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SIMULTANEOUS QUANTITATION OF KREBS CYCLE AND RELATED ACIDS BY MASS FRAGMENTOGRAPHY

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SUMMARY

Methods are described for simultaneous quantitation of Krebs cycle and related acids by gas chromatography–mass spectrometry using deuterium-labelled acids and *n*-butyl-d_9-esters of the organic acids as internal standards. Using sulphuric acid as esterification catalyst, only lactic, succinic, fumaric, malic, maleic and citric acids were found to be stable to hydrogen exchange and could be used as reference standards in the deuterated form. In contrast, pyruvic, oxalacetic, α-ketoglutaric and malonic acids were found to exchange their deuterium readily and could not be employed for this purpose. All the acids could be quantitated using *n*-butyl-d_9-esters of reference organic acids as internal standards, following a separate preparation of the *n*-butyl derivatives of the unknown acids. The method is suitable for routine analysis of organic acids at the picogram level in perchloric acid extracts of tissues.

INTRODUCTION

A method for a specific, sensitive and rapid identification and quantitation of Krebs cycle and related acids is desireable for various analytical and metabolic studies. The techniques currently employed for this purpose involve mainly spectrophotometric assays based on group reactions catalyzed by various enzyme preparations[1,2], although chromatographic methods have also been proposed and occasionally employed[3–5]. The latter techniques, although of great value, are limited by their non-specificity for the absolute identification of any structure responsible for the chromatographic peak. Of greater interest are the techniques based on gas chromatographic–mass spectrometric (GC–MS) fragmentography[6–9], but specific applications to the identification and quantitation of Krebs cycle acids have been made infrequently[10]. Furthermore, definitive methods of analysis involving stable isotope derivatization in combination with GC–MS fragmentography have not been developed or proposed. About ten years ago, Pereira *et al.*[11] reported the simultaneous quantitation of ten amino acids in soil extracts by means of mass fragmentography, but a similar method has not been available for the determination of the Krebs cycle and related acids, although the advantages would seem to be obvious. The present study shows that such a technique can be developed in principle, but that many of

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the organic acids are subject to extensive hydrogen exchange and that quantitative studies based on deuterium-labelled internal standards must be executed with great caution.

MATERIALS

α-Ketoglutaric acid was obtained from Aldrich (Milwaukee, WI, U.S.A.) and oxalacetic acid from Nutritional Biochemicals (Cleveland, OH, U.S.A.), while the other natural organic acids were from Sigma (St. Louis, MI, U.S.A.). Succinic-d_4, fumaric-d_2 and maleic-d_2 acids were purchased from Merck, Sharp & Dohme (Montreal, Canada). Likewise, perdeuterated *n*-butanols ($C^2H_3C^2H_2C^2H_2C^2H_2OH$ and $C^2H_3C^2H_2C^2H_2C^2H_2O^2H$) were also obtained from Merck Sharpe & Dohme. $^2H_2SO_4$ was prepared in the laboratory by repeated dilution of H_2SO_4 with deuterium oxide and evaporation to dryness. Other reagents and solvents were of Certified Reagent grade quality supplied by Fisher Scientific (Don Mills, Canada). Deuterium oxide was supplied by Atomic Energy of Canada (Chalk River, Canada).

METHODS

Preparation of standard n-butyl esters

The *n*-butyl esters were prepared by treating the free acids or their sodium salts (5–15 mg) with sufficient 3–6% (v/v) sulphuric acid in *n*-butanol to cover the dry residue (0.25–0.5 ml). The esterification was completed by heating the tightly closed vials at 80°C for 15–240 min[12]. After cooling, the contents of the vials were diluted with water and the butylation products extracted with chloroform. *n*-Butyl-d_9-esters were prepared similarly. The reaction products were diluted to a known volume (100 mg per 100 ml). The extent of hydrogen exchange during the *n*-butylation was determined using $^2H_2SO_4$ as the catalyst.

Determination of standard curves

To each of four tubes containing 2.5 ml of the deuterated organic acid standard mixture (100 mg in 100 ml of 0.1 *N* H_2SO_4) was added 2.5–1250 μl of standard organic acid solution (2.5 μg of each organic acid per ml). The solutions were mixed and evaporated to dryness. Each residue was derivatized by the above method and an aliquot of each (1 μl) injected into the gas chromatograph, which was operated under the conditions described below. This procedure was used to construct a standard curve for quantitation of each carboxylic acid. A similar procedure was used to obtain standard curves for the quantitation of the organic acid *n*-butyl esters by reference to the *n*-butyl-d_9-esters, which were added following a separate derivatization of the undeuterated acids with undeuterated *n*-butanol.

Gas chromatography and mass spectrometry

Combined GC–MS analyses of the *n*-butyl esters of the organic acids were made with a Varian MAT CH-5 single-focusing mass spectrometer coupled to a Varian Data 620i computer previously described for work with fatty acid methyl esters[13,14]. The GC separations were made with a Varian Model 2700 Moduline gas chromatograph equipped with a 180 × 0.3 cm I.D. glass column filled with 3% Silar

5 CP on Gas Chrom Q (100–120 mesh) (Applied Science Labs., State College, PA, U.S.A.). The gas chromatograph, which did not contain a separate detector, was temperature programmed from 100 to 270°C at 6°C per min, with the interface at 250°C. The transfer line was maintained at 275°C.

All spectra taken over the GC peak were corrected for background by the computer using Varian Module SUB. Alternatively, GC–MS spectra were obtained for some of the compounds on the Hewlett-Packard Model 5985B quadrupole mass spectrometer coupled via a jet separator to a 5840A gas chromatograph, equipped with a packed 3% Silar 5 CP column or with a 25-m SP-2100 fused-silica capillary column. The mass spectrometer was operated in the electron-impact mode. It was coupled to an HP-1000E computer, HP 7906 disk drive, HP 2648A graphics terminal and a Tectronix 4632 video hard copy unit. The instrument was operated at 70 eV in a cyclic manner.

Preparation of extracts of natural organic acids

Krebs cycle and related acids were extracted from natural sources (rat liver and muscle) by perchloric acid as previously described[3].

The residue was dissolved in water and made up to volume of 5 ml. To 2 ml of the prepared solution was added 2 ml of the deuterated organic acid solution (succinic, fumaric and maleic acids) and the mixture was evaporated to dryness. The residue was heated at 80°C with 6% H_2SO_4 in *n*-butanol (1 ml) for 240 min and the *n*-butyl esters were recovered as described above for standard acids. Alternatively, the unknown acids were esterified with *n*-butanol and the esters diluted with known amounts of the standard acid (lactic, β-hydroxybutyric, malonic, fumaric, succinic, maleic, malic, α-ketoglutaric and citric) esters of *n*-butanol-d_9, evaporated to small volume and an aliquot was injected into the gas chromatograph.

RESULTS

Stability of hydrogen during esterification

Complete chemical stability of the deuterium in the organic acid standards is a prerequisite for their use in GC–MS quantitation by mass fragmentography. In the present study it was investigated by subjecting either the deuterium-labeled acids to butylation in the presence of normal sulphuric acid or by exposing normal organic acids to *n*-butylation in the presence of deuterated sulphuric acid. Only succinic, fumaric, maleic, lactic and citric acids were found to be stable to hydrogen exchange under the esterification conditions for up to 240 min. The keto acids and malonic acid suffered extensive hydrogen exchange. Fig. 1 shows the mass spectra recorded for the *n*-butyl ester of malonic acid as obtained following esterification in the presence of natural and deuterated sulphuric acid. It can be seen that, in the presence of the 6% deuterated sulphuric acid, 15% of the malonate acquired 1 or 2 deuterium atoms per molecule and established the expected atom-percent deuterium excess corresponding to that in the esterification medium (7.1%). The mass spectrum of the *n*-butyl malonate is characterized by ions at *m/z* 87, 105, 143 and 161 corresponding to M − 129, M − 111, M − 73 and M − 55, respectively, as expected from the mass spectra previously recorded for its methyl and *n*-butyl esters[15]. There was no introduction of deuterium into the methylene group of the malonate esters during esterification with *n*-butanol-d_9 in the presence of undeuterated sulphuric acid.

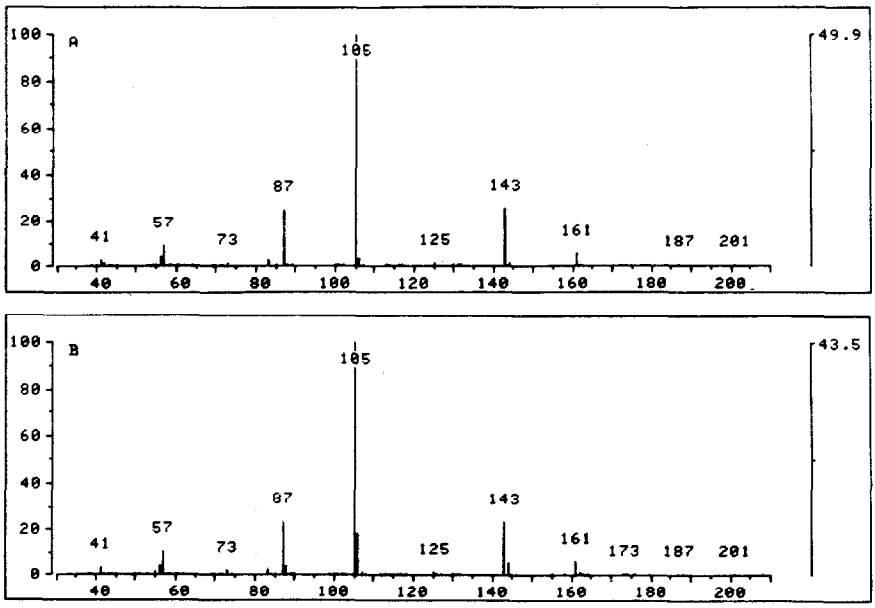

Fig. 1. GC–MS spectra of *n*-butyl esters of malonic acid prepared in the presence of H_2SO_4 (A) and $^2H_2SO_4$ (B) as esterification catalysts. Reaction and analysis conditions as described in text.

Comparable exchange of hydrogen took place when pyruvic, α-ketoglutaric and oxalacetic acids were subjected to *n*-butylation in the presence of deuterated sulfuric acid. There was evidence for an increase by up to 3 mass units in the characteristic ions $[M - 101 + 56]^+$ and $[M - 101]^+$ of the α-ketoglutarate, which indicates that in addition to the α-methylene unit adjacent to the carbonyl group also the β-methylene unit was capable of exchanging its hydrogens. Again the average atom % excess of deuterium found in the α-ketoglutarate was consistent with the atom % excess of deuterium in the reaction medium. The exchange of hydrogen in the pyruvic and oxalacetic acids during *n*-butylation in the presence of deuterated sulfuric acid has been demonstrated previously[12].

TABLE I

CHARACTERISTIC FRAGMENT IONS SELECTED FOR MASS FRAGMENTOGRAPHY OF UNDEUTERATED AND DEUTERATED *n*-BUTYL-d_0-ESTERS OF KREBS CYCLE AND RELATED ACIDS

Organic acid	*Fragment ion (m/z)*	
	Natural compounds	*Deuterated compounds*
Fumaric	99, 117*, 155, 173	101, 119*, 157, 175 (d_2)
Maleic	99*, 117	101*, 119 (d_2)
Succinic	101*, 157	105*, 161 (d_4)

* Base peak.

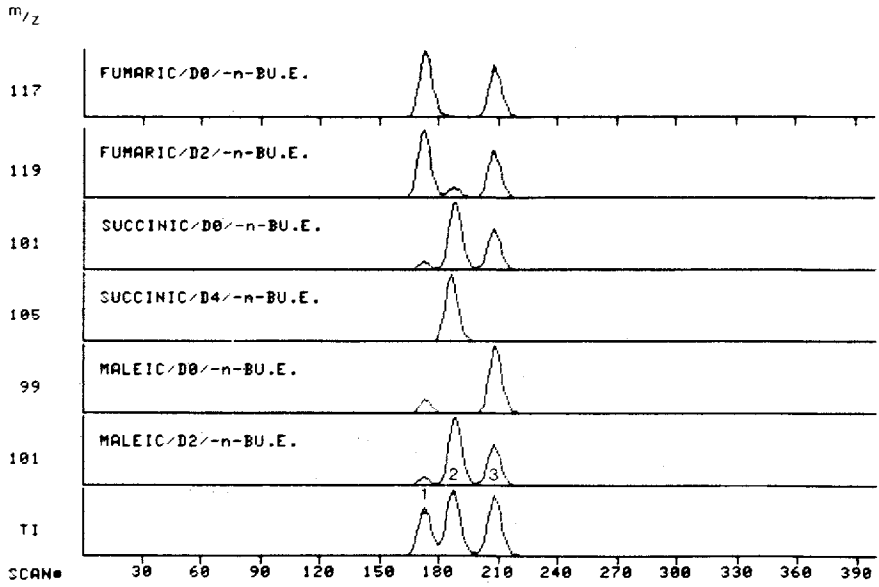

Fig. 2. Mass chromatograms of a mixture of *n*-butyl esters of fumarate-d_2, maleate-d_2 and succinate-d_4 in a 1:1 ratio with the corresponding d_0-acid esters. GC–MS conditions and fragment ions as given in text. TI = Total ion current profile.

Quantitation of known acids

Table I gives the characteristic ions monitored for quantitation in the mass spectra of selected deuterated and undeuterated organic acids. Although some of the major ions overlap, they are still usable because these acids are effectively resolved in the gas chromatograph prior to their ionization. Fig. 2 shows the mass chromatograms recorded for quantitation with fumarate-d_2, maleate-d_2 and succinate-d_4. Ob-

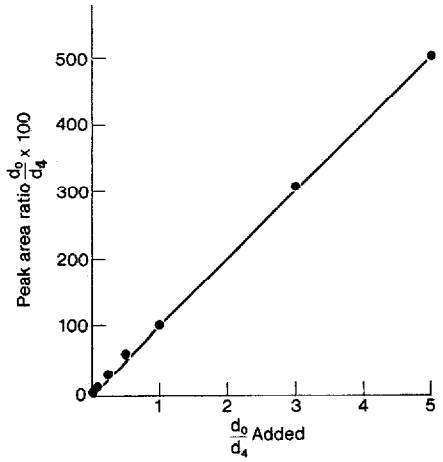

Fig. 3. Standard curve for the quantitation of succinic acid: d_0 and d_4 refer to the natural and deuterated succinic acids, respectively.

TABLE II

CHARACTERISTIC FRAGMENT IONS SELECTED FOR MASS FRAGMENTOGRAPHY OF UNDEUTERATED AND DEUTERATED *n*-BUTYL-d_9-ESTERS OF KREBS CYCLE AND RELATED ACIDS

Organic acid	*Fragment ion (m/z)*	
	n-Butyl-d_0	*n-Butyl-d_9*
Lactic	45*, 57**,***, 75, 85	45*, 66**,§, 76, 94
Pyruvic	43*, 144 ($M+$)	43*, 153 ($M+$)
β-Hydroxybutyric	87*,**, 145	87*,**, 154
Oxalic	57**,***	66**,§
Malonic	87, 105**, 143	88, 108**, 152
Fumaric	99, 117**, 155, 173	100, 120**, 164, 184
Succinic	101**, 157	102**, 166
Maleic	99**, 117, 155, 173	100**, 120, 164, 184
Malic	89**, 145	90**, 154
α-Ketoglutaric	101**, 157	102**, 166
Aconitic	112**, 157, 213	113**, 159, 223
Citric	129**, 185, 259	130**, 194, 277

* Common fragment in both *n*-butyl-d_0- and *n*-butyl-d_9-esters.
** Base peak.
*** *n*-Butyl-d_0-fragment at appropriate retention time.
§ *n*-Butyl-d_9-fragment at appropriate retention time.

viously, other acids could be similarly monitored, provided they possess non-exchangeable hydrogen atoms. Appropriate analogues of such acids could be chemically synthesized from suitable precursors. In such instances at least two deuterium atoms should be incorporated in non-exchangeable positions so that for the deuterium ion chosen the $P + 2$ peak is separate from the ^{13}C isotope contribution of the unlabeled organic acid. The deuterium substitution need not be quantitative, provided the same characteristic ion of the deuterated analogue is used for the calibration of a standard curve. Fig. 3 gives a typical standard curve obtained for the succinic acid. The lowest detection limit (characteristic intensity twice the noise level) for the pure acids was 20 ng/ml (20 pg/μl injected), which is about ten times lower than that obtained by hydrogen flame ionization.

Table II gives the characteristic ions monitored for quantitation in the mass spectra of the d_0 and d_9-*n*-butylation products of various organic acids. This method of quantitation does not require extensive collections of deuterated organic acids, since any standard acid mixture can be converted into a deuterated *n*-butyl ester mixture by treatment with *n*-butanol-d_9 in the presence of sulphuric acid. Fig. 4 gives the mass chromatograms recorded for quantitation of the *n*-butyl-d_0-esters by means of *n*-butyl-d_9-esters of the acids. In this instance the corresponding deuterated and non-deuterared esters are much more extensively resolved and care must be taken in avoiding overlaps between acids of different original molecular weight. Overlap of deuterated and non-deuterated *n*-butyl esters of the common organic acids, however, is not a problem (see below). Fig. 5 gives a typical standard curve obtained for the

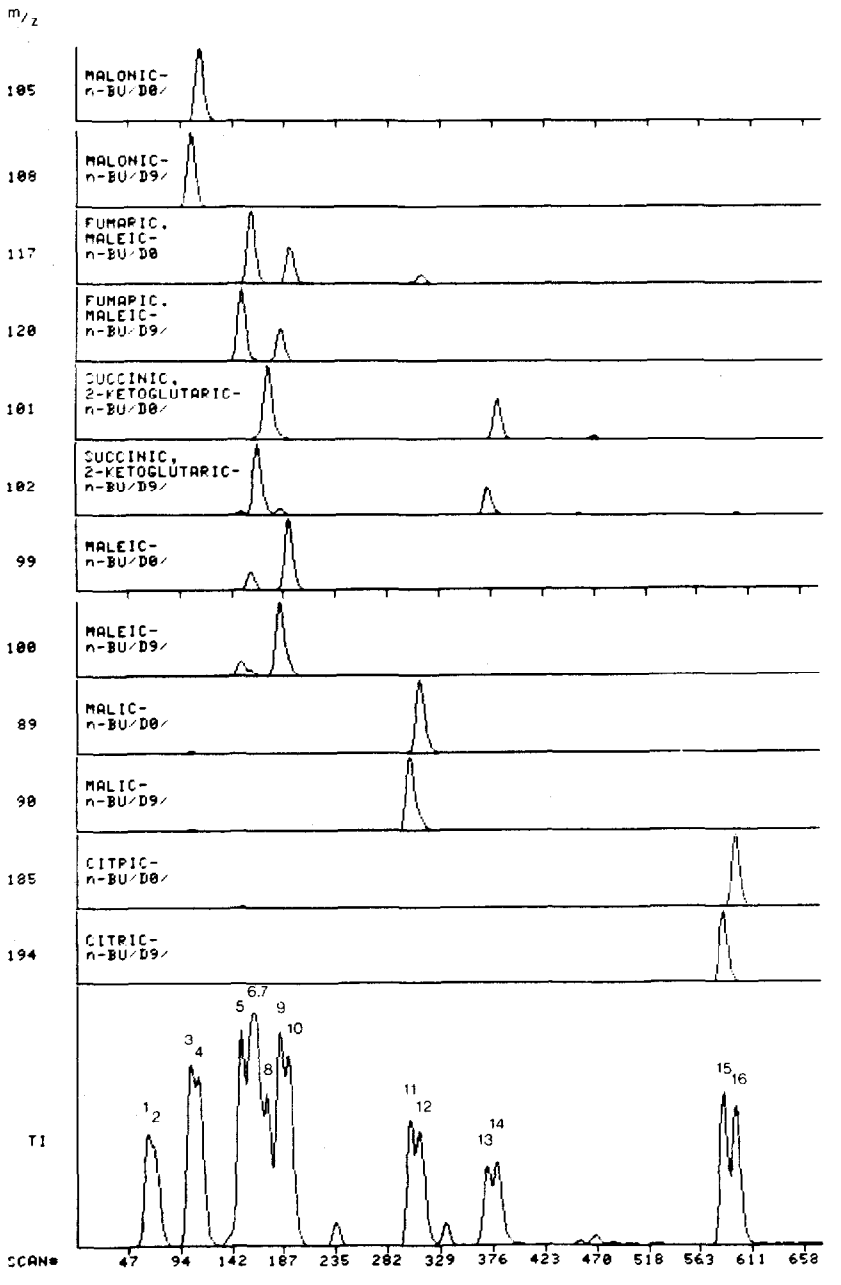

Fig. 4. Mass chromatograms of a 1:1 mixture of *n*-butyl-d_0- and *n*-butyl-d_9-esters of standard organic acids. GC–MS conditions and fragments ions as given in text. TI = Total ion current profile.

deuterated and non-deuterated esters of standard organic acids. The selected ions yield reproducible linear responses over the entire concentration range tested. The lowest amount of acid detected is 20 ng/ml (20 pg/μl injected) of solution. Not all acids, however, are detected at the same level (see below). This level of detection is more than ten times lower than that commonly realized by GC with flame-ionization detection. In many instances mass chromatography has detected peaks no longer reliably discerned by the gas chromatograph.

The ion intensities obtained in the mass spectrometer differ for different acids and require calibration for quantitative work. Table III gives the response factors

TABLE III

RESPONSE FACTORS RELATIVE TO *m/z* 129 OF ADIPIC ACID AS INTERNAL STANDARD

Organic acid	*Fragment ion (m/z)*	*Natural compounds*	*Deuterated compounds*
Lactic	45	0.35 0.34	
Oxalic	57*	0.72 0.68	
Malonic	105	2.10 2.05	
Fumaric	117	1.07 1.01	1.02 1.10 (d_2)
Succinic	101	3.56 3.65	3.68 3.67 (d_4)
Maleic	99	1.52 1.44	1.76 1.81 (d_2)
Malic	89	1.23 1.23	
α-Ketoglutaric	101	1.35 1.33	
Citric	185	2.68 2.76	

* Butyl group.

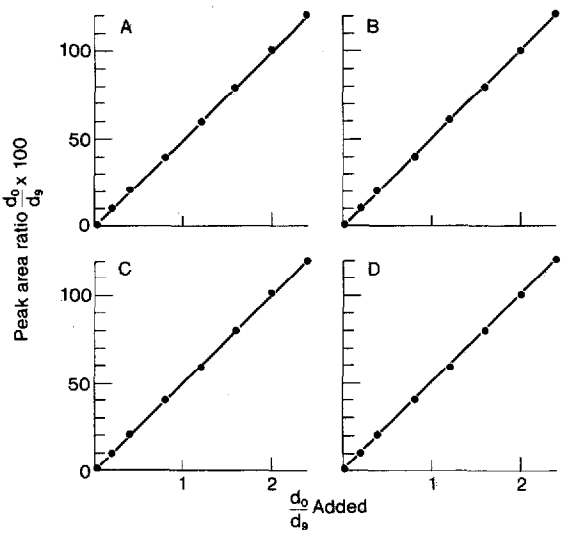

Fig. 5. Standard curve for the quantitation of malonic (A), succinic (B), maleic (C) and citric (D) acids as the *n*-butyl-d_9-esters: d_0 and d_9 refer to the natural and deuterated *n*-butyl esters of the organic acids, respectively.

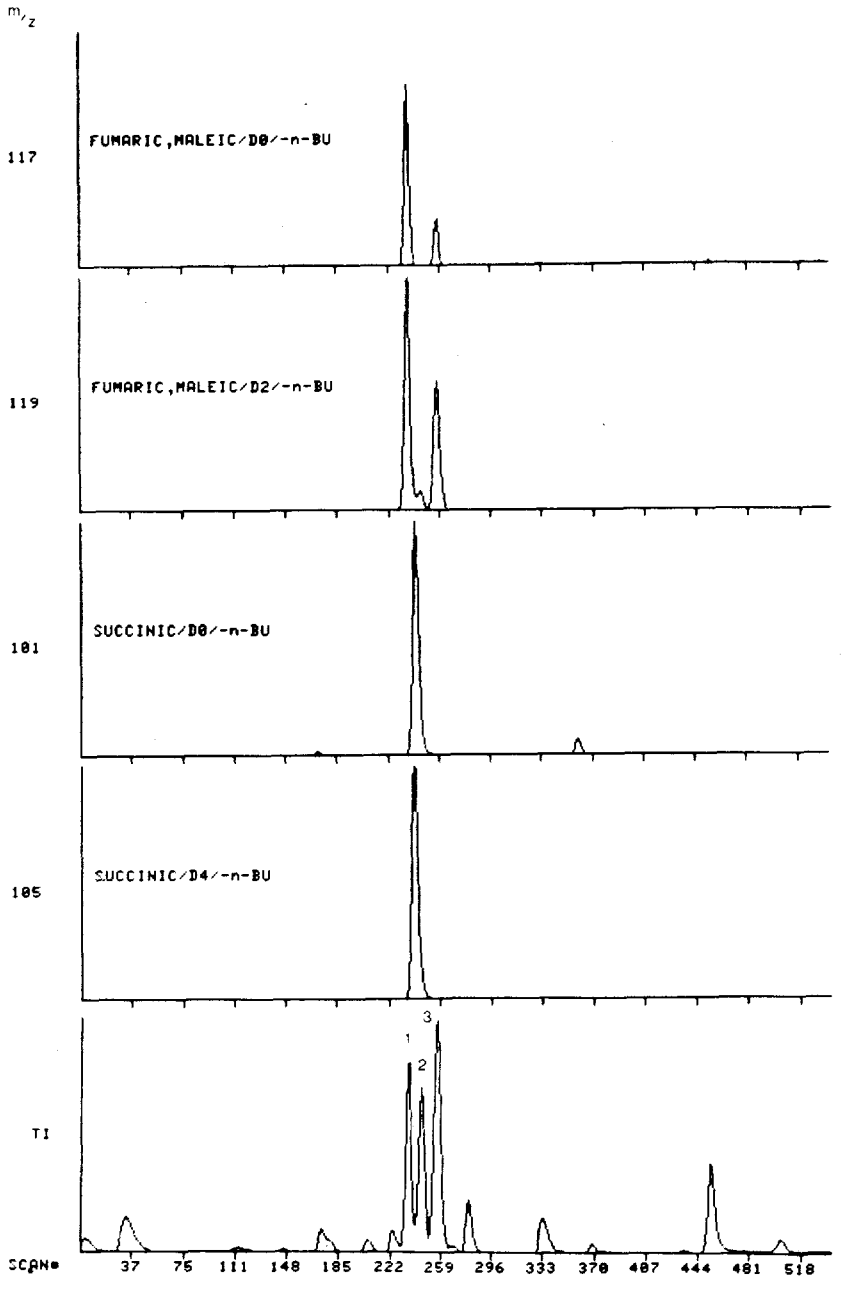

Fig. 6. Mass chromatograms of *n*-butyl esters of Krebs cycle and related acids of rat liver as obtained in the presence of furmaric-d_2 and succinic-d_4 acids as internal standards. GC–MS conditions and ion fragments as given in test. TI = Total ion current profile.

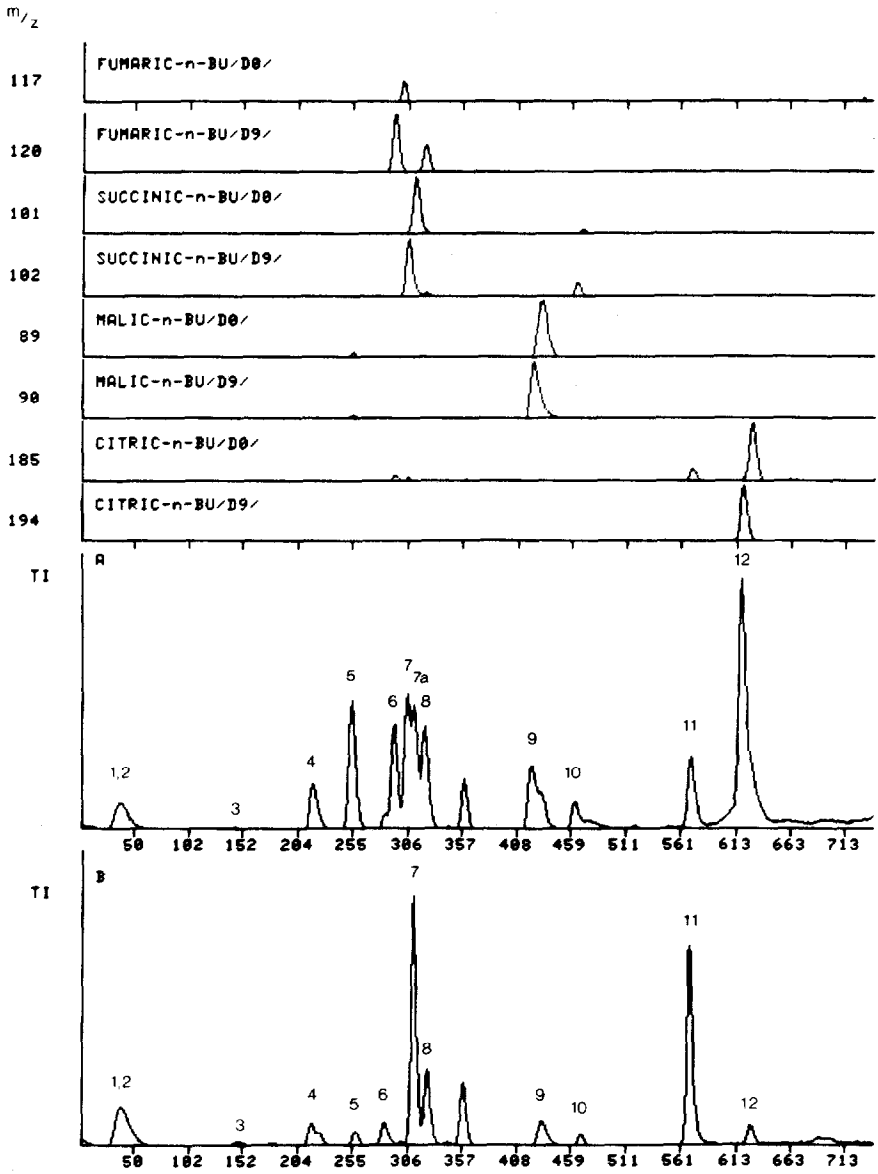

Fig. 7. Mass chromatograms of *n*-butyl esters of Krebs cycle and related acids of rat liver as obtained in the presence of *n*-butyl-d_9-esters of fumaric, succinic, maleic and citric acids as internal standards. GC–MS conditions and ion fragments as given in text. A = total ion current profile of unknown acids and internal standards; B = total ion current profile of unknown acids.

relative to the *m/z* 129 of adipic acid for all the acids examined in the present study. The corresponding mass ratios were determined from the hydrogen flame ionization response using mixtures of organic acid esters in mass quantities sufficiently large for accurate weighing. It is seen that for some acids the response is less than 10% of that of the other acids, although many acid esters give comparable responses in the mass

spectrometer. In all instances the relative ion yields were found to be readily reproducible and provided reliable quantitation.

Quantitation of unknown acids

Fig. 6 shows a mass chromatogram obtained for a mixture of Krebs cycle and related acids of rat liver in the presence and absence of deuterated organic acids as internal standards. Only a minor distortion of the total ion current profile results from the presence of the deuterated analogs. However, the presence of both deuterated and undeuterated acids can be seen from the selected ion plots and the corresponding areas can be quantitatively related as shown below. Fig. 7 shows a mass chromatogram obtained for a mixture of the Krebs cycle and related acids of rat liver in the presence and absence of the *n*-butyl-d_9-ester standards. In this case a marked distortion is seen for the total ion profile due to the resolution of many of the deuterated and non-deuterated *n*-butyl esters. However, the presence of each deuterated and non-deuterated acid can be clearly discerned by means of the single ion plots also shown in the figure. As in Fig. 6 the corresponding areas can be quantitatively related to each other and to any internal mass standards that may be included in addition. Table IV gives the results obtained for the Krebs cycle and related acids of rat liver by means of the two stable isotope dilution methods of analysis along with the data obtained by conventional analyses reported in the literature. The values obtained by GC–MS for fumaric, malic, α-ketoglutaric, β-hydroxybutyric and citric acids on the basis of the mass chromatograms are comparable to those obtained by the enzymatic[1,2,16–18] and GC[3] methods. However, the succinic acid content in these livers was about six times higher than that previously reported[1,3]. Other tissues,

TABLE IV

ANALYSIS OF KREBS CYCLE AND RELATED ACIDS IN RAT LIVER (μg/g WET WEIGHT)

Organic acid	*Conventional analysis**	*Mass fragmentography*						
		*Deuterated acids***		*Deuterated esters****				
Lactic	42.2–428.0			621.4				
Pyruvic								
β-Hydroxy-butyric	18.2–38.5				11.7	11.4		
Oxalic				43.3				
Malonic				3.0	3.0	3.0		
Fumaric	3.0–10.0	8.1	8.1	7.8	8.0	8.2		
Succinic	25.8–40.1	167.5	168.3	163.2	163.6	165.0	170.9	170.9
Maleic		5.6	5.9	4.8	5.8	6.1	6.0	
Malic	36.8–120.0			53.3	53.4	53.0		
α-Ketoglutaric	18.6–93.4			21.7	21.9			
Citric	37.8–79.8			12.6	12.8	12.9		

* Values for conventional analyses taken from various authors[1–3,16–18], who analyzed one or more of the organic acids. Tissue contents given in the literature on a dry weight basis have been divided by a factor 4.94[18].

** Duplicate analysis using deuterated acids as internal standards.

*** Replicate analyses using deuterated *n*-butyl esters as internal standards.

e.g. heart (results not shown), gave closely comparable values by the GC and GC–MS methods. There have been no previous reports on the separate estimation of maleic acid in rat liver.

DISCUSSION

GC estimation of Krebs cycle and related acids in the form of their butyl ester derivatives has proved to be a distinct advantage because of their lower volatility, more uniform weight response in the flame-ionization detector[19], and the possibility of resolution of enantiomers via diastereoisomers, when employing enantiomeric isobutanol in the derivatization[20]. In addition, the butyl ester derivatives of these acids yield characteristic mass spectra, which can be used for their unambiguous identification in natural extracts[12,15,20]. However, because of artifact formation with keto acids, quantitation becomes difficult[12,21]. In recent years, the Krebs cycle and related acids have been resolved by high-performance liquid chromatography, which has overcome the problem of volatility, but quantitation has remained a problem because of a lack of sensitive detector[5,22]. A conversion of the acids into ultraviolet-absorbing or fluorescent derivatives requires the exposure to strong acids and again leads to artifact formation comparable to that experienced during butylation[23,24]. The present study shows that many of these difficulties may be overcome by the use of deuterium-labelled acids or *n*-butyl-d_9-esters as internal standards. The use of the deuterated acids is limited to the few stable species, such as succinic, fumaric, maleic, citric, lactic and malic acids, but caution may still be needed when adding these acids directly to hot acid extracts. Previously, Pereira *et al.*[11] have shown that, when deuterated amino acid mixtures are added directly to soil samples, extensive hydrogen–deuterium exchange occurred during acid hydrolysis of the soil extract. The removal of the isotopic label was catalyzed by the hot inorganic acid in the presence of excess mineral in the soil-hydrolysis step. Kvenvolden *et al.*[25] have reported a similar finding concerning decomposition of amino acids in soil upon direct acid hydrolysis. However, when the deuterated amino acid mixture was added just before derivatization (*i.e.* after hydrolytic extraction of the soil) the problem was avoided. Furthermore, Pereira *et al.*[11] have been able to quantitate the free amino acid content of complex mixtures, such as those in serum and urine samples, by adding the deuterated amino acid mixtures directly to the sample before processing without any deleterious effects. The exchange of hydrogen in the keto acids has been previously demonstrated by Cronholm[26] and by Marai and Kuksis[12]. Clearly, the use of deuterated acids as internal standard for the quantitation of organic acids may lead to loss of the isotopic label and must be guarded against by appropriate preliminary experiments.

The method proposed for overcoming the difficulty requires the use of deuterium-containing alcohol esters, *e.g.* *n*-butyl-d_9-derivatives. It allows an apparently effective identification and quantitation of all or most of the known organic acids of interest including the by-products and artefacts of the esterification procedure. Although this method of quantitation gives results similar to those obtained with the deuterated acids as internal standard, it is somewhat less satisfying theoretically, as it does not allow to take the internal standard through all the steps of sample preparation. However, this may not be a major problem when isolating the acids as salts and avoiding solvent evaporations until after the butylation has been completed and

the internal standard ester added. Potentially more serious may be the problem of differential yields of the simple esters and the mono- and diketals of the keto acids under the two separate esterification reactions. However, this problem may be minimized by employing identical reaction conditions for both unknown sample and standard derivatization, including the addition of potassium perchlorate to the standard ester-preparation mixture, in amounts comparable to those present in the unknown organic acid extract.

In principle, the problems arising from the use of deuterium-labeled internal standards could be overcome by employing ^{13}C-labeled acids instead. For the time being this approach must be ruled out as impractical because of the expense involved and the unavailability of the ^{13}C-standard acids. The use of the *n*-butyl-d_9-derivatives appears to be an inexpensive and effective approach to quantification of organic acids, which combines the advantages of mass fragmentography and gas chromatography of these esters The use of quaternary ammonium salts for butylation and mass spectral identification of volatile organic acids[27] deserves exploration with deuterium-containing derivatives.

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