Pyrethroid metabolites (cis-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; 3-phenoxybenzoic acid; 4-fluoro-3-phenoxybenzoic acid)

Application	Determination in urine
Analytical principle	Capillary gas chromatography with mass selective detector
Completed in	June 1998

Summary

The concentration of five pyrethroid metabolites in urine can be sensitively and reliably determined in one analytical run using the gas chromatographic/mass spectrometric method described here. Due to its sensitivity this method is suitable for the detection of pyrethroid exposure which occurs in indoor areas as well as in concentrations relevant to occupational medicine.

After acidification with concentrated sulphuric acid and addition of 2-phenoxybenzoic acid as an internal standard, the urine sample is then subjected to hydrolysis. The mixture is subsequently drawn through a C18-column on which the pyrethroid metabolites are enriched (alternatively, the metabolites can also be extracted using n-hexane, see Addendum). After elution, the metabolites are converted to their derivatives using methanol/sulphuric acid. The determination is carried out by means of capillary gas chromatography with mass selective detection (GC/MSD).

Calibration curves are obtained by analysing pooled urine, to which known amounts of the pyrethroid metabolites have been added, and which is processed and analysed in the same manner as the samples. The resulting peak areas of the pyrethroid metabolites are expressed in relationship to the peak area of the internal standard.

cis-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*cis*-Cl_2CA)

Within-series imprecision:	Standard deviation (rel.)	s_w = 1.7 or 1.6%
	Prognostic range	u = 3.9 or 3.7%
	at a concentration of 4 or 10 g *cis*-Cl_2CA per litre urine	
	and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 7.7%
	Prognostic range	u = 17.1%
	at a concentration of 10 g *cis*-Cl_2CA per litre urine	
	and where n = 12 days	
Accuracy:	Recovery rate 83.3 or 86.7%	
Detection limit:	0.2 g *cis*-Cl_2CA per litre urine	

trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*trans*-Cl_2CA)

Within-series imprecision:	Standard deviation (rel.)	s_w = 2.7 or 1.6%
	Prognostic range	u = 6.2 or 3.7%
	at a concentration of 4 or 10 g *trans*-Cl_2CA per litre urine	
	and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 8.7%
	Prognostic range	u = 19.3%
	at a concentration of 10 g *trans*-Cl_2CA per litre urine	
	and where n = 12 days	
Accuracy:	Recovery rate 77.5 or 85.1%	
Detection limit:	0.2 g *trans*-Cl_2CA per litre urine	

cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (Br_2CA)

Within-series imprecision:	Standard deviation (rel.)	s_w = 2.5 or 1.7%
	Prognostic range	u = 5.8 or 3.9%
	at a concentration of 4 or 10 g Br_2CA per litre urine	
	and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 6.9%
	Prognostic range	u = 15.3%
	at a concentration of 10 g Br_2CA per litre urine	
	and where n = 12 days	
Accuracy:	Recovery rate 82.2 or 88.0%	
Detection limit:	0.1 g Br_2CA per litre urine	

3-Phenoxybenzoic acid (3-PBA)

Within-series imprecision:	Standard deviation (rel.)	s_w = 1.9 or 1.3%
	Prognostic range	u = 4.4 or 3.1%
	at a concentration of 4 or 10 g 3-PBA per litre urine	
	and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 6.2%
	Prognostic range	u = 13.8 %
	at a concentration of 10 g 3-PBA per litre urine	
	and where n = 12 days	
Accuracy:	Recovery rate 103.1 or 106.8%	
Detection limit:	0.5 g 3-PBA per litre urine	

4-Fluoro-3-phenoxybenzoic acid (F-PBA)

Within-series imprecision:	Standard deviation (rel.)	s_w = 1.6 or 1.7%
	Prognostic range	u = 3.7 or 3.9%
	at a concentration of 4 or 10 g F-PBA per litre urine	
	and where n = 10 determinations	
Between-day imprecision:	Standard deviation (rel.)	s = 6.2%
	Prognostic range	u = 13.8 %
	at a concentration of 10 g F-PBA per litre urine	
	and where n = 12 days	
Accuracy:	Recovery rate 93.1 or 92.8%	
Detection limit:	0.2 g F-PBA per litre urine	

Pyrethroids

General data

Appearance	Oily to crystalline substances with a weak characteristic odour
Purity	Approx. 90–99%
Use	Plant protection, primarily as an insecticide
Quantity used	Approx. 10–20 g/ha compared with several hundred g/ha in the case of other insecticides
Production Germany:	Approx. 44 tonnes per year
Worldwide:	Approx. 500 tonnes per year [1]

Pyrethroids are aliphatic ester compounds derived from pyrethrum. Since 1949 the cyclopropane carboxylic acid and alcohol structures of natural pyrethrum have been modified in many ways to form the synthetic pyrethroids. In the meantime, type I pyrethroids with short-term effects (T(remor) syndrome) are differentiated from type II pyrethroids causing longer lasting effects (CS(choreo-athetosis, salivation) syndrome) with an α-cyano substitution, and a mixture of type I and type II [1].

In contrast to pyrethrum, the synthetic pyrethroids have a more potent and longer effect in the insect organism, and in some cases their stability in the environment is also higher [2]. The metabolites of the pyrethroids exhibit no specific physiological effects [3].

Whereas their toxicity for warm-blooded animals is distinctly lower, pyrethroids are significantly more effective against a broad range of economically important pests than organochloro, organophosphate and carbamide insecticides. This has favoured their worldwide application in almost all external and internal areas of vector control [4].

Pyrethroids are lipophilic and have a low vapour pressure. However, intake is primarily by inhalation, generally by way of airborne dust as a vehicle, and to a lesser extent by absorption through the skin. No data are currently available on the exposure of the general public to pyrethroid residues in food [4, 5]. Butte et al. [6] suggest a reference value of 0.7 g/L (95th percentile) for each of the permethrin metabolites *cis*- and *trans*-Cl_2CA and 3-PBA. Metabolite concentrations of <0.5–277 g per litre urine were observed in the urine of pest controllers who had been working in enclosed areas [7, 8]. Depending on the quality of their protective equipment, farm and forestry workers exhibited metabolite concentrations as high as approx. 300 g/L urine [9–11].

In general, the absorbed pyrethroids are rapidly detoxified by means of ester hydrolysis and hydroxylation and, after conjugation, they are excreted as glucuronides, sulphates or acetates primarily via the kidneys, though a part is also eliminated in the faeces. Their metabolic conversion takes place in the blood, the liver and other organs including the nervous system [1, 12, 13]. The main pathways of pyrethroid metabolism are illustrated schematically in Figure 1.

The biological half-lives of the various pyrethroids were determined in animal studies. They are between 2.5 and 12 hours in blood and plasma, between one and 30 days in fatty tissue and between 9 and 23 hours in the brain [1]. The concentration-time curve of the elimination rate frequently exhibits a biphasic form [12, 14].

Half-lives of 6.44 0.64 hours were found for the urinary excretion of the metabolites *cis*- and *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid and 4-

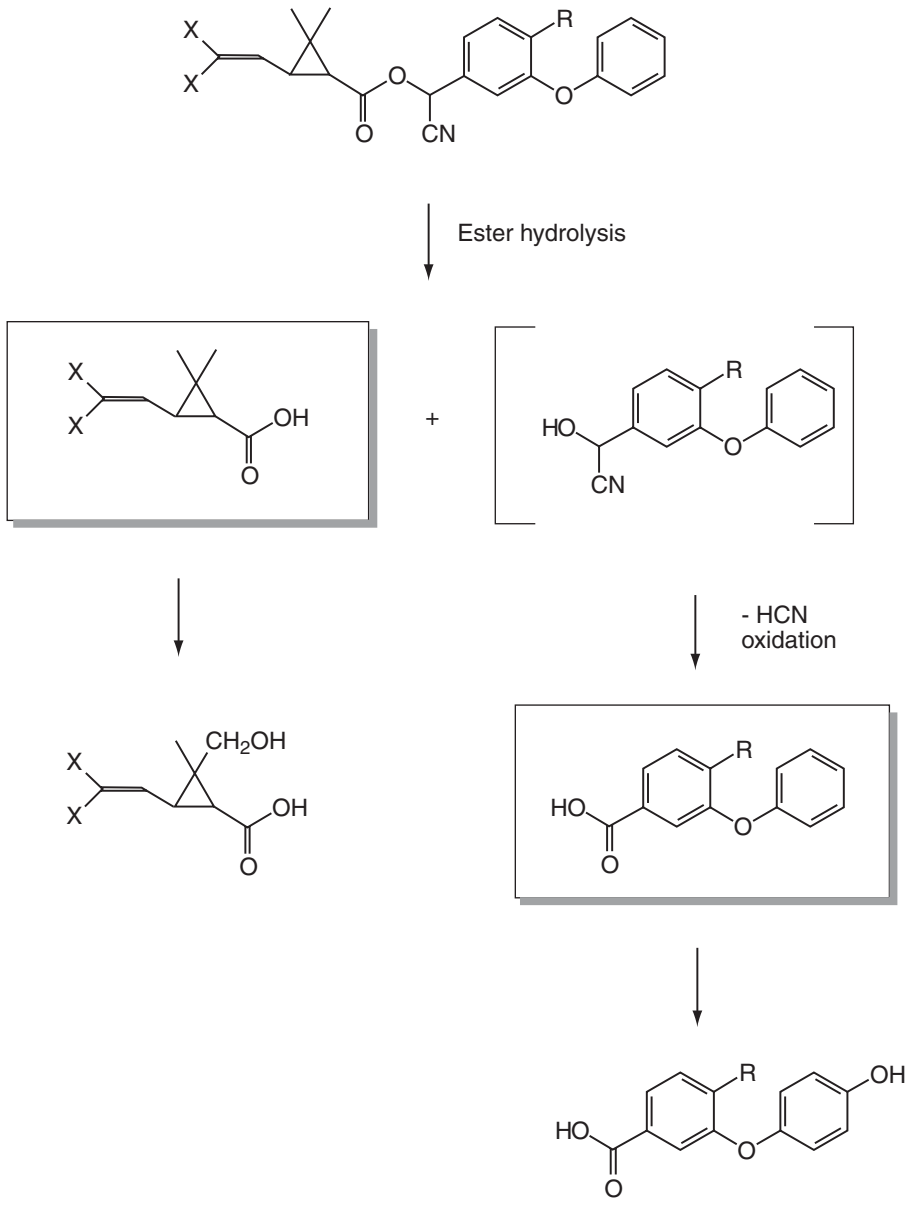

Fig. 1. Main pathways of the pyrethroid metabolism in the human body (X = C1, Br; R = H, F).

fluoro3-phenoxybenzoic acid after administration of an individual oral dose of cyfluthrin of 30 g/kg body weight to volunteers. 94% of the metabolites were excreted renally during the first 48 hours after intake [7, 15].

Similar results were observed for cypermethrin [16].

When the toxicity of the pyrethroids is assessed in humans

1. the dose-dependent effect on the central nervous system must be differentiated from
2. the local, neuro-excitatory (sensory) effect at potential contact points such as the skin, the eyes, the upper respiratory tract, etc.

Users who are exposed to high levels of pyrethroids not only exhibit characteristic neuro-excitatory effects with abnormal facial sensations but also symptoms resembling irritation at the sensory peripheral nervous system of the directly exposed skin, the mucous membranes of the eyes and respiratory tract, e.g. burning and itchiness of the skin, paraesthesiae, formation of blisters, erythema, coughing, irritation of the eyes, etc. [9, 12, 13, 17–22]. Symptoms resembling contact dermatitis , especially in the face, have also been reported [2, 19, 21].

The sensory disorders begin a few minutes after contamination and disappear completely within a maximum period of 24 hours. All acute toxic effects were rapidly reversible.

Indications of chronic or irreversible changes could not be determined, even after massive pyrethroid doses [19, 23]. In individual cases, the sensory effects have been reported to lead to manifold angst syndromes, even to serious neurosis, including somatic complaints [24].

Pyrethroids lengthen the duration of opening of the sodium channels in the nerve membranes of the central and peripheral nerve system and cause a reversible membrane depolarisation. Repeated nerve impulses induce a temporary repetitive firing of the superficial sensory nerve endings [1, 12, 25–27].

Long-term administration of pyrethroids in animal studies showed neither accumulation of the substances nor a cumulative effect. Moreover, there was no evidence of reproductive or embryonic toxicity, nor could a teratogenic or carcinogenic effect be established [4, 28, 29].

In contrast to the allergic effect on the skin caused by natural pyrethrum, no specific dermal or humoral sensitization could be observed when the synthetic pyrethroids were handled [2, 3].

The toxicity of the pyrethroids is classed as low by the WHO [14]. Nevertheless, their health hazard potential to humans is a controversial subject, especially in Germany [30, 31].

In principle, two approaches to biomonitoring are possible: determination of the unchanged pyrethroids in plasma or the detection of their excreted metabolites in urine, especially the cyclopropane carboxylic acids and the phenoxybenzoic acids. The first approach offers the advantage that more consideration is taken of the inherent critical toxicity of the pyrethroids. On the other hand, the pyrethroid metabolites are superior with regard to their diagnostic sensitivity and their analytical detectability.

The permanent conference of company doctors in the chemical industry has set an empirical value acceptable to occupational medicine (arbeitsmedizinischer Erfahrungswert, AEW) of 10 g F-PBA per gram creatinine for the internal pyrethroid stress limit [46].

The AEW value is justified as follows:
In 1990 Vrjverberg et al. discussed local superficial doses of about 5 g pyrethroid/ cm^2 skin for type II pyrethroids as the threshold dose of the receptor stimulation potential [27]. Such exposure controls are unsuitable in practice. However, the peripheral sensory irritations (nasal irritation, trigeminal stimulation, etc.) must be regarded as early clinical symptoms of adverse pyrethroid effects. An AEW value for cyfluthrin should also be oriented towards the threshold dose of the receptor potential of peripheral nerve endings.

A cyfluthrin study showed that the local threshold doses of the peripheral sensory nerve endings are reached at an exposure level of 75 g cyfluthrin per m^3 air. The responses to the stimulation were generally reversible and after a period without exposure (<5 min) they rapidly disappeared [15].

Therefore if inactive pyrethroid metabolites are found in the urine of pyrethroid users without symptoms, it can be assumed that neither the local threshold dose of 5 g of unchanged pyrethroid per cm^2 skin nor the air concentration of 75 g/m^3 was exceeded during the application.

The preventive approach takes into account that neither local nor sensory systemic effects were observed at workplaces contaminated by up to 10 g/m^3 cyfluthrin. An external exposure at this level is equivalent to an urinary excretion of 10 g F-PBA per gram creatinine in the urine sample at the end of the night shift.

The cyfluthrin concentrations which cause systemic effects are unknown, but they are above the threshold dose of the receptor potential of the peripheral nerve endings.

Urine is used for biomonitoring on account of its ready availability, although the metabolites do not directly indicate the inner stress due to pyrethroid exposure [8].

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Pyrethroid metabolites (cis-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; cis-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid; 3-phenoxybenzoic acid; 4-fluoro-3-phenoxybenzoic acid)

Application	Determination in urine
Analytical principle	Capillary gas chromatography with mass selective detector
Completed in	June 1998

Contents

1 General principles

After acidification with concentrated sulphuric acid and addition of 2-phenoxybenzoic acid as an internal standard, the urine sample is then subjected to hydrolysis. The mixture is subsequently drawn through a C18-column on which the pyrethroid metabolites are enriched (alternatively, the metabolites can also be extracted using n-hexane, see Addendum). After elution, the metabolites are converted to their derivatives using methanol/sulphuric acid. The determination is carried out by means of capillary gas chromatography with mass selective detection (GC/MSD).

Calibration curves are obtained by analysing pooled urine, to which known amounts of the pyrethroid metabolites have been added, and which is processed and analysed in the same manner as the samples. The resulting peak areas of the pyrethroid metabolites are expressed in relationship to the peak area of the internal standard.

2 Equipment, chemicals and solutions

2.1 Equipment

Capillary gas chromatograph with split/splitless injector, mass selective detector (MSD) and data processing system

Gas chromatographic column:
Length 60 m, inner diameter 0.25 mm, stationary phase 5% phenylmethyl polysiloxane, film thickness 0.25 m (e.g. from Chrompack)

5 L Syringe for gas chromatography, preferably an autosampler

Workstation for vacuum extraction (e.g. from Baker)

6 mL C18-separation column (e.g. from Baker)

25 mL crimp top vials with PTFE-coated stoppers and crimping tongs

250 L Microvial insert

Drying cupboard

Water bath

2 mL Autosampler vial

Centrifuge

10, 20, 50 and 100 mL Volumetric flasks

Microlitre pipettes, adjustable between 10 and 100 L, and between 200 and 1000 L (e.g. from Eppendorf)

Millilitre pipette, adjustable between 1 and 10 mL (e.g. from Eppendorf)

22 mL Threaded vial with teflon-coated seal and screw cap

2.2 Chemicals

cis-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*cis*-Cl_2CA, 99.8%) [1]

cis-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid methyl ester (*cis*-Cl_2CA-Me, 98.9%) [1]

trans-3-(2,2-Dichlorovinyl)-2,2-dirnethylcyclopropane-1-carboxylic acid (*trans*-Cl_2CA, 99.8%) [1]

trans-3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid methyl ester *(trans*-Cl_2CA-Me, 97.5%) [1]

4-Fluoro-3-phenoxybenzoic acid (F-PBA 99.9%) [1]

[1] BAYER AG kindly supplied the substances.

cis-3-(2,2-Dibromovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (Br_2CA, 98.8%) [2]

3-Phenoxybenzoic acid (3-PBA, 99%: e.g. from Acros Chimica)

2-Phenoxybenzoic acid (2-PBA, 98%: e.g. from Acros Chimica)

98% Sulphuric acid for trace analysis (e.g. from Merck)

95–97% Sulphuric acid for trace analysis (e.g. from Merck)

n-Hexane for trace analysis (e.g. from Merck)

Methanol for trace analysis (e.g. from Merck)

Acetone for trace analysis (e.g. from Merck)

Toluene for trace analysis (e.g. from Merck)

Ethyl acetate for trace analysis (e.g. from Merck)

Ultrapure water (equivalent to ASTM type 1) or double distilled water

Helium for gas chromatography

2.3 Solutions

Solution of the internal standard:
Approx. 50 mg 2-PBA are weighed exactly in a 50 mL volumetric flask. Then the flask is filled to its nominal volume with methanol (1 g/L). 1 mL of this solution is pipetted into a 100 mL volumetric flask which is subsequently filled to its nominal volume with ultrapure water (10 mg/L).

This solution can be stored in the refrigerator at 4 C for about 4 weeks.

2.4 Calibration standards

Starting solutions:
About 50 mg *cis*-Cl_2CA, *trans*-Cl_2CA, F-PBA, Br_2CA and 3-PBA are each weighed in individual 50 mL volumetric flasks. The volumetric flasks are subsequently filled to their nominal value with acetone (1 g/L).

[2] HOECHST AG kindly supplied the substance.
These substances can be bought in the form of methanolic standard solutions, e.g. from Ehrenstorfer.

Stock solution 1:
100 L of each of the starting solutions are pipetted into a 20 mL volumetric flask. The flask is then filled to its nominal volume with methanol (5 mg/L).

Stock solution 2:
1 mL of stock solution 1 is pipetted into a 10 mL volumetric flask. The flask is then filled to its nominal volume with methanol (0.5 mg/L).

The starting solutions and the stock solutions can be kept in the refrigerator at 4 C for at least 8 weeks.

Calibration standards containing between 1.0 and 200 g of the individual pyrethroid metabolites per litre are prepared from stock solutions 1 and 2 by means of dilution with pooled urine. The following pipetting scheme shows the preparation procedure:

Table 1. Pipetting scheme for the preparation of the calibration standards.

Designation of the calibration standard	Volume of the stock solution [L]		Final volume of the calibration standard [mL]	Concentration of the calibration standard [g/L]
	Stock solution 1	Stock solution 2		
1	–	100	50	1.0
2	–	200	50	2.0
3	100	–	50	10
4	250	–	50	25
5	500	–	50	50
6	1000	–	50	100
7	1500	–	50	150
8	2000	–	50	200

The calibration standards can be stored in the deep-freezer at –18 C for at least 6 months.

2.5 Preparation of the C18-column

Prior to use, the C18-columns are each conditioned in a vacuum station with 3 mL ethyl acetate, 3 mL n-hexane, 6 mL methanol and finally with 9 mL ultrapure water in that order. The column thus prepared must still be moist when the urine is introduced into it.

3 Specimen collection and sample preparation

Urine samples are collected in plastic bottles at the end of a working shift. The first urine excreted in the morning should be used for analytical investigations in the field of

environmental medicine. The urine samples are acidified with acetic acid (1 mL glacial acetic acid per 100 mL urine) and, if they cannot be immediately processed, they are stored in the deep-freezer at -18 C until they can be further processed. For processing the samples are thawed in a water bath at 40 C and subsequently brought to room temperature. Before an aliquot is taken the samples are thoroughly shaken. For the analytical investigation 10 mL of the urine sample are pipetted into a 25 mL crimp top vial (alternatively 22 mL threaded vials can be used), then 200 L of the internal standard solution and 2 mL of 95–97% sulphuric acid are added. The vial is sealed with a crimp cap and the mixture is heated in a drying cupboard at 90 C for one hour. After the sample has cooled the crimp top vial is opened. The hydrolyzed urine sample is drawn by a slight vacuum through the columns prepared as described in Section 2.5. The columns are then washed with 6 mL water and sucked dry for about 5–10 min under the vacuum created by a rotary pump. It must be ensured that there are no longer any drops of water on the end of the columns.

The columns are subsequently eluted with 3 mL methanol. A 25 mL crimp top vial, into which 1 mL of 98% sulphuric acid has been previously filled, serves as a recipient vessel (caution: strong generation of heat!). The columns are sucked dry once again. The eluate is heated in the water bath for 1 hour at 75 C to ensure complete derivatization. After cooling to room temperature, 2 mL n-hexane and 3 mL saturated sodium chloride solution are then added and the vial is sealed with a crimp top. The vial is shaken mechanically for 10 min and subsequently centrifuged for 5 min at 3000 rpm. Approximately 1.7 mL of the hexane phase is drawn out of the open crimp top vial and transferred to a 2 mL autosampler vial, into which 100 L toluene is added as a keeper for the volatile esters of the pyrethroids. The solution is subsequently evaporated to about 100 L in a stream of nitrogen.

A reagent blank is included in each analytical series. In this case ultrapure water is subjected to the sample processing described above instead of urine. Instead of a crimp top vial a 22 mL threaded vial can be used.

4 Operational parameters for gas chromatography and mass spectrometry

Capillary column:	Material:	Quartz glass (fused silica)
	Stationary phase:	5% phenylmethyl polysiloxane
	Length:	60 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 m
Temperatures:	Column:	1 min at 90 C; then increase 6 C/min to 150 C; 7 min isothermal; then increase 10 C per min to 200 C; 12 min isothermal; then increase 20 C/min to 270 C; 10 min at the final temperature
	Injector:	250 C

Carrier gas:	Helium with a precolumn pressure of 21 psi
Splitless time:	2 min
Detector:	Quadrupole MSD (EI mode)
MSD Transfer line:	300 C
Electron-impact ionization:	70 eV
Electron multiplier:	1400 V (+600 rel.)
Mode:	SIM
Dwell time/mass:	100 ms
Sample volume:	1 L

5 Analytical determination

In order to carry out the gas chromatographic analysis, 1 L of each of the prepared samples is injected into the gas chromatograph.

The analytical determination is carried out with a mass selective detector (MSD) in the SIM (selected ion monitoring) mode. The following conditions were selected on the MSD for the determination of the methyl esters of pyrethroid metabolites:

Table 2. Conditions for the determination of the methyl esters of the pyrethroid metabolites with the aid of the MSD.

Methyl esters of the analytes	Retention time [min]	Detected mass [*m/e*]
cis-Cl_2CA	18.6	222 187 163
trans-Cl_2CA	18.9	222 187 163
Br_2CA	24.9	312 253 231
F-PBA	33.2	246 215
3-PBA	34.1	228 197
2-PBA (internal standard)	32.1	228 197

The given retention times serve only as an orientation. The user of this method must optimize the adjustments on the instrument used.

The underlined masses were used for the quantitative evaluation.

If the measured values are above the linear range of the calibration curve, the urine samples are diluted and processed anew. A quality control sample and a reagent blank are included in each analytical series.

Figure 2 shows a total ion gas chromatogram of the methylated pyrethroid metabolites.

Figure 3 illustrates a GC-MS-SIM gas chromatogram of the methyl esters of *cis*-Cl_2CA and *trans*-Cl_2CA, and the mass spectra of each compound.

Figure 4 shows the chromatograms of a processed urine sample of a person who had been exposed to permethrin.

6 Calibration

Pooled urine of people who were not exposed to pyrethroids was used to plot a calibration curve. The calibration standards (Section 2.4) are processed in the same manner as the urine samples (Section 3) and analyzed by gas chromatography/mass spectrometry in accordance with Section 4 and 5. Calibration curves are obtained by plotting the quotients of the peak areas of the individual pyrethroid metabolites and that of the internal standard as a function of the concentrations used. It is unnecessary to plot a complete calibration curve for every analytical series. It is sufficient to analyse one calibration standard for every analytical series. The ratio of the value obtained for this standard and the value obtained for the equivalent standard in the complete calibration curve is calculated. Using this quotient, each of the results read off the calibration curve is adjusted for each series. A new calibration curve should be plotted if the analytical conditions change or if systematic deviations are observed in the quality control results.

The calibration curve is linear between the detection limit and 200 g of the pyrethroid metabolites per litre urine.

7 Calculation of the analytical result

The resulting peak areas of the pyrethroid metabolites are divided by the peak area of the internal standard. The quotients thus obtained are used to read off the equivalent concentrations of the pyrethroid metabolites in g per litre urine from the relevant calibration curve. The results are adjusted as described in Section 6. Possible dilution of the urine must be corrected by appropriate multiplication. If a reagent blank value is detected, it must be subtracted.

8 Standardization and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the Bundes rztekammer (German Medical Association) [32, 33] and in the special preliminary remarks in Volume 1 of this series. A urine sample containing a constant concentration of the pyrethroid metabolites is analysed in order to determine the precision of the method. As material for quality control is not commercially available, it must be prepared in the laboratory. For this purpose, urine is spiked with a defined quantity of the pyrethroid metabolites. A six-month supply of this control material is prepared, divided into aliquots in pierceable ampoules and stored in the deep-freezer. The concentration of this control material should lie in the middle of the most frequently occurring concentration range. The theoretical value and the tolerance range for this quality control material is determined in the course of a pre-analytical period (one analysis of the control material on 20 different days) [32, 34].

External quality control can be achieved by participating in round-robin experiments. The round-robin experiments carried out in the fields of occupational and environmental-toxicological medicine in Germany include in their external quality assurance programme the analysis of the pyrethroid metabolites *cis*-Cl_2CA, *trans*-Cl_2CA, Br_2CA and 3-PBA in urine in the concentration range of interest to environmental medicine [35, 36].

9 Reliability of the method

9.1 Precision

Pooled urine from persons who had not been exposed to the pyrethroids was spiked with defined amounts of the pyrethroid metabolites in order to determine the precision in the series. Urine solutions were obtained containing the pyrethroid metabolites at concentrations of 4 and 10 g per litre. Determination of these urine samples ten times resulted in relative standard deviations between 1.3 and 2.7%, equivalent to prognostic ranges between 3.1 and 6.2% (cf. Tab. 3).

The individual urine samples of people who had not been exposed to the pyrethroids were spiked with different concentrations of pyrethroid metabolites to test the influence of the urine matrix on the analytical results. Concentrations between 8.3 and 11.5 g per litre were found. These samples were processed and analyzed in one analytical run. The relative standard deviations were between 1.2 and 6.3% (cf. Tab. 4).

In addition, the precision from day to day was determined (Tab. 5). Pooled urine from persons who had not been exposed to pyrethroids was spiked with a defined quantity of pyrethroid metabolites for this purpose. This resulted in urine solutions containing the pyrethroid metabolites at a mean concentration of 10 g per litre. These urine solutions were processed and analyzed on 12 different days. This resulted in relative standard deviations between 6.2 and 8.7%, equivalent to prognostic ranges between 13.8 and 19.3%.

Table 3. Precision in the series for the determination of the pyrethroid metabolites.

Substance	Standard deviation [%]		Prognostic range [%]	
	4 g/L	10 g/L	4 g/L	10 g/L
cis-Cl_2CA	1.7	1.6	3.9	3.7
trans-Cl_2CA	2.7	1.6	6.2	3.7
Br_2CA	2.5	1.7	5.8	3.9
3-PBA	1.9	1.3	4.4	3.1
F-PBA	1.6	1.7	3.7	3.9

Table 4. Precision in the series for seven spiked individual urine samples.

Substance	Standard deviation [%]	Prognostic range [%]
cis-Cl_2CA	6.3	15.4
trans-Cl_2CA	5.2	12.7
Br_2CA	5.7	13.9
3-PBA	1.2	2.9
F-PBA	1.8	4.4

Table 5. Precision from day to day for the determination of the pyrethroid metabolites.

Substance	Standard deviation [%]	Prognostic range [%]
cis-Cl_2CA	7.7	17.1
trans-Cl_2CA	8.7	19.3
Br_2CA	6.9	15.3
3-PBA	6.2	13.8
F-PBA	6.2	13.8

9.2 Accuracy

Recovery experiments were carried out to test the accuracy of the method. Urine samples from people who had not been exposed to pyrethroids were spiked with two different defined amounts of the pyrethroid metabolites. This resulted in urine solutions contain-

Table 6. Recovery rates for the determination of the pyrethroid metabolites.

Substance	Recovery rate [%]	
	10 g/L	60 g/L
cis-Cl_2CA	83.3	86.7
trans-Cl_2CA	77.5	85.1
Br_2CA	82.2	88.0
F-PBA	93.1	92.8
3-PBA	103.1	106.8

ing pyrethroid metabolites at concentrations of 10 and 60 g per litre. Each of these solutions was subsequently processed and analyzed three times in accordance with Section 3. Mean recovery rates between 77.5 and 106.8% were found (cf. Tab. 6).

In addition, the losses due to sample processing during the analysis of *cis*- and *trans*-Cl_2CA were determined. For this purpose, two solutions with concentrations of 4 and 10 g per litre of the appropriate methyl ester in n-hexane were prepared and analyzed twice by means of gas chromatography without further processing. The analytical results were compared with those for the urine samples containing the same *cis*-and *trans*-Cl_2CA concentrations and which were also each subject to the same processing twice. No significant losses could be ascertained under these conditions.

9.3 Detection limit

Under the conditions for sample preparation and gas chromatographic determination described here, the detection limit was between 0.1 and 0.5 g of the individual pyrethroid metabolites per litre urine (Tab. 7). As no reagent blank value was measured the detection limit was calculated as three times the signal/background ratio.

Table 7. Detection limits obtained for the determination of the pyrethroid metabolites.

Metabolites	Detection limits [g/L]
cis-Cl_2CA	0.2
trans-Cl_2CA	0.2
Br_2CA	0.1
F-PBA	0.2
3-PBA	0.5

9.4 Sources of error

When 3-phenoxybenzoic acid is quantitatively evaluated, an interfering background occasionally occurs which does not exceed three times the signal-noise ratio in most cases. It has not been definitively clarified whether this background originates from the analyte or from other substances, as only an ion trace can be recorded in this analytical range. A possible, very slight excretion of 3-PBA in the normal population could originate from the food chain.

Matrix effects play only a minor role in the determination of 3-PBA and F-PBA. These effects are more obvious in the assay of the cyclopropane carboxylic acids. This can be mainly attributed to the fact that the internal standard 2-PBA differs more strongly from the cyclopropane carboxylic acids in its chemical behaviour than from 3-PBA and F-PBA.

The use of a second internal standard, such as a halogenated cyclopropane carboxylic acid could be helpful.

10 Discussion of the method

This capillary gas chromatographic analytical method with mass selective detection is based on a method described by Angerer and Ritter [47]. It permits the simultaneous determination of five pyrethroid metabolites in urine. Using this method the pyrethroid exposure can be ascertained, even in the concentration range relevant to environmental medicine. Liquid-solid extraction is used for sample preparation. It can be replaced by a liquid-liquid extraction without detracting from the quality (see Addendum). In all the previously published analytical methods, the urine samples were subjected to hydrolysis because of the conjugate formation of the pyrethroid metabolites. This was normally carried out with sulphuric acid at 100 C under reflux conditions for 1 to 2.5 hours [37–42]. One of the authors of the method was able to prove that no appreciable analyte losses of the free pyrethroid metabolites occurred despite the oxidizing properties of sulphuric acid. Based on urine samples of persons who had been exposed to pyrethroids, he was also able to show that a hydrolysis temperature of 90 C for 1 hour was sufficient to ensure complete cleavage of the conjugates. K hn et al. [43] confirm these results. On the other hand, higher temperatures and longer periods of hydrolysis have an adverse effect on the recovery.

In two further analytical procedures described in the literature, enzymes were employed to achieve a mild cleavage of the conjugates at low temperatures [44, 45]. However, in the experience of one of the authors of the present method, losses of the analytes resulted under the conditions for enzymatic hydrolysis. The enzyme possibly attacks the pyrethroid metabolites.

Methanol is used as the elution agent in the method described here. The use of methanol as an eluent offers the advantage that, in contrast to other elution agents, it does not have to be removed before the derivatization step, but can directly function as a reactant for the esterification. Thus the sample processing is shortened and the practicability of the method is enhanced. This is especially important if the method is to be used in

routine analysis. Moreover, carcinogenic derivatizing agents (e.g. diazomethane, methyl iodide, etc.) need not be used for this type of derivatization with sulphuric acid/methanol.

The use of a mass selective detector permits detection of pyrethroid exposure at levels which can be caused by contamination in enclosed spaces. In addition, the mass selective detector ensures a high degree of specificity.

A calibration curve which extends to a concentration of 50 g/L is sufficient to determine exposure to pyrethroids which occurs in indoor areas.

Instruments used:

Gas chromatograph HP 5890A, mass selective detector HP 5970B with Pascal workstation and autosampler HP 7673A from Hewlett-Packard.

11 Addendum: Alternative method of sample preparation

A modified operating procedure was developed as an alternative to the method described above. In this case, a liquid-liquid extraction of the pyrethroid metabolites was performed instead of a solid phase extraction. The following changes in the sample preparation were made:

5 mL urine are acidified with 1 mL conc. hydrochloric acid in a 22 mL threaded vial with a teflon-coated seal and a screw cap, 100 L of the internal standard are added and the mixture is heated in the sealed vessel for 60 min at 90 C in the drying cupboard. After cooling the urine sample to room temperature, 4 mL n-hexane are added, the mixture is vigorously shaken (mechanical shaker) for 5 min and then centrifuged at 3500 rpm. The n-hexane phase is drawn up as completely as possible and transferred to a 10 mL glass vial with a screw cap. This extraction step is repeated once more. The combined n-hexane phases are evaporated in a vacuum centrifuge to about 1–2 mL, and then carefully evaporated to dryness under a stream of nitrogen. The residue is taken up in 3 mL freshly prepared methanolic sulphuric acid (90 mL ice-cold methanol are carefully added to 10 mL conc. sulphuric acid), and the mixture is heated for 1 hour at 75 C. After cooling to room temperature, 3 mL of saturated sodium chloride and 2 mL n-hexane are added to the mixture. Then this mixture is shaken for 3 min, centrifuged at 3500 rpm and 1.5 mL of the n-hexane phase is transferred to a 2 mL autosampler vial. After the addition of 100 L toluene as a keeper, the solution is evaporated to about 100 L in a stream of nitrogen. The residue is transferred to a 250 l microvial insert. 2 L of this solution is injected into the gas chromatograph.

The results obtained using this modified procedure are comparable to those achieved by the previously described method.

12 References

[1] *W.N. Aldridge*: An Assessment of the Toxicological Properties of Pyrethroids and their Neurotoxicity. Crit. Rev. Toxicol. *21,* 89–104 (1990).

[2] *K.E. Appel* and *H. Michalak*: Zur Toxikologie der Pyrethroide. VDI-Berichte *1122,* 401– 424 (1994).

[3] *L.H. Machemer*: Hintergrundgespr ch: ,,Pyrethroide in Haushaltsinsektiziden – Nutzen und Risiken . Ringpress, Frankfurt/Main 1992.

[4] *J. Pauluhn, W. Steffens, J. Haas, L.H. Machemer, L.K. Miksche, H. Neuhauser* and *S. Schüle*: Toxikologische Bewertung von Pyrethroiden in Innenr umen: Aufgezeigt am Cyfluthrin und Permethrin. Gesundheitswesen *58,* 551–556 (1996).

[5] *H. Fromme*: Anwendung von Pestiziden in Innenr umen unter besonderer Ber cksichtigung der Pyrethroide. ff. Gesundh. Wes. *53,* 662–667 (1991).

[6] *W. Butte, G. Walker* and *B. Heinzow*: Referenzwerte der Konzentration von Permefhrin-Metaboliten Cl_2CA [3-(2,2-Dichlorvinyl)-2,2-dimethylcyclopropancarbons ure] und 3-PBA [3-Phenoxybenzoes ure] im Urin. Umweltmed. Forsch. Prax. *3 (1),* 21–26 (1998).

[7] *G. Leng, K.-H. Kühn* and *H. Idel*: Biological Monitoring of Pyrethroids in Blood and Pyrethroid Metabolites in Urine: Applications and Limitations. Sci. Total Environ. *199*, 173–181 (1997).

[8] *G. Leng, B. Wieseler, K.-H. Kühn* and *H. Idel*: Pyrethroide und Gesundheit – Wie gef hrlich lebt der Sch dlingsbek mpfer? Bundesgesundheitsblatt *6,* 250–253 (1998).

[9] *B. Kolmodin-Hedman, A. Swensson* and *M. Akerblom*: Occupational Exposure to some Synthetic Pyrethroids (Permethrin and Fenvalerate). Arch. Toxicol. *50,* 27–33 (1982).

[10] *B. Kolmodin-Hedman, M. Akerblom, S. Flato* and *G Alek*: Symptoms in Forestry Workers Handling Conifer Plants Treated with Permethrin. Bull. Environ. Contam. Toxicol. *55,* 487–493 (1995).

[11] *G.H. Prinsen* and *N.J. van Sittert*: Exposure and Medical Monitoring Study of a new Synthetic Pyrethroid after one Season of Spraying on Cotton in Ivory Coast. In: *W.F. Tordoir, E.A.H. van Heemstra-Lequin* (eds.): Field Worker Exposure during Pesticide Application. Elsevier, Amsterdam 105–120 (1980).

[12] *K.E. Appel* and *S. Gericke:* Zur Neurotoxizit t und Toxikokinetik von Pyrethroiden. Bundesgesundhbl. *6,* 219–252 (1993).

[13] *S.A. Flannigan, S.B. Tucker, M.M. Key, C.E. Ross, E.J. Fairchild II, B.A. Grimes* and *R.B. Harris*: Synthetic Pyrethroid Insecticides: A Dermatological Evaluation. Br. J. Ind. Med. *42,* 363–372 (1985).

[14] *K.E. Appel* and *H. Michalak*: Vom Umgang mit chemischen Sch dlingsbek mpfungsmitteln. BgVV, Pressestelle, Berlin 1996.

[15] *G. Leng, A. Leng, K.-H. Kühn, J. Lewalter* and *J. Pauluhn*: Human Dose-excretion Studies with the Pyrethroid Insecticide Cyfluthrin: Urinary Metabolite Profile following Inhalation. Xenobiotica *27,* 1273–1283 (1997).

[16] *B.H. Woollen, J.R. Marsh, W.J.D. Laird* and *J.E. Lesser*: The Metabolism of Cypermethrin in Man: Differences in Urinary Metabolite Profiles following Oral and Dermal Administration. Xenobiotica *22,* 983–991 (1992).

[17] *S.A. Flannigan* and *S.B. Tucker*: Variation in Cutaneous Sensation between Synthetic Pyrethroid Insecticides. Contact Dermatitis *13,* 140–147 (1985).

[18] *F. He, J. Sun, K. Han, Y. Wu* and *P. Yao*: Effects of Pyrethroid Insecticides on Subjects engaged in Packaging Pyrethroides. Br. J. Ind. Med. *45,* 548–551 (1988).

[19] *F. He, S. Wang, L. Liu, S. Chen, Z. Zhang* and *J. Sun*: Clinical Manifestation and Diagnosis of Acute Pyrethroid Poisoning. Arch. Toxicol. *63,* 54–58 (1989).

[20] *J.E. Lessenger*: Five Office Workers inadvertently exposed to Cypermefhrin. J. Toxicol. Environ. Health *35,* 261–267 (1992).

[21] *A. Moretto*: Indoor Spraying with the Pyrethroid Insecticide lambda-Cyhalothrin: Effects on Spraymen and Inhabitants of sprayed Houses. WHO *69,* 591–594 (1991).

[22] *Z. Zhang, J. Son, S. Chen, Y. Wu* and *F. He*: Levels of Exposure and Biological Monitoring of Pyrethroids in Spraymen. Br. J. Ind. Med. *48,* 82–86 (1991).

[23] *D.J. Ecobichon*: Toxic Effects of Pesticides. In: *M.O. Amdur, J. Doull, CD. Klaassen* (eds.): Casarett and Doull s Toxicology. The Basis Science of Poisons, 4th ed. Pergamon Press 565–622 (1991).

[24] *W. Schimmelpfennig*: Begutachtung umweltbedingter toxischer Gesundheitssch den, Bundesgesundheitsblatt 9, 377–385 (1994).

[25] *T. Narahashi*: Nerve membrane Na-channels as Targets of Insecticides. TIPS *13,* 236–241 (1992).
[26] *H.P.M. Vijverberg* and *J. van der Bercken*: Action of Pyrethroid Insecticides on the Vertebrate Nervous System. Neuropathol. Appl. Neurobiol. *8,* 421–440 (1982).
[27] *H.P.M. Vijverberg* and *J. van der Bercken*: Neurotoxicological Effects and the Mode of Action of Pyrethroid Insecticides. Crit. Rev. Toxicol. *21,* 105–126 (1990).
[28] *IPCS International Programme on Chemical Safety*: Environmental Health Criteria 94: Permethrin. World Health Organization, Geneva 1990.
[29] *WHO Evaluations*: Pesticide Residues in Food, Sponsored jointly by FAO and WHO, Part II. Toxicology (1987).
[30] *C. Götzinger, H. Müller-Mohnssen* and *A. Dohmen*: Pyrethroide – Widerspr chliches aus erster Hand. Umwelt Gesundheit *1,* 6–7 (1994).
[31] *H. Müller-Mohnssen*: ber die Wirkung von Pyrethroiden auf die Nervenfunktion. GSF-Jahresbericht Oberschlei heim 1984.
[32] *Bundesärztekammer*: Qualit tssicherung der quantitativen Bestimmungen im Laboratorium. Neue Richtlinien der Bundes rztekammer. Dt. rztebl. *85,* A699–A712 (1988).
[33] *Bundesärztekammer*: Erg nzung der „Richtlinien der Bundes rztekammer zur Qualit tssicherung in medizinischen Laboratorien . Dt. rztebl. *91,* C159–C161 (1994).
[34] *J. Angerer* and *K.H. Schaller*: Erfahrungen mit der statistischen Qualit tskontrolle im arbeitsmedizinisch-toxikologischen Laboratorium. Arbeitsmed. Sozialmed. Pr ventivmed. *1,* 33–35 (1977).
[35] *J. Angerer* and *G. Lehnert*: Anforderungen an arbeitsmedizinisch-toxikologische Analysen. Dt. rztebl. *37,* C1753–C1760 (1997).
[36] *G. Lehnert, J. Angerer* and *K.H. Schaller*: Statusbericht ber die externe Qualit tssicherung arbeits- und umweltmedizinisch-toxikologischer Analysen in biologischen Materialien. Arbeitsmed. Sozialmed. Umweltmed. *33 (1),* 21–26 (1998).
[37] *F. Asakawa, F. Jitsunari, K. Miki, J.O. Choi, N. Takeda, T. Kitamado, S. Suna* and *Y. Manabe*: Agricultural Worker Exposure to and Absorption of Permethrin applied to Cabbage. Bull. Environ. Contam. Toxicol. *56,* 42–49 (1996).
[38] *C. Aprea, A. Stridori* and *G. Sciarra*: Analytical Method for the Determination of Urinary 3-Phenoxybenzoic acid in Subjects occupationally exposed to Pyrethroid Insecticides. J. Chromatogr. *695,* 227–236 (1997).
[39] *B.H. Woolen, J.R. Marsh, W.J.D. Laird* and *J.E. Lesser*: The Metabolism of Cypermethrin in Man: Differences in Urinary Metabolite Profiles following Oral and Dermal Administration. Xenobiotica *22,* 983–991 (1992).
[40] *A. Tuomainen, J. Kangas, J. Liesivuori* and *A. Manninen*: Biological Monitoring of Deltamethrin Exposure in Greenhouses. Int. Arch. Occup. Environ. Health *69,* 62–64 (1996).
[41] *G. Chester, L.D. Hadfield, T.B. Hart, B.C. Leppert, H. Swaine* and *O.J. Tummon*: Worker Exposure to, and Absorption of, Cypermethrin during Aerial Application of an Ultra Low Volume Formulation to Cotton. Arch. Environ. Contam. Toxicol. *16,* 69–78 (1987).
[42] *C.V. Eadsforth* and *M.K. Baldwin*: Human Dose-excretion Studies with the Pyrethroid Insecticide Cypermethrin. Xenobiotica *13,* 67–72 (1983).
[43] *K.H. Kühn, G. Leng, K.A. Bucholski, L. Dunemann* and *H. Idel*: Determination of Pyrethroid Metabolites in Human Urine by Capillary Gas Chromatography-Mass Spectrometry. Chromatographia *43,* 285–292 (1996).
[44] *M. Nassif, J.P. Brooke, D.B.A. Hutchinson, O.M. Kamel* and *E.A. Savage*: Studies with Permethrin against Bodylice in Egypt. Pestic. Sci. *11,* 679–684 (1980).
[45] *P.P. Yao, Y.W. Li, Y.Z. Ding* and *F. He*: Biological Monitoring of Deltamethrin in Sprayers by HPLC Method. J. Hyg. Epidemiol. Microbiol. Immun. *36,* 31–36 (1992).
[46] *J. Lewalter*: Personal communication (1998).
[47] *J. Angerer* and *A. Ritter*: Determination of Metabolites of Pyrethroids in Human Urine using Solid-Phase Extraction and Gas Chromatography-Mass Spectrometry. J. Chromatogr. B. Biomed. Sci. Appl. *695(2),* 217–226 (1997).

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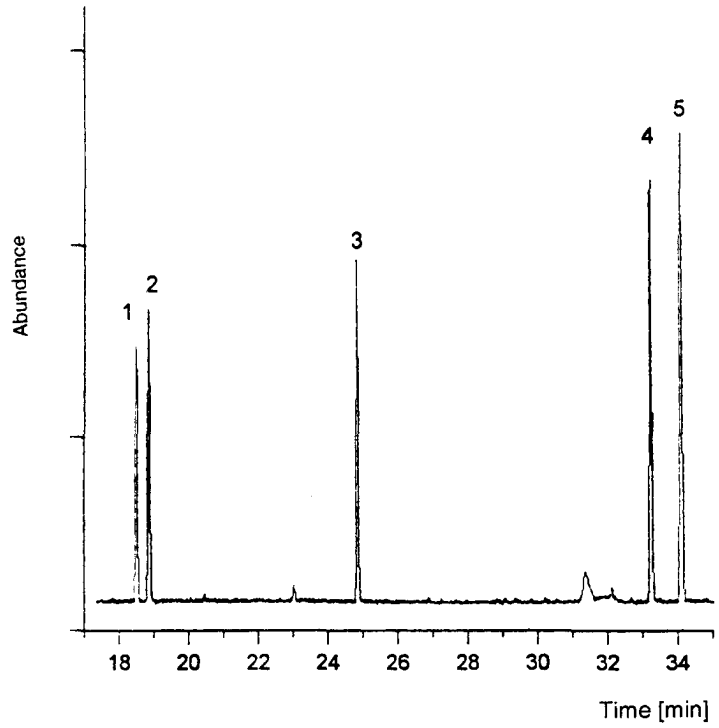

Fig. 2. Total ion gas chromatogram of the methylated pyrethroid metabolites in urine (5 mg/L): (1) cis-Cl_2CA, (2) trans $C1_2$CA, (3) Br_2CA, (4) F-PBA and (5) 3-PBA.

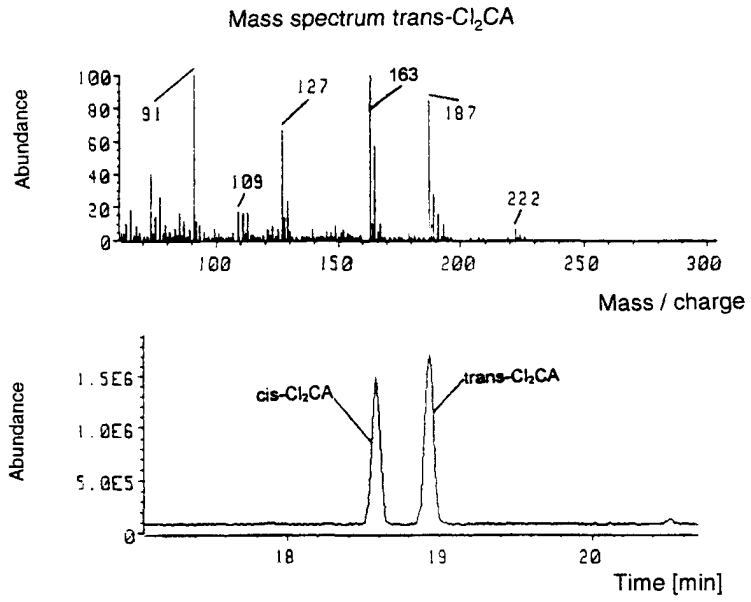

Fig. 3. GC-MS-SIM gas chromatogram of the methyl ester of cis-Cl_2CA and trans $C1_2$CA as well as the mass spectrum of trans-Cl_2CA.

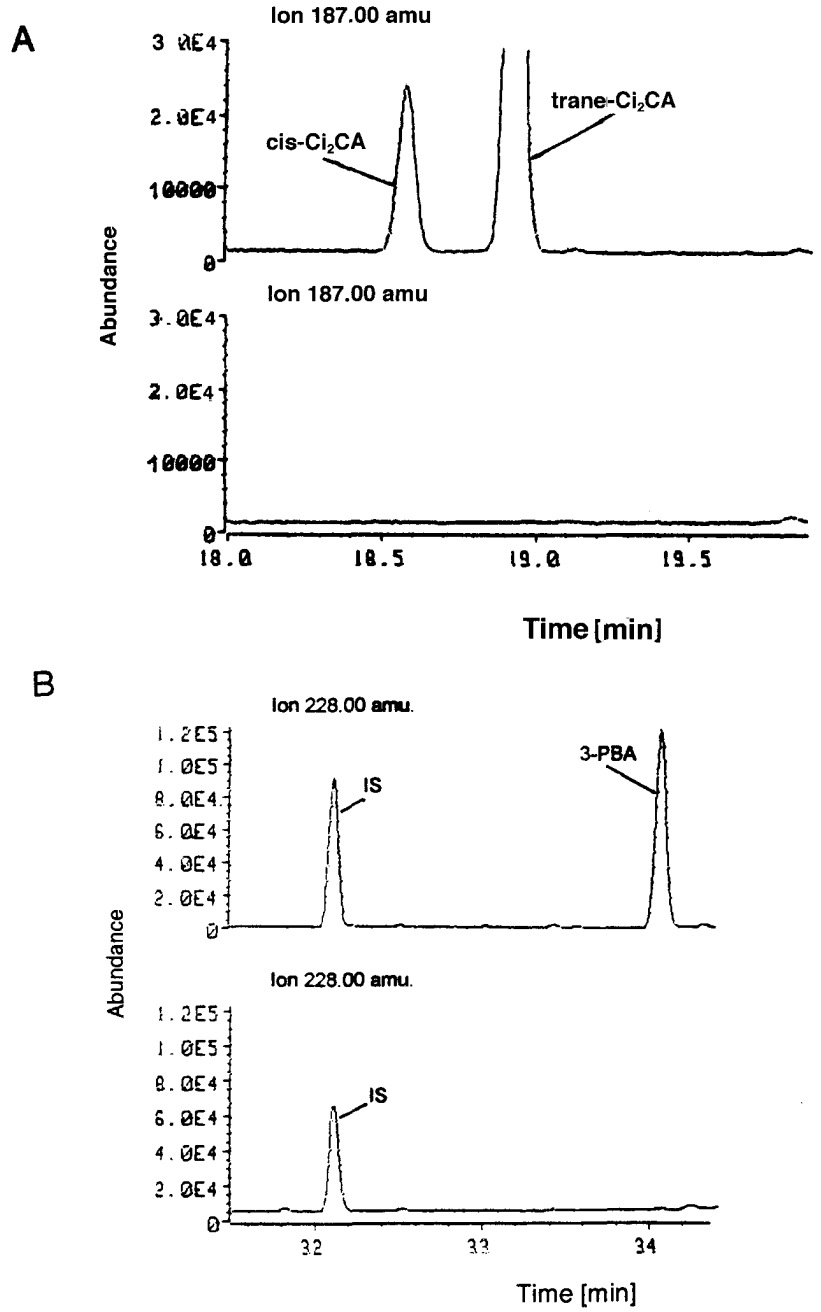

Fig. 4. Processed urine sample of a person who had been exposed to permethrin. (A) above: cis-Cl_2CA (82.4 g/L) and trans-Cl_2CA (87.3 g/L); (B) above: 3-PBA (33.0 g/L). (A) and (B) below: processed urine sample of a person who was not exposed to permethrin.