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Determination of dichloroanilines in human urine by GC–MS, GC–MS–MS, and GC–ECD as markers of low-level pesticide exposure

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Abstract

Methods for the determination of 3,4-dichloroaniline (3,4-DCA) and 3,5-dichloroaniline (3,5-DCA) as common markers of eight non-persistent pesticides in human urine are presented. 3,5-DCA is a marker for the exposure to the fungicides vinclozolin, procymidone, iprodione, and chlozolinate. Furthermore the herbicides diuron, linuron, neburon, and propanil are covered using their common marker 3,4-DCA. The urine samples were treated by basic hydrolysis to degrade all pesticides, metabolites, and their conjugates containing the intact moieties completely to the corresponding dichloroanilines. After addition of the internal standard 4-chloro-2-methylaniline, simultaneous steam distillation extraction (SDE) followed by liquid–liquid extraction (LLE) was carried out to produce, concentrate and purify the dichloroaniline moieties. Gas chromatography (GC) with mass spectrometric (MS) and tandem mass spectrometric (MS–MS) detection and also detection with an electron-capture detector (ECD) after derivatisation with heptafluorobutyric anhydride (HFBA) were employed for separation, detection, and identification. Limit of detection of the GC–MS–MS and the GC–ECD methods was 0.03 and 0.05 μg/l, respectively. Absolute recoveries obtained from a urine sample spiked with the internal standard, 3,5-, and 3,4-DCA, ranged from 93 to 103% with 9–18% coefficient of variation. The three detection techniques were compared concerning their performance, expenditure and suitability for their application in human biomonitoring studies. The described procedure has been successfully applied for the determination of 3,4- and 3,5-DCA in the urine of non-occupationally exposed volunteers. The 3,4-DCA levels in these urine samples ranged between 0.13 and 0.34 μg/g creatinine or 0.11 and 0.56 μg/l, while those for 3,5-DCA were between 0.39 and 3.33 μg/g creatinine or 0.17 and 1.17 μg/l.

Keywords: Pesticides; Dichloroanilines

1. Introduction

In the European Union residues of ~350 active pesticide substances are expected to be found in and on products of plant origin [1]. Under the existing

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regulations of the German Food Law (LMBG) [2] monitoring of food has to be carried out in the context of official food control measures. Thus, an effective instrument to expand the knowledge of the occurrence of these pesticides in food is available, which can contribute to preventive health protection. However, knowledge about the fate of the residues of these non-persistent compounds in the human body is still scare. Research in the field of human biomonitoring on this subject is still in its infancy [3]. Biological monitoring of exposure to pesticides is aimed at estimating the effective internal dose according to the fate of the pesticide in the body. This monitoring can be divided into four areas [4]: direct measurement of unchanged pesticides in biological media, determination of their metabolites in urine or blood, quantification of biological effects related to the internal dose, and measurement of active chemicals interacting with the target- or non-target molecules.

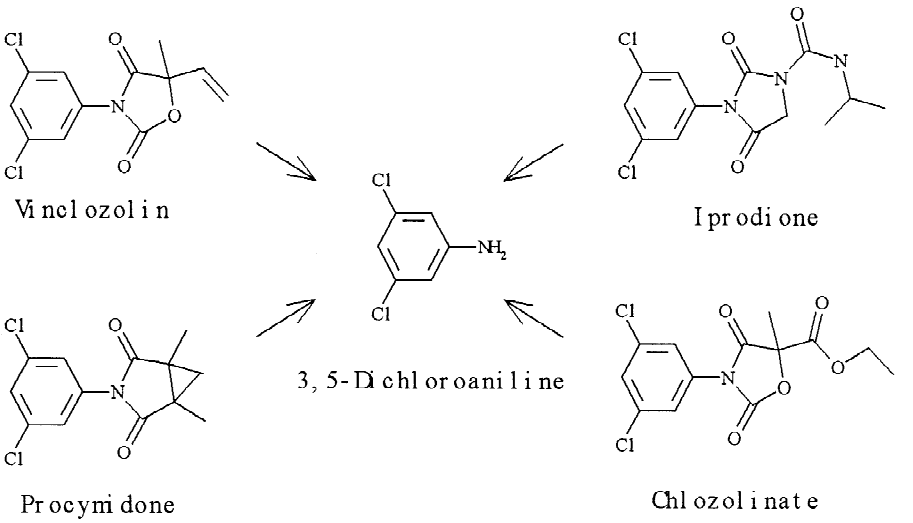
Due to the lower concentration levels and the only small sample amounts available, analytical procedures for the determination of pesticides or their metabolites in human body fluids have to be more powerful than those for food or workplace monitoring.

According to publications of the German BgVV (1995–1998) [5] and DGE (1988–1996) [6,7] dealing with the nationwide monitoring of foodstuffs in Germany, pesticide residues have been detected in ~60% of fruit and 40% of all vegetable samples. From 1995 to 1998 the maximum levels or guide values [8] were exceeded in ~5% of all samples. Concerning pesticide residues in foodstuffs, currently no significant trends are observable. However, in several harvested goods some individual pesticides appear frequently. About 15 pesticides were determined in more than 10% of the analysed food samples. The fungicides vinclozolin, procymidone, and iprodione belong to these most frequently occurring pesticides. These pesticides are all dicarboximide fungicides containing the common moiety 3,5-DCA. Exceeded guide values of these fungicides from foodstuff monitoring studies were mainly obtained in the case of vegetables and fruits such as lettuce, tomatoes, sweet peppers, grapes, strawberries, apples, peaches, and carrots which are consumed daily uncooked in relatively high amounts [9]. Additionally, in nearly all samples of wine which were analysed in the context of foodstuff monitoring studies in 1997 in Germany [10], considerable amounts of the mentioned fungicides were found. Thus, the consumer of these products may be relatively highly exposed to these substances. Due to the formation of the 3,5-DCA moiety, the fungicide chlozolinate is included in the study as well. However, this pesticide was not found frequently in previous monitoring studies.

Regarding the toxicological potential of these pesticides, the mentioned metabolites are very interesting for human biomonitoring studies concerning assessment of the non-occupational exposure of the general population. Even low-level exposures may be associated with adverse health effects such as endocrine disruption, chemical sensitivity, and cancer [11]. Vinclozolin, for instance, is a proven endocrine disruptor [12,13] causing anti-androgenic effects. Following prenatal and perinatal exposure to low doses of vinclozolin, male rats developed sex organ changes including retained nipples, reduced ejaculated sperm numbers, and reduced ventral prostate weight [14,15]. Young male rats exposed to vinclozolin showed delayed puberty [16]. These anti-androgenic effects are caused by two of its metabolites, which are able to bind to the androgen receptor and block its activity [17,18]. Due to the fact that vinclozolin was found to develop tumours in animal experiments, its genotoxicity is also discussed [19–22]. Moreover, procymidone is an anti-androgen with anti-maleness properties similar to vinclozolin. It is able to block androgen binding to the human androgen receptor. Male offspring of rats exposed to procymidone during pregnancy and early lactation showed a range of reproductive deformities such as permanent nipples and malformed penises [22]. The urea based herbicide linuron was found to have a weak affinity for the androgen receptor. A multi-generation study with rats exposed to linuron revealed a range of male reproductive tissue problems in offspring including testicular malformations and reduced size of androgen-dependent tissues [23]. Finally, the herbicide propanil, which is widely used in rice production, has been shown to produce methaemoglobinaemia in animals and in occupationally exposed humans through the action of its metabolite 3,4-DCA [24].

According to current knowledge, there is no acute risk to the general population as a result of dietary exposure to these pesticides.

In the present paper the moiety 3,5-DCA is used as marker for the fungicides vinclozolin, iprodione, procymidone, and chlozolinate and the moiety 3,4-DCA as marker for the herbicides diuron, linuron, neburon, and propanil. The chemical structures of these pesticides are shown in Fig. 1. As listed in Table 1, the method may be extended to other aniline



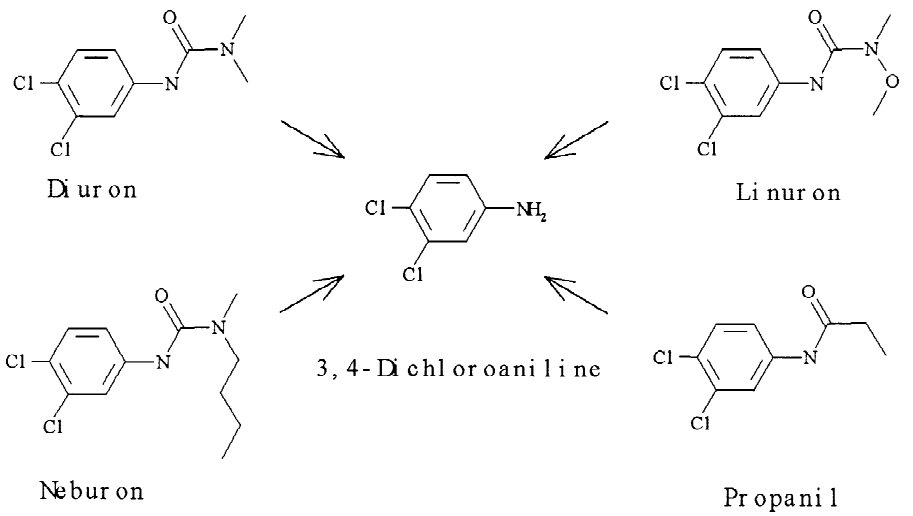


Fig. 1. Chemical structures of selected non-persistent pesticides and their common dichloroaniline metabolites.

Table 1
Pesticides with their common aniline metabolites

Common metabolite	Pesticide	Used as[a]	Class
3,5-Dichloroaniline	Vinclozolin	F	Dicaroximide
	Iprodione	F	Dicaroximide
	Procymidone	F	Dicaroximide
	Chlozolinate	F	Dicaroximide
3,4-Dichloroaniline	Diuron	H	Phenylurea
	Linuron	H	Phenylurea
	Neburon	H	Phenylurea
	Propanil	H	
Aniline	Propham	H	Carbamate
	Dichlofluanid	F	Aniline-derivate
	Fenfuram	F/B	Carbonacidanilide
	Carboxin	F	Oxathiin-derivate
	Carbetamid	H	Carbamate
3-Chloroaniline	Barbam	H	Carbamate
	Chlorpropham	H	Carbamate
3-Methylaniline	Phenmedipham	H	Biscarbamate
4-Chloroaniline	Monuron	H	Phenylurea
	Monulinuron	H	Phenylurea
	Buturon	H	Phenylurea
	Diflubenzuron	H	Phenylurea
3-Chloro-4-methylaniline	Chlortoluron	H	Phenylurea
	Pentanochlor		Amide
4-Isopropylaniline	Isoproturon	H	Phenylurea
2,4-Dimethylaniline	Amitraz	I/A	Formamidine
2,6-Dimethylaniline	Metazachlor	H	Acetanilide
3-Chloro-4-methoxyaniline	Metoxuron	H	Phenylurea
2,4-Difluoro-3,5-dichloraniline	Teflubenzuron	I	Phenylurea
3-Trifluoromethylaniline	Fluometuron	H	Phenylurea
4-(4-Methoxy)-phenoxyaniline	Difenoxuron	H	Phenylurea
4-(4-Chlorophenoxy)-aniline	Chloroxuron	H	Phenylurea
4-Bromo-3-chloraniline	Chlorobromuron	H	Phenylurea
4-Chloro-2-methylaniline	Chlordimeform	A	Foramidine
4-Bromoaniline	Metobromuron	H	Phenylurea
3-(Trifluoromethoxy)-aniline	Triflumuron	I	Phenylurea

[a] A, acaricide; B, stain; F, fungicide; H, herbicide; I, insecticide.

metabolites as well [25]. Care should be taken if the origin of the moiety is not specific for the presence of individual pesticides. For instance, aniline derivatives are widely uses as intermediates for pharmaceuticals, dyestuffs, rubber chemicals, cosmetics and pigments [26]. Furthermore the reduction of nitrobenzene derivatives should be considered.

Only a few papers were available concerning dichloroanilines as common pesticide metabolites in foodstuffs [27,28] and in urine of occupationally [29,30] or non-occupationally exposed persons [35]. Regarding the detection power, only the method applied by Weiß et al. [35] is suitable for application in human biomonitoring studies at low-level exposure. These authors used acidic hydrolysis followed by solvent extraction in combination with derivatization and GC–MS for the determination of 3,5-DCA in urine samples from the general population.

The aim of the present work was to develop analytical methods for the determination of 3,4- and 3,5-DCAs as markers of exposure to non-persistent pesticides in non-occupationally exposed persons to assess low-level exposure. The pesticides were selected according to their presence in vegetable foodstuffs and their toxicological potential. Two independent analytical procedures were described and compared, which can both be used in biological monitoring studies to gather information about exposure of the general population.

2. Experimental

2.1. Reagents

3,4- and 3,5-DCA were obtained from Dr Ehrendorfer (Augsburg, Germany). The internal standard (I.S.) 4-chloro-2-methylaniline was purchased from Riedel-de Haen (Hannover, Germany) and heptafluorobutyric anhydride (HFBA) from Macherey-Nagel (Düren, Germany). Water was purified using a Milli-Q system (Millipore, Eschborn, Germany). The Silicone-Antifoam emulsion was purchased from Fluka (Deisenhofen, Germany). The sodium hydroxide solution (10 mol/l) was prepared by dissolving 200.0 g of sodium hydroxide (p.a. quality; Merck, Darmstadt, Germany) in deionised water to a final volume of 500 ml. Sulphuric acid solution (5 mol/l) was produced by diluting 52 ml of concentrated sulphuric acid (p.a. quality, 95–97%; Merck, Darmstadt, Germany) with deionised water to a final volume of 200 ml. Anhydrous sodium sulphate (for residue analysis) was purchased from Merck (Darmstadt, Germany). All used organic solvents were for residue analysis as well.

Stock solutions of 3,4-, 3,5-DCA, and the internal standard containing 500 mg/l each were prepared in methanol (Merck, Darmstadt, Germany). Working standard solutions containing both analytes at concentrations of 10, 100, and 1000 μg/l, respectively, were prepared by appropriate dilution with methanol. The working standard solution of the internal standard (200 μg/l) was produced in the same way. For recovery experiments, solutions of 3,4-, 3,5-DCA, and the internal standard containing 50 and 250 μg/l were prepared using cyclohexane (Fluka, Deisenhofen, Germany).

2.2. Sample preparation

Urine was collected in acid-washed polypropylene bottles and stored in a freezer at −20°C. For preparation the samples were thawed in a water bath (40°C) and adapted to room temperature. Prior to aliquotation the samples were carefully shaken to distribute the sediment as homogeneously as possible.

For simultaneous steam distillation extraction (SDE) the Bleidner device [31] illustrated in Fig. 2 was filled with 10 ml of water and 10 ml cyclohexane. PTFE sockets (Bohlender, Lauda-Königshofen, Germany) were put onto the ground surface. Afterwards 100 ml urine (smaller volumes were adjusted with water to a final volume of 100 ml) were filled into a 250-ml round-bottom flask. After addition of 500 μl of the internal standard solution (200 μg/l in methanol), 20 ml of sodium hydroxide solution (10 mol/l) and 5 ml of the antifoam, the flask was coupled to the lower end of the distillation device. The upper end was coupled to a 50-ml round-bottom receiver flask which was filled with ~25 ml of cyclohexane from Fluka (Deisenhofen, Germany). Finally, the device was heated for at least 3 h under reflux. The relation of both reflux rates should be equal. For clean-up and preconcentration purposes 10 ml of the SDE extract were then filled

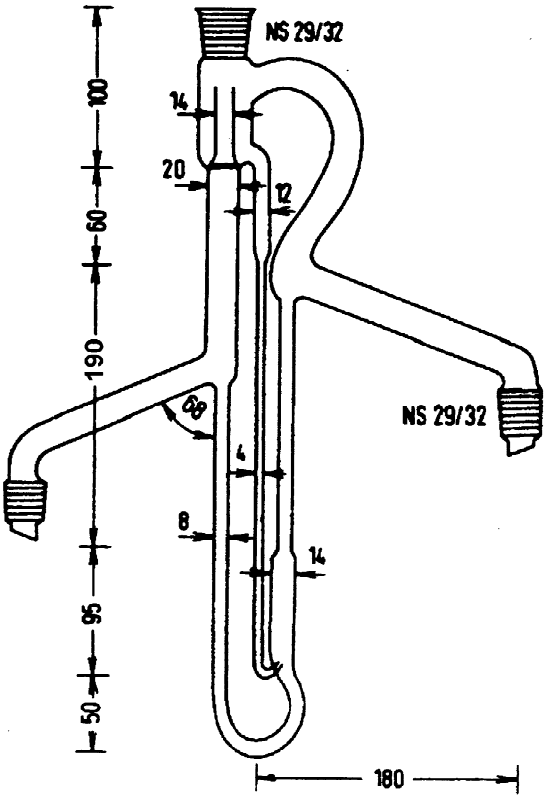


Fig. 2. Bleidner device (according to W. Heizler, modified for cyclohexane by K. Wittke).

into a 15-ml glass tube. After addition of 1 ml sulphuric acid (5 mol/l) the tubes were vortexed for at least 15 s. The organic layer was discarded and 400 μl of cyclohexane and 1.5 ml of sodium hydroxide solution (10 mol/l) were added to the tube. The extract was then cautiously vortexed again under the already mentioned conditions. Prior to the GC–MS or GC–MS–MS analysis the organic layer was dried over anhydrous sodium sulphate and transferred into a 1-ml brown-coloured autosampler vial containing a 200-μl glass insert (Macherey-Nagel, Düren, Germany).

For GC–ECD analysis 300 μl of the cyclohexane extract were derivatized by addition of 10 μl HFBA followed by mixing and incubation in a tube heater (15 min, 60°C). After addition of 1 ml phosphate buffer solution (pH 7.0; Merck, Darmstadt, Germany), the tubes were vortexed for at least 30 s. The organic layer was then dried over anhydrous sodium sulphate and transferred into a 1-ml brown-coloured autosampler vial with a 200-μl glass insert.

2.3. Calibration

Standard solutions in the concentration range from 0.1 to 10 μg/l were prepared by spiking 100 ml water with 1 ml of the appropriate working standard solutions containing 10, 100, and 1000 μg/l 3,4- and 3,5-DCA in methanol.

2.4. Quality control

For quality control investigations pooled urine was spiked with the working standard solution in methanol to final concentrations of 0.1, 0.5 and 5.0 μg/l of 3,4- and 3,5-DCA, respectively. Since no urine samples with 3,4- und 3,5-DCA concentrations below the limit of determination were available, blank values of the used urine sample pools have to be subtracted. For a valid determination of the accuracy the native amount of 3,4- and 3,5-DCA in the pooled urine should be equal or lower than the spike. For this reason we used bovine urine for the preparation of the low-level quality control urine (0.1 μg/l) which fulfils this requirement.

2.5. Capillary gas chromatography with ECD

Analysis of the HFBA derivatives was performed with a Hewlett-Packard (Palo Alto, CA, USA) Model 5880A gas chromatograph equipped with a split/splitless injector and a ^{63}Ni ECD detector. Helium (purity: 5.5; Air Products, Hattingen, Germany) was used as carrier gas with 25-psi head pressure. A sample volume of 1 μl was injected into the split/splitless injector in the splitless mode at an injector temperature of 250°C. Then 10 s after injection the split opened to a ratio of 1:40. The chromatographic separation was performed using a 60-m DB-5 fused-silica capillary column (0.32 mm I.D., 1-μm film thickness; J&W Scientific, Köln, Germany). The oven temperature program started isothermally at 150°C for 1 min, increased then at a rate of 1.5°C/min to 175°C and was finally heated for 15 min at 300°C in the post time mode. The detector temperature was held at 320°C. N_2 (ECD grade; Air

Products, Hattingen, Germany) served as make-up gas with a flow-rate of 30 ml/min.

2.6. Capillary gas chromatography with MS and MS–MS

Analysis of the dichloroanilines was done with a GC–MS system from Varian Instruments (Sunnyvale, CA, USA) consisting of a Saturn 2000 ion trap mass spectrometer coupled to a gas chromatograph type 3800 equipped with a split/splitless PTV injector type 1079 and an autosampler type 8200. A sample volume of 1 μl was injected at an injector temperature of 250°C applying the splitless mode. After 60 s the split was opened to a ratio of 1:50. Helium (purity 5.5; Air Products, Hattingen, Germany) was used as carrier gas at a constant flow of 1.5 ml/min. The gas chromatographic separation was performed with a 30-m fused-silica capillary column DB-5MS (0.25 mm I.D., 0.25-μm film thickness; J&W Scientific, Köln, Germany). The oven temperature program started isothermally at 120°C for 15 min, was then increased at a rate of 20°C/min to 240°C and was finally held for 1 min at the final temperature.

Besides the described non-polar column (DB-5MS), the more polar SB-11 (30 m, 0.25 mm I.D, 0.20-μm film thickness; WGA, Krefeld, Germany) was also tested for the separation of the DCAs. For this investigation the HRGC 5300 (Carlo Erba, Hofheim, Taunus, Germany) coupled to an ion trap detector (ITD) model 700 (Finnigan, San Jose, CA, USA) was used. Manifold, transfer-line, and trap temperatures were 50, 170, and 200°C, respectively. The ionisation was in all cases done in the electron impact mode (EI) at ~70 eV. Scan function with automatic gain control (AGC), using a scan rate of 1 scan/s, was applied. The MS–MS operation is subdivided into four basic parts: the ion formation and matrix ion ejection, parent ion isolation, product ion formation, and product ion mass scanning. The optimised parameters are summarized in Table 2. The MS–MS section is subdivided into two different segments for the determination of the internal standard and the DCAs.

2.7. Calculation

For GC–ECD analysis the peak areas were used as a basis for calculation applying the integrator of the Hewlett-Packard terminal 5889A (Palo Alto, CA, USA). The concentrations were calculated via the peak area ratios from the analyte to the internal standard. For GC–MS and GC–MS–MS analysis, the calculation was done by Varian Instruments (Sunnyvale, CA, USA) GC–MS Workstation version 5.40. Calculation of the concentrations was per-

Table 2
Method section parameters for ion trap MS–MS conditions

Mode	Parameter	Segment 1	Segment 2
General	Analyte	ISTD	3,4- and 3,5-DCA
	Time (min)	4–8	8–14
	Technique	MS–MS	MS–MS
	Low mass (m/z)	72	85
	High mass (m/z)	146	166
	Ionisation mode	EI/AGC	EI/AGC
Ionisation	Ionisation storage level (m/z)	67.0	80.0
	Ejection amplitude (V)	0.6	0.6
Isolation	Parent ion (m/z)	141	161
	Product ions[a] (m/z)	106	134/126/125/90/99
	Isolation window (m/z)	3	3
Dissociation	Waveform (m/z)	Resonant	Resonant
	Excitation storage level (V)	80	67
	Excitation amplitude (V)	0.6	0.6

[a] Used for quantification.

formed via the peak height ratios from the analyte to the internal standard.

2.8. Cross-validation of GC–ECD and GC–MS–MS technique

Since GC–ECD is a chromatographic technique that possibly looses selectivity due to interferences from other matrix components, reagents or other sources, the method was validated by comparison with the tandem mass spectrometric technique. For this purpose the same urine samples (spiked and unspiked) were analysed with both methods.

2.9. Reliability criteria

The precision (inter-assay precision) and accuracy of the methods were evaluated using the quality control samples already described in this section. The precision is expressed by the mean coefficient of variation of all quality control samples on different days (C.V. (%)=100*SD/mean). Accuracy is the mean relative deviation of the found concentration in quality control samples from the nominal value. Maximum permitted deviation of precision accuracy was fixed to 25% relative error. The limit of quantification (LOQ) is defined as the lowest concentration of the calibration curve which could be measured with a precision and accuracy below 30%. A signal at a signal-to-noise ratio of ~3:1 is defined as the limit of detection (LOD) [33].

The calibration functions were constructed using $1/y^2$-weighted linear regression for GC–MS–MS and polynomic regression (2nd grade) for GC–ECD using the peak intensity ratios from analyte to the internal standard. For calibration acceptance the following criteria should be fulfilled: a minimum of six calibration samples (e.g. three concentrations with two replicates) with a relative error below 20%, up to 30% relative error at the LOQ level should be available and not more than 25% of the calibration samples might be eliminated. Recovery studies were carried out using the GC–MS–MS technique. Recovery is defined as the peak-area or peak-height ratio between the analyte signals from the matrix samples (after complete clean-up process) and the mean signal from standard solutions in cyclohexane of the corresponding concentrations, given in %. For evaluation, samples of 0.5 and 2.5 μg/l of the internal standard, 3,4- and 3,5-DCA were prepared and compared using regression analysis of corresponding concentrations with 50 and 250 μg/l from cyclohexane standard solutions.

2.10. Correction for creatinine content

In order to correct for the influence of the variation in diuresis, the creatinine content of the urine samples was analysed using a test kit (Boehringer, Ingelheim, Germany) based on the Jaffe' reaction.

2.11. Calculation of pK_a-values

The pK_a-values of the dichloroanilines and the internal standard were calculated by the Interactive Lab from Advanced Chemistry Development (Toronto, Canada).

3. Results and discussion

A substantial merit of the introduced methods is that all pesticides, metabolites and their conjugates (e.g. glycosides) containing the intact DCA moieties can be determined as common analyte. Consequently, the sensitivity is increased. The preparation of the moieties by alkaline degradation from the pesticides, metabolites and conjugates (e.g. glycosides), the clean-up by steam distillation, solvent extraction and extract concentration, were carried out in a very elegant way by simultaneous steam distillation extraction (SDE) in just one single step. Moreover, SDE is a very selective tool for the separation and enrichment of the analyte from the matrix. As a result of the described extraction procedure, clean cyclohexane extracts were obtained. After an additional liquid–liquid extraction step, the DCAs were once again cleaned and concentrated. This extract was used for the determination of the anilines with GC–MS, GC–MS–MS, and, after HFBA derivatisation, with GC–ECD. Compared with existing methods using the DCA moieties for determination of pesticide residues, the advantages of the developed GC–MS–MS and GC–ECD methods are obvious. The determination of 3,5-DCA metabolites in human urine by HPLC with electrochemical detection [29]

is, for instance, 50 times less sensitive, the determination of the HFBA derivatives of 3,5-DCA in vegetable foodstuffs by GC–ECD [27] is ~80 times less sensitive, and the determination of 3,5-DCA metabolites in vegetable food by GC–MS [28] is even 100 times less sensitive than the presented method.

3.1. Sample preparation

SDE has proved to be a powerful technique for extraction and clean-up of the complex urine matrix. The critical stage of this process is the release of the dichloroaniline moieties. According to the publication of Newsome and Collins [27], reflux extraction under strong alkaline conditions (1.7 N NaOH) for at least 3 h is necessary for a complete degradation of the dicarboximides, and for the quantitative hydrolysis of procymidone an extraction time of up to 4 h is needed. They also stated that even refluxing with 6 N HCl is not suitable for the hydrolysis of iprodione. Additionally, anilines are not steam distillable under acidic conditions.

To guarantee a complete release of the 3,5-DCA markers from all residues containing the DCA moieties, in this work alkaline hydrolysis (1.7 N NaOH) was done for at least 3 h. These conditions were also applied by Hemmerling et al. [28], who used SDE as well, to determine the common 3,5-DCA residues in food of plant origin by GC–MS. If the time for hydrolysis should be reduced the hydrolysis conditions have to be investigated for each individual pesticide.

Although SDE produces clean and concentrated extracts, a selective enrichment technique with LLE at variable pH values was added. Due to the acidity of the analytes, especially of the 3,5-DCA ammonium ion, the addition of at least 1 ml (5 mol/l) sulphuric acid per 10 ml of the SDE extract is necessary to achieve a complete transfer of the DCAs into the aqueous layer. The pK_a-values from the corresponding ammonium ions ranged from 2.48 for 3,5-DCA, 2.90 for 3,4-DCA and 3.81 for the internal standard. The DCAs are finally ~25 times more concentrated than in the SDE extract and additionally purified from interfering compounds such as hydrocarbons, phenols, aldehydes, and ketones. These clean extracts are very suitable for GC–MS and GC–MS–MS analysis resulting in an enhanced long-term stability of the capillary column, the glass liner, and the MS system. Furthermore, they can be also subjected directly to the derivatisation with HFBA for GC–ECD analysis.

The DCAs can in principle be analysed by GC–ECD without any derivatisation. The additional derivatisation step using HBFA is recommended because it results in a better resolution of the analyte peaks and in an enhanced ECD sensitivity of the analytes [32]. Compared with the unchanged analytes the signals are improved by a factor of ~100 for 3,5-DCA and 200 for 3,4-DCA in standard solutions after derivatisation with HBFA. The removal of the surplus HFBA can be performed in a simple final extraction step with phosphate buffer. The results are reproducible chromatograms with distinct peak shapes. Unfortunately, after derivatisation the semi-selective effect of the ECD gets partly lost due to the non-specific formation of interfering compounds. For example, amines are not accessible to ECD detection, but after derivatisation with HBFA these amines are also detected very sensitively by ECD.

3.2. Reliability criteria

The analytical ranges and limits of detection and determination obtained for the GC–ECD, GC–MS and GC–MS–MS based analytical procedures are summarized in Table 3. Due to its lower sensitivity, the GC–MS technique was not validated. Identical limits of determination for the GC–ECD and the GC–MS–MS procedures were achieved for both analytes. The detection limits of 3,5-DCA for the GC–ECD and GC–MS–MS procedures described in this paper, were in good accordance with the detection limit given by Weiß et al. [35]. Applying

Table 3
Method specifications for the determination of 3,4- and 3,5-DCA in human urine by capillary gas chromatography

	3,4- and 3,5-DCA		
	GC–ECD	GC–MS	GC–MS–MS
Analytical range (μg/l)	0.1–5.0	NC[a]	0.1–10.0
LOQ (μg/l)	0.10	NC	0.10
LOD (μg/l)	0.05	0.50	0.03
Specificity	No	Medium	High

[a] NC, not calculated.

Table 4
Inter-assay precision and accuracy obtained from spiked urine samples, prepared by spiking urine with appropriate solutions of 3,4- and 3,5-DCA in methanol

	Low (0.1 μg/l)		Medium (0.5 μg/l)		High (5.0 μg/l)	
	3,4-DCA	3,5-DCA	3,4-DCA	3,5-DCA	3,4-DCA	3,5-DCA
GC–ECD						
n	4	5	5	5	4	4
Precision (%)	19.7	11.7	8.4	17.9	8.3	5.2
Accuracy (%)	1	22.3	−1.4	7.5	−19.6	−20.7
GC–MS–MS						
n	4	4	4	4	4	4
Precision (%)	18.5	21.6	16.3	21.3	25.2	23.8
Accuracy (%)	11.7	23.5	15.9	8.5	−6.6	−18.7

The blank values of the native urine samples were subtracted.

acidic hydrolysis and liquid extraction followed by derivatization with pentafluoropropionic anhydride and GC–MS, they achieved a detection limit for 3,5-DCA of 0.05 μg/l. The precisions and accuracies given in Table 4 are within the acceptable range. The results of the recovery investigations (Table 5) show that the extraction was reproducible and nearly quantitative. Stability investigations using DCA standards in urine and in cyclohexane have shown that the DCAs were stable for at least 3 months at −18°C in urine while the DCAs in cyclohexane were stable for at least 6 months even at 4°C. The HFBA derivatives in cyclohexane were stable at room temperature for at least 2 weeks, provided that they were stored in brown-coloured auto sampler vials.

3.3. GC–MS and –MS–MS

During method development an optimisation of the GC and MS–MS working conditions was carried out. No significant advantage was obtained when the more comprehensive PTV injection technique was used instead of the conventional spilt/splitless injection. Moreover, peak shapes got worse if oven temperature gradients were used in combination with the DB-5MS column. Using the more polar SB-11 column coated with a modified polyethylene glycol a substantially better separation concerning the peak shapes and resolution was obtained. Furthermore, signal amplification resulting in decreasing peak width and shorter run times was achieved. Although there are numerous stationary phases available, most of the applications are usually carried out with 100% methylsiloxane or 5% phenyl-, 95% methylsiloxane coatings. However, it is well known that peak tailing often occurs if polar analytes such as primary and secondary amines (e.g. anilines) are separated on columns with non-polar phases. In spite of the considerably better peak shape with the SB-11 column, we used the DB-5MS column because the column bleed was lower than for the SB-11 column. Additionally, the GC–MS–MS device was also used for other applications, for which the DB-5MS column is recommended. Thus, no working capacity was wasted by frequently changing columns.

The 3,4- and 3,5-DCA isomers show characteristic and similar MS spectra. Three parent ions occur at

Table 5
Recoveries and coefficients of variation of 3,4- and 3,5-DCA and the internal standard (given in %) obtained by GC–MS–MS determination using calibration samples

	0.5 μg/l		2.5 μg/l		Mean (μg/l)	C.V. (%)
I.S.	72.9	88.6	99.6	110.4	92.9	17.2
3,5-DCA	89.5	79.1	116.8	111.6	99.2	18.0
3,4-DCA	88.8	104.7	107.0	109.9	102.6	9.2

C.V., coefficient of variation.

$m/z = 161$, 163, and 165 with a relative abundance of 100, 72, and 12%, respectively, which are caused by the ^{35}Cl and ^{37}Cl isotopes of the DCAs. Main product ions in the MS and in MS–MS mode were formed by elimination of hydrogen cyanide, chlorine and hydrogen chloride from the parent ions [34]. Due to interferences from co-eluting matrix compounds in the MS mode, only the m/z ion 161 was suitable for quantification in urine samples, applying the described GC conditions. An attempt to increase the sensitivity by adding further fragment ions failed due to losses in selectivity. Caused by the relatively high background noise, peak identification using the MS reference spectra was also not possible in the MS mode in the lower calibration range. As a consequence, the GC–MS procedure was inferior to the GC–MS–MS procedure.

GC–MS–MS turned out to be a very powerful technique considering sensitivity and selectivity. The ion trap provided full scan MS–MS data even at trace levels. The obtained MS–MS spectra from both analytes were characteristic and comparable considering their product ions and their relative abundance. For the purpose of signal amplification, the object of fragmentation was to generate MS–MS spectra in which the chosen parent ion (m/z 161) was nearly completely degraded. The sum of the intensities of the five most striking product ions with m/z 134, 126, 125, 99 and 90 was used for quantification. For GC–MS–MS analysis no significant differences between calibration and urine samples were observed considering the chromatograms as well as the resulting spectrums. Blank samples (water was used instead of urine) which were processed within the entire clean-up process were clean, indicating that no carry-over was observed.

The obvious advantage of detection with MS–MS is the enhancement in sensitivity and selectivity due to the elimination of interferences from co-eluting compounds and the quantification of characteristic product ions from one selected parent ion.

In the MS chromatogram given in Fig. 3, which shows the urine sample from an environmentally exposed person, merely two small peaks corresponding to the retention times of the internal standard and 3,5-DCA are present, although only the m/z ratios 106 for the internal standard and 161 for 3,5-DCA were considered. Due to interferences, an acceptable fit to the reference spectrum cannot be achieved. As shown in Fig. 3, in contrast to the MS mode the baseline noise and interferences are almost eliminated in the MS–MS mode. Referring to the ratio from signal-to-noise, in the MS–MS mode we got more than ten orders of magnitude better sensitivity. As a result quantification is possible at concentrations down to 0.1 μg/l and, as shown in Fig. 3, we got equal spectral matches to the MS–MS reference spectra even for 3,4-DCA which is not detectable in the MS mode. In all processed urine samples, interfering peaks were not detected and a specific detection of the analytes was possible. The benefit of monitoring five specific product ions in MS–MS mode is obvious. Due to identical parent and product ions of the six possible DCA isomers, there is still a need for sufficient GC separation power and as a result total run time cannot be reduced further.

3.4. GC–ECD analysis

As shown in Fig. 4, a satisfactory separation of the HBFA derivatives from the urine matrix and a complete gas chromatographic resolution were achieved under the conditions given in the experimental section. Accurate results concerning the resolution were also obtained with the SB-11 fused-silica capillary column. Interfering compounds or background from the complex urine matrix did not influence the determination. This finding was confirmed by cross-validation with the GC–MS–MS technique.

3.5. Cross-validation results from urine samples

By analysis of identical native urine and spiked urine samples with the GC–ECD and GC–MS–MS methods, a good correspondence of the results of these independent techniques was achieved. The results are summarized in Table 6.

3.6. Applications

Urine samples from five non-occupationally exposed volunteers and a bovine urine were investigated to prove the applicability of the described

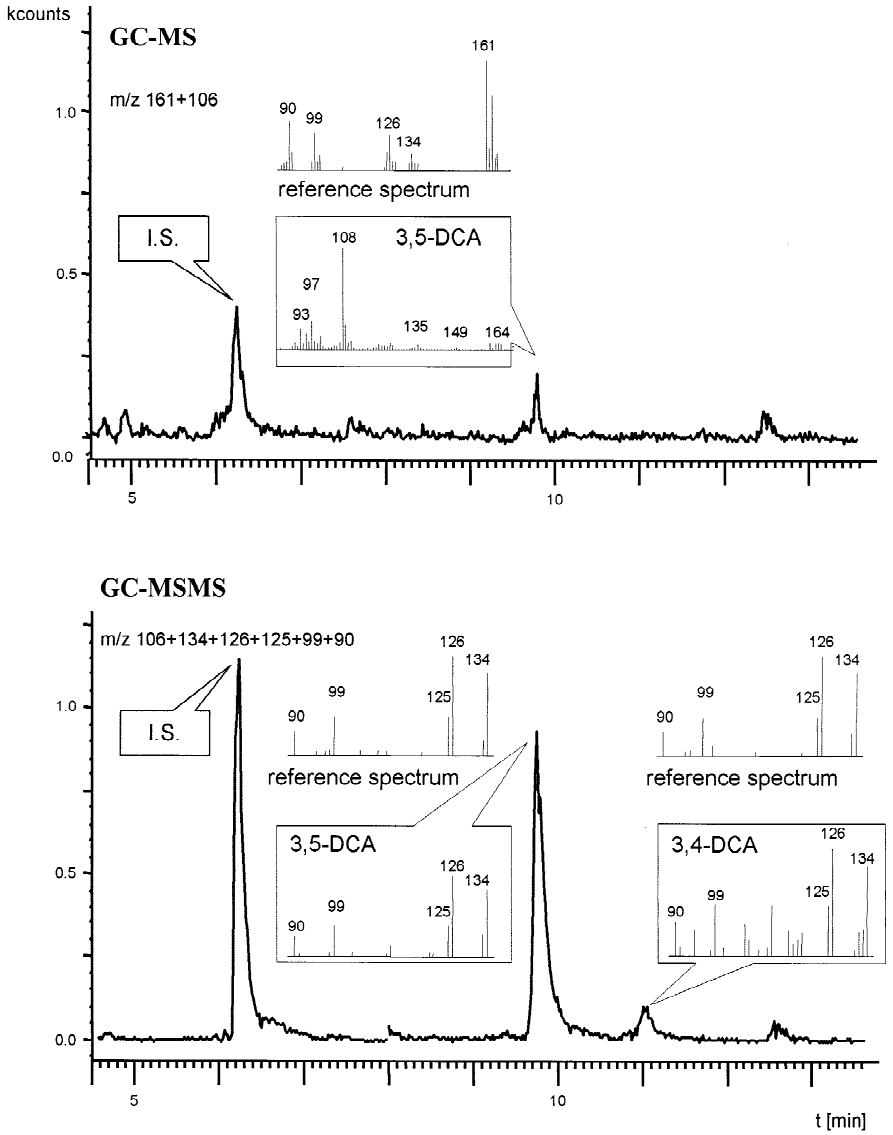


Fig. 3. Comparison of the analysis of an identical reinjected urine sample by GC–MS and GC–MS–MS (urine sample containing 1.000 μg/l I.S., 0.712 μg/l 3,5-DCA and 0.050 μg/l 3,4-DCA). The reference spectrums were obtained by analysis of a cyclohexane standard solution containing 1 mg/l of 3,4- and 3,5-DCA.

analytical procedure. The results are summarized in Table 7. 3,5-DCA was found in all urine samples, while in two urine samples the concentrations of 3,4-DCA were below the limit of quantification. The 3,5-DCA levels in the human urine samples ranged between 0.172 and 1.166 μg/l and were thus about a factor of 6 higher than the corresponding 3,4-DCA levels. Our results for 3,5-DCA were in good accordance with those of Weiß et al. [35], who found 3,5-DCA levels from below the limit of detection (0.05 μg/l) up to 54.9 μg/l in the urine samples of 84 people. They found a corresponding median of

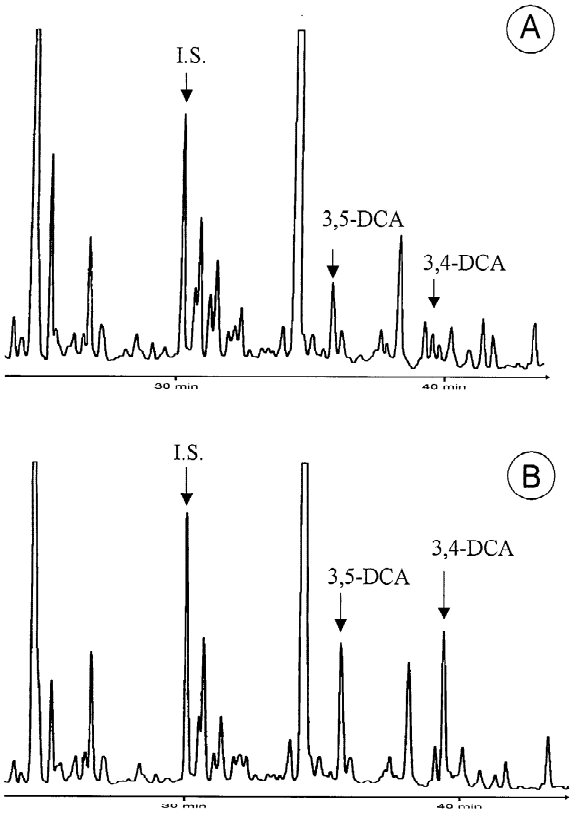


Fig. 4. GC–ECD chromatograms from HFBA derivatives of a urine sample (A) and a urine sample spiked with 0.5 μg/l 3,4- and 3,5-DCA (B).

0.54 μg/l with a 95 percentile of 3.9 μg/l, and they were able to detect 3,5-DCA in 88% of the investigated urine samples.

A human volunteer study which investigates the low-level exposure of the general population considering different dietary behaviours is in preparation.

4. Conclusions

Sensitive and reliable methods are urgently needed to monitor the internal exposure of the general population to the non-persistent pesticides vinclozolin, procymidone, iprodione, chlozolinate, diuron, linuron, neburon, and propanil. In this paper methods based on GC–MS–MS and GC–ECD are described which permit the low-level determination in urine of the common markers of these pesticides, 3,5- and 3,4-dichloroaniline. The described GC–MS–MS and GC–ECD procedures are more sensitive than those procedures which are available from the literature [27–30] and are both suitable for the determination of the DCAs in urine samples from non-occupationally exposed persons. Although both analytical methods can in principle be used for applications in human biomonitoring studies, GC–MS–MS is recommended, because of its higher selectivity and because the derivatisation procedure can be omitted.

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Table 6
Comparison of the GC–MS–MS and GC–ECD results of 3,4- and 3,5-DCA in identical urine quality control samples

3,5-DCA			3,4-DCA		
GC–MS–MS (μg/l)	GC–ECD (μg/l)	Deviation (%)	GC–MS–MS (μg/l)	GC–ECD (μg/l)	Deviation (%)
0.554	0.713	28.7	0.128	0.147	14.8
0.861	0.842	−2.2	0.313	0.262	−16.3
1.096	1.251	14.1	0.708	0.640	−9.6
4.928	4.809	−2.4	4.985	4.279	−14.2

Each result represents the mean of at least four repetitions.

Table 7
Concentrations of 3,4- and 3,5-DCA in the urine samples of five non-occupationally exposed persons and in a bovine urine sample

	3,4-DCA (μg/g creatinine)	3,5-DCA (μg/g creatinine)	3,4-DCA (μg/l)	3,5-DCA (μg/l)
Volunteer 1	0.134	0.485	0.303	1.097
Volunteer 2	0.340	0.715	0.555	1.166
Volunteer 3	–	0.390	<LOD[a]	0.314
Volunteer 4	0.160	3.332	0.050[b]	1.040
Volunteer 5	0.267	0.419	0.109	0.172
Bovine	0.251	0.253	0.115	0.116

[a] <LOD (limit of detection) = 0.03 μg/l.
[b] <LOQ (limit of quantification) = 0.10 μg/l.

Limberg from Bayer AG (Wuppertal, Germany) for calculation of the pK_a-values.

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