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Analysis of volatile organic compounds in human urine by headspace gas chromatography–mass spectrometry with a multipurpose sampler

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Abstract

A multipurpose sampler (Gerstel MPS), designed for liquid large volume, gaseous and headspace samples was used for the GC–MS analysis of organic volatiles in human urine. Headspace sampling with a volume-, temperature- and speed-controlled gas-tight syringe was combined with a temperature-controlled cold injection system (CIS) for cold trapping, enrichment and focusing of analytes. Regular 2-ml GC vials filled with 1 ml acidified urine were used as headspace sampling vials. A 100-vial autosampler tray was equipped with an additional temperature and heating time controlled ''preheating station'' for five vials. Profiles of organic volatiles in human urine were determined and 34 components identified. Trimethylamine (TMA) and 4-heptanone as two metabolites of medical interest were quantified. Calibration curves and intra assay imprecision for 4-heptanone concentrations in the range of 40 to 800 ng/ml showed a correlation coefficient of $r=0.9980$ and a relative standard deviation (RSD) between 3.0 and 3.4%. Calibration curves and intra-assay imprecision for TMA concentrations in the range of medical interest from 0.5 to 20 μg/ml showed a correlation coefficient of $r=0.9968$ and a RSD between 4.1 and 6.8%. The high practicability of the multipurpose sampler for both gaseous and liquid samples together with the here shown good reproducibility and sensitivity make this single CIS-GC–MS system very attractive for routine clinical use in metabolic profiling of organic volatiles (headspace) and non-volatiles (liquid).

Keywords: Headspace analysis; Injection methods; Sample handling; Volatile organic compounds

1. Introduction

The concept of ''metabolic profiling'' has been widely applied in general to different kinds of biological fluids such as urine, serum, cerebrospinal fluid, amniotic fluid, breast milk and to tissue homogenates [1–3]. In addition to the analysis of organic acids, amino acids and fatty acids in urine and serum for the diagnosis of inborn metabolic diseases, the profiles of organic volatiles have been intensively studied and linked to metabolic disorders [4–10]. The profile of organic volatiles in urine covers a diverse group of different polarities: alcohols, aldehydes, ketones, *O*- and *N*-heterocycles, sulfur-containing compounds (isocyanates, sulfides) and hydrocarbons are found regularly and may be derived from nutrients, intermediates or environmental contaminants [11]. Mercaptans such as methanthiole, ethanthiole, dimethyl sulfide and dimethyl disulfide can result from the enterobacterial degra-

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dation of methionine in the state of hepatic encephalopathy, but may also in some extent be due to sulfur compounds (methanthiole, dimethyldisulfide) found in coffee [12].

Pattern recognition of profiles [5] and especially the concentrations of several ketones [6,13], such as 4-heptanone [8,14], were linked to diabetes mellitus. In diabetic patients elevated levels of 4-heptanone in urine were found and tentatively related to more specific stages of the disease [8,14]. A possible relationship also was found between endogenous volatile urinary metabolites with structures similar to certain neurotoxins [15] and the development of diabetic polyneuropathy [10,16].

Trimethylamine (TMA) and its *N*-oxide (TMAO) are normal components of human urine. They are present in the diet as such (in marine fish) and also originate from the enterobacterial metabolism of precursors such as food derived carnitine and cholin (egg, liver, soybean) [17]. TMA is readily oxidized to TMAO by a flavin containing monooxygenase in the liver. Healthy young volunteers were reported to excrete about 1 mg TMA and 40 mg TMAO per day in the urine under normal dietary intake [18]. Whereas the majority of individuals excrete more than 90% of total TMA in form of TMAO [18] there is a rare inborn error of TMA oxidation called fish-odor syndrome. Affected individuals have an impaired TMA oxidation and excrete TMA in their breath, sweat, urine and other body fluids, causing a characteristic rotting fish odor. The standard analysis has been headspace gas chromatography–mass spectrometry (GC–MS) of TMA in urine before and after reducing TMAO to calculate the percentage of TMA to total TMA (TMA+TMAO) in addition to the absolute TMA concentration [19].

The sampling techniques used for the analysis of organic volatiles include static and dynamic headspace with condensation in a cryogenic trap [20,21], adsorption onto the hydrophobic porous polymer Tenax [poly (2,6-diphenyl-*p*-phenylene oxide)] [5,8,22,23], solvent extraction [4,14], the early use of a transevaporator [24–26] or several other modifications concerning the instrumentation [27,28]. The most effective method for the interpretation of complex chromatograms is the combination of GC with MS, which can be used for identification and quantification. Also, selective detectors for complex sulfur, nitrogen, phosphorous or halogen have been applied succesfully in headspace analysis of complex matrices [29]. Urine analysis by nuclear magnetic resonance has evolved as a useful method for the screening of inborn errors of metabolism where it is available [30]. Next to the high cost of this technique the relatively poor sensitivity is one of its major drawbacks [30]. Solid-phase microextraction has been used for GC/GC–MS analysis of volatiles and semivolatiles in biological samples. Good results have been reported for aromatic amines [31], polycyclic aromatic hydrocarbons [32] and especially drug monitoring [33–36].

Metabolic profiling in a clinical chemistry laboratory is predominantly done by GC–MS analysis of organic acids and high-performance liquid chromatography (HPLC) of amino acids. Only a few headspace analyses of volatiles are needed for inborn errors of metabolism, but quite a few more in environmental and occupational medicine. It would be of great advantage if the different analyses could be performed on the same instrument. We therefore tested a single GC–MS system with a multipurpose sampler for both liquid and gaseous samples for its suitability in routine clinical analysis. Sample preparation and analysis conditions were optimized for identification of volatiles in human urine and quantification of both trimethylamine and 4-heptanone, two metabolites of medical interest, were performed.

2. Experimental

2.1. Sample preparation

A total of 189 urine samples (spontaneous and 24 h collecting period) were taken from healthy controls, patients with diabetes, liver and kidney diseases. Two-ml GC vials were filled with 1-ml aliquots of urine and after acidification or alkalization sealed with gas-tight polytetrafluoroethylene (PTFE)-lined rubber septum caps. Acidification was done with 10, 30 and 50 μl of conc. HCl to optimize the quantification of 4-heptanone in urine. For the quantification of trimethylamine the addition of 30, 50 and 70 μl of 1 *M* NaOH was tested. The addition of either base or acid was done directly into the GC vial, which was sealed immediately thereafter. Am-

monium sulfate, sodium chloride, ammonium chloride and potassium carbonate were tested for increasing headspace sensitivity by saturating the urine sample.

2.2. Instrumentation and operation principles

The applied system consisted of a Multi Purpose Sampler MPS (Gerstel, Mülheim an der Ruhr, Germany), operated in headspace-mode and equipped with a 1000 μl gas-tight syringe, a HP-7673 tray for 2-ml standard vials (Hewlett-Packard, Waldbronn, Germany) plus an additional pre-heating module for five vials with control of temperature and heating-time (Gerstel). A temperature-controlled cold injection system CIS-3 (Gerstel) was used as interface for the subsequent GC–MS combination (HP 5890 series II, HP 5972, Hewlett-Packard). Cryogenic cooling of the CIS-3 at −150°C was done with liquid nitrogen.

Each sample was heated for the same period of time at the same temperature in the pre-heating module. Prewarm temperatures tested were 70 and 80°C with prewarm times of 2, 5, 7, 10 and 30 min for highest sensitivity and reproducibility of the headspace analysis. Solvent flushing of the MPS with helium was done by injecting the specially designed syringe into the CIS-3 for 8 min at a flow-rate of 30 ml/min. The heated syringe was filled with 1000 μl of helium and injected into the headspace vial. After 2 min of equilibration 1000 μl of the gaseous phase was removed from the headspace vial and injected into the cooled CIS-3 for focusing and enrichment. The needle remained within the CIS until its final temperature was reached. The syringe was then flushed again with helium for 2 min before it was removed from the injector. The depth of injection was controlled for both the position in the vial and in the injector. The CIS was first in split mode for the injection of 1000 μl headspace sample (solvent venting) and then in splitless mode for the transfer from the CIS into the GC column.

2.3. Final sample preparation.

Quantification of 4-heptanone in urine: 1 ml urine was filled into a 2-ml GC vial, acidified with 30 μl of conc. HCl and immediately sealed with a gas-tight PTFE-lined septum cap.

Quantification of trimethylamine in urine: 1 ml urine was filled into a 2-ml GC vial, alkalinized with 50 μl of 1 *M* NaOH and immediately sealed with a gas-tight PTFE-lined septum cap.

2.4. Final analysis conditions.

Headspace sampler parameters: Fill volume: 1000 μl, fill speed: 5000 μl/min. Injection volume: 1000 μl, injection speed: 10 000 μl/min. Turret temperature: 70°C, syringe temperature: 70°C. Prewarm time: 10 min, equilibration time: 2 min. Preflush time: 8 min, postflush time: 2 min.

CIS parameters: (I) Glass liner: 91 mm×1.4 mm I.D., 20 mm Carbotrap (Supelco), 20–40 mesh. Initial temperature: 10°C (Peltier effect), initial time: 1.0 min. Rate: 12.0°C/sec, final temperature: 300°C, final time: 3.0 min. Split mode solvent venting: 1.0 min, splitless time: 1.1 min. Temperature: 10°C to 300°C with 12°C/s. (II) Glass liner: 91 mm×1.4 mm I.D., glass wool. Initial temperature: −150 °C (liquid nitrogen), initial time: 0.6 min. Rate: 12.0 °C/s, final temperature: 300°C, final time: 3.0 min. Split mode solvent venting: 0.6 min, splitless time: 1.1 min.

GC columns: (I) 30 m×0.25 mm I.D. HP-5, d_f= 0.33 μm, (II) 60 m×0.25 mm I.D. DB-5 (J&W), d_f=0.25 μm. GC oven temperature: 60°C to 100°C with 5°C/min; to 240°C with 25°C/min.

Pneumatics: He, p_i=100 kPa.

Detector: Mass-selective detector, scan mode 10–260 u for peak identification, selected ion monitoring (SIM) mode for quantification.

2.5. Metabolic profiling

Urine samples from healthy controls, patients with different metabolic diseases or organ malfunctions were analysed using the above optimized conditions. Identification was done with the help of MS libraries (NIST, Wiley PBM Library) and standards when available.

2.6. Quantification of 4-heptanone in urine

Acidified pooled urine samples spiked with 4-heptanone (Sigma, Germany) were used for the

calibration curves in a concentration range from 40 to 800 ng/ml [37,38]. The ion *m/z* 71 was used for quantification and the ions *m/z* 43 and *m/z* 114 were used as qualifier ions for the identification. Intra-assay imprecision of the method was determined for different urine samples in the observed concentration range by measuring 10 aliquots from each sample in a row. Standard recoveries were calculated from spiked urine samples.

2.7. Quantification of trimethylamine in urine

Pooled urine samples spiked with TMA (Sigma, Germany) were used for the calibration curves in a concentration range from 0.5 to 20 μg/ml. The ion *m/z* 58 was used for quantification and the ions *m/z* 42 and *m/z* 59 were used as qualifier ions for the identification. Intra-assay imprecision of the method was determined for different urine samples in the observed concentration range by measuring 10 aliquots from each sample in a row. Standard recoveries were calculated from spiked urine samples. For the determination of the TMA/(TMA+ TMAO) ratio TMAO was reduced with Ti(III)Cl to TMA as previously described [19]. Briefly, 0.2 ml of Ti(III)Cl (30%, w/v) was added to 2 ml urine in a screw-capped vial and left for 30 min at room temperature. The reduced urine was then diluted 10-fold with distilled water and analysed.

3. Results and discussion

Sensitivity of headspace analysis can basically be improved by lowering the values of the distribution coefficients of the analytes in the investigated system, by increasing the volume of the headspace sample and by adsorbing or cryogenically trapping the volatiles before introducing them into the chromatographic column. The value of the distribution coefficients can be lowered by increasing the equilibrium temperature and by adding mineral salts into the solution. Mineralization is especially of great advantage for determining polar volatiles in aqueous solutions where depending on the nature of the salt and analyte a 10-fold increase in sensitivity can be achieved [39]. Human urine is an aqueous matrix with a fairly high salt content and most of the headspace analyses is therefore done with either untreated urine samples or after the addition of an acid (HCl, H_2SO_4) or base (KOH, NaOH) [17,18,40]. The question of adding salts cannot be answered in general for all components present in urine but has to be discussed for each single component or group of similar components. In the case of 4-heptanone we did not see an increase in acidified urine samples (30 μl conc. HCl) when adding either sodium chloride or ammonium chloride. Without acidifying the urine the number of peaks became significantly smaller, but on the other hand some peaks became more prominent (e.g., allylisothiocyanate and some amines). In the case of trimethylamine the sensitivity could even be more improved when the urine was alkalinized (see quantification of trimethylamine). Increasing the temperature above 70°C (temperature limit of the heated syringe was 80°C) and prolonging the preheating time for more than 10 min did not further improve sensitivity. The final conditions for screening of volatiles were 1 ml urine acidified with 30 μl conc. HCl and thermostatted at 70°C for 10 min. Fig. 1 shows the chromatogram (analysis condition I, scan 10–260 u) from an acidified urine sample of a healthy person. The identified volatiles are listed in Table 1. Identification was done with the help of MS libraries (NIST, Wiley PBM Library) and standards (reference r) when available. As this GC–MS system with the multipurpose sampler was used not only for headspace analysis but also for liquid samples in clinical routine use [methylesters of organic and fatty acids, trimethysilyl (TMS) ethers of glycerol, boronate derivatives of glucose] a versatile 30-m column had to be used (GC column I). For better separation especially for early eluting volatiles a 60-m column should be used (analysis condition II).

3.1. Quantification of 4-heptanone

Acidifying the urine increased the headspace concentration of 4-heptanone. Addition of 30 μl of conc. HCl showed an increase by 42.8 % compared to 10 μl, whereas the addition of 50 μl showed no further increase (−2.5 % compared to 30 μl) at a concentration of 145 ng/ml. A prewarm time of 7 min showed an increase of 1.2 % compared to 5 min and after 10 min no further increase was found at a

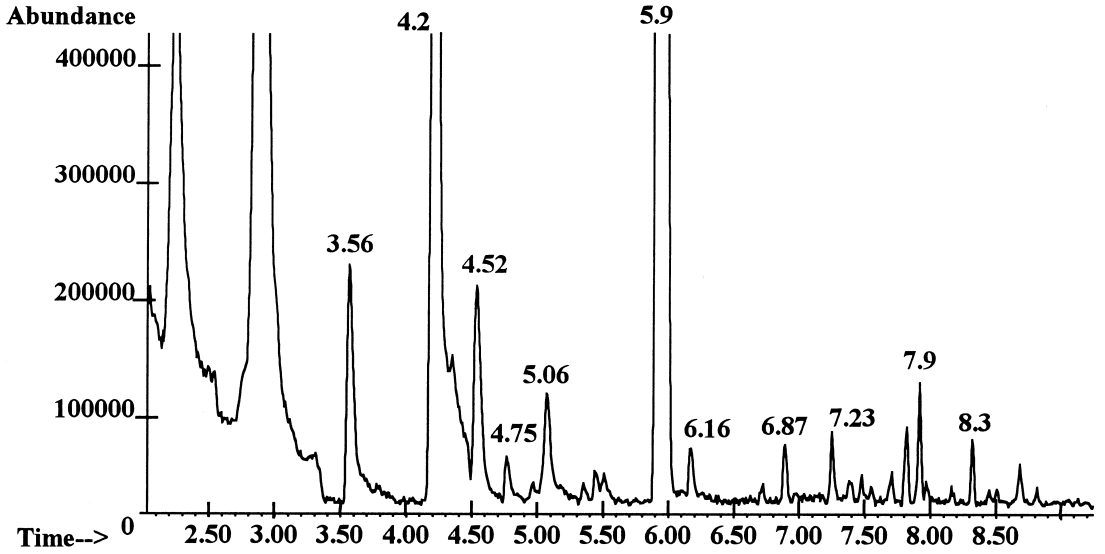

Fig. 1. Chromatogram of acidified urine sample from healthy person (analysis condition I, identified volatiles see Table 1). Time in min.

temperature of 70°C. The temperature limit of the heated syringe is 80°C and operating at this temperature did not significantly improve the results. Fig. 2a shows the mass spectrum of 4-heptanone and Fig. 2b the ion chromatogram of an acidified urine sample (analysis condition II). The ion m/z 71 was used for quantification and the ions m/z 43 and m/z 114 were used as qualifier ions for the identification. The calibration curve for 4-heptanone in urine was linear in the range from 40 to 800 ng/ml with a correlation coefficient of $r=0.9980$. The determination of intra-assay imprecision showed a relative standard deviation (RSD) between 3.0 and 3.4% for the total concentration range. The signal-to-noise ratio (m/z 71) was calculated as $S/N = 12$ for a 4-heptanone concentration of 15 ng/ml. Standard recoveries calculated from spiked urine samples (original concentration of 47 ng/ml) were 91 to 101% in a final

Table 1
Identified volatiles in urine (analysis condition I, r=reference)

Organic volatile	t_R (min)	Organic volatile	t_R (min)
Acetone (r)	2.23	Cyclohexanol (r)	6.07
2-Butanone (r)	2.73	Allylisothiocyanate	6.10
Chloroform	2.87	Chlorocyclohexane	6.13
Benzene	3.29	2-Heptanone (r)	6.16
Cyclohexene	3.45	2,4-Dimethylthiophene	6.36
2-Pentanone (r)	3.56	Methyl-2-propenyldisulfide	6.56
2,5-Dimethylfuran (r)	3.78	Methylpropyldisulfide	6.71
Phenol (r)	3.98	3-Methyl-2-heptanone	6.87
3-Methyl-1-butanol	4.11	Dimethyltrisulfide	7.23
Dimethyldisulfide	4.20	Phellandrene	7.66
Toluene (r)	4.52	1,4-Dichlorobenzene	7.69
3-Hexanone (r)	4.75	α-Terpinene	7.80
2-Ethyl-5-methylfuran	4.94	1-Methyl-2-(1-methylethyl)benzene	7.90
Tetrachlorethylene	5.06	γ-Terpinene	8.31
5-Methyl-3-hexanone	5.43	4-Methylphenol	8.50
3-Heptanone (r)	5.45	α-Terpinolene	8.64
4-Heptanone (r)	5.90	3-Methylhexane-2-one	8.86

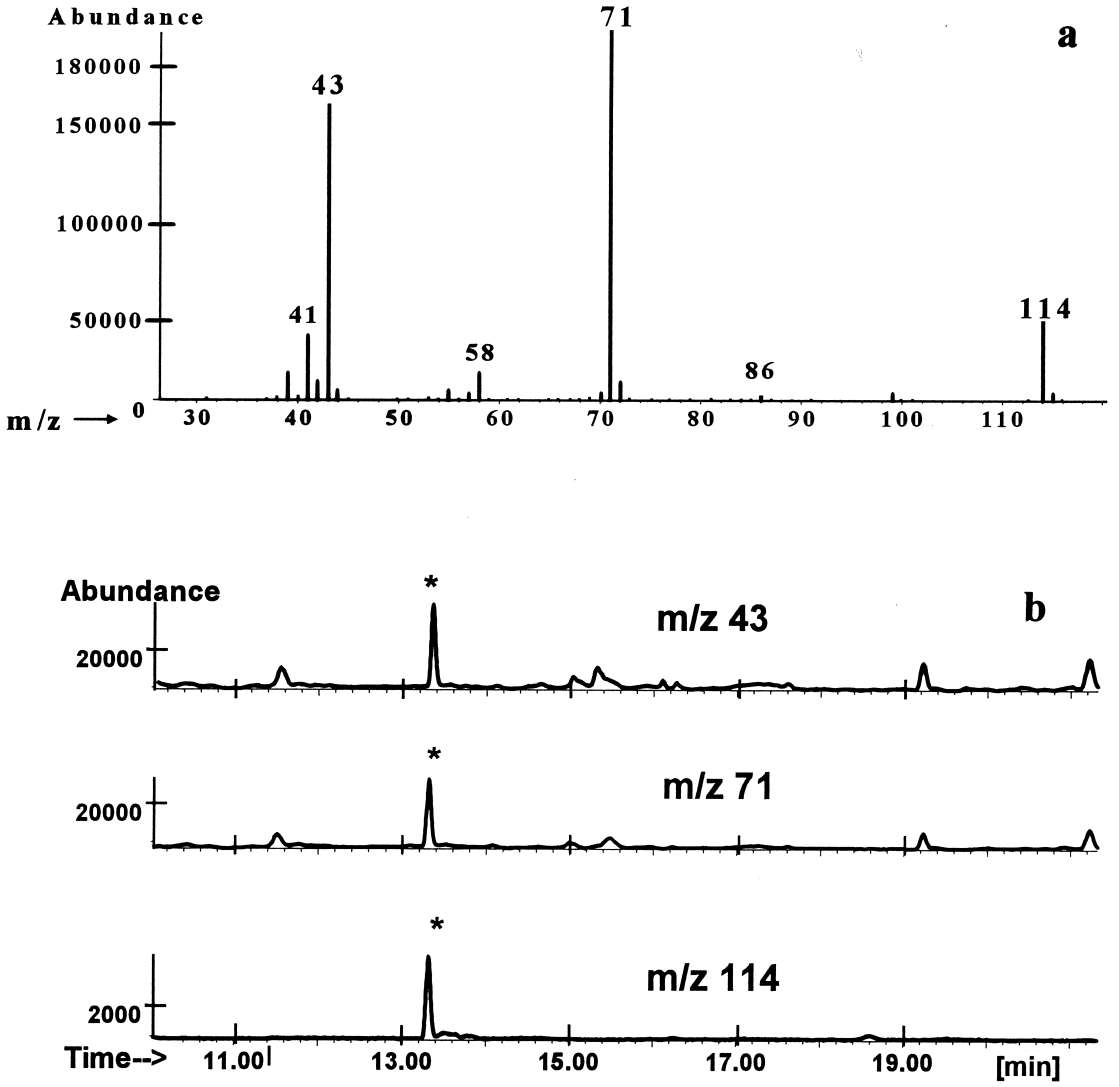

Fig. 2. (a) Mass spectrum of 4-heptanone. (b) Ion chromatogram of acidified urine sample from healthy control (ions m/z 43, 71 and 114 for 4-heptanone, analysis condition II).

concentration range from 104 to 852 ng/ml. The median concentration of 4-heptanone in urine from the healthy control group ($n=51$) was 188 ng/ml, ranging from 27 to 1044 ng/ml compared to 179 ng/ml, ranging from 17 to 978 ng/ml in the diabetic patient group ($n=92$). As there seems to be no difference in the median concentration of 4-heptanone between the diabetic and the control group, and considering the highly elevated levels in a few controls the source of 4-heptanone seems to be exogenous and further studies with more patients are needed.

3.2. Quantification of trimethylamine

For the liberation of amines the urine sample has to be carefully alkalinized in order not to have significant losses: 1 ml urine was placed in a 2-ml GC vial, alkalinized with 50 μl of 1 *M* NaOH and immediately sealed with a gas-tight PTFE-lined

septum cap. Fig. 3a shows the mass spectrum of trimethylamine and Fig. 3b the ion chromatogram of an alkalinized urine sample (analysis condition II). The ion m/z 58 was used for quantification and the ions m/z 42 and m/z 59 were used as qualifier ions for the identification. The calibration curve for TMA in urine was linear in the range from 0.5 to 20 μg/ml with a correlation coefficient of $r=0.9968$. The signal-to-noise ratio (m/z 58) was calculated as $S/N=21$ for a trimethylamine concentration of 0.05 mg/l. Standard recovery calculated from spiked urine samples were between 92 and 106% in a final concentration range from 1.21 to 3.48 mg/l. The determination of intra assay imprecision showed a RSD between 4.1 and 6.8% for the total concentration range. The greater imprecision for TMA

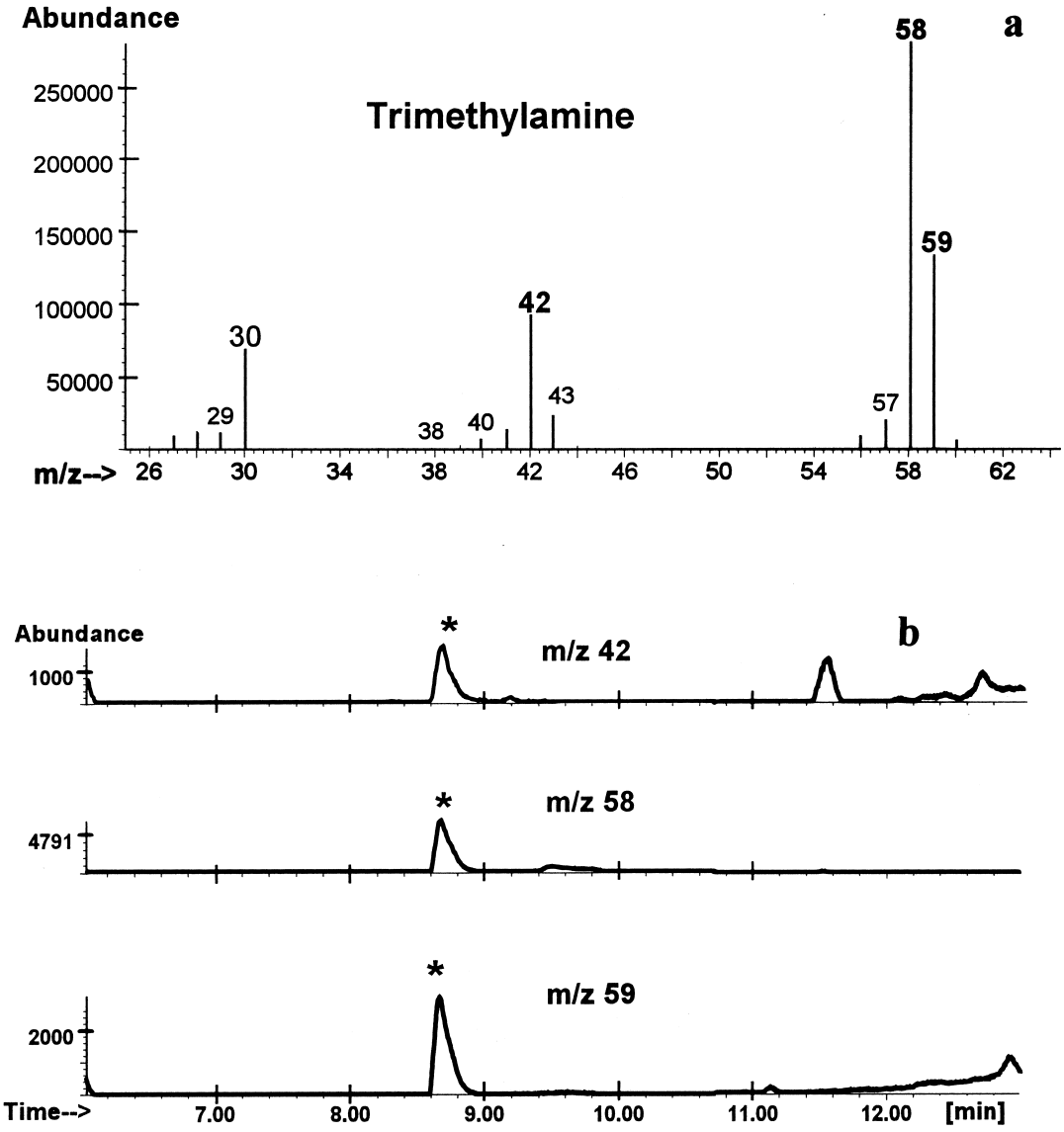

Fig. 3. (a) Mass spectrum of trimethylamine. (b) Ion chromatogram of alkalinized urine sample from healthy control (ions m/z 42, 58 and 59 for trimethylamine, analysis condition II).

compared to 4-heptanone might partly be due to the peak tailing (Fig. 3b) seen for the amine on the here used GC column (DB-5, J&W). Another source could be the addition of the alkaline solution into the open GC vial before sealing which might lead to amine losses. With the method and instrumentation described we were able to diagnose fish odor syndrome in a 10-month-old baby. The absolute concentration of TMA in urine was 1.5 μg/ml and the free TMA/total TMA ratio of 53% under normal dietary intake increased to 74% after administration of one hard boiled egg.

4. Conclusions

With the instrumentation and method described here, organic volatiles in human urine can now be easily analysed with the same system used for non-volatiles. Applications are metabolic profile studies in a qualitative as well as quantitative way. The high precision and sensitivity of the method was shown for trimethylamine and 4-heptanone as two examples of metabolites with diagnostic relevance.

Metabolic profiling by means of nuclear magnetic resonance (NMR) analysis or solid-phase microextraction (SPME) headspace GC–MS might be possible alternatives where it is available (NMR) or the application spectrum is broadened (SPME). For the moment, the high practicability of the multipurpose sampler for both gaseous and liquid samples together with the here shown good reproducibilty and sensitivity make this single CIS-GC–MS system very attractive for routine clinical use in metabolic profiling of organic volatiles (headspace) and non-volatiles (liquid).

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