram of acids on DEGS column at 130°. Key: L = propyl lactate; BHB = propyl β-hydroxybutyrate; AP = acetophenone internal standard; S = dipropyl succinate.

was useful for special applications such as the high-temperature analyses described in this paper.

Reagents

Solvents. All were reagent grade quality. Anhydrous diethyl ether containing less than 0.05 % ethanol was used to avoid the formation of ethyl esters.

Calcium lactate standard. 0.171 g of N.F. grade calcium lactate pentahydrate was dissolved in distilled water and diluted to 100 ml (1 mg/ml as lactic acid).

Lactic acid standard. 0.100 g of crystalline L(+) lactic acid (Sigma Chemical Co.) was dissolved in anhydrous ether and diluted to 100 ml (1 mg/ml).

Succinic acid standard. 0.100 g of reagent grade succinic acid was dissolved in water and diluted to 100 ml (1 mg/ml). For an ether solution (1 mg/ml), 0.100 g of the acid was dissolved in anhydrous ether by gentle heating and shaking and diluted to 100 ml.

Sodium β-hydroxybutyrate standard. 0.121 g of the salt was dissolved in distilled water and diluted to 100 ml (1 mg/ml as β-hydroxybutyric acid).

Acetophenone standard. 0.800 g of acetophenone, 99 mole %, Chromatoquality Reagent (Matheson, Coleman and Bell), was dissolved in 1-propanol and diluted to 100 ml.

Diluting solution. 20.0 ml of 1-propanol, 10.0 ml of acetophenone standard solution and 20.0 ml of chloroform were pipetted into a 125 ml separatory funnel. 40 ml of a saturated ammonium sulfate solution was added and the funnel was stoppered and shaken for 1 min. The aqueous layer was discarded and the organic layer was dried over about 5 g of anhydrous sodium sulfate. The solution was made fresh daily.

Boron trifluoride–propanol reagent. A laboratory preparation of 10 % (w/w) boron trifluoride in 1-propanol[6] or the commercial 14 % (w/v) reagent (Applied Science Laboratories, Inc.) can be used. The latter can be used undiluted or diluted 1:0.6, v/v, with 1-propanol.

Calibration procedures

While the isolation of β-hydroxybutyric acid from eggs presents no special problem, precautions are required in preparing standard solutions of esters for calibrating the gas chromatographic column. The procedure varies, depending upon the kind and numbers of acids being esterified. The two calibration procedures described in this report were designed for application to the analysis of eggs containing β-hydroxybutyric, lactic and succinic acids. The procedures may require modification if additional acids are encountered in other applications.

Procedure A permits the simultaneous calibration for lactic, succinic and β-hydroxybutyric acids. In procedure B, the calibration for β-hydroxybutyric acid is performed separately.

Calibration procedure A. The number of calibration solutions used depends upon the precision required and the linearity of the system. See Table I for typical amounts of acids. Aliquots of the standard solution of sodium β-hydroxybutyrate were pipetted into 250 ml round-bottom ₹ 24/40 flasks and taken to dryness in a rotary evaporator at 50° and a pressure of 30 mm Hg or less. Aliquots of standard ether solutions of lactic and succinic acids were added to the flasks and the ether was removed in a

rotary evaporator at 30°. Two milliliters of the BF_3–propanol reagent was added to the dry residues and the flask was heated on a steam bath for 30 min with an air condenser attached. The flask was clamped on concentric rings so that only the lower half was immersed in steam. The flask was swirled after the reaction was initiated to insure contact between the solid residue and the reagent. When the esterification was completed, 4 ml of a saturated ammonium sulfate solution was added and the contents were cooled to room temperature. One milliliter of the acetophenone standard solution was pipetted into the flask, followed by 2 ml of chloroform. The flask was swirled to mix the contents and they were transferred to a 30 ml separatory funnel and shaken for 1 min. The lower layer (aqueous phase) was discarded and the organic phase was dried over 3 g of anhydrous sodium sulfate in a 4 dram screw-cap vial. A piece of aluminum foil was placed over the top of the vial before affixing the cap. When sampling the solution the syringe needle was inserted through the foil into the liquid. Duplicate injections of 3 μl each were made into the gas chromatograph and each peak height was measured to the nearest 0.5 mm. Peak height ratios (r) were calculated according to r = height of ester peak: height of acetophenone peak. For duplicate injections of the same solution of esters r values agreed within 5 % of their mean. A calibration graph was obtained by plotting r *versus* mg acid esterified (Fig. 2). The solutions of propyl esters should be stored at 4° if they are retained beyond the day of preparation.

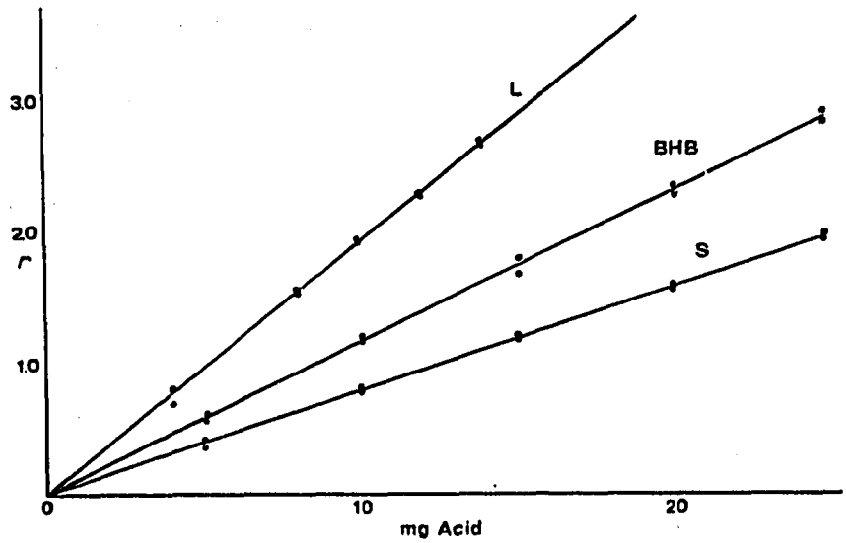


Fig. 2. Calibration data on DEGS column at 130° from solutions prepared in duplicate by Procedure A. Key: L = lactic acid; BHB = β-hydroxybutyric acid; S = succinic acid.

Calibration procedure B. Aliquots of the standard solution of sodium β-hydroxybutyrate were pipetted into 250 ml round-bottom $\overline{\$}$ 24/40 flasks and taken to dryness in a rotary evaporator as in procedure A. The residues were esterified for 10 min on a steam bath; the esters were isolated and chromatographed as in procedure A. Calibration solutions of propyl lactate and dipropyl succinate were prepared by combining aliquots of the calcium lactate and aqueous succinic acid solutions, evaporating to dryness and esterifying for 10 min. The esters were isolated and chromatographed as in procedure A.

Precautions in calibrations. The aliquots of sodium β-hydroxybutyrate were dried under vacuum to a white residue. In procedure A, when the ether was evaporated at 30° after the addition of lactic and succinic acids, the final residue no longer had a "dry salt" appearance but the esterification still proceeded quantitatively. The dried residues could not be stored and the BF_3–propanol was added immediately in order to convert them to their more stable propyl esters. The reaction mixtures were vigorously heated for the required length of time. The concentric rings of the steam bath served to keep the neck of the flask relatively cool by deflecting the steam.

Application to the analysis of egg. In the analysis of egg the organic acids were extracted according to the AOAC official method[8]. The ether extraction apparatus was checked for its efficiency by extracting a weighed amount of calcium lactate and quantitating the acid by titration. Recovery was greater than 98 %. When the acids were extracted from samples, reagent grade anhydrous ether containing less than 0.05 % ethanol was used to prevent the formation of ethyl esters in the subsequent BF_3-catalyzed esterification. The esters were prepared for chromatography as in calibration procedure A after the ether extract (with the omission of the 5 ml of water specified in AOAC 16.040) was evaporated to near dryness in a rotary evaporator at 30°. Two milliliters of BF_3–propanol were added, a 35 mm funnel or an air condenser was inserted in the neck of the flask and the reaction mixture was heated on a steam bath for 10 min. The BF_3–propanol reagent was added to the residue from the ether evaporation shortly after removing the flask from the rotary evaporator. It has been found that storage of the concentrated extract can result in polymerization between the organic acids and consequently in low recoveries.

When the ester concentration in a sample gave a recorder deflection greater than full scale for a 3 μl injection, 1.0 ml of the sample solution and 1.0 ml of the diluting solution were mixed in a screw cap vial, about 0.2 g of anhydrous sodium sulfate was added and the mixture was chromatographed. Additional diluting solution was added, when necessary, until the ester peak was on-scale. The amount of acid was calculated by multiplying the final result (obtained from calibration graph) by the dilution factor (F) where F is the volume after dilution: volume before dilution.

RESULTS AND DISCUSSION

Accuracy and precision

Fig. 2 shows the linearity of calibration procedure A over the ranges 0–14 mg lactic acid, 0–25 mg β-hydroxybutyric acid and 0–25 mg succinic acid. Similar results have been obtained on wider ranges (0–20 mg lactic acid, 0–40 mg β-hydroxybutyric acid and 0–40 mg succinic acid). Table I shows the close agreement in the calibration data obtained by procedures A and B and the reproducibility of each procedure on duplicate solutions. Table II is a further comparison of the precision of procedures A and B for β-hydroxybutyric acid at the 20 mg level. The S.D.'s of the mean r values were, respectively, 0.06 and 0.02.

Stability of calibration solutions

The solutions of propyl esters can be stored at 4° for periods of at least four weeks (Table III). The changes in the r values also reflect any change in the chromatographic system over the period of time. The aluminum foil lining and screw cap of the sample

TABLE I

r VALUES FOR CALIBRATION SOLUTIONS PREPARED IN DUPLICATE BY PROCEDURES A AND B
DEGS column at 130°; conditions as described in text.

Acid[a] (*mg*)	*r values*[b]					
	Lactic acid		*β-Hydroxybutyric acid*		*Succinic acid*	
	A	*B*	*A*	*B*	*A*	*B*
4:5:5	0.76	0.78	0.58	0.56	0.41	0.40
4:5:5	0.65	0.76	0.52	0.58	0.34	0.41
8:10:10	1.50		1.13		0.79	
8:10:10	1.53		1.16		0.78	
10:15:15	1.90	1.92	1.60	1.67	1.16	1.14
10:15:15	1.92	1.88	1.74	1.64	1.20	1.17
12:20:20	2.22		2.28		1.56	
12:20:20	2.25		2.20		1.52	
14:25:25	2.61	2.66	2.88	2.78	1.94	1.86
14:25:25	2.65	2.62	2.82	2.78	1.89	1.95

[a] Lactic acid–β-hydroxybutyric acid–succinic acid.
[b] r = height of ester peak: height of internal standard peak.

TABLE II

REPRODUCIBILITY OF r VALUES FOR β-HYDROXYBUTYRIC ACID ON SIX SOLUTIONS AT THE 20 mg LEVEL
DEGS column at 130°; conditions as described in text.

	r values	
	Procedure A	*Procedure B*
	2.28	2.26
	2.27	2.31
	2.20	2.28
	2.12	2.31
	2.30	2.28
	2.25	2.27
Mean	2.24	2.28
S.D.	0.06	0.02

vial are loose enough to permit some loss of chloroform during storage, but the presence of acetophenone as an internal standard avoids reliance upon solvent volumes.

Aqueous systems

When calibrations were attempted on aqueous systems containing sodium β-hydroxybutyrate, calcium lactate and succinic acid, recoveries of the propyl esters were low because of polymerization reactions during removal of water (Table IV). When one of the esterified mixtures was chromatographed at high temperature, the presence of several polymeric esters was observed (Fig. 3). Although the structures have not been determined, it has been established that peak A results from the reaction of lactate with β-hydroxybutyrate, peak B is the dimer ester shown below and peak C results from the reaction of succinate with β-hydroxybutyrate. In addition, much of the lactate-β-hydroxybutyrate polymer is insoluble in the reaction

TABLE III

STABILITY AT 4° OF CALIBRATION SOLUTIONS OF PROPYL ESTERS PREPARED BY PROCEDURE A

Acid[a] (*mg*)	*r values*					
	Lactic acid		*B-Hydroxybutyric acid*		*Succinic acid*	
	1[b]	*2*[b]	*1*	*2*	*1*	*2*
4:5:5	0.76	0.75	0.58	0.58	0.41	0.40
4:5:5	0.65	0.64	0.52	0.52	0.34	0.34
8:10:10	1.50	1.44	1.13	1.11	0.79	0.78
8:10:10	1.53	1.44	1.16	1.16	0.78	0.78
10:15:15	1.90	1.83	1.60	1.55	1.16	1.16
10:15:15	1.92	1.79	1.74	1.71	1.20	1.17
12:20:20	2.22	2.16	2.28	2.25	1.56	1.53
12:20:20	2.25	2.16	2.20	2.24	1.52	1.53
14:25:25	2.61	2.54	2.88	2.82	1.94	1.86
14:25:25	2.65	2.52	2.82	2.76	1.89	1.86

[a] Lactic acid–β-hydroxybutyric acid–succinic acid.
[b] 1 is freshly prepared; 2 is four weeks after preparation.

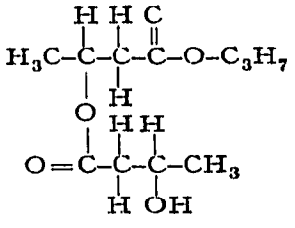
TABLE IV

RECOVERIES FROM AQUEOUS STANDARDS OF CALCIUM LACTATE, SODIUM β-HYDROXYBUTYRATE AND SUCCINIC ACID

Acid[a] (*mg*)	*Recovery*[b] (%)		
	Lactic acid	*β-Hydroxy-butyric acid*	*Succinic acid*
Mixture of three standards			
20:30:30	98	83	65
35:50:50	87	87	74
35:50:50	51	42	26
Mixture of three standards plus H_2SO_4			
20:30:30	99	99	96
35:50:50	99	100	99
35:50:50	97	100	94

[a] Lactic acid–β-hydroxybutyric acid–succinic acid.
[b] By comparison with procedure B.

mixture and thus remains in the flask. The existence of dimers of β-hydroxybutyric acid is known[9].



The interactions among the acids in aqueous solution could be minimized by the addition of a small amount of sulfuric acid (Table IV). However, the improvement

in results was not as dependable as that obtained with calibration procedure A in which the aqueous solution of sodium β-hydroxybutyrate is evaporated under vacuum to a dry residue before adding the other acids in ether solution.

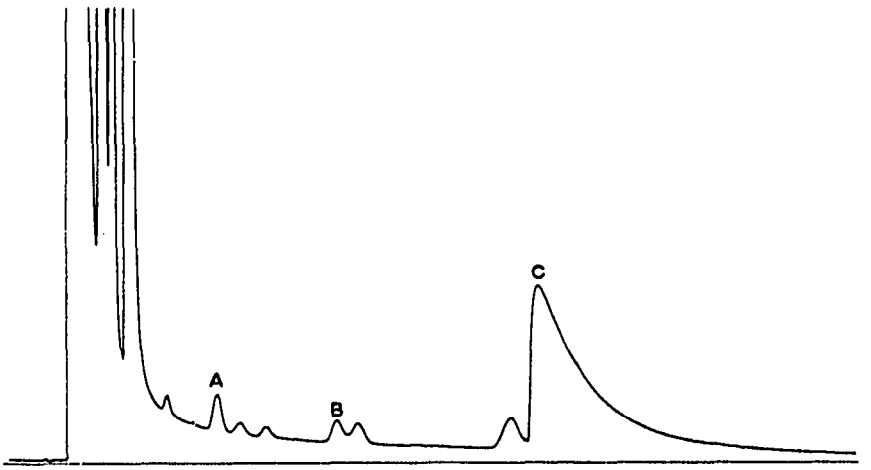


Fig. 3. Chromatogram of polymers from esterification of mixture of calcium lactate, sodium β-hydroxybutyrate and succinic acid on FFAP column at 225°. Key: A = β-hydroxybutyrate-lactate; B = β-hydroxybutyric acid dimer ester; C = β-hydroxybutyrate-succinate.

Aqueous systems could be avoided by using an ether solution of β-hydroxybutyric acid. However, the acid could not be completely esterified until the reaction time was extended to 90–120 min. At shorter times, recoveries of β-hydroxybutyric acid were low even when lactic and succinic acids were absent. Neutralization of the free acid, which is a syrup, by direct titration and by saponification indicated that it existed largely in polymer form. NMR spectroscopy also indicated the presence of polymeric forms. GC examination of the syrup after partial esterification with BF_3–propanol disclosed the presence of a compound which eluted at high temperature (BHB dimer, Fig. 4). The mass spectrum of the compound collected from the gas

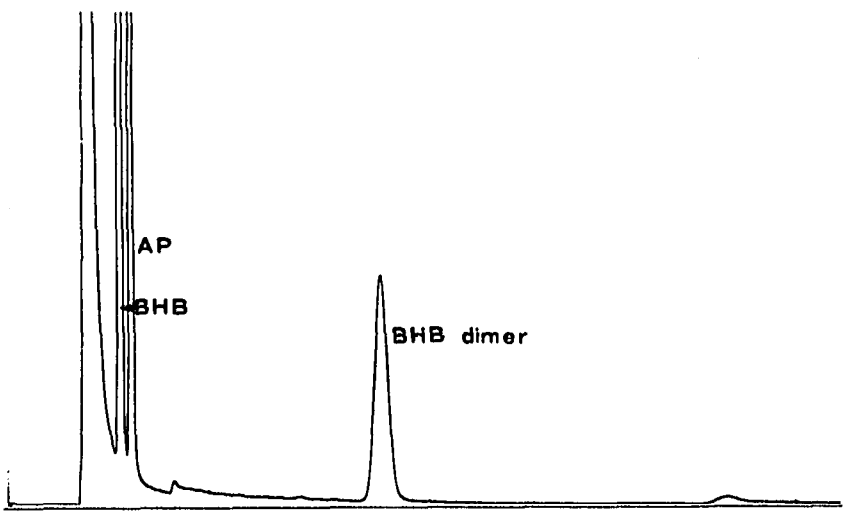


Fig. 4. Chromatogram on FFAP column at 185° of propyl esters of monomer and dimer forms of β-hydroxybutyric acid. Key: BHB = propyl β-hydroxybutyrate; AP = acetophenone internal standard; BHB dimer = propyl ester of dimer of β-hydroxybutyric acid.

chromatographic column was consistent with the dimer structure above. Peak B in Fig. 3 represents the same dimer. The esterification probably proceeds through a two-step process in which the dimer ester is first formed followed by cleavage and formation of the monomer ester.

Time of esterification

The esterification time is 30 min in calibration procedure A and 10 min in all other applications. The shorter time is adequate for converting combined residues of lactic acid (or its calcium salt) and succinic acid to their propyl esters[6]. Dry sodium β-hydroxybutyrate, in the absence of the other acids, can also be esterified in 10 min (procedure B). In like manner, a mixture of the three acids extracted from eggs can be esterified in 10 min. In that case, the ether solution of the free acids is evaporated with a minimum of heat, and interactions among the acids are not encountered. On the other hand, the stipulated conditions regarding the preparation of dry residues is more critical with the standard solutions. The reaction time was therefore extended to 30 min in procedure A in order to insure complete esterification despite occasional interactions among the acids.

Interference by other esters

The retention time of acetophenone is such that it does not interfere with the esters of a number of other low molecular weight acids. Table V lists some acids that were chromatographed as their propyl esters. The acids were used as received from commercial sources without further purification. Although β-hydroxypropionic acid could not be distinguished from acetophenone on a DEGS column it was separated on a 10 ft. by 4 mm I.D. column coated with 10 % FFAP at 150°. Under those conditions, the retention time relative to acetophenone was 1.40.

TABLE V

RELATIVE RETENTION DATA OF PROPYL ESTERS OF SOME LOW MOLECULAR WEIGHT ACIDS
DEGS column at 130°; conditions as described in text.

Acid	*Retention relative to acetophenone*
Crotonic	0.14
Pyruvic	0.27, 0.48
Lactic	0.34
Acetoacetic	0.64
β-Hydroxybuytric	0.77
α-Hydroxybutyric	0.39, 0.60
γ-Hydroxybutyric	0.48, 1.28, 2.55
β-Hydroxypropionic	1.04
Succinic	1.85
Fumaric	2.00
Maleic	2.91

Recovery of β-hydroxybutyric acid from eggs

Apparently, β-hydroxybutyric acid is extracted from incubated eggs as the monomer because, with an esterification time of 10 min, no significant amount of the dimer ester (BHB dimer, Fig. 4) was ever observed on the gas chromatogram. Furthermore, there was no increase in the amount of monomer ester when the esterification

time was increased. Thus, a 10 min esterification is adequate for esterifying the β-hydroxybutyric acid extracted from eggs. On the other hand, when the acid in syrup form was added to eggs it was recovered in part as the monomer ester and in part as the dimer ester. With an esterification time of 90 min, the acid was recovered entirely as the monomer ester.

Table VI shows recovery data for the sodium salt of β-hydroxybutyric acid added to passable eggs and to incubator-reject eggs. Quantitative recovery was obtained with the prescribed esterification time of 10 min. The recoveries of lactic and succinic acids were also quantitative. Extensive collaboration has already been accomplished on the application of the method to the determination of lactic and succinic acids in eggs[10,11]. It is now possible to quantitate the three acids from a single sample.

TABLE VI

RECOVERY OF β-HYDROXYBUTYRIC ACID FROM EGG

mg β-Hydroxybutyric acid/100 g egg		
Added as sodium salt	*Found*	*Recovery (%)*
Passable egg		
0	0	—
10.0	10.8	108
10.0	10.3	103
15.0	15.2	101
25.0	23.5	94
50.0	49.6	99
50.0	51.3	103
Incubator-reject egg		
0	13.0	—
10.0	23.2	102
10.0	24.0	110
15.0	28.0	100

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