A STUDY OF THE QUANTITATIVE MEASUREMENT OF CERTAIN METABOLIC ACIDS BY GAS–LIQUID CHROMATOGRAPHY*,**

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INTRODUCTION

In recent years, gas–liquid chromatography (GLC) has been utilized for the separation and identification of organic acids involved in the tricarboxylic acid (TCA) cycle and certain other organic acids which perform vital functions in carbohydrate, fat, and protein metabolism[1,6,12,14]. The acids have been readily chromatographed as their methyl or ethyl ester derivatives. The GLC technique appears to be more applicable than previous methods which are time consuming, require meticulous attention to details, and are limited by compounds which interfere with the analytical measurement of these acids in biological material. Unfortunately, there is a paucity of reports[4,5,8,13] in the literature pertaining to the measurement of these metabolic intermediates by GLC, possibly because of the considerable amount of time required to study the quantitative aspects of the technique. If a useful and efficient GLC method was developed for quantitatively measuring these acids in biological material, it would be a useful tool with which to ascertain the complexities of intermediary metabolism in both plants and animals.

The objective of this research was to develop a quantitative GLC method for measuring certain metabolic acids, which could be readily applied to biological samples on a routine basis. Because the authors have previously reported on the separation and identification of the acid esters involved in this study[12], the qualitative aspects of the GLC technique will not be discussed in this paper except to outline the GLC instrumentation and columns used. None of the operating conditions for chromatographing the acid esters were changed from those specified in the previous report; however, it was necessary to change the esterification procedure to increase analytical precision.

EXPERIMENTAL

Chromatography

The instrumentation for chromatographing the organic acids consisted of a model 600 Aerograph Hy-Fi§ gas chromatograph equipped with a gold-plated

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§ Wilkens Instrument and Research, Inc., P.O. Box 313, Walnut Creek, Calif.

hydrogen flame ionization detector and a Speedomax H* Leeds and Northrup recorder. Hydrogen gas for the detector was supplied by a model A-650 hydrogen generator** and a commercial source of high purity nitrogen was used as the carrier gas. The hydrogen gas line was kept dry by periodically regenerating the molecular sieve filter at 100° and an ultrasonic water bath was used to clean the flame detector. A loosely packed 1.5-in. long glass wool plug was placed inside the glass liner of the injection block next to the chromatographic column. A clean liner and plug were inserted periodically to minimize contamination of the system.

Methyl lactate, methyl oxalate, dimethyl malonate, dimethyl fumarate, and dimethyl succinate were separated with a 9-ft., 1/8-in. O.D. stainless steel column containing 15 % diethylene glycol succinate (DEGS) on 60–80 mesh, acid-washed Chromosorb W. These esters were separated isothermally at 140°. The same column at 200° resolved dimethyl malate and dimethyl α-ketoglutarate. Trimethyl citrate was most conveniently separated with a 5-ft. column containing the same packing material operated isothermally at 200°. The detector response for each ester appeared on the recorder chart as an individual, symmetrical peak.

Complete details of the GLC resolution and identification of these organic acid esters have been reported[12], including retention times and examples of representative chromatograms.

Methylation

The organic acids were converted to the corresponding methyl esters using concentrated hydrochloric acid (HCl) as the esterifying catalyst. Solutions of individual organic acids and of organic acid mixtures were prepared by dissolving the undissociated acids in absolute methanol (Fisher Scientific, 99.9 %, ACS). Ten-milliliter aliquots of the acid solutions were placed in dry reaction tubes (20 × 125 mm culture tubes with screw caps), 0.5 ml concentrated HCl was added to each tube, and the tubes were sealed and shaken in a shaker water bath at 55° for 4 h. At the end of the reaction period, the samples were stored in a refrigerator in the tightly capped reaction tubes until chromatographed.

A 3-μl aliquot of a sample was injected at each isothermal setting using a 10-μl Hamilton No. 701 N syringe. Prior to each injection the attenuator of the electrometer was set at 250 and was kept at this position until approximately 0.75 min after the solvent (methanol) peak. The attenuator was then adjusted to settings of 2, 4, 8, or 16, depending on the amount of acid esters. Attenuation changes were made only between peaks and the recorder pen was adjusted to the baseline before the next peak appeared, thus minimizing the error in peak area as determined with a Disc integrator***.

Titration

The undissociated acids used as standards were of the highest purity available commercially, but absolute purity values did not accompany the acids. Rather than assuming the acids to be 100 % pure, their purities were estimated by titrating them with a standardized sodium hydroxide (NaOH) solution. Titration was also used to determine the per cent methylation of each organic acid by comparing the

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*** Disc Instruments, Inc., 3014-B South Halladay St., Santa Ana, Calif.

amount of organic acid left after esterification to the original amount. This technique for determining per cent methylation was reported to compare favorably with a direct determination which involved weighing the esterified acids[11]. The estimates of purity and per cent methylation were used to determine the m*M*/l of acid ester measured by the gas chromatograph.

Quantification

A standard solution of a known concentration for each organic acid and a solution containing a mixture of the acids were prepared by dissolving the acids in absolute methanol. A series of dilutions was then prepared from each solution. Triplicate 10-ml samples were taken at each concentration from the solutions containing single acids and duplicate samples from solutions containing a mixture of acids. All samples in a given series were chromatographed once, then rechromatographed on the following day. The data from the first day were compared with those of the second day to detect any effects or changes in the operating conditions of the chromatograph and column with time. These data were also used to study the relationship between peak area and methyl ester concentration and to study the effect of methylating the organic acids individually and in a mixture.

Commercial esters were obtained from Eastman Kodak for all acids except lactic. A standard solution of each ester and a solution containing an ester mixture were prepared by dissolving the esters in absolute methanol. A series of dilutions for each solution was prepared, chromatographed, and standard curves constructed. These data were compared to the previous data, obtained from standard acids methylated in the laboratory, to study the effect of methylation on the quantification of these acids.

Lyophilization

The direct esterification of organic acids is a reversible reaction, with water as one of the end-products. Thus, the presence of excess water during the methylation reaction will prevent maximum production of the acid esters. This problem must be considered when adapting this method to biological samples and aqueous extracts. In the present study, lyophilization was investigated as a means of dehydrating and concentrating samples prior to methylation. It was necessary to determine if any organic acids were lost during lyophilization and the effect of pH on these losses.

A water solution and a methanol solution containing 10 m*M*/l of each acid were prepared. The water solution was adjusted to pH 7.0 with NaOH to dissolve the acids. Ten 20-ml samples of the aqueous solution were placed in 8-oz. narrow-mouth polyethylene bottles. Five were adjusted to pH 10.0 with NaOH and 5 to pH 4.5 with HCl. Four layers of cheesecloth were placed over the opening of each bottle. The bottles were closed with screw caps and frozen in liquid nitrogen. While frozen, the screw caps were removed and the bottles were placed in the vacuum chamber of a Stokes model 21 lyophilizer.* The cheesecloth acted to trap any flakes of material that might float to the mouth of the bottle while in the lyophilizing chamber.

Following lyophilization, 20 ml of methanol and 0.5 ml of concentrated HCl were added to the dried residue of each sample. The bottles were tightly capped and shaken in a shaker water bath at 55° for 4 h. After methylation, each sample was

* F. J. Stokes Corp., Philadelphia 20, Pa.

chromatographed in duplicate. The results were compared to similar samples from the alcohol solution which had not been lyophilized.

RESULTS AND DISCUSSION

Methylation

The first requirement for quantitatively measuring these organic acids was to establish a reliable method for converting the acids to their esters. Methanol and HCl were tried as a possible methylating agent because it has received wide application for methylating low molecular weight carboxylic acids with no apparent difficulties.[3] This method was used by earlier workers[9] for separating acids involved in the TCA cycle by fractional distillation and it was found equally applicable in the present study. Although the qualitative phase of this procedure used concentrated sulfuric acid as the methylating catalyst, changing to concentrated HCl did not pose any chromatographic problems. On the basis of comparisons with standard acids, the chromatographic results of samples methylated with these two catalysts were identical except that analytical variation was less when HCl was used.

The conditions for methylation as outlined in the experimental section were optimal for the organic acids studied. They were determined by varying the amount of concentrated HCl added and the length of time in the water bath at 55°. Maximal methylation of the organic acids occurred when sufficient HCl was added to lower sample pH below the pK's of each acid. Also, maximal methylation was not affected by adding HCl in an amount which lowered the pH to 1.0. The lowest pK was for oxalic acid (pK = 1.19). Thus, methylation of all acids at pH 1.0 permitted carrying out the procedure under the same conditions. At pH 1.0, maximal methylation of the organic acids occurred within 4 h at 55° in a shaker water bath.

Under the conditions in our laboratory, this method was superior to the sodium-methylate method[2] and the boron-trifluoride method.[7] Also, when 2,2-dimethoxypropane was used as a water scavenger, as reported by RADIN *et al.*[11] and PRICE[10], no measurable benefit was obtained. This suggested that under the conditions of the present study, the water endogenous to the reagents, and that produced during methylation, had an insignificant effect on the efficiency of esterification.

Titration

Once the optimal conditions for methylation were established, acid titration was used to quantitate the amount of each acid which was converted to its ester. The percentage of esterification of each acid is presented in Table I. The range values represent the lowest and highest of three determinations. Table I also contains estimates of acid purity as determined by titration. The standard errors of titration were 0.36 and 0.90 for per cent methylation and per cent purity, respectively. Although these percentage estimates may be subject to error, most of them were within a realistic range and, because of their constancy, they were used to develop the quantitative aspects of measuring these organic acids by GLC. Different lots of acids were not used in the study.

Quantification

Investigations of the quantitative aspects of measuring the organic acids consisted of studying each acid individually and as part of a mixture of acids. Thus,

TABLE I

PER CENT METHYLATION AND PURITY OF ORGANIC ACIDS AS DETERMINED BY TITRATION

Acid	*Methylation*		*Purity*	
	Average	*Range*	*Average*	*Range*
Lactic	98.7	98.5– 98.9	75.1	74.1– 75.5
Oxalic	42.6	42.6– 45.5	84.4	82.9– 85.5
Malonic	97.6	97.5– 98.2	77.4	76.6– 78.3
Fumaric	93.3	93.2– 93.4	104.0	103.1–104.8
Succinic	99.4	99.0– 99.8	97.6	96.8– 98.5
Malic	100.5	100.2–100.8	93.8	92.8– 94.1
α-Ketoglutaric	83.6	82.8– 84.1	94.8	94.0– 95.7
Citric	91.6	91.4– 91.8	103.1	101.5–103.4

any differences between methylating and chromatographing the organic acids separately and in a mixture could be defined. The per cent methylation and per cent purity values obtained by titration were used to determine the methyl ester concentration of each acid so that the data could be subjected to statistical analyses and standard curves could be established. Table II summarizes the statistical significance of the effects of ester concentration and time interval on the chromatographic response measured as peak area by a Disc integrator. This information represents the type of concentration *versus* response curve obtained for the methyl ester of each acid, the curves for the esters in a mixture being more important because biological samples contain organic acid mixtures. The fact that very similar curves were obtained for the esters measured individually and in a mixture indicated that the organic acids were chemically independent of each other when carried through the procedure as a mixture.

Linear curves fit by the least squares procedure were obtained for lactic, malonic, fumaric, succinic, and citric acids. Although the actual data tended to be non-linear at the lower concentrations, this deviation from linearity was not significant at the $P = 0.05$ level. Plotting the curve for α-ketoglutarate revealed a linear relationship between 6 and 24 mM/l. A cubic relationship was obtained when the entire concentration range was considered because of the significant curvilinear response below 6 mM/l which was the lower quarter of the concentration range. A similar non-linear response in the lower portion of the curve for individual methyl citrate explains its cubic relationship between concentration and response.

The curves for methyl malate and methyl oxalate obtained from the acid mixture were quadratic. This agrees with data on oxalic acid reported by SHARPLESS[13], who also reported non-linear curves for malonate and succinate but did not report any statistical treatment of the data. In the present study the type of curvilinear response for oxalic acid measured individually and in a mixture did not agree; however, when samples of commercially prepared methyl oxalate were analyzed, both individually and as part of a mixture, they followed a quadratic trend. Although the curves for malic acid were non-linear, there was considerable variation not accounted for by regression and the curves may have been linear.

It is difficult to make direct comparisons of this study with other studies found in the literature because of the differences in instrumentation and column materia

TABLE II

STATISTICAL SUMMARY OF THE EFFECTS OF CONCENTRATION AND DAYS ON THE CHROMATOGRAPHIC RESPONSE TO ORGANIC ACID ESTERS

Source	*Lactic*		*Oxalic*		*Malonic*		*Fumaric*		*Succinic*		*Malic*		*α-Ketoglutaric*		*Citric*	
	I[a]	*M*[b]	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*	*I*	*M*
Concentration	**[c]	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Linear	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**	**
Quadratic	o[d]	o	**	**	o	o	o	o	o	o	**	**	o	**	**	o
Cubic	o	o	**	o	o	o	o	o	o	o	o	o	o	*	**	o
Quartic	o	o	o	o	—	o	—	o	o	o	o	o	o	o	o	o
Quintic	o	—[f]	—	—	—	—	—	—	o	—	o	—	o	—	o	—
Days	*[e]	o	o	o	o	o	o	o	o	o	o	o	o	o	o	**
Days × concentration	*	*	o	o	o	o	o	o	o	o	o	**	o	*	o	**
Linear	**	**	o	o	o	o	o	o	o	o	*	**	o	**	o	**
Quadratic	o	*	*	o	o	*	*	o	o	o	o	**	o	**	o	o
Cubic	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Quartic	o	o	o	o	—	o	—	o	o	o	o	o	o	o	o	o
Quintic	o	—	—	—	—	—	—	—	o	—	o	—	o	—	o	—

[a] Organic acids methylated and chromatographed individually.
[b] Organic acids methylated and chromatographed as part of a mixture.
[c] $P < 0.01$
[d] Statistically non-significant at $P = 0.05$.
[e] $P < 0.05$.
[f] Sources of variation which could not be tested with the data.

which play an important role in the analysis of compounds by GLC. SHARPLESS[13] obtained alterations in peak area response as increasing amounts of the methyl esters of oxalate, malonate, and succinate were chromatographed. The esters of these acids were separated using DEGS as the liquid phase and measured with an argon B-ray detector. MIROCHA AND DEVAY[8] found this same non-linear response between peak area and concentration with fumaric acid using DEGS as the liquid phase and a hydrogen flame ionization detector; however, fumarate was separated as its diethyl ester. A linear response for the methyl esters of succinate, oxalacetate, malate, α-ketoglutarate, *cis*-aconitate, citrate, and fumarate was obtained by KUKSIS AND VISHWAKARMA[4] using a flame-ionization detector and Carbowax as the liquid phase. It appears that because of the differences in chromatographic systems, one should be concerned with the type and reproducibility of the standard curves in establishing a quantitative procedure and should not expect the ideal linear relationship between concentration and chromatographic response in all cases. However, it may be possible to choose concentration ranges within which the response is linear.

TABLE III

REGRESSION EQUATIONS REPRESENTING THE STANDARD CURVES FOR ORGANIC ACID ESTERS OBTAINED FROM AN ACID MIXTURE

Ester	*Concentration range (mM/l)*	*Day*	*Equation*
Lactic	1.19–19.06	1	$Y^a = -258.47 + 212.95(X)^b$
		2	$Y = -443.64 + 253.61(X)$
Oxalic	0.45– 1.81	1	$Y = -151.30 + 592.55(X)$
		2	$Y = -148.76 + 588.74(X)$
	1.81– 7.25	1	$Y = -461.25 + 752.47(X)$
		2	$Y = -552.44 + 798.52(X)$
Malonic	1.14–18.29	1	$Y = -153.17 + 394.89(X)$
		2	$Y = -192.56 + 400.15(X)$
Fumaric	1.45–23.20	1	$Y = -527.44 + 605.01(X)$
		2	$Y = -391.77 + 580.92(X)$
Succinic	1.38–22.20	1	$Y = -231.48 + 599.94(X)$
		2	$Y = -172.85 + 588.39(X)$
Malic	1.43– 5.73	1	$Y = -184.78 + 141.91(X)$
		2	$Y = -134.32 + 123.36(X)$
	5.73–22.92	1	$Y = 35.43 + 114.62(X)$
		2	$Y = -200.00 + 133.00(X)$
α-Ketoglutaric	1.50– 6.00	1	$Y = -116.77 + 114.02(X)$
		2	$Y = -52.05 + 90.67(X)$
	6.00–24.00	1	$Y = -276.24 + 147.71(X)$
		2	$Y = -546.76 + 168.34(X)$
Citric	1.37–21.98	1	$Y = -234.89 + 243.32(X)$
		2	$Y = -581.55 + 327.40(X)$

[a] Y = peak area.
[b] X = ester concentration.

Linear equations which represent the standard curves for the acid esters are shown in Table III. For the esters which gave a linear response with concentration by showing no significant (at $P = 0.05$ level) departure from linearity, an equation is presented which covers the entire range. For the esters giving a non-linear response the concentrations were divided into linear segments and linear equations are presented for each segment. In all cases, an equation representing each concentration range is presented for each of two days. Thus, these equations can be used to plot the standard curves obtained for the mixture of esters in this study to give an idea of the variation obtained due to analyzing the same set of standard solutions on different days. There were some differences due to days which is shown in Table II by the significant concentration–day interactions. This day effect was due primarily to changes in the operating conditions of the chromatograph or changes within the chromatographic columns.

Commercially prepared esters for all the acids except lactic were obtained and standard curves prepared with these esters compared favorably with the esters prepared in the laboratory. This indicated that any curvilinear response was due to the chromatographic instrumentation and not the esterification of the acids.

The coefficients of variation for lactic, oxalic, malonic, fumaric, succinic, malic, α-ketoglutaric, and citric acids when analyzed in a mixture were 8.1, 2.7, 3.1, 2.8, 1.7, 1.8, 2.0, and 3.6, respectively. These coefficients were determined using the average peak area of the concentration range of each acid studied. Above the mid-point of each concentration range, the coefficient of variation was constant; but as the concentrations were decreased below the mid-points, the proportional amount of random variation increased. The coefficients of variation range from 5 to 10 % at the lower contration levels.

Lyophilization

Before applying this method to biological tissues and fluids which contain large amounts of water, a method had to be developed for removing the water prior to the esterification step. The use of lyophilization was suggested by THIMANN AND BONNER[15]

TABLE IV

EFFECT OF pH ON LOSSES DURING LYOPHILIZATION

	Loss in mM/l					*Recovery* (%)
	Non-lyophilized		*Lyophilized*		$\bar{S}x$	
	pH 10.0	*pH 4.5*	*pH 10.0*	*pH 4.5*		
Lactic	10.0	10.5	10.2	9.9	0.71	98.3
Oxalic	10.0	9.7	11.0	9.7	0.32	105.2[b]
Malonic	10.0	9.6	6.7	6.3	0.29	66.3[c]
Fumaric	10.0	9.4	10.1	9.9	0.28	103.0
Succinic	10.0	9.3	9.7	9.5	0.23	99.4
Malic[a]	—	10.0	—	10.2	0.49	101.5
α-Ketoglutaric	—	10.0	—	9.6	0.64	95.7
Citric	10.0	9.8	10.1	9.5	0.38	98.4

[a] The effect of pH on malic acid was studied later with α-ketoglutaric acid.
[b] $P < 0.05$.
[c] $P < 0.01$.

for drying biological materials with a minimum of organic acid loss. Table IV contains a summary of the data from the present investigation comparing lyophilized to non-lyophilized samples at a high and low pH. All recoveries during lyophilization were near 100 % except for malonic acid, which was consistently lost in a significant amount. One sample at a pH of 10.0, which gave an unusually high detector response, caused an apparent increase in oxalic acid during lyophilization; but an error in its preparation could not be found. Although there was a tendency for the recoveries of the other acids to be lower at pH 4.5, the differences attributable to pH were insignificant.

TABLE V

EFFECT OF pH ON THE LOSS OF MALIC AND α-KETOGLUTARIC ACIDS DURING LYOPHILIZATION

Acid	*Loss in mM/l*					
	pH 4.5	*pH 6.0*	*pH 7.0*	*pH 8.5*	*pH 10.0*	$S\bar{x}$
Malic	10.0	9.6	10.4	12.8	11.8	0.68
α-Ketoglutaric	10.0	9.3	10.7	—	—	1.14

α-Ketoglutaric acid was completely lost at a pH of 10.0 during lyophilization. Therefore, this acid was studied at pH's of 4.5, 6.0, 7.0, 8.5, and 10.0 and found to be completely lost above pH 7.0 (Table V). From pH 4.5 to 7.0, α-ketoglutarate was not significantly lost. Also, under the conditions of this study, malic acid was not lost by changing the pH. Malic acid was studied with α-ketoglutaric acid instead of the other organic acids because it was chromatographed with α-ketoglutarate.

Thus, the data indicated that freeze-drying was applicable for removing water from biological samples and extracts. Although α-ketoglutarate was completely lost at pH's above 7.0, there was no loss between 4.5–7.0, which represents a wide working range.

This technique also allowed standard acid solutions to be made with water instead of methanol. Therefore, pure organic acid salts, which were easily obtained for some of the organic acids, could be incorporated into standard solutions and used for subsequent quantitative measurements. It was also noted that purities of the free organic acids as determined by titration were in close agreement with their purities determined chromatographically by comparing the free acids to the salts. The purities of lactic, oxalic, fumaric, succinic, and citric acids were 75.1, 84.4, 104.0, 97.6, and 103.1 % respectively when determined by titration and 81.9, 86.8, 97.6, 95.6 and 101.1 % when determined chromatographically.

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SUMMARY

Various factors in the quantitative measurement of metabolic organic acids by GLC were studied. Conditions were established for quantitatively converting lactic,

oxalic, malonic, fumaric, succinic, malic, α-ketoglutaric, and citric acids to their methyl esters using methanol and concentrated HCl, and the esters were measured by GLC using a DEGS column. Not all of the esters gave a linear response with increasing concentration levels, and the non-linearity appeared to be an effect of the GLC system. Lyophilization was suitable for dehydrating aqueous samples. This method of drying represents a convenient means of adapting the GLC method to the analysis of biological samples or aqueous extracts.

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