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Simultaneous determination of pyrethroid and pyrethrin metabolites in human urine by gas chromatography–high resolution mass spectrometry

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Abstract

A new developed gas chromatographic–high resolution mass spectrometric method for the sensitive simultaneous determination of *trans*-chrysanthemumdicarboxylic acid, *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid, *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid, 3-phenoxybenzoic acid and 4-fluoro-3-phenoxybenzoic acid in human urine is presented. These metabolites are biomarkers for an exposure to pyrethrum, allethrin, resmethrin, phenothrin, tetramethrin, cyfluthrin, cypermethrin, deltamethrin or permethrin. Therefore, with the help of this method for the first time a complete assessment of exposure to pyrethroid and pyrethrin insecticides is possible. After acid hydrolysis and extraction with *tert*-butyl-methyl-ether the residue is derivatized with 1,1,1,3,3,3-hexafluoroisopropanol and analyzed by GC/HRMS in electron impact mode (detection limits < 0.1 μg/l) as well as in negative chemical ionization mode (detection limit < 0.05 μg/l urine).

Keywords: Biomonitoring; Pyrethroids; Pyrethrum; Urine; GC/HRMS; Derivatization

1. Introduction

Pyrethrins as one of the six natural esters of pyrethrum (pyrethrin I/II, jasmolin I/II and cinerins I/II) as well as synthetic pyrethroids such as cyfluthrin, cypermethrin, deltamethrin and permethrin are among the insecticides most often used worldwide. For humans, these insecticides are much less toxic than other insecticides [1,2]. The ADI values (acceptable daily intake) vary between 0.01 mg/kg body weight per day (deltamethrin), 0.04 mg/kg body weight per day (pyrethrum) and 0.05 mg/kg body weight per day (permethrin) [3]. Nevertheless, after exposure, a variety of reversible symptoms such as paraesthesia, irritations of the skin and mucosa, headache, dizziness and nausea are reported [4,5].

Human metabolism studies with cyfluthrin, cypermethrin and pyrethrin I have shown that after oral, inhalative or dermal exposure these substances are metabolized rapidly by hydrolytic cleavage of the ester bond, followed by oxidation and mainly glucuronization yielding the non-toxic metabolites *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*-DCCA and *trans*-DCCA), 3-phenoxybenzoic acid (3-PBA), 4-fluoro-3-phenoxybenzoic acid (F-PBA) or *trans*-chrysanthemumdicarboxylic acid (CDCA) as shown in Table 1 and Fig. 1. The half-life times for urinary excretion differ between 4 and 13 h [6–9]. Half-lives of 6.44 h were found for the urinary excretion of the metabolites *cis*-DCCA, *trans*-DCCA and F-PBA after oral or inhalative exposure to cyfluthrin in volunteers. Ninety-four percent of the metabolites were excreted renally during the first 48 h after exposure [9]. Following a (*S*)-bioallethrin exposure, maximum peak excretion of *trans*-CDCA was within the first 24 h after exposure. Seventy-two hours later the concentration of *trans*-CDCA was below the limit of detection [10]. After oral intake of pyrethrin I, *trans*-CDCA was eliminated rapidly mainly during the first 6 h with the half-life time being 4.2 h [11]. Because of this rapid metabolism the determination of

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Table 1
Pyrethroid insecticides and their metabolites in urine covered by new method

Substance	Biomarker in urine
Allethrin, phenothrin, pyrethrum, resmethrin, tetramethrin	*trans*-Chrysanthemumdicarboxylic acid (*trans*-CDCA)
Cypermethrin, deltamethrin permethrin	*cis*- and *trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*- and *trans*-DCCA)
Deltamethrin	*cis*-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*-DBCA)
Cyfluthrin, cypermethrin, deltamethrin, permethrin	3-Phenoxybenzoic acid (3-PBA)
Cyfluthrin	4-Fluoro-3-phenoxybenzoic acid (F-PBA)

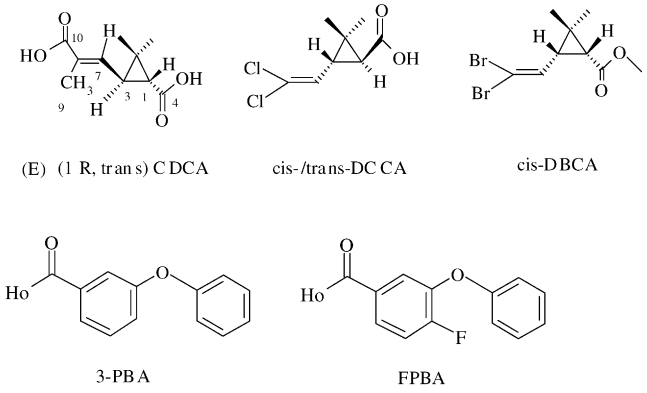

Fig. 1. Molecular structures of pyrethrum and pyrethroid metabolites. CDCA, *trans*-chrysanthemumdicarboxylic acid; *cis*-/*trans*-DCCA, *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid; *cis*-DBCA, *cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid; 3-PBA, 3-phenoxybenzoic acid; and F-PBA, 4-fluoro-3-phenoxybenzoic acid.

urinary metabolites is preferred to blood analysis for the estimation of pyrethroid exposures [12,13].

By now several methods have been published for the determination of pyrethroid metabolites in human urine, which can be divided into GC/MS methods [13–19], LC/MS methods [20,21] or immunoassay methods [22]. The disadvantage of some of these methods is that sample preparation involves time-consuming enzymatic hydrolysis procedures [20,21]. Baker et at. [20] reported selectivity problems determining *cis*-DCCA, *trans*-DCCA and *cis*-DBCA by LC–MS/MS because of unfavorable fragmentation patterns. Immunoassay methods are on the one hand comparable cheap and easy to perform, but on the other hand they do not achieve the sensitivity and specificity of analytical methods. For the determination of the internal pyrethrum, allethrin, resmethrin, phenothrin and tetramethrin burden, *trans*-chrysanthemumdicarboxylic acid (CDCA) turned out to be the relevant biomarker in the urine [10,23]. Regarding the detection of *trans*-CDCA in human urine there are only two publications available [10,17]. Compared to Leng et al., the further developed method of Elflein et al. is much more sensitive (detection limit 0.05 μg/l urine). On the whole it can be concluded that up to now there were only two separate analytical methods available either for detecting the pyrethrum or the major pyrethroid metabolites. Therefore, the need existed to develop a sensitive, reliable and practical method for the analysis of all relevant pyrethrum and pyrethroid metabolites in one analytical run, which safes analysis time and costs and makes it possible to obtain data very fast which is essential for risk evaluation.

Here, a GC/HRMS method is presented, which enables for the first time a quick, sensitive and selective determination of all pyrethrum and pyrethroid metabolites in one analytical run with detection limits until 0.05 μg/l urine in electron impact mode (EI+-mode) being even more sensitive in negative chemical ionization mode (NCI-mode). The possibility to use both detection techniques leads to more flexible sample handling in the daily laboratory routine.

2. Materials and methods

2.1. Chemicals

1,1,1,3,3,3-Hexafluoroisopropanol (HFIP), *N*,*N*-diisopropylcarbodiimide (DIC), 3-phenoxybenzoic acid (3-PBA, 98%) and 2-phenoxybenzoic acid (2-PBA, 98%) used as internal standard were purchased from Sigma-Aldrich, Taufkirchen, Germany.

Acetonitrile, *tert*-butyl-methyl-ether (*t*-BME) and *iso*-octane (all supra solv), hydrochloric acid (37%), sodium hydrogen carbonate (all analysis quality) were purchased from Merck, Darmstadt, Germany.

cis- and *trans*-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*-DCCA, 99.8% and *trans*-DCCA, 99.8%) were synthesized at Bayer Crop Science, Monheim, Germany.

cis-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid (*cis*-DBCA, 98.8%) was obtained from Roussel-Uclaf, Romainville Cedex, France.

4-Fluoro-3-phenoxybenzoic acid (F-PBA, 99.9%) and *trans*-chrysanthemumdicarboxylic acid (*trans*-CDCA, 99.6%) were synthesized at Bayer Crop Science, Monheim, Germany.

2.2. Equipment

2.2.1. Laboratory equipment

Microlitre pipettes, adjustable between 1 and 2000 μl and pipette tips (Eppendorf, Hamburg, Germany); 10 ml glass tubes with Teflon sealed screw caps (Schütt, Germany); volu-

metric flasks (Fisherbrand, Germany), transfer pipette (Sarstedt, Nümbrecht, Germany); centrifuge (Hettich, Tuttlingen, Germany); block heater for hydrolysis (Techne, Cambridge, UK); Vibrax VXR-Mixer (IKA, Staufen, Germany); nitrogen evaporator (Zymark, Engelskirchen, Germany); microvials (Agilent, Palo Alto, CA, USA).

2.2.2. Gas chromatography–high resolution mass spectrometry

A HP 5890II gas chromatograph (Agilent, Palo Alto, CA, USA,) equipped with a split/splitless-injector (split 40 ml/min, septum purge 3 ml/min, purge time 1 min off) and a CTC A 200S autosampler (CTC Analytics, Zwingen, Switzerland) was coupled to an AutoSpec Ultima high resolution mass spectrometer (Micromass/Waters, Manchester, UK). EI with perfluorokerosine (PFK) as calibration gas was used as ionization mode (electron energy 70 eV, ion source temperature 250 °C, interface temperature 250 °C, accelerating voltage 8000 V, multiplier voltage 350 V, resolution 10,000, filament 0.3 mA). Helium (99.999%) was used as carrier gas and the sample separation was performed on a 30 m × 0.25 mm × 0.25 μm Rtx 65 medium polar fused silica capillary column (Restek, Bad Homburg, Germany).

One microliter was injected splitless (1 min) in a 280 °C isothermal heated split–splitless injector equipped with a deactivated double gooseneck liner (Restek, Bad Homburg, Germany). A pressure pulsed injection technique was used with the carrier gas pressure program starting with 120 kPa for 1 min, then decreasing with 100 kPa/min to 80 kPa, which was held till the end of the run. The oven temperature program was ramped from 60 °C (1 min hold time) to 150 °C at 8 °C/min and finally ramped at 30 °C/min to 300 °C with a hold time of 5 min.

In case of determination in negative chemical ionization mode (NCI-mode), ammonia (99.98%) was used as reactant gas at a source pressure of 2×10^{-5} kPa. Optimum ionization conditions were also obtained with PFK as calibration gas for optimum sensitivity and selectivity (electron energy 100 eV, source temperature 180 °C, interface temperature 250 °C, accelerating voltage 8000 V, multiplier voltage 350 V, resolution 10,000, filament 0.5 mA).

2.3. Preparation of standard solutions

About 10 mg (or proportionally more if purity is less than 100%) of *trans*-CDCA, *cis*-DCCA, *trans*-DCCA, *cis*-DBCA, 3-PBA and F-PBA were weighted into separate 10 ml glass volumetric flasks. Each flask was diluted to the mark with acetonitrile. The concentrations of these starting solutions were 1000 mg/l. From these starting solutions, a multi-component stock solution was prepared by diluting 100 μl of each starting solution to the mark with acetonitrile in a 10 ml glass volumetric flask (10 mg/l). From this stock solution, three working solutions were prepared in acetonitrile.

Working solution A: 1000 μl of the stock solution was diluted to the mark in a 10 ml glass volumetric flask (1 mg/l).
Working solution B: 100 μl of the stock solution was diluted to the mark in a 10 ml glass volumetric flask (0.1 mg/l).
Working solution C: 1000 μl of the stock was diluted to the mark in a 10 ml glass volumetric flask (0.01 μg/l).

2.4. Preparation of internal standard solution

The preparation of the stock solution for the internal standard was done by diluting 10 mg 2-PBA in a 10 ml glass volumetric flask with acetonitrile (1000 mg/l). A 100 μl aliquot of this stock solution was diluted to the mark in a 100 ml glass volumetric flask (1 mg/l). This 2-PBA solution was used for the sample preparation as described in Section 2.5.

2.5. Sample preparation

2.5.1. Acid hydrolysis

Two milliliters urine were transferred in a screw cap test tube. Twenty microliters of the internal standard solution (2-PBA, 10 μg/l urine) and 500 ml hydrochloric acid (37%) was added. The test tubes were covered with screw caps and hydrolyzed at 100 °C for 2 h in a block heater.

Acid hydrolysis was used to allow the determination of total (conjugated as well as free) metabolites [24]. No experiments were done regarding the determination of only free metabolites because the focus of this work was the quantitative analysis.

2.5.2. Liquid–liquid extraction and derivatization

Four milliliters of *tert*-butyl-methyl-ether were added to the cold sample, then the urine samples were shaked vigorously for 10 min at a roller mixture, followed by centrifugation for 5 min at 2000 × *g*. With an Pasteur pipette the upper organic phase was transferred in a new screw cap test tube, whilst the lower urine phase was discarded. The organic phase was dried for 10 min in a nitrogen evaporator with a stream of 0.5 kPa N_2 and a water bath temperature of 40 °C.

The residue was dissolved in 250 μl acetonitrile. For derivatization, 30 μl of HFIP and 20 μl of DIC was added. The solution was slightly mixed for 10 min at room temperature on a roller mixture. Then 1 ml of an 1 M sodiumhydrogencarbonate solution, which was used for eliminating excess derivatization reagents and 250 μl *iso*-octane (for determination in NCI-mode 2 ml *iso*-octane) were added. The test tubes were covered and vigorously mixed for 10 min to extract the HFIP-esters. The sample was then centrifugated for 5 min at 2000 × *g* for phase separation. The *iso*-octane phase was finally transferred in a microvial.

2.5.3. Analytical determination

One microliter of the sample was injected into the GC/MS-system; the corresponding SIM-masses and retention times are given in Table 2.

Table 2
Signal-to-noise ratio for all metabolites at a standard concentration of 0.1 μg/l in EI+-mode and 0.02 μg/l in NCI-mode

Pyrethroid metabolite	Retention time (min)	Target mass in EI+-mode (*m/z*)	Signal-to-noise ratio (S/N)	Target mass in NCI-mode (*m/z*)	Signal-to-noise ratio (S/N)
trans-CDCA	6:18	331.077	10:1	330.069	10:1
cis-DCCA	7:32	323.027	20:1	322.020	10:1
trans-DCCA	7:43	323.027	20:1	322.020	12:1
cis-DBCA	10:50	368.975	3:1	365.969	3:1
2-PBA	14:27	364.053	(ISTD)	213.055	(ISTD)
3-PBA	14:38	364.053	20:1	213.055	20:1
F-PBA	14:05	382.044	20:1	231.046	50:1

3. Results and discussion

3.1. Instrumental parameters

The advantage of high mass resolution is the ability to differentiate between ions of interest and interfering compounds with different chemical structures and the same nominal masses. Therefore, this routine method enhances the accuracy of analytical results. In Fig. 2 for instance, a stereoselective resolved chromatogram of each pyrethrin and pyrethroid metabolite is shown. Another advantage is the introduction of electronegative fluorine, which makes it possible to use electron capture processes for optional determinations in NCI-mode (Figs. 3 and 4). The choice of NCI enables higher sensitivity with detection limits achieving the lower ng/l range [10]. Based on the weak fragmentation process in NCI-mode, the stability or reproducibility is higher influenced by the sample matrix and source conditions, which plays a minor rule in EI+ detection. Residues of samples with high matrix interferences such as urine may cause rapid contamination of the NCI source. This depends on the reactant gas used. Ammonia was found to be a good compromise to methane. However, routine work on a magnetic sector MS requires well trained staff, especially if NCI is used.

This method used 2-PBA as internal standard for all compounds in both ionization techniques. Although 2-PBA eluates at a later retention time it compensates all the influences of the analysis procedure as well as GC/MS determination, which is shown in Table 3. It was found that only a dilution of the sample extract enabled the use of 2-PBA as internal standard for all compounds in NCI-mode, too. A very good alternative could be the use of deuterated or ^{13}C-labelled analogues as internal standards [15,18].

Alternatively to the applied Rtx 65 capillary column a 30 m × 0.25 mm × 1 μm DB5 and a 30 m × 0.25 mm × 0.25 μm DB5 column was tested [10]. All columns gave similar results but the Rtx 65 column was the column of choice because of its better resolution of *cis*-/*trans*-DCCA and 2-PBA/3-PBA. Moreover, a good chromatographic resolution of *cis*-CDCA was achieved with this column. Although here it has to be emphasized that *cis*-CDCA does not seem to be present in human urine [5,12]. Acid hydrolysis was used to allow quantitative analysis of total levels of the metabolites. No experiments were performed regarding the determination

Table 3
Quality control data for precision within-day (n = 6) and between-day (n = 6) in EI+ as well as NCI-mode for *trans*-CDCA, *cis*-/*trans*-DCCA, *cis*-DBCA, 3-PBA and F-PBA spiked in urine with concentrations 0.2, 1.0 and 10 μg/l

Analyte	EI+-mode						NCI-mode					
	0.2 μg/l		1.0 μg/l		10.0 μg/l		0.2 μg/l		1.0 μg/l		10.0 μg/l	
	Recov. (%)	R.S.D. (%)	Recov. (%)	R.S.D. (%)	Recov. (%)	R.S.D. (%)	Recov. (%)	R.S.D. (%)	Recov. (%)	R.S.D. (%)	Recov. (%)	R.S.D. (%)
Within-day												
trans-CDCA	90	10	95	5.3	102	2.7	114	3.8	90	4.1	102	5.1
cis-DCCA	106	4.7	104	5.2	97	3.4	104	7.6	94	4.5	87	8.7
trans-DCCA	108	5.2	97	4.4	97	2.1	96	3.8	106	3.0	97	7.6
cis-DBCA	94	6.6	102	3.5	99	1.9	94	6.0	94	4.6	74	9.3
3-PBA	101	7.8	100	3.0	101	1.3	83	7.3	96	7.0	105	3.6
F-PBA	101	5.1	104	3.0	101	2.1	94	1.6	93	3.6	103	1.2
Between-day												
trans-CDCA	86	13.3	103	3.2	88	10.7	103	8.1	107	6.5	105	13.1
cis-DCCA	104	13.9	87	7.0	94	6.4	90	11.2	90	13.3	99	13.0
trans-DCCA	105	13.0	86	9.0	93	6.4	83	13.4	102	12.7	93	10.0
cis-DBCA	95	9.2	96	10.9	100	5.4	95	10.9	94	6.2	97	10.7
3-PBA	99	6.9	99	3.2	92	1.7	76	10.7	96	7.4	101	11.7
F-PBA	104	6.7	101	4.7	101	2.5	91	6.0	110	5.9	99	8.8

Notes: R.S.D.: relative standard deviation; Recov.: Recovery.

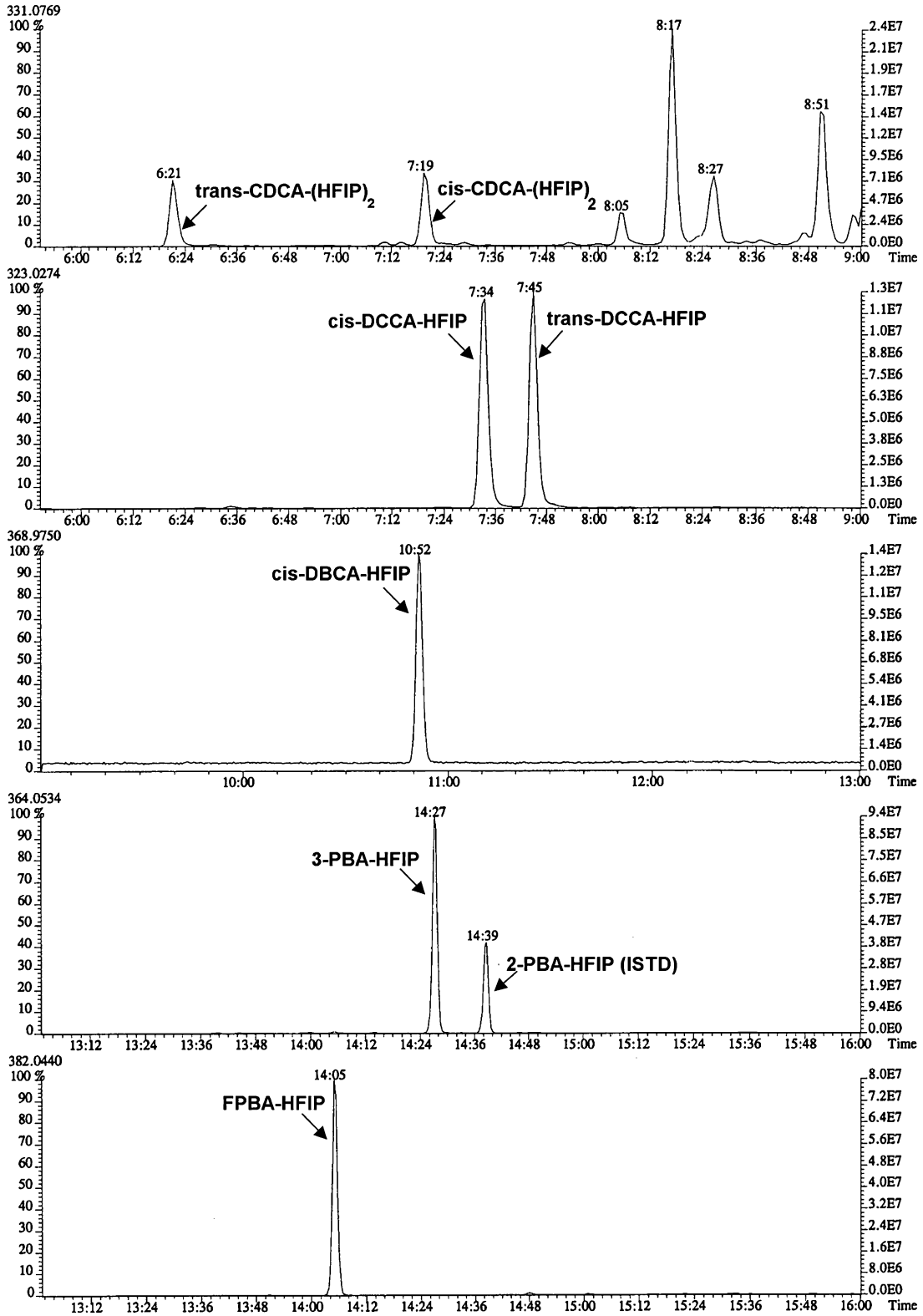

Fig. 2. High resolution EIMS chromatogram of a blank urine sample spiked with 20 μg/l *trans*-CDCA, *cis*-/*trans*-DCCA, *cis*-DBCA, 3-PBA, and F-PBA (2-PBA served as internal standard).

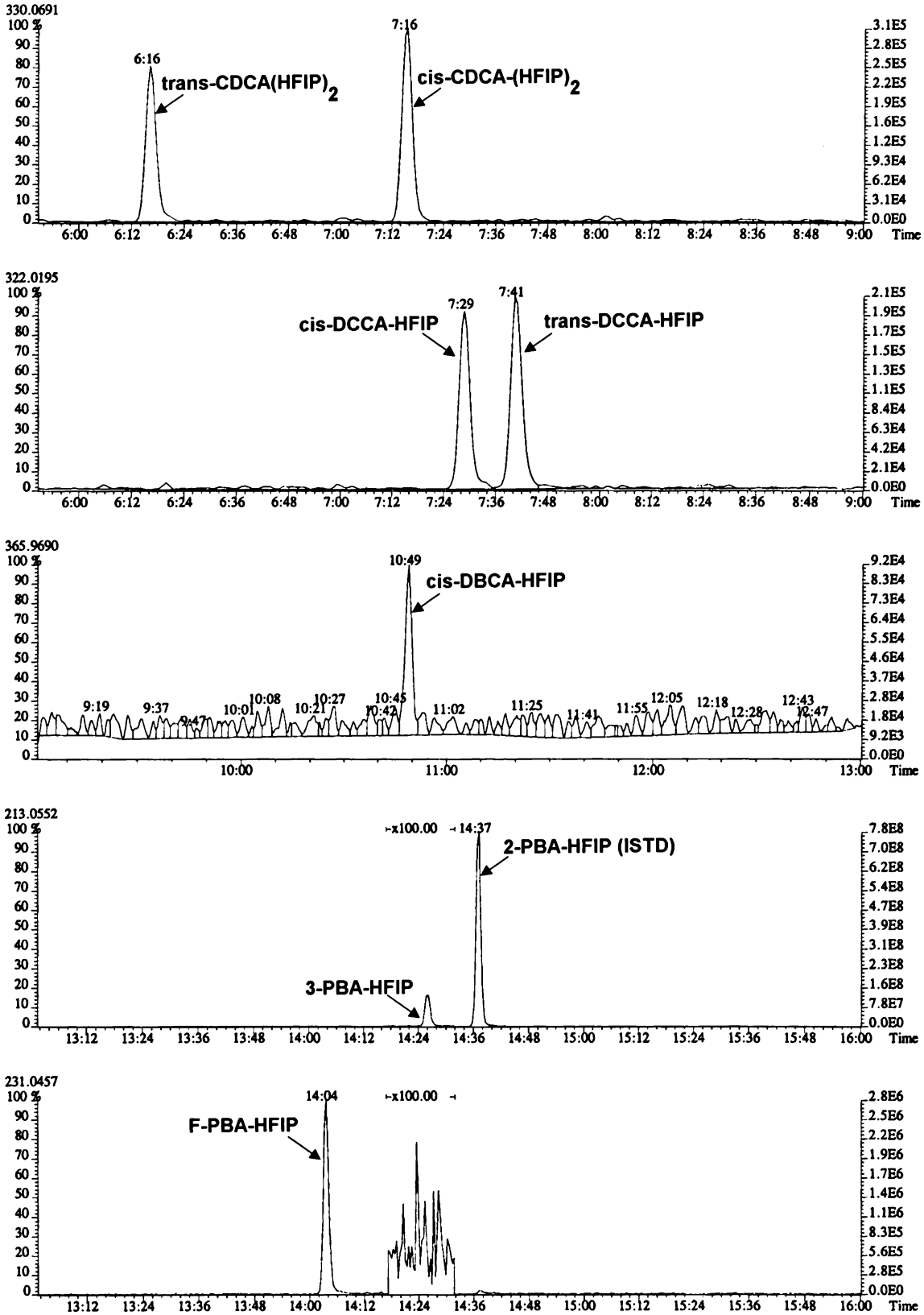

Fig. 3. High resolution NCIMS chromatogram of a blank water sample spiked with 0.02 μg/l *trans*-CDCA, *cis*-/*trans*-DCCA, *cis*-DBCA, 3-PBA, and F-PBA (2-PBA served as internal standard).

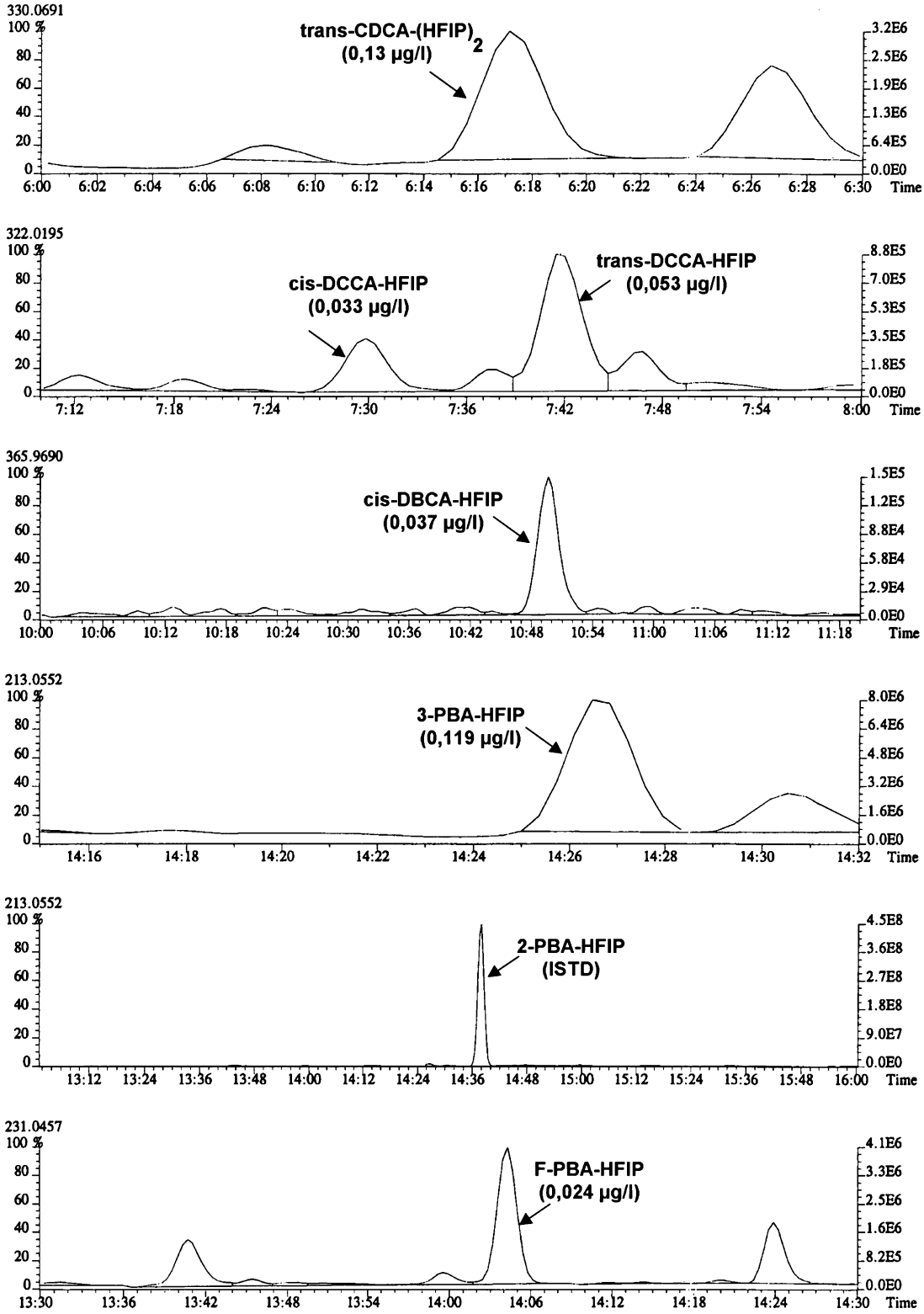

Fig. 4. High resolution NCIMS chromatogram of a native human urine with background exposure to pyrethrum and pyrethroids.

of free metabolites because the hydrolyzation step enabled the determination of conjugated as well as free metabolites [20].

3.2. Calibration procedure

Calibration curves were prepared with urine of non-exposed subjects. In EI+-mode the linearity of each metabolite was tested in a range between 0.05 and 100 μg/l at 11 calibration points (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0 μg/l). The calibration curves were linear with correlation coefficients being more than 0.995.

In case of determination in NCI-mode, the same calibration procedure was used. Based on the high sensitivity of this technique only a calibration range between 0.02 and 10 μg/l was considered. Higher concentrations led to non-linearity because of saturation of the ion source. Therefore, nine calibration points were prepared at 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0 μg/l. The correlation coefficients found for these curves were similar to those in EI+-mode.

As an internal standard 2-PBA was used for all tested compounds. Calibration curves were calculated for each compound by plotting the quotient of the analyte area and the area of the internal standard against the analyte concentration. The prepared calibration solutions were aliquoted in four portions of 50 μl for each calibration level in separate microvials and were used for several days. A calibration curve was measured and calculated in each analytical run. Each run was controlled with two or three quality control samples, which were analyzed simultaneously with the samples.

3.3. Reliability of the method

3.3.1. Extraction efficiency

To get an overview regarding the recovery of the *t*-BME extraction procedure three urine samples were spiked with 1 μg/l and three with 10 μg/l of each metabolite. In addition to that procedure, six blank urine samples were analyzed parallel as described in Section 2.5. Following the *t*-BME extraction, three of the blank urines were spiked with 1 μg/l and three with 10 μg/l. The extraction step seemed to work quantitatively as there was no significant difference between the response of the direct spiked samples and the ones after *t*-BME extraction.

3.3.2. Influence of urine creatinine concentration

One important influence on the analysis results could be attributed to different urine matrices. For the evaluation of these effects six individual urines with different creatinine concentrations (0.5, 0.67, 1.3, 1.48, 1.6 and 2.1 g/l) were spiked three times with 10 μg/l of each metabolite. The samples were analyzed as described in Section 2.5. It turned out that the recovery rates of all samples were similar to those of the within series determinations without any exception.

3.3.3. Influences of the derivatization yield

Several publications suggested that the derivatization only works quantitatively in non-polar solvents [17,25]. We had the problem that after evaporation of *t*-BME the urine residue could not be solved in non-polar but only in polar solvents. In contrast to literature, acetonitrile was found to be a good compromise because, on the one hand, the urine residue could be solved, and on the other hand, the described derivatization procedure could be performed. Another advantage was the efficiency of the sample clean up based on the solvation of most parts of the sample matrix in the alkaline water phase, whereas the yielded HFIP-ester was extracted with *iso*-octane. For the estimation of possible influences several derivatization procedures were tested. Blank urine samples were analyzed as described in Section 2.5. After the extraction with *t*-BME all samples were spiked with 10 μg/l of each metabolite. The following procedures were done three times: First of all, it was tested if there was a difference between a derivatization in acetonitrile and in *iso*-octane. Because the urine residue could not be solved in *iso*-octane it was first solved in 30 μl HFIP and 20 μl DIC and then 250 μl *iso*-octane was added. After 10 min reaction time the sample was washed as described and the *iso*-octane phase was transferred to a microvial. This sample was used as standard sample. The color of the *iso*-octane extract showed that the major part of the urine matrix was not washed out in the alkaline water phase. In comparison to this, the samples solved in acetonitrile were only slightly colored. The results of this procedure showed that the recovery yield of the *iso*-octane derivatization was 30% higher than the one in acetonitrile [14]. The lower clean up efficiency of the *iso*-octane derivatization was found to be a major disadvantage because the high matrix level caused a rapid contamination of the GC-injector and the capillary column.

In the next step several concentrations of HFIP/DIC (10/15, 15/20, 30/20, 30/50 and 50/75 μl) were tested in several acetonitrile volumes (50, 100, 250 and 500 μl). The highest recovery was found in case of low volumes of acetonitrile (50 or 100 μl) combined with low HFIP/DIC ratios (10/15 and 15/20 μl). Another result was that the volume of DIC had no influence on the recovery rate. Moreover, each combination of acetonitrile, HFIP and DIC gave a linear calibration curve for each metabolite with similar standard deviations as demonstrated in Table 3. As a consequence of these results the combination of 250 μl acetonitrile, 30 μl HFIP and 20 μl DIC turned out to be the most appropriate solution obtaining the cleanest analysis sample.

The derivatization with HFIP works only in water free samples. Therefore, it is important to separate *tert*-butyl-methyl-ether carefully from the lower water phase. HFIP is a very powerful reagent which reacts spontaneously with carboxylic acids, whilst DIC was used as a catalyst eliminating water by reacting to *N*,*N*′-diisopropylcarboxydiamin [15,17,25].

3.3.4. Method precision

By spiking blank pooled urine of laboratory personnel with three concentration (0.2, 1.0 and 10 μg/l) of quality control standards, the within-series precision was determined six times as recoveries and relative standard deviations (R.S.D.). The recovery was calculated against the calibration curves in a range from 0.05 to 10 μg/l for each metabolite in EI+ and NCI-mode (Table 3). At a spiked concentration of 0.2 μg/l R.S.D. varied between 1.6% (F-PBA) and 7.6% (*cis*-DCCA) in NCI-mode.

Between-day precision was also tested with three quality control standards (0.2, 1.0 and 10.0 μg/l urine) on six different days in a week varying at 0.2 μg/l between 6% (F-PBA) and 13.4% (*trans*-DCCA) in NCI-mode (Table 3).

3.3.5. Limit of detection

The limit of detection was defined as a signal-to-noise ratio of three. In EI+-mode, the detection limit was 0.05 μg/l for *trans*-CDCA, *cis*-DCCA and *trans*-DCCA, 0.1 μg/l for *cis*-DBCA and 0.02 μg/l for 3-PBA and F-PBA. If NCI-mode was used, lower detection limits were achieved: 0.05 μg/l for *trans*-CDCA, 0.02 μg/l for *cis*-DCCA, *trans*-DCCA and *cis*-DBCA, 0.01 μg/l for 3-PBA and 0.005 μg/l for F-PBA. Table 2 demonstrates the power of GC–HRMS if standard solutions were measured.

3.3.6. External quality control

The results obtained by the new method were assured by regular participation at interlaboratory comparison programs organized in Germany for the determination of pyrethroid metabolites in human urine. Our results corresponded very well with the theoretical values of the spiked urine samples.

3.3.7. Storage stability

The starting solutions and stock solutions can be stored in a deep freezer at −18 °C for at least 6 months. Longer times were not tested. Urine samples can be stored for more than a year at −21 °C in a deep freezer [12]. The stability of the derivatized final sample was also tested. It was found that they were stable for 1 month in a refrigerator at 4 °C. Longer times were not checked.

3.4. Results of biological monitoring

In order to check the practicability of the method for use in case of low exposure, metabolite concentrations were measured in urine samples of 30 persons, who applied commercially available vaporizer plates or sprays containing pyrethrum. Twenty-four-hour urine was collected in polyethylene bottles and then stored at −20 °C until analysis. *trans*-CDCA was found above detection limit in 90% of the samples with the mean concentration being 1.1 μg/l urine and the maximal concentration reaching 54 μg/l urine (Table 4). Besides CDCA, pyrethroid metabolites *cis*-DCCA, *trans*-DCCA and 3-PBA were found in most subjects with mean concentrations in the range of the background burden of the general population with one exception (maximal value).

Moreover, to demonstrate the suitability of the method for quantifying values around the detection limit safely, urine samples of 15 persons not knowingly exposed to pyrethrum or pyrethroids were investigated. Table 5 shows that there was a general background level of pyrethroid metabolites as well as to a less extent to *trans*-CDCA, which was found in 4 out of 15 urine samples in concentrations between 0.05 and 0.13 μg/l urine. The 95th percentiles were comparable higher than those mentioned in literature (for *cis*-DCCA, *trans*-DCCA and 3-PBA: 0.5–0.6, 0.9 and 0.2 up to 0.9 μg/l, respectively) [26–30]. Background values for CDCA, *cis*-DBCA and F-PBA were quantified for the first time. In general, food is assumed to be a major source for pyrethroid intake. This is also confirmed by person "L" being a vegetarian emphasizing on tropical fruits.

In Fig. 4, an example chromatogram showing background levels from one subject is shown.

3.5. Comparison with other methods

Using GC/MS methods Arrebola et al. [19] reported recovery ranges at 10 μg/l between 89.9 and 121.1% (R.S.D. 4.8–14.5%), Schettgen et al. [18] found at 0.4 μg/l recovery rates between 79.3 and 93.4% (R.S.D. 2.0–5.4%) and Elflein et al. [17] found at 1 μg/l a recovery rate of 104 ± 7%. Baker et al. [20] reported similar results on a LC–MS/MS System. All these methods cover only pyrethroid or pyrethrum metabolites. The quality control data of the presented method is comparable to these findings. Moreover, it has to be emphasized that with the help of this method it is for the first time possible to evaluate the body burden of all relevant pyrethroids and pyrethrins in one analytical run, which saves analysis time and costs. In a normal 8 h working day it is possible for one lab assistant to routinely handle 48 samples in two 24-sample blocks. This method is easy to handle and

Table 4
Biological monitoring of 30 subjects exposed to pyrethrum (number (*N*) of samples above detection limit (%) as well as mean ± S.D., minimum, median and maximum concentrations of *trans*-CDCA, *cis*- and *trans*-DCCA and 3-PBA in urine samples are given)

	trans-CDCA (μg/l urine)	*cis*-DCCA (μg/l urine)	*trans*-DCCA (μg/l urine)	3-PBA (μg/l urine)
N > LOD (%)	90	69	91	97
Mean ± S.D.	1.10 ± 4.35	0.46 ± 1.30	1.00 ± 2.58	1.24 ± 2.81
Minimum	<0.05	<0.02	<0.02	<0.01
Median	0.23	0.15	0.29	0.49
Maximum	54	11.3	23.4	25.6

Table 5
Background concentrations of pyrethroid and pyrethrum metabolites in urine of 15 not-exposed subjects (for calculation values below detection limit were considered with 1/2 detection limit)

Urine-sample	*trans*-CDCA (μg/l urine)	*cis*-DCCA (μg/l urine)	*trans*-DCCA (μg/l urine)	*cis*-DBCA (μg/l urine)	3-PBA (μg/l urine)	F-PBA (μg/l urine)
A	<0.05	0.048	0.068	<0.02	0.133	0.021
B	<0.05	0.046	0.087	0.038	0.103	<0.01
C	<0.05	0.055	0.075	0.074	0.109	<0.01
D	<0.05	0.018	0.022	<0.02	0.027	<0.01
E	<0.05	0.031	0.052	<0.02	0.096	<0.01
F	<0.05	0.024	0.028	<0.02	0.054	<0.01
G	0.12	0.069	0.100	0.046	0.155	<0.01
H	0.05	0.212	0.330	0.051	0.470	<0.01
I	<0.05	0.038	0.060	<0.02	0.033	<0.01
J	<0.05	0.362	0.656	0.036	0.702	0.010
K	<0.05	0.070	0.095	<0.02	0.137	<0.01
L[a]	0.11	2.33	3.35	0.040	3.063	<0.01
M	0.13	0.033	0.053	0.037	0.119	0.024
N	<0.05	0.027	0.047	0.177	0.142	<0.01
O	<0.05	0.074	0.079	0.037	0.132	<0.01
$N >$ LOD	4	15	15	9	15	3
Mean ± S.D.	0.05 ± 0.04	0.23 ± 0.56	0.34 ± 0.85	0.04 ± 0.04	0.37 ± 0.77	0.01 ± 0.01
Median	0.03	0.05	0.08	0.04	0.13	0.01
95th percentiles	0.12	0.95	1.46	0.11	1.41	0.02

[a] Vegetarian.

does not consist of time consuming clean up procedures as published in some methods covering only part of the parameter spectrum. This method enriches the existing methods by applying alternative analysis as well as detection techniques.

4. Conclusions

A gas chromatographic–high resolution mass spectrometric method, which enables for the first time a very sensitive and reliable determination of natural pyrethrum as well as synthetic pyrethroid metabolites in urine in one analytical run, is presented. This method is adequate for simultaneously evaluating the internal burden of pyrethrum, allethrin, resmethrin, phenothrin, tetramethrin, cyfluthrin, cypermethrin, deltamethrin and permethrin.

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