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Profiling of organic acids by capillary gas chromatography–mass spectrometry after direct methylation in urine using trimethyloxonium tetrafluoroborate

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Abstract

Trimethyloxonium tetrafluoroborate (TMO) is applied as derivatising reagent to transform urinary organic acids into their methyl esters. The method is suggested as an alternative to the use of diazomethane which is carcinogenic and explosive. In contrast to other methods avoiding diazomethane, such as derivatizations with acetyl chloride–methanol and boron trifluoride–methanol, which require an organic reaction medium and therefore an extraction of the organic acids from the urine, TMO efficiently reacts with the acids in an aqueous solution and can therefore be directly applied to native urine. The use of TMO simplifies and improves the sample preparation in the profile analysis of urinary organic acids by capillary GC–MS and hereby increases the speed of analysis. The method gives reproducible results which are comparable with the data obtained using conventional solid-phase extraction with strong anion-exchange cartridges prior to derivatisation.

Keywords: Derivatization, GC; Methylation; Organic acids; Trimethyloxonium tetrafluoroborate

1. Introduction

The analysis of organic acids continues to be an important diagnostic tool to recognize and characterize a number of metabolic disorders, in particular inherited defects of amino acid metabolism connected with organic acidurias [1]. Many of these inborn errors of metabolism show no apparent abnormalities at birth, but in early childhood they lead to severe clinical manifestations, such as mental and growth retardation, neurologic defects and different organ failures, sometimes resulting in life-threatening complications. Since in many cases clinical manifestations can be prevented if the disorder is diagnosed early and appropriate treatment, for instance dietary protein and amino acid restriction, is started soon, recognition of the disorder is essential. In newborn screening profile analysis of urinary organic acids is decisive in excluding or confirming and identifying a disorder leading to an organic aciduria.

Also in acquired metabolic and other diseases abnormal concentrations of certain organic acids are found. In patients with diabetes mellitus an increased excretion of hydroxycarboxylic and dicarboxylic acids in urine is observed [2,3]. Elevated serum levels of furancarboxylic acids, especially 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid, are found in patients with chronic renal failure [4], and phenol-

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carboxylic acids are increased in urine of patients with severe liver diseases [5].

Commonly the organic acids are analysed by gas chromatography (GC) or GC–mass spectrometry (GC–MS). Applications using high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been described [6,7] with the advantage of shorter analysis time as compared to GC. However, due to the lower resolving power of HPLC and limitations in the detection sensitivity of CE, GC methods are more suitable for profile analysis of organic acids. In urine and serum the organic acids occur in a wide range in structure and polarity, and their analysis by GC requires sample preparation, in particular derivatisation of the carboxyl group in order to increase the volatility of the acids [8–10]. The most common derivatives are silyl products and methyl esters. Methyl esters have the advantage that they are more stable than silyl products, particularly against moisture, and their mass spectra are often easier to interpret.

Diazomethane is a very effective methylating reagent for organic acids [8]. However, because of its carcinogenic and explosive properties, working with diazomethane involves certain risks. Therefore other methylating procedures are of interest. Acetyl chloride–methanol and boron trifluoride–methanol are common reagents to transform fatty acids into their methyl esters [11,12] and could be considered for the analysis of organic acids, too. Because enrichment of the analyte and purification of the sample are often necessary, and because derivatisation usually requires an organic reaction medium, the organic acids are conventionally isolated by liquid phase or solid-phase extraction prior to derivatisation. This step in the sample preparation procedure increases the total analysis time and bears the risk of introducing impurities and producing artifacts. We propose trimethyloxonium tetrafluoroborate (TMO) as the methylating agent.This substance was first synthesized and characterized by Meerwein et al. [13,14]. TMO has the advantage that the acids can be transformed into their methyl esters directly in the aqueous urine. By omitting the extraction of the organic acids from urine before derivatisation, a simplification of the sample preparation is reached. The results of the direct methylation method are compared with a procedure using strong anion-exchange (SAX) cartridges for solid-phase extraction (SPE) prior to methylation.

2. Materials and methods

2.1. Chemicals

TMO and ethyldiisopropylamine were obtained from Aldrich (Steinheim, Germany), sodium carbonate, sodium hydrogencarbonate, chloroform, methanol, phosphate buffer pH 7, and conc. HCl were purchased from Merck (Darmstadt, Germany). Bond Elut LRC cartridges containing 500 mg SAX were received from ICT (Frankfurt, Germany).

2.2. Sample preparation applying direct methylation with TMO in urine

Two ml of urine were transferred into a glass vial (100 mm in length and 16 mm in diameter) containing a magnetic stirring bar. The glass vial was placed in a heatable stirring unit, Model Reactitherm with Reactibloc (Bender and Hobein, Bruchsal, Germany). Under stirring at room temperature about 20 mg of sodium carbonate were added to alkalise the sample. Under continuous stirring derivatisation with TMO was performed in five steps. In step one approximately 30 mg of solid TMO were added by spatula within 4 min in five nearly equal portions. After a reaction time of 1 min the solution was neutralised with about 15 mg of sodium hydrogencarbonate. Steps two and three were exact repetitions of step one, whereas step four involved alkalisation (pH 8) of the reaction mixture with about 20 mg of sodium carbonate instead of neutralisation. In the last step, after the addition of the final 30 mg of TMO and reaction for 1 min, the reaction mixture was neutralised with about 20 mg of sodium hydrogencarbonate. Then the glass vial was stoppered and its content incubated for 2 min at 100°C. After cooling, 0.5 ml of chloroform were added, and the sample was thoroughly mixed in a vortex mixer and then centrifuged. The organic phase was transferred into a GC vial, concentrated to approximately 70 μl

under a stream of nitrogen and subjected to GC or GC–MS analysis.

2.3. Sample preparation applying solid-phase extraction and methylation with TMO

For SPE the SAX cartridges were placed in a Vac Elut SPS 24 work station with 24 positions (ICT) and conditioned in the following manner. Under a flow-rate of 5 ml/min two portions of 3 ml of methanol and two portions of 3 ml of water were passed through the cartridges, followed by 20 ml of phosphate buffer, pH 7 (0.336 *M* potassium dihydrogenphosphate and 0.665 *M* disodium hydrogenphosphate), five portions of 3 ml of water and finally 2.5 ml of diluted phosphate buffer, pH 7 (0.013 *M* potassium dihydrogenphosphate and 0.020 *M* disodium hydrogenphosphate). After conditioning, 4 ml of centrifuged urine were applied and passed through the cartridge within 20 to 25 min under slightly reduced pressure. The cartridges were dried by sucking air through them for 10 min. Elution of the adsorbed organic acids was performed with two 0.7 ml portions of aqueous HCl in methanol (4.16 ml of conc. HCl adjusted with methanol to 100 ml).

For derivatisation with TMO the heatable stirring unit was used. Under stirring at room temperature the eluate was mixed with 150 μl of 1 *M* sodium carbonate and 18 μl of ethyldiisopropylamine. Derivatisation was performed in three steps. In step one 80 mg of TMO were added to the mixture by spatula within 8 min in five nearly equal portions. After a reaction time of 2 min the solution was neutralised with about 15 mg of sodium hydrogencarbonate. Step two involved alkalisation of the reaction mixture with 15 mg of sodium hydrogencarbonate and 6 μl of ethyldiisopropylamine instead of neutralisation. In the third step, after the addition of the final 80 mg of TMO and reaction for 2 min, the reaction mixture was neutralised with sodium hydrogencarbonate. Then 1 ml of water was added, the glass vial was stoppered and its content incubated for 2 min at 100°C. After cooling, 0.5 ml of chloroform were added, and the sample was thoroughly mixed and then centrifuged. The organic phase was concentrated to about 40 μl and subjected to the analysis.

2.4. GC and GC–MS conditions

GC instrumentation, Vega 6130 (Carlo Erba, Hofheim, Germany), equipped with flame ionisation detector; column, 25 m×25 mm fused-silica column, coated with OV1701 (Macherey–Nagel, Düren, Germany), carrier gas, helium; head pressure, 102 kPa; injector temperature, 280°C; detector temperature, 300°C; temperature was programmed from 40°C to 260°C at 2°C/min, then held at 260°C for 30 min; split, 1:3. GC–MS instrumentation, TSQ 70 (Finnigan MAT, Bremen, Germany); ionization mode, electron impact (EI); temperature of ion source, 150°C; pressure, 1.33 Pa; emission current, 200 μA; multiplier, 1400 V; mass scan from m/z 40 to m/z 400; scan time, 0.7 s.

3. Results and discussion

3.1. Derivatisation of organic acids with TMO

Depending on the structures of the organic acids, the reactivities of different classes of acids towards methylating reagents are varying. Diazomethane appears to be the most effective methylating substance and reacts with carboxylic groups and phenolic OH-groups regardless of the steric surrounding. Following the goal of replacing diazomethane because of its adverse properties, the comparison of acetyl chloride–methanol, boron trifluoride–methanol and TMO as methylating reagents reveals the superiority of TMO. Experiments with reference substances show that for fatty acids, dicarboxylic acids, hydroxycarboxylic acids and aromatic acids with the carboxyl group in an aliphatic side chain all three derivatizing agents are equally effective and the yields are comparable with the yields obtained with diazomethane. However, when the carboxylic group is located directly at the aromatic ring, for acetyl chloride–methanol and boron trifluoride–methanol the methyl ester formation is only about 50% of that obtained with diazomethane. Using TMO, the yield of methyl esters of these acids is ca. 100% as compared to diazomethane. Among the three alternatives TMO appears to be the best replacement for diazomethane.

A further advantage of TMO as methylating reagent is that in contrast to acetyl chloride–methanol and boron trifluoride–methanol which require an organic reaction medium, under controlled conditions TMO can be used as a powerful methylating reagent in aqueous solutions such as urine samples. Also under this aspect TMO can be suggested as a replacement for diazomethane, for which derivatisation in an aqueous medium has been described, too, however only for some reference components and not for urinary organic acids [15].

3.2. Derivatisation with TMO in native urine

TMO reacts with the carboxyl group of the organic acid and particularly with the carboxylate anion to form the organic acid methyl ester:

$$RCOO^- + CH_3–O–(CH_3)_2^+ \rightarrow RCOOCH_3 + CH_3–O–CH_3$$

Water does not react instantaneously with TMO. However, because it is a concurrent reaction, it is essential to choose experimental conditions, under which the reaction between TMO and water is minimised. Because of the higher reactivity of the carboxylate anion as compared to the undissociated organic acid, the urine is alkalised with sodium carbonate. To keep the reaction with water low, derivatisation is carried out at room temperature, and the TMO is added in small portions. During the course of the derivatisation the reaction medium turns acidic due to hydrolysis of the tetrafluoroborate ion. Therefore during the derivatisation the reaction mixture is neutralised several times with sodium hydrogencarbonate and alkalised with sodium carbonate. Under these conditions the reaction of TMO with the organic acids is strongly favored, and the reaction with water occurs only to a small extent.

There are other side reactions, also occurring to a minor extent, for example the reaction of the hydroxide, carbonate and hydrogencarbonate ions with TMO. These reactions are favored in an alkaline and reduced in an acidic medium. Fluoride ions, which are also formed from tetrafluoroborate ions, do not react with TMO, as Meerwein et al. found [14].

The yields of the reaction with TMO in native urine has not been determined for the broad spectrum of organic acids, because the present study is qualitative or semiquantitative in nature. Also for quantitative analysis using calibration curves on the basis of external standards for each component, the yield would not be necessary for calculation. For diagnostic purposes quantitative data from profile analysis are frequently not necessary.

3.3. Profiles of urinary organic acids

When the derivatisation of the organic acids in urine with TMO is standardised on the basis of the described analytical conditions, the method is very useful for the analysis of urinary organic acid profiles. Even though the derivatising reagent has to be added in several steps, sample preparation is simplified as compared to methods including isolation of the organic acids, and the risk of introducing impurities and producing artifacts is reduced. Analysis time is saved, because no isolation, for instance solid-phase extraction of the organic acids from the urine prior to derivatisation, is necessary. The addition of several portions of solid TMO by spatula is not problematic. The method is rugged, and the time required for the entire sample preparation is only about 40 min. The price for the total sample preparation is reduced, because no solid-phase cartridges are required.

Figs. 1 and 2 demonstrate analysis A and B of the profile of organic acids in urine of a healthy individual. The same urine was analysed on two separate days. The given example is characteristic for the degree of repeatability of the applied analytical method. The methodical variations observed in Figs. 1 and 2 are well below interindividual variations in different urines. The components of the profiles identified by GC–MS are summarised in Table 1. Identification is based on mass spectra of reference compounds, on mass spectra of previously identified components [8] and on a library. The homogeneity of the peaks in the profile is determined by mass spectrometric scanning.

In addition to the carboxylic groups of the organic acids, TMO reacts with phenolic OH groups but not with aliphatic OH groups. For complete reaction of phenolic OH groups and also carboxylic groups of

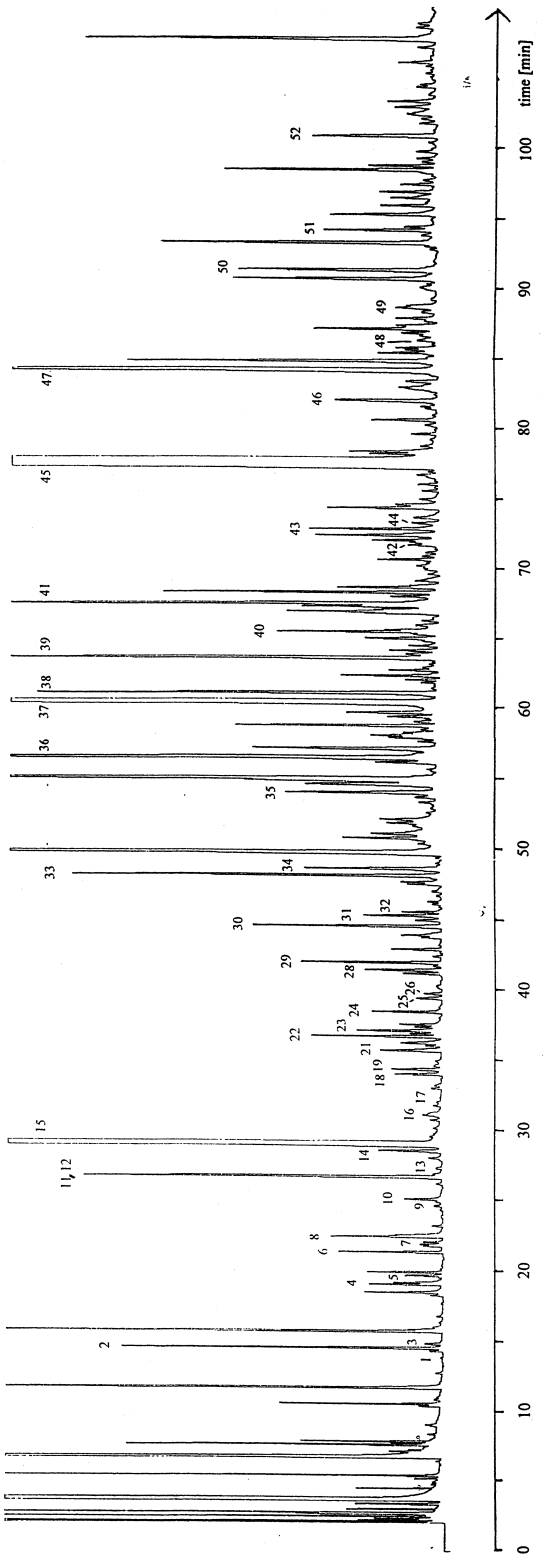

Fig. 1. Gas chromatogram of the methyl esters of the organic acids in urine of a healthy individual. Direct methylation with TMO in native urine. Analysis A.

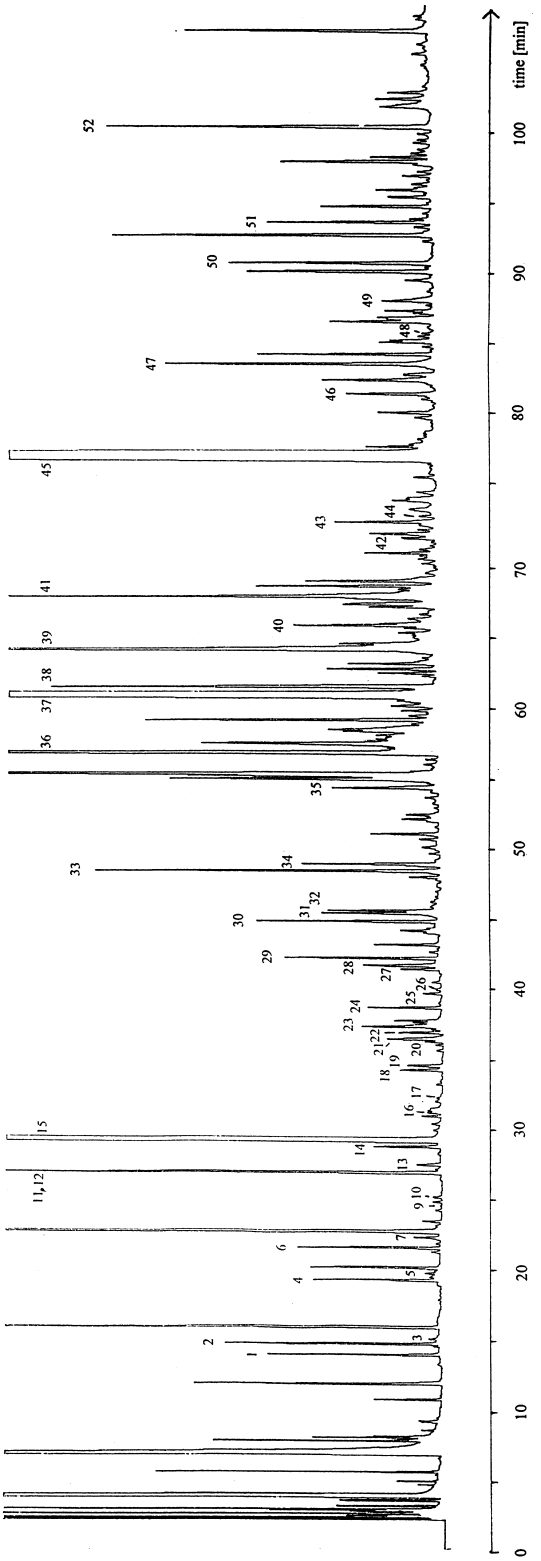

Fig. 2. As in Fig. 1. Analysis B.

Table 1
Substances identified by mass spectrometry: the numbers indicate the peaks in the chromatograms in Figs. 1–3

1	Oxalic acid
2	3-Hydroxyisovaleric acid
3	3-Hydroxybutyric acid
4	3-Hydroxy-2-methylbutyric acid
5	Malonic acid
6	Methylmalonic acid
7	Furan-3-carboxylic acid
8	Phosphoric acid
9	Fumaric acid
10	2-Ethylhydracrylic acid
11	Succinic acid
12	Ethylmalonic acid
13	Maleic acid
14	Methylsuccinic acid
15	Benzoic acid
16	Mesaconic acid
17	Citraconic acid
18	2-Hydroxy-2-methylsuccinic acid
19	Glutaric acid
20	*O*-Methylmalic acid
21	Phenylacetic acid
22	3-Methylglutaric acid
23	*p*-Cresol
24	3-Methylglutaconic acid
25	2,3-Methyleneglutaric acid
26	2-Hydroxy-2-ethylsuccinic acid
27	3-Methylglutaconic acid
28	3-Hydroxy-3-methylglutaric acid
29	Adipic acid
30	2-Hydroxy-2-isopropylsuccinic acid
31	3-Methyladipic acid
32	2-Hydroxyglutaric acid
33	3,4-Methyleneadipic acid
34	Pimelic acid
35	Suberic acid
36	3-Methylsuberic acid
37	Citric acid
38	Azelaic acid
39	Isocitric acid
40	Furoylglycine
41	4-Hydroxyphenylacetic acid
42	Saccharine
43	3-Hydroxyphenylhydracrylic acid
44	3-Carboxy-4-methyl-5-propyl-2-furanpropionic acid
45	Hippuric acid
46	3-Carboxy-4-methyl-5-pentyl-2-furanpropionic acid
47	3-Indoleacetic acid
48	Hydroxyhippuric acid
49	5-Carboxyfuroylglycine
50	Hydroxyhippuric acid
52	*N*-Phenylacetylpyroglutamic acid
52	*N*-Phenylacetylglutamic acid

organic acids with lower reactivity, it is essential that during the derivatisation the reaction mixture is reneutralised and alkalised.

An advantage of the methylation of the organic acids in the native urine is, that the acids are derivatised in their original concentration ratios. Due to the different polarities of the organic acids and different affinities toward liquid and solid phases, isolation of the acids from the urine can easily falsify the concentrations. The extraction of the methyl esters with chloroform at the end of the derivatisation is less critical, because the methyl esters are more hydrophobic than the organic acids themselves and because the differences in the polarities are less pronounced.

Among the materials used for solid-phase extraction such as C_8, C_{18} and SAX phases, SAX as an anion-exchange material appears to be most suitable for a uniform extraction of organic acids with different polarities. We used solid-phase extraction with SAX cartridges and subsequent derivatisation with TMO to compare direct methylation of the organic acids in native urine with methylation of the acids after their isolation from the urine. Fig. 3 shows the urinary acid profile of the same urine as in Figs. 1 and 2, however analysed by the SAX procedure. Profiles and components identified by GC–MS (Table 1) demonstrate good comparability of the results from the two methods. Certainly, as it is true for all analytical methods applied to long series of samples and in routine, it is advisable to use one procedure for a longer period of time.

4. Conclusion

We conclude that for the methylation of organic acids TMO can be considered as a good replacement for the hazardous diazomethane, and that derivatisation of the organic acids with TMO can be reliably performed in native urine as an aqueous medium. Extraction of the acids from the urine prior to derivatisation is not required. This simplification leads to a higher speed of analysis which is required for instance in newborn screening of urinary organic acids. The characteristics of the method make it also very suitable for a combination with solid-phase microextraction (SPME) [16]. When SPME tech-

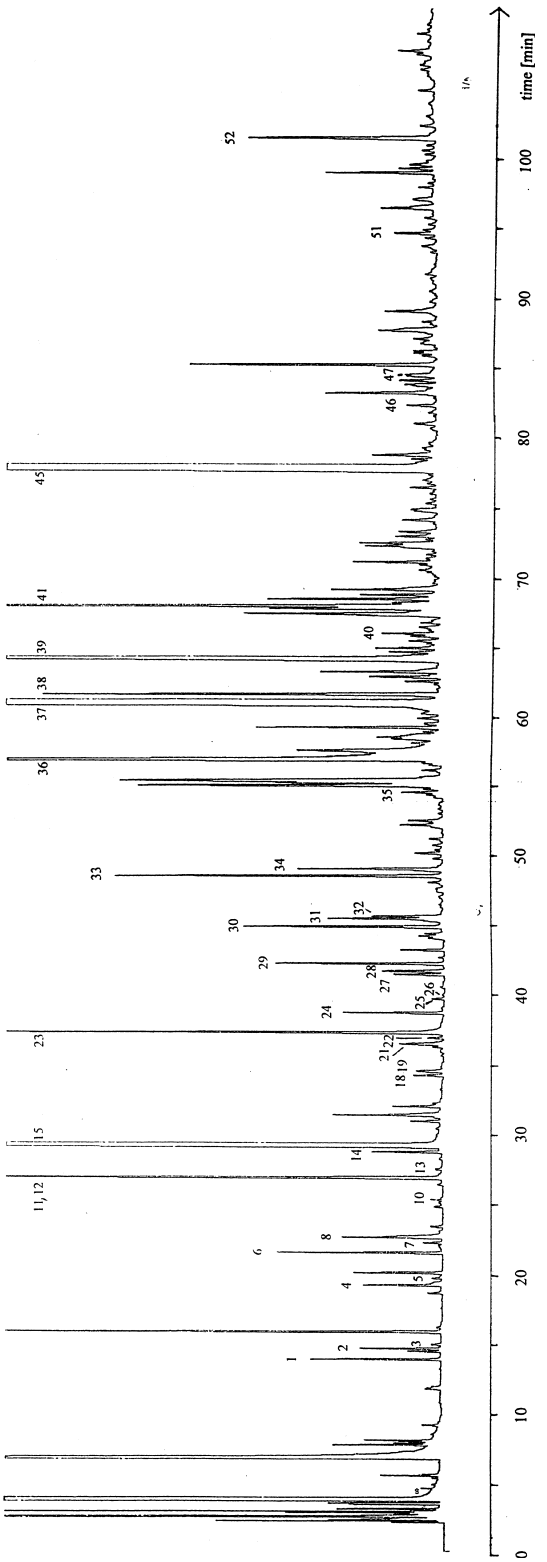

Fig. 3. Gas chromatogram of the methyl esters of the organic acids in urine of the same healthy individual as in Figs. 1 and 2. Methylation with TMO after solid-phase extraction on SAX.

niques are applied to liquid samples, an aqueous medium is required.

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