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Human biomonitoring of pyrethrum and pyrethroid insecticides used indoors: Determination of the metabolites *E-cis*/*trans*-chrysanthemumdicarboxylic acid in human urine by gas chromatography-mass spectrometry with negative chemical ionization

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Abstract

This work describes a gas chromatographic-mass spectrometric method employing negative chemical ionization (NCI) for the determination of *E-cis*/*trans*-chrysanthemumdicarboxylic acid (CDCA) in human urine used as a biomarker for the exposure to pyrethrum and/or certain pyrethroids in insecticide formulations applied indoors. Mixed-mode solid phase extraction was utilized for sample cleanup. Extraction recoveries ranged from 92 to 104% (2–9% R.S.D.). The acids were esterified with 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) allowing both their gas chromatographic separation and their sensitive mass spectrometric detection under NCI conditions. Detection limits of ca. 0.05 μg/l urine were achieved.

Keywords: Derivatization, GC; Pyrethrum; Pyrethroid insecticides; Chrysanthemumdicarboxylic acid

1. Introduction

Chrysanthemumdicarboxylic acid (CDCA) (Fig. 1) is a mammalian metabolite of the pyrethroids allethrin, resmethrin, phenothrin, tetramethrin and of the pyrethrins (i.e. pyrethrin I + II, cinerin I + II, jasmolin I + II) which are the six naturally occurring esters of pyrethrum. Pyrethrum and the mentioned synthetic pyrethroids, which are derived from natural pyrethrum, are commonly used in residential and horticultural pest control operations as insecticides with a flushing, rapid knock-down and kill effect. Many methods for the analysis of these insecticides in (indoor) air, house dust, surfaces or greenhouses

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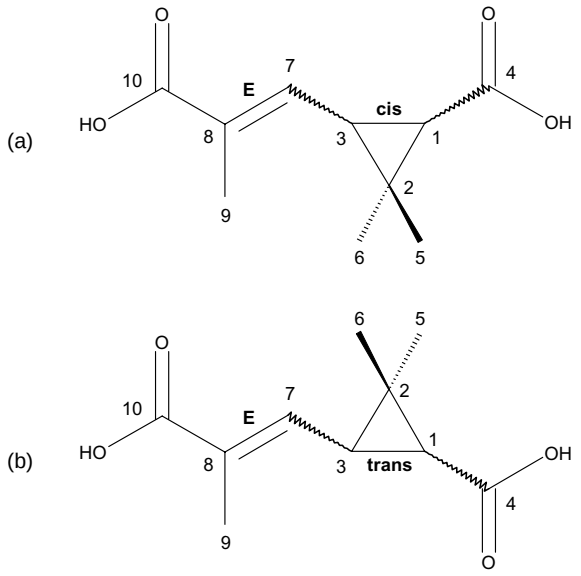

Fig. 1. Molecular structures and designations of *E-cis*/*trans*-chrysanthemumdicarboxylic acid. (a) *E-cis*-chrysanthemumdicarboxylic acid, IUPAC name: (1*RS*,3*SR*)-3-[(*E*)-2-carboxy-propenyl]-2,2-dimethylcyclopropanecarboxylic acid; (b) *E-trans*-chrysanthemumdicarboxylic acid, IUPAC name: (1*RS*,3*RS*)-3-[(*E*)-2-carboxy-propenyl]-2,2-dimethylcyclopropanecarboxylic acid.

during and after application of pesticide formulations have been published [1–16], allowing to determine the external exposure levels for humans living or working in treated areas. The comparatively small dermal and oral uptakes, the rapid metabolism of the above-mentioned insecticides and their low acute toxicity for birds, mammals and humans explains why these agents are considered to be relatively save [17–20]. However, it is important to know the internal burden due to inhalation (as the main pathway for their uptake) caused by a given external exposure level in order to assess any health effects of these insecticides for humans. Sensitive methods with low detection limits are thus required for the determination of biomarkers such as the metabolite chrysanthemumdicarboxylic acid. Examples for biological monitoring of various pesticides and their metabolites in body fluids have been reported in the literature [21–43]. Only one method for the determination of chrysanthemumdicarboxylic acid as diethyl or dipropyl ester using gas chromatography-mass spectrometry (GC-MS) with electron impact ionization has been published so far [17]. However, the reported detection limits were rather high (10–20 μg/l), making this method hardly suitable for the monitoring of lower insecticide levels as expected particularly in the indoor environment. The use of an electron capture detector (ECD) or a mass spectrometer (MS) with negative chemical ionization (NCI) has proved to be more sensitive for detection [15,33,39,44–46], because matrix interferences are reduced and the sensitivity can be enhanced by incorporating one or more electronegative substituents in the molecule by derivatization. The new method presented combines the advantages of the NCI-MS technique after sample derivatization with an effective sample enrichment by a newly developed mixed-mode solid phase extraction method. It offers enhanced sensitivity and robustness and is particularly suited for the routine biomonitoring of low levels of pyrethrins and several pyrethroids after indoor application.

2. Materials and methods

2.1. Chemicals

The *cis*/*trans*-CDCA was synthesized by Bachem AG, Weil am Rhein, Germany, according to method b described in [17] (synthetic racemic mixture). Synthesis led to a mixture of the two chrysanthemumdicarboxylic acids and additional unknown impurities. The fractionation of this mixture is described below.

p-Phenylenediacetic acid (PDAA, internal standard), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), *N*,*N*′-diisopropylcarbodiimide (DIC) and Sylon-CT silylation reagent were obtained from Sigma-Aldrich, Munich, Germany.

Ethyl acetate (EtAc), methanol (MeOH), acetonitrile (AcCN), toluene, 1,4-dioxane (99.0%) and *n*-hexane were purchased from Riedel-de-Haen, Seelze, Germany.

Potassium carbonate, potassium hydroxide (KOH), hydrochloric acid (HCl), concentrated ammonium hydroxide solution (NH_4OH, 25%) and formic acid (HCOOH) were obtained from VWR International, Darmstadt, Germany.

Dimethylsulfoxide-d6 (100%), chloroform-d (100%) and cyclohexane-d12 (99.5%) were received from Deutero GmbH, Kastellaun, Germany.

Maleic acid (99.907%) was obtained from the Federal Institute for Materials Research and Testing, Berlin, Germany.

2.2. Equipment

2.2.1. Laboratory equipment

Sonorex RK100H ultrasonic bath (Bandelin Electronic, Berlin, Germany); microliter pipettes and pipette tips (Eppendorf, Hamburg, Germany); silanized 250 μl glass flat bottom inserts and 2 ml autosampler vials (Agilent Technologies, Waldbronn, Germany); glass beakers, Pasteur pipettes, 12 ml glass centrifuge tubes with screw caps containing a PTFE sealing, and volumetric flasks (Omnilab, Gehrden, Germany); Vibrax-VXR roller-mixer (Janke & Kunkel, Staufen i. Br., Germany).

Note: Always use silanized glassware! It prevents the $(HFIP)_2$ esters from adhering to the glass surface. The glassware was treated with Sylon-CT silylation reagent according to the accompanying document.

2.2.2. Solid phase extraction equipment

Waters Oasis® MAX cartridges, 6 ml/150 mg (Waters, Eschborn, Germany); Visiprep SPE vacuum manifold (Supelco, Taufkirchen, Germany).

2.2.3. Instrumental methods

2.2.3.1. Gas chromatography-mass spectrometry (GC-MS). An Agilent 6890 gas chromatograph equipped with split/splitless-injector, Agilent 7683 autosampler and HP-5MS column (60 m length, 250 μm i.d., 0.25 μm d_f bonded phase of 5% diphenyl/95% dimethylpolysiloxane on fused silica), was coupled to an Agilent 5973 mass selective detector (MSD). NCI with methane (40%) as reagent gas was used as ionization mode (electron energy 96 eV, ion source temperature 150 °C). The injection port temperature was set to 250 °C, the transfer line temperature to 280 °C and the quadrupole temperature to 106 °C. The electron multiplier voltage was 2518 V. The carrier gas flow was adjusted to 1.4 ml/min helium (constant flow mode). The oven temperature program was ramped from 50 °C (1 min holdup time) to 190 °C (10 °C/min) and finally to 280 °C (25 °C/min), with a holdup time of 5 min. The injection volume was 1 μl.

The MSD was run in selected ion monitoring (SIM) mode. The following target and qualifier ions (*m*/*z*) were monitored: *cis*/*trans*-chrysanthemumdicarboxylic acid (498/330), *p*-phenylenediacetic acid (298/326, internal standard).

The underlined masses (*m*/*z*) given in parentheses were used for quantification (target ions), the other masses (*m*/*z*) for confirmation of a specific compound (qualifier ions). Every compound was identified by retention time (±0.2 min) and target/qualifier ion response ratio (with a maximum acceptable error of ±20%).

Note: Always use silanized injection port liners (see Section 2.2.1)!

2.2.3.2. High performance liquid chromatography with UV detection (HPLC-UV). A Waters Alliance 2690 HPLC separation module equipped with a Nucleosil 120-C18, 250 mm × 3 mm analytical column (Macherey-Nagel, Düren, Germany) and a Waters 996 photodiode array detector (190–400 nm) were used for the fractionation of both the purchased synthetic racemic mixture of CDCA and its $(HFIP)_2$ esters (Section 2.3).

The gradient program for the separation of the free acids was as follows: 0.2% HCOOH + MeOH + AcCN (95 + 5 + 0 vol.%) → 0.2% HCOOH + MeOH + AcCN (70 + 12 + 18 vol.%) in 35 min → 0.2% HCOOH + MeOH + AcCN (67.8 + 13.3 + 18.9 vol.%) in 11 min. The flow rate was kept at 0.5 ml/min and oven temperature at 30 °C, detection at 260 nm. For fractionation, about 100 μg of the synthetic racemic mixture, dissolved in eluent, were injected onto the column.

The gradient program for the cleanup of the $(HFIP)_2$ esters (Section 2.3) was: water + MeOH + AcCN (67.8+13.3+18.9 vol.%) → water+MeOH+ AcCN (0 + 0 + 100 vol.%) in 45 min. Flow rate, oven temperature and detection were the same as above.

The $(HFIP)_2$-ester of the internal standard PDAA was purified with the following isocratic method: water + AcCN (35 + 65 vol.%), detection at 192 nm.

2.2.3.3. Proton nuclear magnetic resonance spectroscopy (^1H NMR). A Bruker DRX 600 NMR instrument equipped with a 2.5 mm $^1H/^{13}C$ inverse-dual probe head with *z*-gradient was used to identify and quantify *E*-*cis*/*trans*-CDCA and its corresponding $(HFIP)_2$ esters. For the quantification of the acids, aliquots of the HPLC fractions were evaporated to

dryness and the residues dissolved in DMSO-d6 with maleic acid as quantification standard. The esters were quantified after addition of 1,4-dioxane as standard to aliquots of the cyclohexane-d12 solutions obtained from the preparation of the $(HFIP)_2$ esters. Quantitative ^1H NMR measurements were carried out using the Bruker standard puls program zg30 with a relaxation delay of 20 s. Chemical shifts were referenced to the signal of the corresponding deuterated solvent (^{TM}DMSO-d6 = 2.49 ppm, ^{TM}cyclohexane-d12 = 1.46 ppm).

2.3. Preparation of $(HFIP)_2$-ester standards of cis/trans-chrysamthemumdicarboxylic acid and p-phenylenediacetic acid

Ester standards were prepared by dissolving 0.1 mmol of the acids in 2.5 mmol of HFIP and adding 1.1 mmol of DIC. The mixture was heated at 80 °C for 2 h. An aliquot of the clear reaction mixture was re-dissolved in the HPLC eluent for cleanup and fractionation (Section 2.2.3.2). The HPLC fractions were extracted with cyclohexane-d12 and the concentration of the HFIP-esters in these extracts determined by ^1H NMR. Standards for gas chromatography were generated from these extracts by dilution with *n*-hexane.

2.4. Preparation of standards, internal and external calibration

2.4.1. External calibration standards

2.4.1.1. Recovery experiments in SPE. Standards for calibration were either prepared by spiking SPE extracts of blank 24 h urine with known concentrations of CDCA and PDAA and subsequent derivatization (method a) or by dissolving solid CDCA and PDAA directly in blank 24 h urine followed by SPE and derivatization (method b). Standard concentrations typically ranged from 0.1 to 2.0 μg/l.

2.4.1.2. Determination of derivatization yields. $(HFIP)_2$-ester standards were prepared by dilution of the HPLC fractionation extracts (quantified by NMR) with *n*-hexane (Section 2.3). Yields in pure standard solutions were determined using external calibration, yields in urine matrix by the standard addition method.

2.4.2. Internal method calibration standards

Method b in Section 2.4.1.1 was also used to prepare standards for internal calibration. The internal standard concentration of PDAA was 1 μg/l, the concentrations for CDCA ranged from 0.1 to 2.0 μg/l.

2.5. Sample preparation

2.5.1. Hydrolysis of urine conjugates

Ten milliliter of urine sample were placed in a centrifuge tube, 1 ml of 10 M KOH were added and the sealed tubes were heated in a drying oven at 70 °C for 15 min. After cooling to room temperature, the sample was acidified with 1 ml of concentrated HCl and finally diluted 1:1 by volume with distilled water.

2.5.2. Solid phase extraction (SPE) of CDCA and PDAA

The extraction cartridges were successively conditioned with 6 ml of EtAc, MeOH, water and 0.1N HCl (pH 1). After sample loading, they were washed with 6 ml of 0.1N HCl (removal of salts) and dried with nitrogen. For anion exchange, the cartridges were washed with 6 ml of NH_4OH solution (a 1:50 dilution of concentrated NH_4OH in water, pH 10), thus binding the analytes to the $–CH_2NR_3^+$ groups of the anion exchanger. Additional washing steps with 6 ml MeOH and 6 ml EtAc were carried out in order to remove non-polar to medium polar matrix components. After drying with nitrogen, the cartridges were re-acidified by flushing with 6 ml of 0.1N HCl. The cartridges were dried once again and the analytes were eluted with 6 ml of 5 vol.% MeOH in EtAc. The eluates were evaporated to dryness with nitrogen for further analysis.

2.5.3. Derivatization of solid phase extracts for gas chromatographic measurement

The residues obtained according to Section 2.5.2 were dissolved in a mixture of 5 ml *n*-hexane, 50 μl HFIP and 75 μl DIC. The solution was shaken for 30 min in a roller-mixer. Excess derivatization reagents were removed by liquid–liquid partitioning with 5 ml of 5% potassium carbonate solution (pH 12) for 5 min. An aliquot of the organic layer was transferred to the autosampler vial containing a *silanized* 250 μl insert (see Notes in Sections 2.2.1 and 2.2.3.1) for further gas chromatographic analysis.

3. Results and discussion

3.1. Description of the synthesis procedure of cis/trans-chrysanthemumdicarboxylic acid

2,5-Dimethylsorbate was synthesized by Reformatzky reaction of ethyl-2-bromopropionate and 3,3-dimethylacrolein followed by elimination of water. In a second step, 2,5-dimethylsorbate was condensed with ethyl diazoacetate, producing the diethylesters of *E-cis*/*trans*-CDCA, which were then hydrolyzed to give the free acids [17].

3.2. HPLC analysis of the synthetic mixtures of E-cis/trans-CDCA and its $(HFIP)_2$ esters

The synthetic racemic mixture was measured by HPLC as described in Section 2.2.3.2. The UV-chromatogram showed five major peaks (Fig. 2). On the basis of their NMR and MS data, the first peak in the chromatogram was identified as *E-trans*-CDCA and the fourth peak as *E-cis*-CDCA; the second and fifth peaks are unknown constitutional isomers, while the third peak was identified as *Z-trans*-CDCA. The UV absorption maxima are at 239 nm for peaks 1, 3 and 4 and at 279 nm for peaks 2 and 5.

The HPLC chromatogram of the synthetic mixture of the $CDCA(HFIP)_2$ esters (Section 2.3) is shown in Fig. 3. Four peaks were observed at a detection wavelength of 260 nm. The first peak was identified as *E-cis*-CDCA (with an impurity from one of the unknown constitutional isomers), the third peak as *Z-trans*-CDCA and the fourth peak as *E-trans*-CDCA. The second peak corresponds to the second peak of the HPLC chromatogram of the free acids.

3.3. Identification and quantification of E-cis/trans-CDCA and its $(HFIP)_2$ esters by NMR

The ^1H NMR data of *E-cis*/*trans*-CDCA and its $(HFIP)_2$ esters are listed in Table 1. The data of the free acids are in good agreement with previously published data for the natural pyrethrins and *E-cis*/*trans*-CDCA [17,47–49], the data of the $CDCA(HFIP)_2$ esters are reported for the first time.

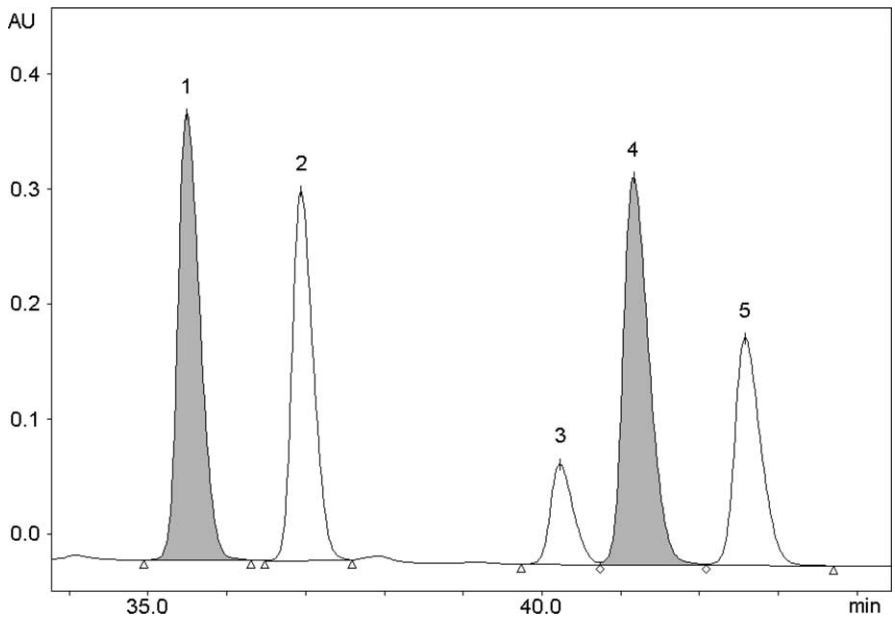

Fig. 2. HPLC chromatogram of the synthetic racemic mixture (ca. 25 ppm). (1) *E-trans*-CDCA, (3) *Z-trans*-CDCA, (4) *E-cis*-CDCA, (2, 5) unknown constitutional isomers, $\lambda = 260$ nm.

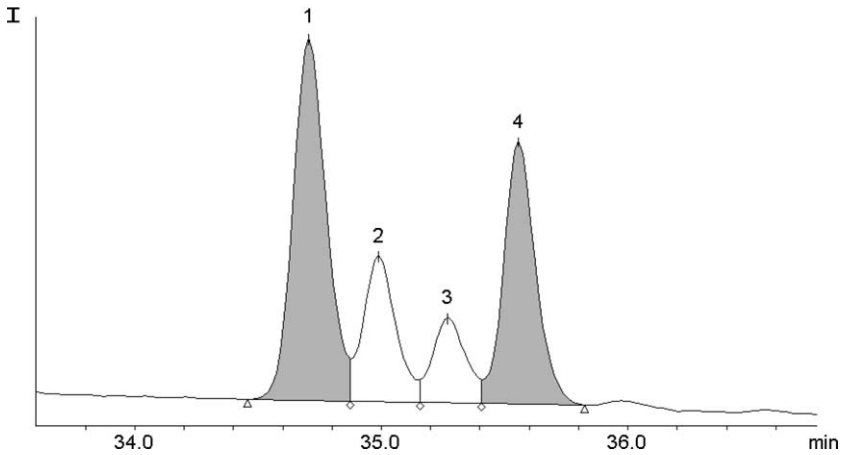

Fig. 3. HPLC chromatogram of the CDCA(HFIP)$_2$ esters; *E-cis*- (1), *Z-trans*- (3), *E-trans*- (4); (2) unknown constitutional isomer, $\lambda = 260$ nm.

Quantitation by NMR spectroscopy was carried out using the following equation [11]:

$$C_X = \frac{C_S N_S A_X M_X}{N_X A_S M_S}$$

where C_X and C_S are the concentrations (mass per volume) of the analyte and a free selectable standard. N_X and N_S are the number of protons giving rise to the respective integral signal areas A_X and A_S. The relative molecular masses of the analyte and standard are M_X and M_S, respectively.

Unlike chromatographic methods, no genuine reference compounds of the analytes are required for quantification. Therefore, NMR can be used in cases where such reference standards are not commercially available. Here, the NMR method was used to determine the concentration of *E-cis*/*trans*-CDCA and *E-cis*/*trans*-CDCA(HFIP)$_2$ esters in solutions that were used later on for the calibration of the GC-MS method. Quantification was achieved in the lower μg/ml level with an accuracy of 2–3%.

The signals at 7.28 ppm (4 aromatic protons), 3.73 ppm (4 methylene protons) and 5.73 ppm (2 HFIP protons) were used for the quantification of the internal standard PDAA(HFIP)$_2$ by ^{1}H NMR.

Table 1
^{1}H NMR data of *E-cis*/*trans*-CDCA and its (HFIP)$_2$ esters in DMSO-d6 and cyclohexane-d12, respectively

Proton	*E-cis*-CDCA	*E-trans*-CDCA	*E-cis*-CDCA(HFIP)$_2$	*E-trans*-CDCA(HFIP)$_2$
Chemical shifts (ppm)				
δ (H-7) dd	6.96	6.43	7.29	6.71
δ (H-1) d	1.86	1.79	2.10	1.94
δ (H-3) dd	2.03	2.01	2.16	2.43
δ (H_3-9) d	1.82	1.82	2.02	2.06
δ (H-HFIP) h	–	–	5.83 + 5.77	5.84 + 5.78
δ (H_3-5, H_3-6)	1.238, 1.222, 1.217, 1.167	1.238, 1.222, 1.217, 1.167	0.94	0.90
Coupling constants (Hz)				
^3J (H-1, H-3)	8.4	5.4	8.4	5.4
^3J (H-3, H-7)	10.2	10.2	9.3	9.6
^4J (H-7, H_3-9)	1.8	1.8	1.5	1.5

δ: chemical shift, *J*: coupling constant, HFIP: protons in the ester moiety, d: doublet, dd: double doublet, h: heptet, proton assignments according to Fig. 1.

3.4. Gas chromatography-mass spectrometry with negative chemical ionization

All five peaks of the HPLC chromatogram (Fig. 2) could be baseline-separated by GC-MS after derivatization with HFIP using the conditions described in Section 2.2.3.1. An additional cleanup for further investigations by HPLC fractionation according to Section 2.2.3.2 was thus not necessary, because the matching peaks in the HPLC and GC chromatograms could be assigned unambiguously. Therefore, the synthetic racemic mixture was used in the subsequent method development for convenience reasons. The mass spectra of CDCA showed three major peaks of *m*/*z* 302, 330 and 498 (details in Fig. 4).

The detection limits of *E*-*cis*/*trans*-CDCA in pure standard solutions were 0.01 μg/l (target ion, *m*/*z* 498) and 0.03 μg/l (qualifier ion, *m*/*z* 330), and of PDAA 0.11 μg/l (target ion, *m*/*z* 298) and 0.34 μg/l (qualifier ion, *m*/*z* 326), defined as a signal-to-noise ratio of 3. The lower sensitivity for PDAA is due to the fact that no molecule ion peak (*m*/*z* 494) could be observed and the fragment signals had to be used instead. Attempts to use 3-methylene-1,2-cyclopropanedicarboxylic acid as internal standard failed due to poor recovery in solid phase extraction and the high volatility of its $(HFIP)_2$-ester.

The reproducibility of repeated injections of a standard (1 μg/ml) was determined to be ±0.02% for the retention time and ±5–7% for the peak area ($n = 10$). The same values were obtained after derivatization of 10 identical standard solutions.

Furthermore, the influence of the amount of derivatization reagents was investigated, as this detail was not described in the previously reported derivatization method [34,39,46]. A 20,000-fold molar excess was found to be optimal, whereas a reduction of this amount results in a rapid decrease of the derivatization yields. The derivatization proceeds very fast, so hardly any dependence of the yields on the reaction time was observed. For pure standard solutions, 5 min are sufficient. The derivatization yields for standard solutions of *E*-*cis*/*trans*-CDCA and PDAA (determined by external calibration with the $(HFIP)_2$-ester

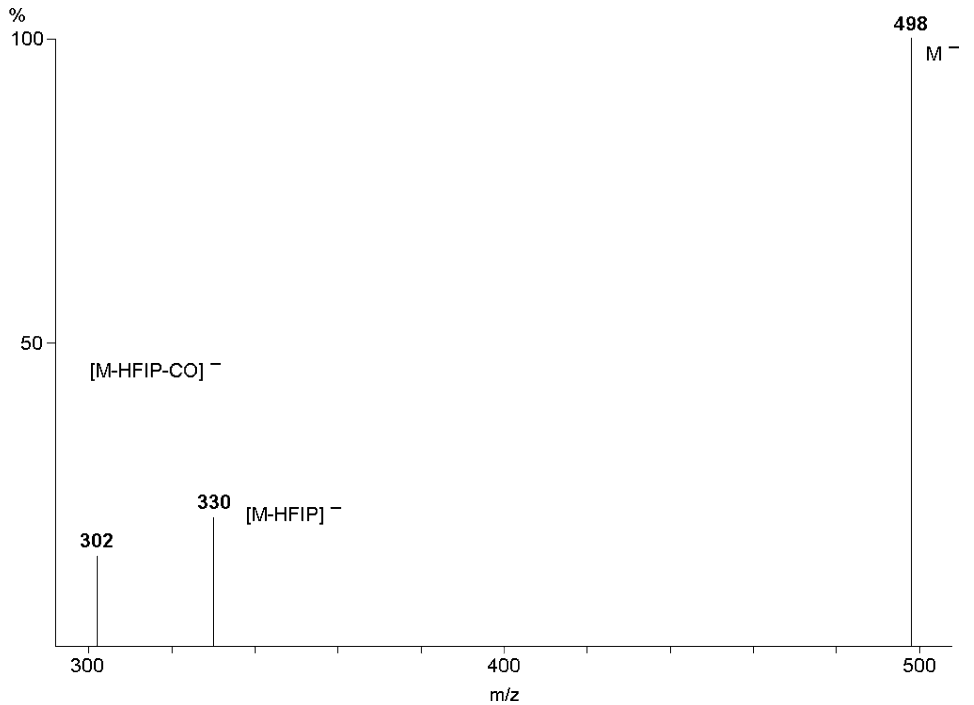

Fig. 4. GC-MS (NCI) mass spectrum of *E*-*cis*/*trans*-CDCA$(HFIP)_2$. M^- is the molecule ion peak, the fragments at *m*/*z* 330 and 302 are due to the elimination of HFIP and carbon monoxide (CO).

Table 2
Recoveries at spiking level 1 (25 μg/l urine) using external calibration methods a or b (Section 2.4.1.1) and internal calibration (b, ISTD calibration)

Compound name	Level 1 (%) (25 ppb) (method a)	Level 1 (%) (25 ppb) (method b)	Level 1 (%) (25 ppb) (method b, ISTD calibration)
E-cis-CDCA	96 ± 9 ($n = 5$)	96 ± 5 ($n = 6$)	96 ± 3 ($n = 6$)
E-trans-CDCA	92 ± 5 ($n = 5$)	95 ± 6 ($n = 6$)	95 ± 2 ($n = 6$)
PDAA	97 ± 6 ($n = 5$)	100 ± 8 ($n = 6$)	–

standards described in Section 2.3) were 92–93 ± 7% ($n = 3$).

External calibration curves of *E-cis*/*trans*-CDCA-$(HFIP)_2$ were linear for concentrations up to 1 mg/l with correlation coefficients >0.999.

3.5. Recoveries and derivatization yields in urine matrix

3.5.1. General aspects of the mixed-mode solid phase extraction procedure

The presence of urine matrix has a strong influence on the derivatization yields. The more matrix components in the sample solution, the lower the derivatization yields. Therefore, an effective sample cleanup for good sensitivity is of particular importance. Oasis® MAX solid phase extraction cartridges proved to be very suitable for this cleanup. These cartridges contain a pH-stable polymeric material which participates both in non-polar and anion exchange interactions. The protonated analytes are loaded onto the cartridges and retained by the non-polar interactions while the salts are washed out. In a second step, the analytes are deprotonated by washing with an alkaline solution and bound by ionic interactions to the $–CH_2NR_3^+$ groups of the sorbent, while non-polar matrix components can be washed out without loss of analytes using organic solvents such as MeOH. In a third step, the analytes can be easily eluted after re-acidification using a volatile solvent such as EtAc. Five percent of MeOH in EtAc are necessary to break hydrogen-bond interactions for complete elution.

Table 3
Recoveries at spiking level 2 (1 μg/l urine) using external calibration method b (Section 2.4.1.1) and internal calibration (b, ISTD calibration)

Compound name	Level 2 (%) (1 ppb) (method b)	Level 2 (%) (1 ppb) (method b, ISTD calibration)
E-cis-CDCA	104 ± 7 ($n = 15$)	104 ± 4 ($n = 15$)
E-trans-CDCA	101 ± 7 ($n = 15$)	100 ± 3 ($n = 15$)
PDAA	101 ± 8 ($n = 15$)	–

3.5.2. Recovery studies for mixed-mode SPE

Recovery studies were carried out at two spiking levels: 25 μg/l (level 1) and 1 μg/l (level 2). The results are listed in Tables 2 and 3, respectively. The choice of the calibration method (method a or b in Section 2.4.1.1) had no impact on the recoveries (as shown by the *F*- and *t*-tests). As the derivatization yields in urine matrix were found to be almost 100% (see Section 3.5.3), it may be concluded from Tables 2 and 3 that the extraction of the analytes from urine is almost quantitative. As expected, the coefficients of variation using internal calibration were somewhat smaller than those for external calibration.

3.5.3. Derivatization yields in urine matrix

The derivatization reaction for *E-cis*/*trans*-CDCA is shown in Fig. 5.

Derivatization yields were determined by spiking three samples at both levels with known amounts of the $(HFIP)_2$-ester standards (standard addition method). The results are listed in Table 4. The low value for *E-cis*-CDCA$(HFIP)_2$ at level 1 is presumably due to incorrect spiking. The spiking procedure causes additional variation from sample to sample

Table 4
Derivatization yields in urine samples at 25 μg/l (level 1) and 1 μg/l (level 2)

Compound name	Level 1 (%)	Level 2 (%)
E-cis-CDCA$(HFIP)_2$	82 ± 13	96 ± 11
E-trans-CDCA$(HFIP)_2$	101 ± 15	102 ± 12
PDAA$(HFIP)_2$	98 ± 15	101 ± 10

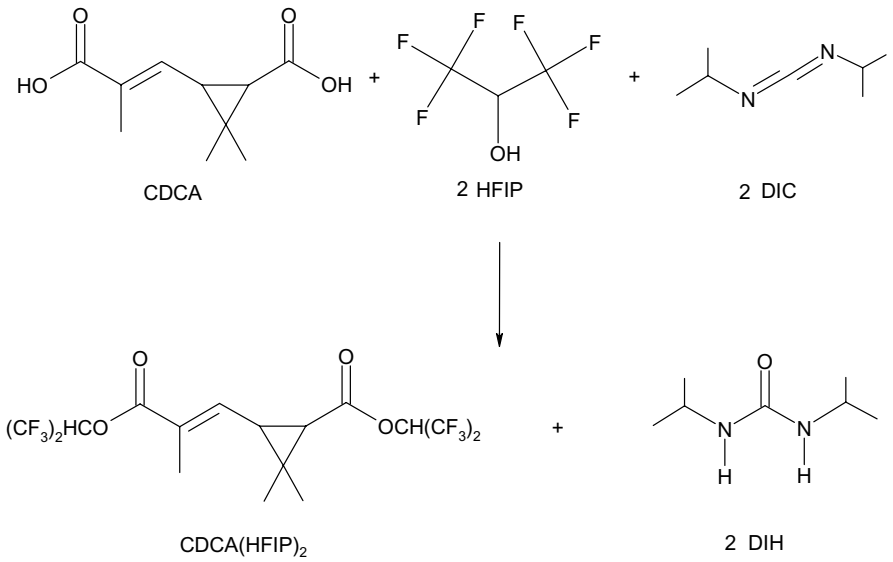

Fig. 5. Derivatization scheme of *E-cis*/*trans*-chrysanthemumdicarboxylic acid.

resulting in somewhat higher R.S.D. values. Nevertheless, the derivatization is apparently quantitative.

The relatively large volume of 5 ml *n*-hexane spiked with 50 μl HFIP and 75 μl DIC is necessary for the complete dissolution of the SPE residue and ensures quantitative derivatization. Derivatization in a more polar solvent (allowing the use of a smaller volume) is not suitable as it leads to secondary reactions [46].

3.6. External and internal calibration; detection limits

3.6.1. Validation of the calibration

Calibration curves for external and internal calibration were generated at concentration levels ranging from 0.1 to 2.0 μg/l urine, which represent the expected levels in real-life samples. Fifteen calibration points were determined by three-fold repetition at five different calibration levels (0.1, 0.3, 0.7, 1.3 and 2.0 μg/l). The results are listed in Table 5. The calibration curves are linear with correlation coefficients $R^2 \geq 0.996$ and relative standard deviations $CV_X \leq 10\%$.

3.6.2. Limits of detection and quantification

Detection limits can be calculated from the calibration curves as described in [50]. Using the internal calibration curves from Section 3.6.1 (obtained by matrix-matched calibration), the detection limits for *E-cis*- and *E-trans*-CDCA are determined to be 0.06 and 0.04 μg/l, respectively ($P = 95\%$ certainty). These detection limits may depend on the origin of the urine used. If necessary, the sensitivity may be increased by further reduction of the sample volume after derivatization (Section 2.5.3). The quantification limits of *E-cis*- and *E-trans*-CDCA were 0.1 μg/l urine.

3.7. Method robustness

The robustness of the method was tested over a period of 8 days. A spiked urine sample (25 μg/l) was injected every 12 h. The absolute values

Table 5
Validation data for external and internal calibration curves

Compound name	External calibration		Internal calibration	
	R^2	CV_X (%)	R^2	CV_X (%)
E-cis-CDCA(HFIP)$_2$	0.996	10.2	0.999	2.3
E-trans-CDCA(HFIP)$_2$	0.998	7.4	0.999	3.8
PDAA(HFIP)$_2$	0.998	6.9	–	–

R^2: square of correlation coefficient, CV_X: relative standard deviation of the calibration curve.

varied by 18% for *E-cis*-CDCA(HFIP)$_2$, 19% for *E-trans*-CDCA(HFIP)$_2$ and 21% for the internal standard PDAA(HFIP)$_2$ (external calibration). Applying internal calibration, the variations of the concentrations were found to be ≤3% for *E-cis*-CDCA(HFIP)$_2$ and ≤4% for *E-trans*-CDCA(HFIP)$_2$. Nearly the same values (22, 20, and 24% with external calibration, 4 and 5% with internal calibration) were obtained at a spiking level of 0.8 μg/l, demonstrating the excellent robustness of the method.

3.8. Gas chromatographic measurement of spiked and real urine samples

Example chromatograms of a blank 24 h urine sample spiked with *E-cis*/*trans*-CDCA and PDAA

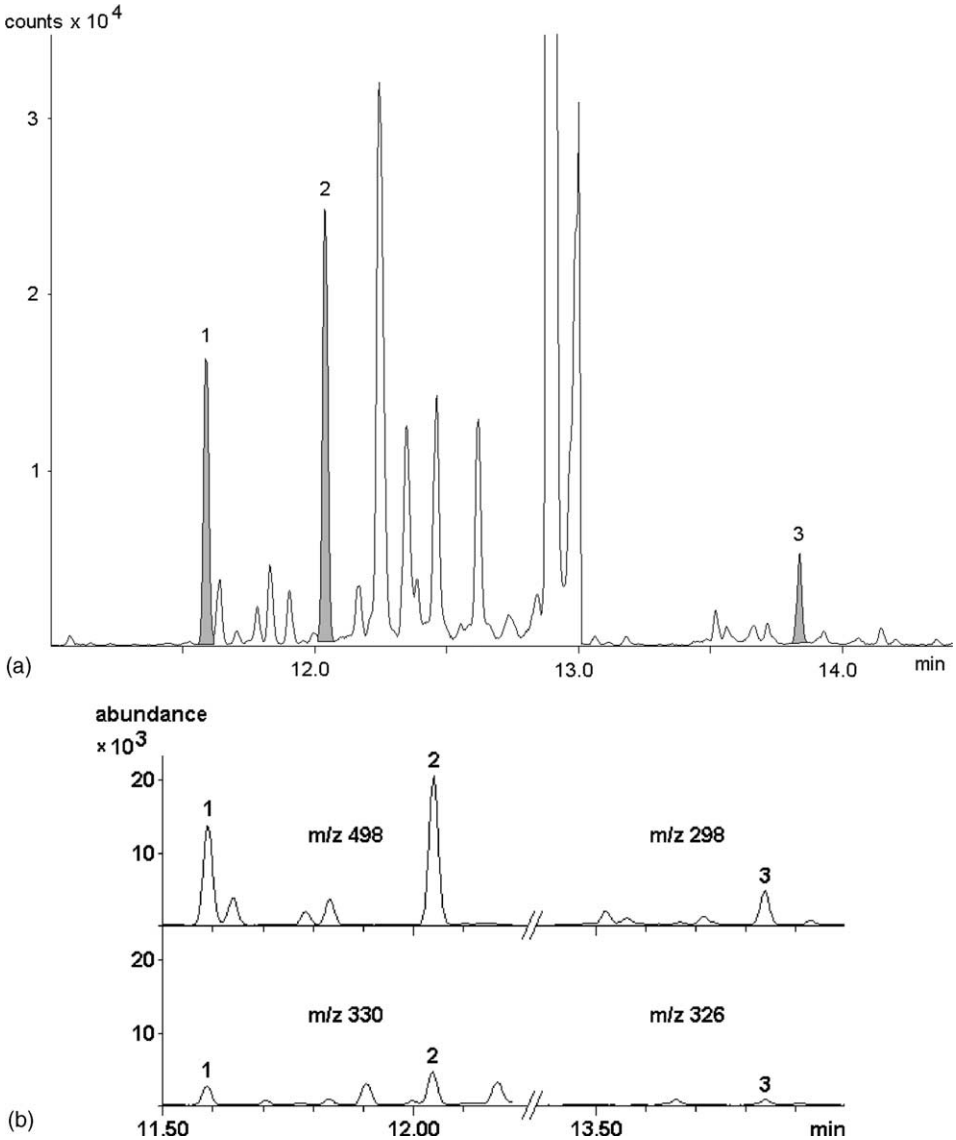

Fig. 6. (a) Total ion chromatogram of a blank urine sample spiked with 1 μg/l *E-cis*-CDCA, *E-trans*-CDCA and PDAA. (1) *E-trans*-CDCA(HFIP)$_2$, (2) *E-cis*-CDCA(HFIP)$_2$, (3) PDAA(HFIP)$_2$. (b) Extracted ion chromatograms of the spiked urine sample from part a. (1) *E-trans*-CDCA(HFIP)$_2$, (2) *E-cis*-CDCA(HFIP)$_2$, (3) PDAA(HFIP)$_2$.

(1 μg/l each) are illustrated in Fig. 6a and b. The peak of *E-cis*-CDCA is slightly higher than that of *E-trans*-CDCA as a result of different response factors. The response factor of PDAA is distinctly lower, because only the ester fragment ions can be measured as described in Section 3.3.

In order to check the method's suitability for practical use, real urine samples were collected from persons after using commercially available vaporizer plates containing d-allethrin in a household. The results are listed in Table 6 and sample chromatograms are shown in Fig. 7a and b. As shown, only *E-trans*-CDCA was detected, whereas *E-cis*-CDCA was not found. This may be due to its lower concentration in the insecticide formulation (the *cis*/*trans* isomer ratio of d-allethrin is 1:4) and due to the different metabolism of *cis* and

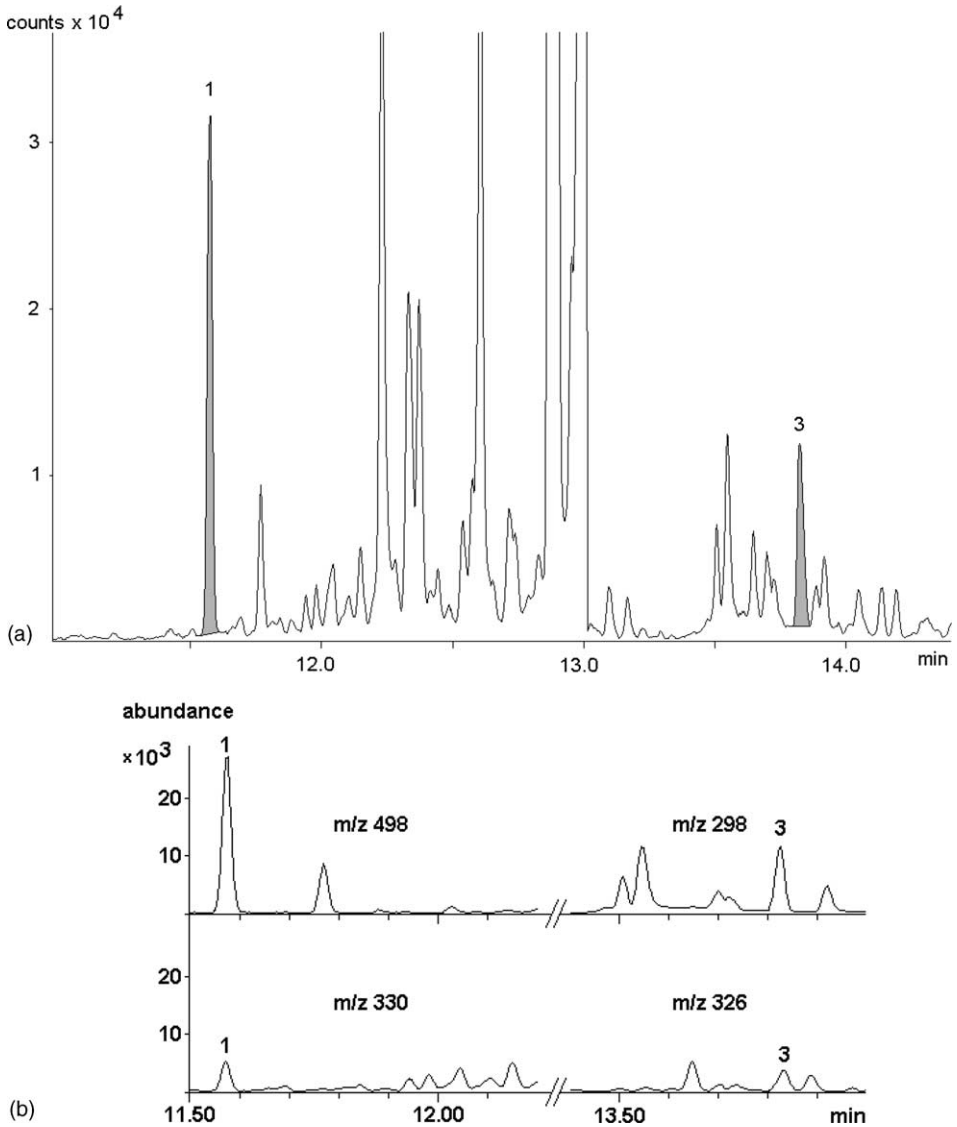

Fig. 7. (a) TIC-chromatogram of a 24 h urine sample (sample a) after exposure to d-allethrin. (1) *E-trans*-CDCA(HFIP)$_2$, (3) PDAA(HFIP)$_2$, *E-cis*-CDCA(HFIP)$_2$ was not detected. (b) Extracted ion chromatograms of the real urine sample from part a. (1) *E-trans*-CDCA(HFIP)$_2$, (3) PDAA(HFIP)$_2$. *E-cis*-CDCA(HFIP)$_2$ was not detected.

Table 6
Concentrations of *E-trans*-CDCA in real urine samples after exposure to d-allethrin

Sample	Volume of 24 h urine (l)	Concentration (μg/l)	Total amount (μg)
A	3.2	2.3	7.4
B	1.3	4.1	5.3
C	1.1	6.1	6.7
D	1.1	6.2	6.8

trans isomers. Although no detailed information on the metabolism differences of *cis*- and *trans*-d-allethrin was found in the literature, general rules of pyrethroids metabolism suggest that ester cleavage (by esterases) occurs mainly with *trans* isomers, whereas *cis* isomers are subject to oxidative degradation [17,51–53]. This is why *trans* isomers are metabolised more rapidly than *cis* isomers. It was found for several pyrethroids, e.g. resmethrin and phenothrin, that metabolites of the *trans* isomers (ester-cleaved) excrete mainly in the urine, but metabolites of the *cis* isomers (ester-form) excrete mainly in the feces [54,55]. Furthermore, ester cleavage of *cis* isomers was observed with oxidase catalyzed reactions leading to the possibility of *cis*/*trans*-isomerization on carbon atom C_3 of the cyclopropane ring. All these facts make it plausible that concentrations of *E-cis*-CDCA in the real urine samples were below the detection limit.

4. Conclusions

CDCA is an important metabolic biomarker for the internal burden of humans exposed to pyrethrum, allethrin, phenothrin, resmethrin and/or tetramethrin, which are common active ingredients in indoor pest control formulations. A gas chromatographic-mass spectrometric method with NCI for the determination of *E-cis*/*trans*-chrysanthemumdicarboxylic acid is described. The newly developed mixed-mode solid phase extraction method for sample enrichment combined with the high resolution capability of capillary gas chromatography and the sensitive and selective detection by negative ionization mass spectrometry allows sub ppb levels of CDCA to be monitored in human urine.

^{1}H NMR was used to characterize and quantify chrysanthemumdicarboxylic acid in the synthetic racemic mixture and, for the first time, its hexafluoroisopropanol esters as well. The extraction of CDCA from urine samples by mixed-mode solid phase extraction and the subsequent derivatization to its $(HFIP)_2$ esters were almost quantitative, thus avoiding a loss of sensitivity during these sample preparation steps.

The determination of the metabolites in real urine samples illustrates the suitability of the method for biomonitoring of CDCA. The measured levels were higher as expected, but below the detection limit of the previously published method [17].

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