differing functionality.

Registry No. *Z*4-10Ac, 67452-27-1; *E*4-10Ac, 69222-16-8; *Z*5-10Ac, 67446-07-5; *E*5-10Ac, 38421-90-8; *Z*7-10Ac, 13857-03-9; Δ9-10Ac, 50816-18-7; *Z*5-11Ac, 67452-29-3; *E*5-11Ac, 67446-06-4; *Z*6-11Ac, 68760-71-4; *Z*9-11Ac, 56218-82-7; Δ10-11Ac, 112-19-6; *Z*2-12Ac, 84801-15-0; *E*2-12Ac, 84801-16-1; *E*3-12Ac, 56218-63-4; *Z*8-12Ac, 28079-04-1; *E*8-12Ac, 38363-29-0; *Z*9-12Ac, 16974-11-1; *E*5-13Ac, 75568-03-5; *Z*6-13Ac, 68760-66-7; *E*6-13Ac, 84801-17-2; *Z*10-13Ac, 64437-24-7; *E*10-13Ac, 64437-33-8; Δ12-13Ac, 84801-18-3; *Z*2-14Ac, 51309-20-7; *E*2-14Ac, 51309-21-8; *Z*4-14Ac, 54897-66-4; *Z*8-14Ac, 35835-80-4; *Z*9-14Ac, 16725-53-4; *Z*11-14Ac, 20711-10-8; *Z*4-15Ac, 65954-21-4; *E*4-15Ac, 65954-22-5; *Z*5-15Ac, 70711-44-3; *Z*10-15Ac, 64437-43-0; *E*10-15Ac, 64437-45-2; *Z*11-15Ac, 35153-25-4; *Z*6-16Ac, 34010-19-0; *E*6-16Ac, 56218-66-7; *E*9-16Ac, 56218-69-0; *Z*11-16Ac, 34010-21-4; *Z*13-16Ac, 56218-74-7; *E*13-16Ac, 69282-67-3; *Z*11-17Ac, 73461-65-1; *E*11-17Ac, 84801-19-4; *E*6-18Ac, 84801-20-7; *Z*9-18Ac, 693-80-1; *Z*11-18Ac, 6186-98-7; *E*11-18Ac, 69282-64-0; *Z*4-10Ac *threo*-DMDS, 84801-21-8; *E*4-10Ac *erythro*-DMDS, 84801-22-9; *Z*5-10Ac *threo*-DMDS, 84801-23-0; *E*5-10Ac *erythro*-DMDS, 84801-24-1; *Z*7-10Ac *threo*-DMDS, 84801-25-2; Δ9-10Ac DMDS, 84801-26-3; *Z*5-11Ac *threo*-DMDS, 84801-27-4; *E*5-11Ac *erythro*-DMDS, 84801-28-5; *Z*6-11Ac *threo*-DMDS, 84801-29-6; *Z*9-11Ac *threo*-DMDS, 84801-30-9; Δ10-11Ac DMDS, 84801-31-0; *Z*2-12Ac *threo*-DMDS, 84801-32-1; *E*2-12Ac *erythro*-DMDS, 84801-33-2; *E*3-12Ac *erythro*-DMDS, 84801-34-3; *Z*8-12Ac *threo*-DMDS, 84801-35-4; *E*8-12Ac *erythro*-DMDS, 84801-36-5; *Z*9-12Ac *threo*-DMDS, 84801-37-6; *E*5-13Ac *erythro*-DMDS, 84801-38-7; *Z*6-13Ac *threo*-DMDS, 84801-39-8; *E*6-13Ac *erythro*-DMDS, 84801-40-1; *Z*10-13Ac *threo*-DMDS, 84801-41-2; *E*10-13Ac *erythro*-DMDS, 84801-42-3; Δ12-13Ac DMDS, 84801-43-4; *Z*2-14Ac *threo*-DMDS, 84801-44-5; *E*2-14Ac *erythro*-DMDS, 84801-45-6; *Z*4-14Ac *threo*-DMDS, 84801-46-7; *Z*8-14Ac *threo*-DMDS, 84801-47-8; *Z*9-14Ac *threo*-DMDS, 84801-48-9; *Z*11-14Ac *threo*-DMDS, 84801-49-0; *Z*4-15Ac *threo*-DMDS, 84801-50-3; *E*4-15Ac *erythro*-DMDS, 84801-51-4; *Z*5-15Ac *threo*-DMDS, 84801-52-5; *Z*10-15Ac *threo*-DMDS, 84801-53-6; *E*10-15Ac *erythro*-DMDS, 84801-54-7; *Z*11-15Ac *threo*-DMDS, 84801-55-8; *Z*6-16Ac *threo*-DMDS, 84801-56-9; *E*6-16Ac *erythro*-DMDS, 84801-57-0; *E*9-16Ac *erythro*-DMDS, 84801-58-1; *Z*11-16Ac *threo*-DMDS, 84801-59-2; *Z*13-16Ac *threo*-DMDS, 84801-60-5; *E*13-16Ac *erythro*-DMDS, 84801-61-6; *Z*11-17Ac *threo*-DMDS, 84801-62-7; *E*11-17Ac *erythro*-DMDS, 84801-63-8; *E*6-18Ac *erythro*-DMDS, 84801-64-9; *Z*9-18Ac *threo*-DMDS, 84801-65-0; *Z*11-18Ac *threo*-DMDS, 84801-66-1; *E*11-18Ac *erythro*-DMDS, 84801-67-2; DMDS, 624-92-0.

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Quaternary Ammonium Salts for Butylation and Mass Spectral Identification of Volatile Organic Acids

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The esterification method described by Greeley (*J. Chromatogr.* 1974, *88*, 229) was modified to overcome the problem of volatile acid evaporation during sample workup procedures. After solvent extraction from an aqueous sample, the acids were converted to their butyl ester derivatives in a two-step process. Firstly, an involatile quaternary ammonium salt was formed which allowed concentration by solvent evaporation without loss of volatile acids. The acid salts were then converted to butyl esters by heating in the presence of *n*-butyl bromide and the esters were separated on a support-coated open tubular (SCOT) OV17 capillary column. The recovery and butylation yield were shown to be quantitative for isobutyric acid and the linearities and limits of detection are given for the five C_4–C_5 aliphatic acids. The mass spectra of butyl esters contain abundant rearrangement ions not present in methyl, ethyl, trimethylsilyl, or aryl esters which enable their rapid identification. The method was applied to the analysis of volatile urinary acids from a patient with Maple Syrup Urine Disease and showed conclusively the presence of both 2-methylbutyric and isovaleric acids.

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An increasingly important requirement for the elucidation of the metabolic defects underlying some diseases, most notably the inborn errors of metabolism associated with branched chain amino acids, is the identification of all the short chain volatile organic acids found in the body fluids of individuals with such diseases. The identification of isovaleric acid led to the discovery of one of the first inborn errors of metabolism, Isovaleric Acidemia (*1*), and an error in identification of isovaleric acid was the cause of a misdiagnosis in another case (*2*).

The identification and quantitation of short chain volatile carboxylic acids are also necessary to characterize the metabolic products of anaerobic bacteria in both microbiological (*3*) and dental (*4*) applications.

The qualitative analysis of organic acids in biological fluids is usually performed by solvent extraction of the acid fraction followed by solvent evaporation, derivatization, and gas chromatography (*5*). The volatility of short chain acids may result in losses during their extraction from biological fluids and subsequent concentrations, so previous workers have used specialized vacuum distillation techniques for these compounds (*6*, *7*). Although physically complex and necessitating specialized equipment, many workers still follow this procedure (*8*) or one with minor variations (*9*) since no effective

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alternative has been available. However, recently L'Emeillat et al. (*10*) have applied extractive alkylation (*11*, *12*) to the determination of these compounds as their *p*-bromophenacyl esters, but the mass spectra of these esters do not contain characteristic ions.

Loss of acids when evaporating the solvent causes irregular recoveries, but this factor may be overcome by reducing the volume of the extract. However in this instance residual water and solvent impurities become more concentrated along with the metabolites, and these contaminants then cause interference in subsequent derivatization and gas chromatography.

Derivatization of organic acids is essential since their polar nature causes interaction with the GC column which results in "ghosting" (reversible adsorption) and tailing, thus destroying both qualitative and quantitative information (*13*). Esterification with either diazomethane or silylating reagents decreases their polarity and thus improves GC characteristics but also increases the volatility of the acids (*14*). This causes some acids to cochromatograph with the solvent and results in excessive losses during workup. Mass spectra of these acid derivatives do not contain many characteristic ions, and this makes unequivocal identification of isomeric compounds difficult.

The method we present in this paper overcomes the problems associated with volatile acid extraction, concentration, and derivatization by using a two-step procedure yielding acid derivatives with excellent chromatographic properties and very characteristic mass spectra. The acids were extracted from urine with diethyl either and were converted to involatile salts by the addition of a quaternary ammonium hydroxide before the excess diethyl ether was evaporated. Thus subsequent evaporation of the extract to dryness without loss of volatile acids was possible. The quaternary salts were then converted to esters prior to GC analysis by heating with butyl bromide in dimethylacetamide (*12*, *15*). Korte (*16*) has also used a modification of Greeley's (*15*) procedure for the benzylation of short chain organic acids; however, extraction of the acids from an aqueous medium was not tested and it is not clear whether the esters are formed prior to GC analysis or by thermal degradation of the quaternary ammonium acid salts on injection into the GC.

EXPERIMENTAL SECTION

Gas Chromatograph. A Varian 1800 dual column, dual flame ionization detector (FID) gas chromatograph was used for this work. The capillary column was connected to the injection port and detector with glass-lined stainless steel tubing (GLT) (0.4 mm i.d.). A zero dead volume "T" piece was placed in the column to FID GLT line inside the detector oven and the "T" outlet was connected with GLT (0.4 mm i.d.) to a microneedle valve (SGE, Melbourne) and thence to the GC/MS interface. A 60-m SCOT OV17 capillary column (SGE, Melbourne, No. 566/85) was used with helium (5 mL/min) as carrier gas.

Mass Spectrometer. The column outlet from the Varian 1800 GC was connected to an Electronics Associates Inc. Quadrupole 300 D mass spectrometer via a single stage jet separator (SGE, Melbourne) and GLT. The MS could be operated manually or by a Data General Nova 1200 minicomputer through an EAI 55 interface (Digital Electronics, Sydney). Machine variables were set as follows: filament emission current, 50 μA; electron energy, 50 V; extractor, 12 V; ion energy, 8 V; electron multiplier 2000 V; resolution, 29; analyzer pressure, 1×10^{-5} torr.

Preparation of Tetrabutylammonium Hydroxide. Tetrabutylammonium bromide (4.5 g, 14 mmol) was dissolved in 50 mL of dry methanol (distilled from MgOEt) and then silver oxide (8.8 g, 39 mmol) was added and the mixture was stirred for 30 min after which dry methanol (50 mL) and ethanol (30 mL) were added to the mixture.

This solution was filtered to remove the silver iodide/silver oxide slurry and centrifuged at 800*g* to remove remaining suspended material. The resulting alcoholic mixture was stored in plastic bottles (as storage in glass leads to degradation of this reagent) and was analyzed by TLC for unreacted tetrabutylammonium bromide. The shelf life of this reagent in plastic bottles was not determined.

Solvent Extraction of Organic Acids from Aqueous Solutions. The urine or standard sample (2 mL) was placed in a stoppered centrifuge tube and HCl (6 M) was added till the pH of the solution was 1. NaCl (600 mg) was added to saturate the solution. Diethyl ether (2 mL) was then added to the sample, and the two phases were mixed thoroughly for 2 min. If a suspension had formed the tube was centrifuged at 800*g* for 2–5 min to separate the aqueous and organic phases before the organic phase was transferred to a test tube. The extraction was repeated twice more and the pooled organic extract was dried by addition of $MgSO_4$ (1 g) and then filtered into a culture tube (10 mL capacity).

Butylation of Organic Acids. The filtered and dried acid extract was adjusted to a pH of 10–12 by addition of tetrabutylammonium hydroxide (TBA-OH) solution. The solvent was then evaporated under a stream of dry nitrogen while the culture tubes were heated to 50 °C. A solution of dimethylacetamide (DMA, 500 μL) and a 10-fold molar excess of *n*-butyl bromide over moles of TBA-OH was added to the culture tube, and the mixture was heated at 100 °C for 60 min.

The mixture was cooled and then extracted three times with pentane (1 mL) and the pooled pentane extract was washed with water (0.5 mL) and then transferred to a small sample vial and the pentane was evaporated under a stream of nitrogen. An aliquot (0.05 μL) of the solution was then injected into the GC.

Calibration Curves. Calibration points were prepared by using 4×30 μL multiples of a solution containing the acids isobutyric, butyric, 2-methylbutyric, isovaleric, and valeric at concentrations of 1.9, 2.0, 1.8, 1.8, and 1.8 μM, respectively. The volumes were adjusted to 2 mL with H_2O and then the extraction and butylation procedure given above was followed save for the addition of cyclohexyl acetate (50 μL, 3.4 mM) as internal standard to the pooled pentane layer before final evaporation. Peak areas were calculated by triangulation and the mean of five determinations of the relative (to the internal standard) areas was used as the dependent variable in a linear regression analysis.

RESULTS AND DISCUSSION

Quaternary alkylammonium salts of organic acids can be injected directly into the GC where they are converted to the alkyl ester and the tertiary alkylamine byproduct in the injection port (*16*).

Although the alkyl ester can be produced in this manner we found that this procedure rapidly degraded the GC column and the tertiary alkylamine byproduct interfered in the GC analysis. We also observed that hexamethylcyclohexasiloxane and octamethylcylcooctasiloxane were liberated when the reaction mixture containing tetramethylammonium salts was injected onto the GC column. This suggested that a reaction between excess quaternary alkammonium hydroxide and the glass column or silylated solid support material occurred. Formation of siloxanes also seemed to occur during storage of the alcoholic quaternary alkylammonium hydroxide in a glass container.

To overcome these problems the quaternary salts were converted to esters prior to the GC analysis by heating with butyl bromide in diemthylacetamide (DMA) (*12*, *15*). Butyl bromide was chosen because the decreased volatility of the resulting butyl esters allowed ready separation from the solvent on low polarity GC columns (packed or capillary). In addition the mass spectra of these compounds contained several diagnostic rearrangement ions involving the butyl side chain which allowed improved mass spectral identification (*17*).

The esters were then extracted from this solution with pentane and concentrated by removing the excess pentane with dry nitrogen. Traces of DMA were removed from the pentane extract by washing with water prior to its evaporation.

Table I. Calibration Curves for Five Volatile Organic Acids[a]

ester	rel peak areas and coefficients of variation (in parentheses) for abcissa points 1	2	3	4	linear regression analysis: Y intercept	X intercept (detection limit)	slope	corr coeff
butyl isobutyrate	0.13 (14)	0.60 (9)	0.94 (3)	1.26 (4)	−0.20	0.306	0.654	0.996
butyl butyrate	0.16 (10)	0.52 (7)	1.29 (4)	1.86 (3)	−0.51	0.521	0.978	0.492
butyl 2-methylbutyrate	0.15 (6)	0.57 (6)	0.91 (3)	1.28 (7)	−0.21	0.304	0.691	0.999
butyl isovalerate	0.13 (5)	0.54 (4)	0.88 (4)	1.39 (5)	−0.30	0.393	0.763	0.997
butyl valerate	0.13 (17)	0.46 (3)	0.80 (9)	1.21 (4)	−0.25	0.377	0.663	0.999

[a] The amounts of each acid used for abcissa points 1-4 are given in the text. Units for the independent variable were 10^{-7} mol.

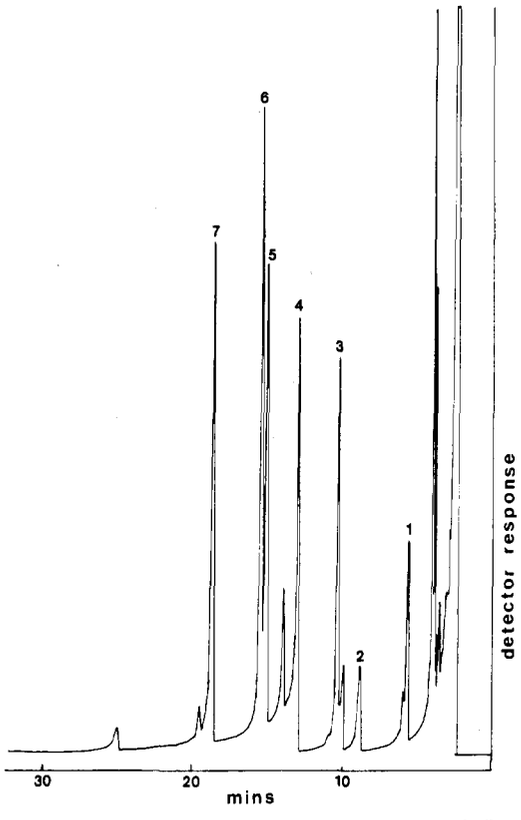

Figure 1. Chromatogram of the butyl esters of seven volatile organic acids extracted from urine. Identifications (and relative retentions) are as follows: 1 = *n*-butyl acetate (0.37), 2 = *n*-butyl propionate (0.59), 3 = *n*-butyl isobutyrate (0.68), 4 = *n*-butyl *n*-butyrate (0.88), 5 = *n*-butyl 2-methylbutyrate (1.00), 6 = *n*-butyl isovalerate (1.03), 7 = *n*-butyl *n*-valerate (1.24). Column oven temperature program was as follows: 70 °C for 4 min, and then 3 °C/min to 214 °C.

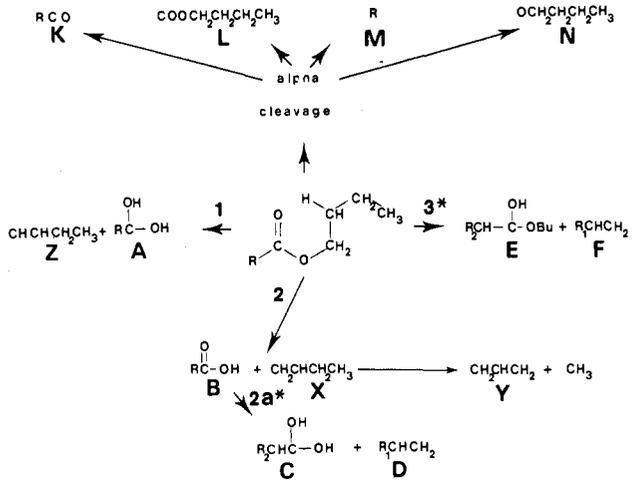

Figure 2. Major framentation pathways of butyl esters of organic acids. The numbers over the arrows show the pathway referenced in the text and the letters under the fragments are references in Table II. *These pathways (i.e., 2a, 3) are only possible when R = R′-CH_2-CH_2-CH_2-, and Bu = butyl moiety (i.e., -$CH_2CH_2CH_2CH_3$).

A chromatographic and detection system other than GC/MS may be used by alkylating the acids with a different alkyl group, e.g., benzyl bromide, to introduce a chromaphore for ultraviolet detection with high-pressure liquid chromatography.

Recovery, Linearity, and Detection Limits. The efficiency of the extraction and butylation procedure was tested by using isobutyric acid as a model with two internal standards. An aqueous solution of isobutyric acid (500 μL, 4.6 mM) was extracted and an equimolar amount of butyric acid (500 μL, 4.6 mM) was added to the extract. The acids were then converted to their butyl esters as described above, and an equimolar amount of butyl 2-methylbutyrate (50 μL, 46 mM) was added. The amount of butyl isobutyrate relative to butyl butyrate (first internal standard) recovered thus gave a measurement of the efficiency of the extraction procedure while the relative amounts of butyl butyrate and butyl 2-methylbutyrate (second internal standard) gave a measurement of the efficiency of the conversion of butyric acid to butyl butyrate. The solvent extraction of the acid was 96.3% efficient ((amt of butyl isobutyrate)/(amt of butyl butyrate) = 0.963, coefficient of variation = 7.4%, n = 5), the butylation was 96.6% efficient (amt of butyl butyrate)/(amt of butyl 2-methylbutyrate) = 0.969, coefficient of variation = 7.2%), and the overall procedure resulted in a 93.1% yield (amt of butyl isobutyrate)/(amt of butyl 2-methylbutyrate) = 0.931, coefficient of variation = 7.8%).

The linearity of the method was tested by constructing calibration curves for the five C_4–C_5 acids over the concentration range of (50–250) × 10^{-9} mol extracted. The results given in Table I show an excellent linear relationship for each acid between the amount detected and the amount used (correlation coefficients 0.994–0.999). The extrapolated detection limits range from 30.4 to 52.1 nmol of acid extracted. The high value for butyl butyrate is due to interference from a closely eluting component.

Figure 1 shows the GC profile obtained by extraction and butylation as described of a normal urine sample to which was added to following at a concentration of approximately 3 μM: acetic, propionic, butyric, isobutyric, 2-methylbutyric, isovaleric, and *n*-valeric acids.

Of the acids tested, the derivatives of 2-methylbutyric and 3-methylbutyric (isovaleric) acids presented the greatest chromatographic resolution problem. When not derivatized the similiarity of the mass spectra (electron impact) and volatilites of these two compounds make their identification in biological extracts particularly difficult. When butylated,

Table II. Mass Values Corresponding to the Characteristic Fragments Shown in Figure 2

name	R-	R_2	fragment[a] A	B	C	E	D/F	K
acetic	CH_3-	na	61	60				43
propionic	CH_3CH_2-	na	75	74				57
butyric	$CH_3(CH_2)_2$-	H	89	88	60	116	28	71
isobutyric	$(CH_3)_2CH_2$-	na	89	88				71
isovaleric	$(CH_3)_2CHCH_2$-	H	103	102	60	116	42	85
2-methylbutyric	$CH_3CH_2CH(CH_3)$-	CH_3	103	102	74	130	28	85
n-valeric	$CH_3(CH_2)_3$-	H	103	102	60	116	42	85
caproic	$CH_3(CH_2)_4$-	H	117	116	60	116	56	99
heptanoic	$CH_3(CH_2)_5$-	H	131	130	60	116	70	113
caprylic	$CH_3(CH_2)_6$-	H	145	144	60	116	84	127

[a] Fragments L, M, N, X, and Y are common to all butyl esters and have mass values of 101, 57, 73, 56, and 41, respectively.

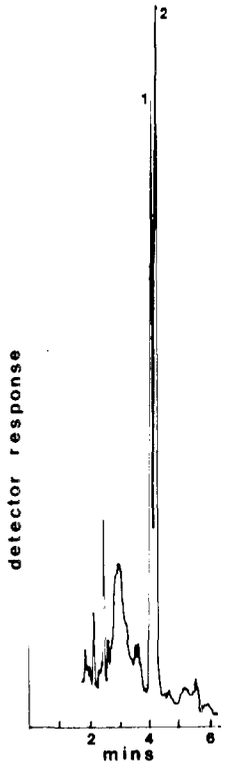

Figure 3. A portion of the chromatogram of the butylated urinary organic acids from a patient with Maple Syrup Disease: 1 = *n*-butyl 2-methylbutyrate, 2 = *n*-butyl isovalerate; column, 20-m SE54, Jaeggi, Switzerland; temperature program, 60 °C, 4 °C/min to 250 °C; instrument, Varian MAT 44; carrier gas, hellum, 2 L/min.

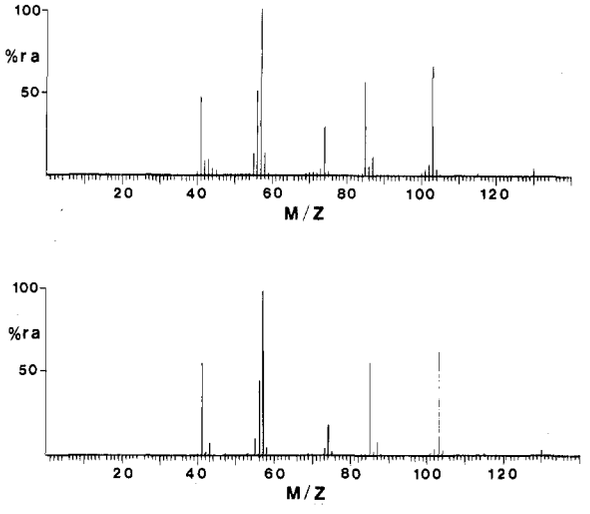

Figure 4. Mass spectra of authentic *n*-butyl 2-methylbutyrate (top) and peak 1, Figure 3 (bottom).

however, their gas chromatographic separation is enhanced and their mass spectra contain two diagnostic ions. The structures and the fragmentation pathways leading to these ions are given in Figure 2.

Mass Spectra of Butyl Esters of Short Chain Fatty Acids. The utility of mass spectrometry in rapidly identifying newly encountered metabolites depends on the extent of reference mass spectra available. When imporved analytical techniques allow the observation of previously unencountered metabolites and their derivatives, reference data may not exist. To allow the use of this method in such areas we have detailed the fragmentation pathways of *n*-butyl esters of saturated monocarboxylic acids and have compiled lists of characteristic mass ions which may act as an aid in the identification of unknown compounds. In addition the gas chromatographic properties and mass spectra of the butyl ester derivatives of some dicarboxylic acids have recently been reported (*18*).

The mass spectra of the butyl esters of the acids used in this work, except for butyl 2-methylbutyrate, have been previously reported (*19*). The mass spectrum of butyl 2-methylbutyrate is presented in the section below.

The mechanisms for the formation of the ions observed in the mass spectra of butyl esters are discussed in detail elsewhere (*20–24*) and summarized in Figure 2. The availability of a γ-hydrogen from the butyl moiety of this derivative enables rearrangements to occur yielding characteristic ions which are not observed in methyl, ethyl, trimethylsilyl, or aryl type esters. The mass values of these ions are shown in Table II. Thus in addition to improving separation of the acids by GC, butylation affords compounds which are readily identified by mass spectrometry. The most diagnostic ion has the formula $(RCOO + 2H)^+$ and results from the rearrangement of two hydrogen atoms (*25–27*). This is shown as fragmentation pathway 1 in Figure 2 yielding the ion A. We found that this was the most useful ion in the spectrum since its high abundance and unusual mass number make it easily recognizable and it immediately identifies the molecular weight of the acid from which the ester is derived.

Application to Maple Syrup Urine Disease. The profile of organic acids in Maple Syrup Urine Disease (MSUD) has been well documented (*28*); however, when investigating the volatiles profile, by collection of the volatiles on Chromasorb 105 and thermal desorption (*29*), associated with this disease, we found evidence for the presence of some short chain volatile acids. To show that these compounds were not artifacts of our volatiles profiling procedure, the method of extracting and butylating volatile acids described above was applied to this case. Figure 3 shows a portion of the chromatogram of volatile acids obtained from an MSUD patient's urine, using the

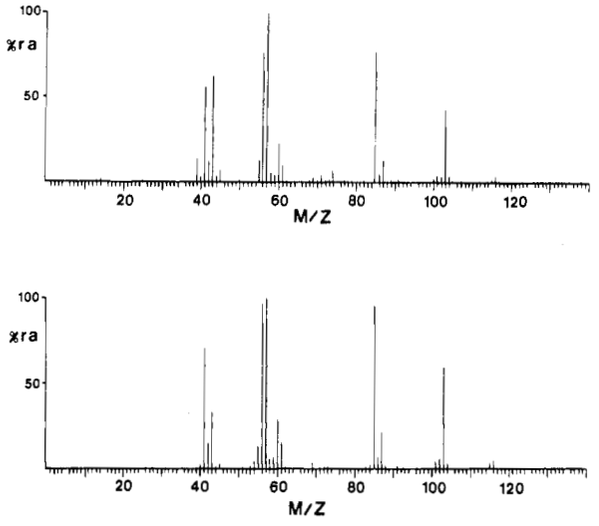

Figure 5. Mass spectra of authentic *n*-butyl isovalerate (ref 18, top) and peak 2, Figure 3 (bottom).

method described in this work.

The mass spectra of the two peaks and standard compounds are given in Figures 4 and 5 and illustrate the application of Table II to the identification of the two components. All the ions common to butyl esters (m/z 101, 73, 57, 56 41) are present in these spectra. The elimination of these common ions leaves only four other significant ions above mass 50 (m/z 74, 85, 103, and 130 for Figure 4 and m/z 60, 85, 103, and 116 for Figure 5). the only volatile acid in Table I for which all the characteristic ions in Figure 4 occur is 2-methylbutyric; thus peak 1 can be identified by using its mass spectrum alone. However, the mass spectra of both isovaleric and *n*-valeric acids contain the characteristic ions observed in the mass spectrum of peak 2, but since these acids are readily separated by GC as their butyl esters (relative retentions to butyl 2-methylbutyrate are 1.03 and 1.24, respectively) peak 2 was identified as isovaleric acid.

Registry No. Butyl bromide, 109-65-9; acetic acid, 64-19-7; propionic acid, 79-09-4; butyric acid, 107-92-6; isobutyric acid, 79-31-2; isovaleric acid, 503-74-2; 2-methylbutyric acid, 116-53-0; valeric acid, 109-52-4; caproic acid, 142-62-1; heptanoic acid, 111-14-8; caprylic acid, 124-07-2; butyl 2-methylbutyrate, 15706-73-7.

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