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Quantification of 4-Heptanone in Urine by Headspace GC-MS Analysis Using a Multipurpose Sampler

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Abstract

A multi-purpose-sampler (Gerstel MPS), designed for liquid large volume, gaseous and headspace samples was tested for its suitability in 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 for cold trapping, enrichment and focussing of analyte. 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 5 vials.

Profiles of organic volatiles were determined and 4-heptanone as a ketone of medical interest presumably related to diabetes was quantified. Calibration curves and imprecision of the method for 4-heptanone concentrations in the range from 40 to 800 ng/ml showed a correlation coefficient of r = 0,999 and a coefficient of variation (CV) between 3.0 and 3.4% respectively. In this pilot study including 51 patients with diabetes mellitus (Type I and II) and 42 controls the median for the diabetic group was 179 ng/ml compared to 188 ng/ml in the control group. Further studies have to show if there actually exsists a relationship between 4-heptanone and diabetes mellitus.

Introduction

The concept of "metabolic profiling" has been widely applied in general to all different kinds of biological fluids such as urine, serum, cerebrospinal fluid, amniotic fluid, breast milk and to tissue homogenates [1-3]. Next to the organic acid fraction in urine and serum 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 polarity: 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]. Patternrecognition of profiles [5] and especially the concentration of several ketones [6,12], such as 4-heptanone [8,13] were related 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 [8,13]. A possible relationship also was found between endogenous volatile urinary metabolites with structures similar to certain neurotoxins [14] and the development of the diabetic polyneuropathy [10,15].

The sampling techniques used for the analysis of organic volatiles include static and dynamic headspace with condensation in a cryogenic trap [16,17] or adsorption onto the hydrophobic porous polymer Tenax (poly 2,6-diphenyl-p-phenylene oxide) [5,8,18,19], solvent extraction [4,13] and the use

of a transevaporator [20-22]. Modifications have also be done concerning the instrumentation [23,24]. Next to the GC-MS other selectiv detectors for complex sulfur, nitrogen, phosphorous or halogen can be very useful in headspace analysis [25]. Changes in the composition of the volatile sulfur containing compounds in the urine of diabetic persons can reliable be registered by use of a sulfur detector [25]. Mercaptanes such as methanthiole, ethanthiole, dimethylsulfide and dimethyldisulfide can result from the enterobacterial degradation of methionine in the state of hepatic encephalophaty, but may also in some extent be due to sulfur compounds (methanthiole, dimethyldisulfide) found in coffee [26].

EXPERIMENTAL

Sample preparation. A total of 139 urine samples (spontaneous and 24h collecting period) were taken from 42 healthy controls and 51 diabetic patients. 2ml GC vials were filled with aliquots of 1 ml acidified (30µl conc. HCL) urine and analyzed in duplicates.

Headspace GC-MS with Gerstel MPS Multi Purpose Sampler. Each sample is heated for the same period of time at the same temperature in the pre-heating module (**Figure 1**, 3). Solvent flushing of the MPS with helium is done by injecting the special designed syringe into the CIS-3 for 8 min. The heated syringe can then be filled with a defined volume of helium and injected into the headspace vial. The depht of injection is controlled for both the position in the vial and in the injector. The sample is injected into the cooled CIS-3 for focussing and enrichment (**Figure 1**, 6)and after heating up to the desired temperature transferred to the capillary column in either split or splitless mode.

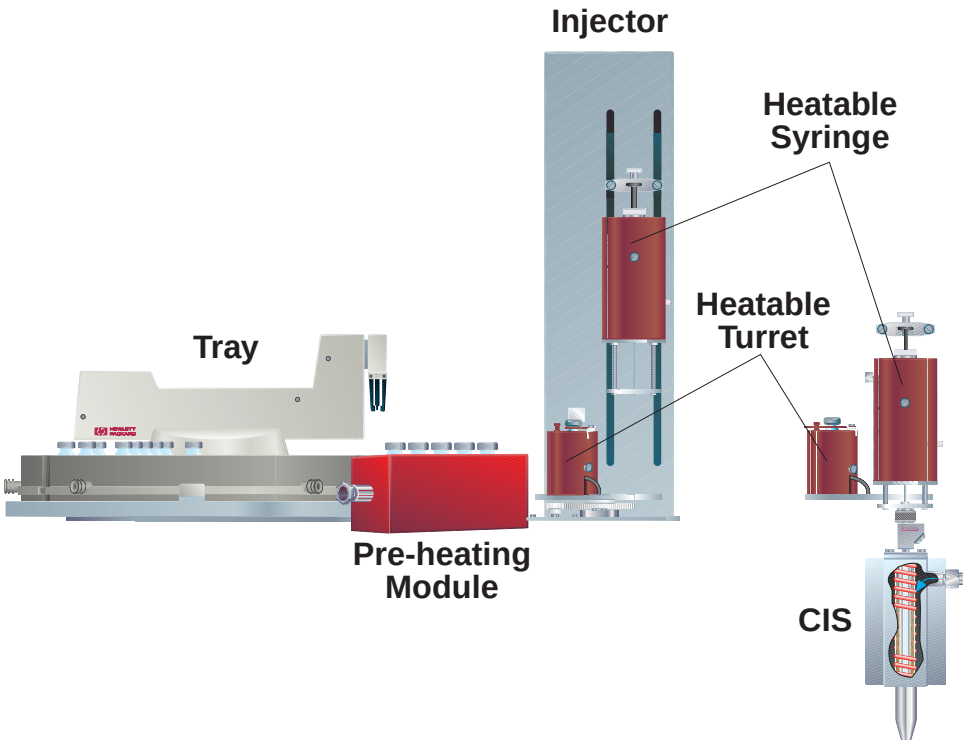

Figure 1. *Gerstel Multi Purpose Sampler in standby (left) and injection mode (right).*

Quantification of 4-heptanone in urine: Acidified pooled urine samples spiked with 4-heptanone were used for the calibration curves in a concentration range from 40 to 800 ng/ml.The ion m/z 71.15 was used for quantification and the ions m/z 43.1 and m/z 114.15 were used als 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.

Instrumentation. The applied system consists of a Multi Purpose Sampler (Gerstel GmbH, Mülheim an der Ruhr, Germany), operated in headspace-mode and equipped with a 1000 µl gas tight syringe, a HP-7673 tray for 100 2ml standard vials (Hewlett-Packard, Avondale, USA) plus an additional pre-heating module for 5 vials with control of temperature and heating-time (Gerstel GmbH, Mülheim an der Ruhr, Germany), a temperature controlled cold injection system CIS-3, (Gerstel GmbH, Mülheim an der Ruhr, Germany) used as interface, cold trap and injection system for the subsequently following GC-MSD combination (HP 5890/5972, Hewlett-Packard, Avondale, USA).

Analysis conditions I.

Columns:	CIS-liner	20 mm Carbotrap (Supelco), 20/40 mesh	
	GC	60 m DB-5 (J&W), d_i=0,25 mm, d_f=0,25 µm	
Pneumatics:	He, p_i =100 kPa, split x:30, splitless 0.1-1.1 min		
Temperatures:	HSS pre-heating module:		70°C (10 min)
	HSS turret:		70°C
	HSS syringe:		70°C
	CIS:		10°C to 300°C with 12°C/s
	Oven:		60°C to 100°C with 5°C/min;
			to 240°C with 25°C/min
	MSD:		280°C
Detector:	MSD	scan 10-260 amu	

Analysis conditions II.

Columns:	CIS-liner	20 mm Carbotrap (Supelco), 20/40 mesh	
	GC	30 m HP-5 MS (HP), d_i=0,25 mm, d_f=0,33 µm	
Pneumatics:	He, p_i =30 kPa, split x:30, splitless 0.1-1.1 min		
Temperatures:	HSS pre-heating module:		70°C (10 min)
	HSS turret:		70°C
	HSS syringe:		70°C
	CIS:		-50°C to 300°C with 12°C/s
	Oven:		35°C (2 min), to 260°C with 15°C/min
	MSD:		280°C
Detector:	MSD	scan 10-260 amu	

Results and Discussion

Identification: **Figure 2** shows the chromatogram from an acidified urine sample of a healthy person, acquired with analysis condition I. Without acidifying the number of peaks is significant smaller, but on the other hand some peaks become more prominent as is the case for allylisothiocyanate. The identified volatiles are listed in **Table I**.

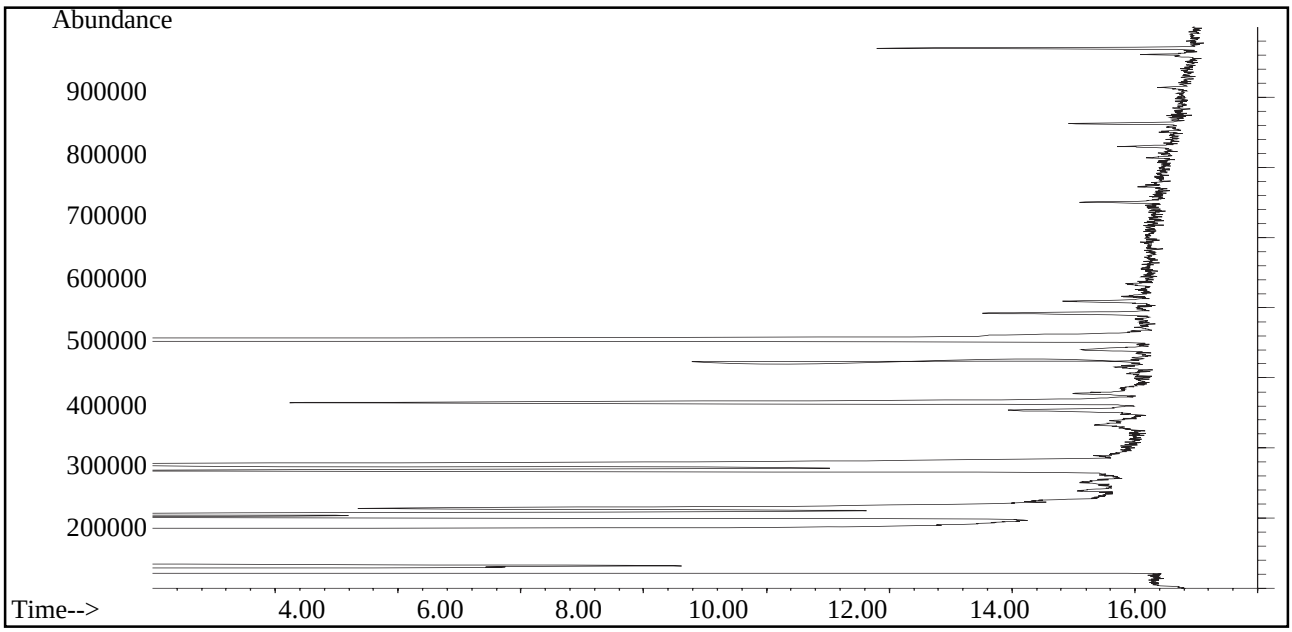

Figure 2. *Chromatogram of acidified urine sample from healthy person.*

organic volatile	RT (min)		organic volatile	RT (min)
methanthiole	3,5		2-hexanone	8,8
trimethylamine	3,8		dimethyldisulfide	9,1
acetone	4,1		toluene	9,9
dihydro-3-methyl-2,5-furandione	4,1		3-hexanone	10,2
dimethylsulfide	4,35		hexanal	10,7
2-butanone	5,3		4-heptanone	13,0
hexane	5,5		allylisothiocyanate	13,4
2-pentanone	7,3		2-ethyl-1-hexanol	17,1
2,5-dimethylfuran	7,9		1-methyl-2-(1-ethylethyl)benzene	17,2

RT: retention time

Table I. *Identified volatiles in urine.*

For a fast analysis of 4-heptanone different conditions were used (*Analysis conditions II*). **Figure 3** and **Table II** show the resulting chromatogram with the identified compounds from a diabetic patient.

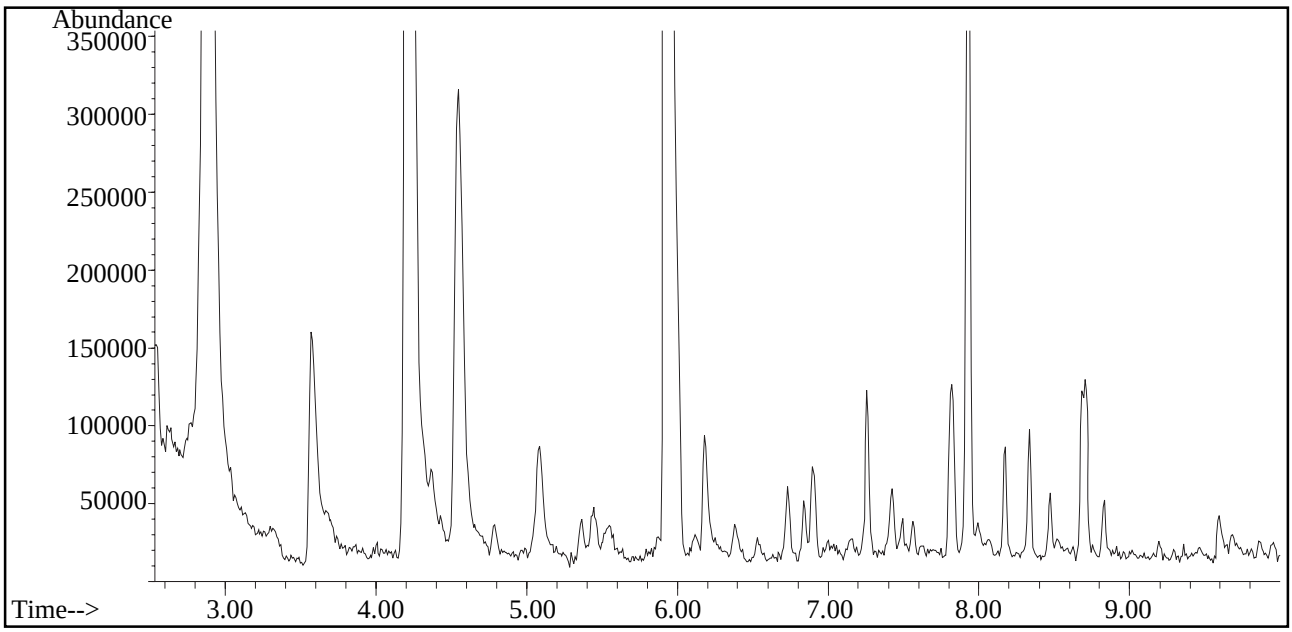

Figure 3. *Chromatogram of acidified urine sample from person with diabetes.*

organic volatile	RT (min)		organic volatile	RT (min)
acetone	2,23		3-heptanone	6,13
2-butanone	2,73		chlorocyclohexane	6,14
chloroform	2,87		2-heptanone	6,16
benzene	3,29		2,4-dimethylthiophene	6,36
cyclohexene	3,45		methyl-2-propenyldisulfide	6,56
2-pentanone	3,56		methylpropyldisulfide	6,71
2,5-dimethylfuran	3,78		propenylmethyldisulfide	6,80
phenol	3,98		3-methyl-2-heptanone	6,87
3-methyl-1-butanol	4,11		dimethyltrisulfide	7,23
dimethyldisulfide	4,20		phellandrene	7,66
toluene	4,52		1,4-dichlorobenzene	7,69
3-hexanone	4,75		α-terpinene	7,80
2-ethyl-5-methylfuran	4,94		1-methyl-2-(1-ethylethyl)benzene	7,90
tetrachloroethylene	5,06		γ-terpinene	8,31
5-methyl-3-hexanone	5,43		4-methylphenol	8,50
4-heptanone	5,90		α-terpinolene	8,64
cyclohexanol	6,07		3-methylhexane-2-one	8,86
allylisothiocyanate	6,10			

RT: retention time

Table II. *Identified volatiles in urine.*

The analysis of ketones is of clinical relevance in metabolic disorders, especially in the case of diabetes mellitus. Next to methylketones, found at elevated levels in ketoacidosis 4-heptanone proved to be an interesting metabolite [8,13]. Figure 4 shows an ion chromatogram (m/z 43, gained from total ion chromatogram) of an urin specimen from a healthy control person, which can be chosen to monitor the following ketones: 2-propanone (a), 2-butanone (b), 2-pentanone (c) and 4-heptanone (d).

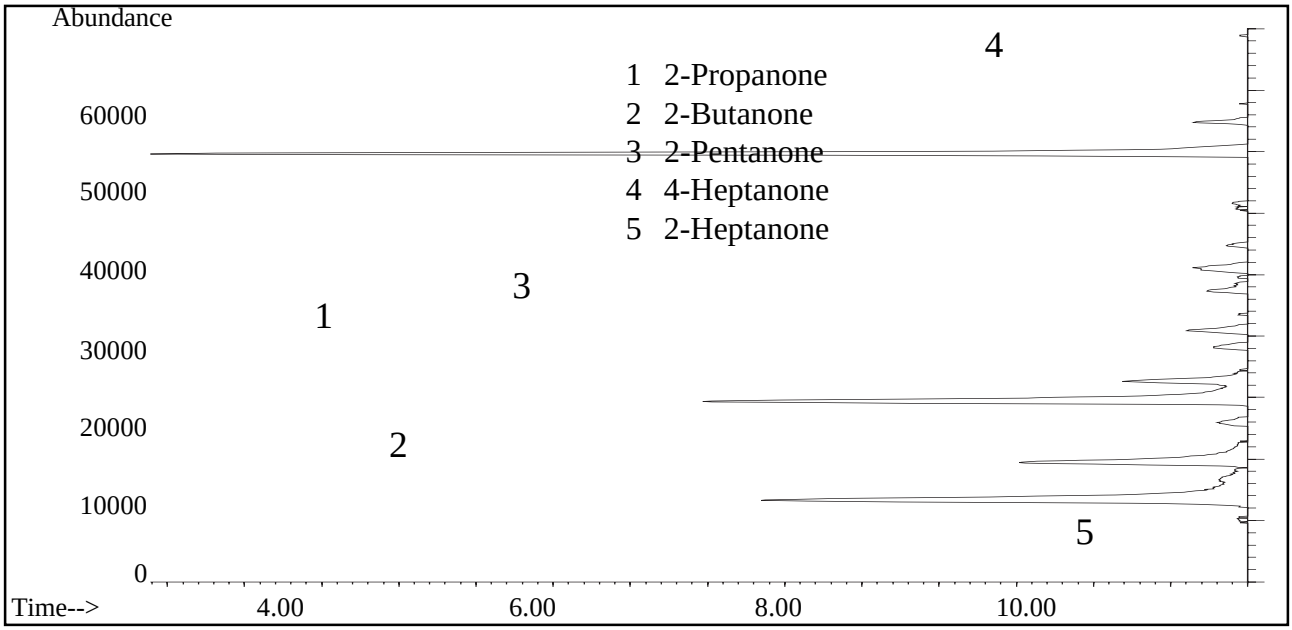

Figure 4. *Healthy person.*

Quantification: 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 = 0,999 (**Figure 5**). The determination of intra assay imprecision showed a coefficient of variation (CV) between 3.0 and 3.4% for the total concentration range.

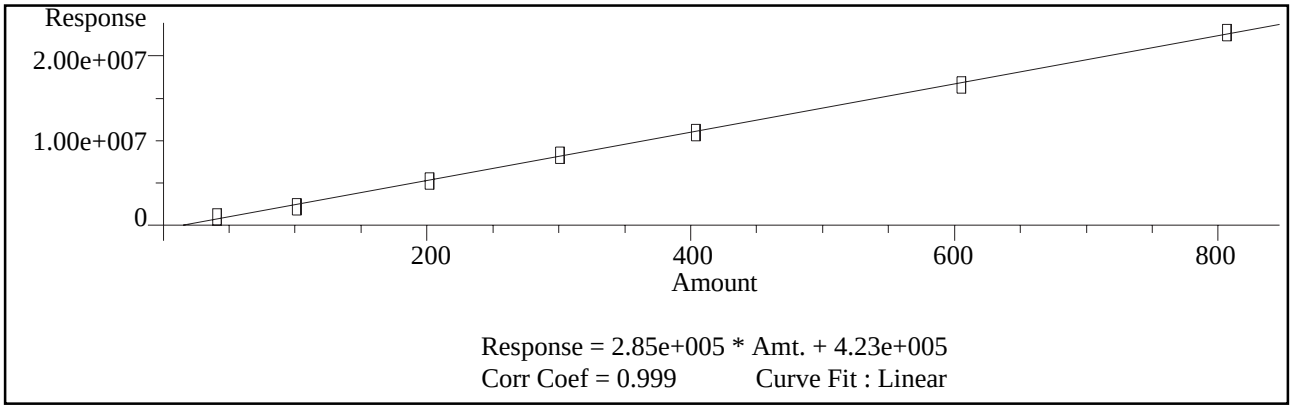

Figure 5. *Calibration curve for 4-heptanone in urine.*

Clinical study: The median concentration of 4-heptanone in urine from the healthy control group 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 (**Figure 6**).

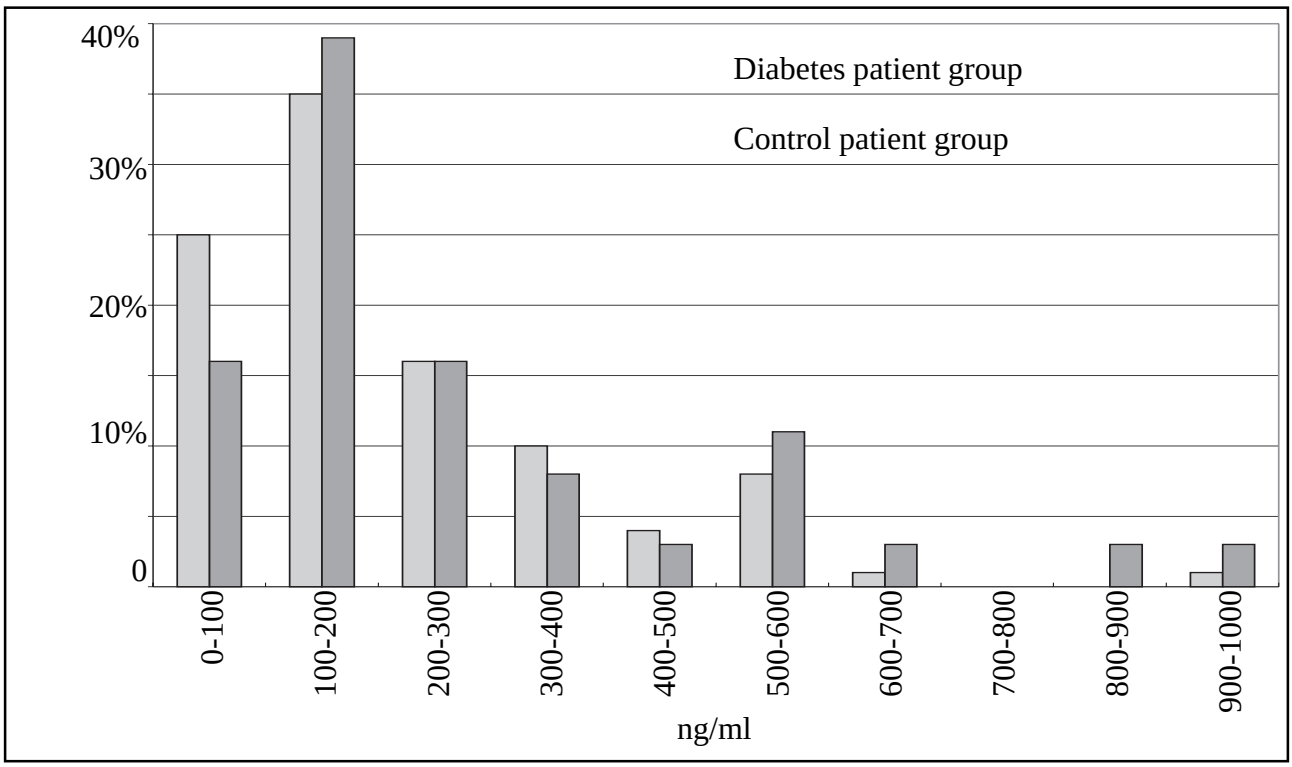

Figure 6. *Distribution of urinary 4-heptanone concentrations in control and diabetic patient group* .

The diabetic patient group was rather inhomogenous regarding duration, metabolic control and type of diabetes. There was no significant correlation to any of these parameters which might at least partially be due to the relative small number of patients (97 samples of 51 patients). In the group of healthy controls 4 persons had very high concentrations of 4-heptanone in urine: 1390 ng/ml, 1650 ng/ml, 1780 ng/ml and 3720 ng/ml. As there is 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 might be solely environmental. One source could be the widespread plasticizer 1,2-benzenedicarboxylic acid-bis-(2-ethylhexyl)-ester, which in vivo could be hydrolyzed and then oxidized to the corresponding ß-keto-acid excreted in urine. Spontaneously and upon heating this acid would yield 4-heptanone. Studies under current investigation will further elucidate the origin of 4-heptanone in human urine and its relation to diabetes mellitus.

CONCLUSION

With the method described organic volatiles in human urine can now be easily analyzed. Applications are metabolic profile studies in a qualitative as well as quantitative way. For 4-heptanone as one example high precision and sensitivity of the method was shown. The high practicality in a day to day routine together with the cost cutting philosophy of a multi purpose sampler makes this automated system very attractive for clinical routine use.

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[26] C.P. Bicchi, A.E. Binello, M.M. Legovich, G.M. Pellegrino, and A.C. Vanni, J Agric Food Chem 41, No. 12, (1993) 2324 -2328.polymer Tenax (poly 2,6-diphenyl-p-phenylene oxide) [5,8,18,19], solvent extraction [4,13] and the use of a transevaporator [20-22]. Modifications have also be done concerning the instrumentation [23,24].

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