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A novel method for the accurate quantification of two isomeric mercapturic acids of 1,3-dichlorobenzene in human urine using isotope dilution online-SPE-LC-MS/MS

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ABSTRACT

1,3-dichlorobenzene (1,3-DCB) is an aromatic solvent that might be formed during thermal decomposition of bis (2,4-dichlorobenzoyl)peroxide used as initiator in silicone rubber production with many workers exposed worldwide. During metabolism of 1,3-DCB, two isomeric mercapturic acids can be formed from ring oxidation of 1,3-DCB in the liver, namely 2,4-dichlorophenylmercapturic acid (24CPhMA) and 3,5-dichlorophenylmercapturic acid (35CPhMA). These urinary mercapturic acids might serve as biomarkers of the toxicologically relevant absorbed dose of 1,3-DCB and have not been determined so far. Thus, we were aimed to develop an analytical method for quantification of these biomarkers.

Authentic standards of both mercapturic acids as well as deuterium-labelled analogues were self-synthesized. A method for the quantification of both CPhMAs in human urine using online-SPE LC/MS/MS was developed and validated with an LOQ of 0.1 ng mL^{-1} for both CPhMAs. The analytes were extracted from urine by online-SPE on a restricted access material phase, transferred to the analytical column and quantified by tandem mass spectrometry. Interday (n = 6) and Intraday (n = 10) precision for both CPhMAs ranged from 1.7 to 4.3 % with accuracies between 99.4 and 109.9 % at concentrations of 0.6 and 3 ng mL^{-1}. We applied the method on post-shift urine samples of 16 workers of the silicone rubber industry with occupational exposure to 1,3-DCB. Both CPhMAs were above LOQ in 15 of 16 urine samples with median levels (range) for 24CPhMA and 35CPhMA of 1.64 ng mL^{-1} (<0.1 – 8.2 ng mL^{-1}) and 3.98 ng mL^{-1} (0.36 – 24.1 ng mL^{-1}), respectively.

This is the first report on specific urinary mercapturic acids of 1,3-DCB in humans. Our results show that ring oxidation of 1,3-DCB is considered to be a toxicologically relevant metabolic pathway in humans. This might improve risk assessment of 1,3-DCB-emissions in silicone rubber industry.

1. Introduction

1,3-dichlorobenzene (1,3-DCB) is a colourless liquid with a boiling point of 173 °C, a comparatively high vapour pressure of 2 hPa (at 20 °C) and a strong odour [1]. Previous studies revealed that 1,3-DCB is formed from thermal decomposition of bis(2,4-dichlorobenzoyl)peroxide (2,4-DCBP), a compound that is used as initiator in the production of silicone rubber [2]. Thus, workers of the silicone rubber industry are exposed to 1,3-DCB to a considerable extent as our previous human biomonitoring study has shown by the determination of the main metabolites in urine [3].

Exposure to 1,3-DCB induced liver and kidney damage in rats accompanied by the induction of xenobiotic-metabolising enzymes of the phenobarbital type [4]. Furthermore, the observed induction of glucuronosyltransferases might lead to a disturbance of the thyroid homeostasis in rats [5]. Unlike its isomer 1,4-dichlorobenzene (1,4-DCB, carcinogen category 4), 1,3-DCB is not considered to be carcinogenic by the Deutsche Forschungsgemeinschaft (DFG), although studies on its genotoxic effect have not yet produced a consistent picture [1,6]. The DFG has evaluated a Threshold Limit Value (MAK) of 2 ppm (=12 mg/m^3) in workplace air to protect workers from potential health effects of 1,3-DCB [6].

Metabolism of 1,3-DCB is characterised by oxidation in the liver by cytochrome *P* 450 enzymes [1]. From the corresponding epoxide the

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main metabolites 3,5-dichlorocatechol as well as 2,4- and 3,5-dichlorophenol are formed, which are excreted via the kidney as glucuronides or sulfates. In a controlled human experiment, we could previously derive biological equivalents for these main metabolites to the MAK value in order to evaluate a Biological Limit Value (BAT) for workers [7].

An alternative metabolic pathway for 1,3-DCB is the conjugation of the epoxide with glutathione, finally leading to the excretion of two isomeric mercapturic acids, 2,4- and 3,5-dichlorophenylmercapturic acid. An overview of the metabolism of 1,3-DCB is given in Fig. 1. The excretion of mercapturic acids of a substance indicates that electrophilic compounds have been formed in the body and that they were able to react with endogenous glutathione [8]. Thus, the excretion of mercapturic acids is regarded as toxicologically relevant absorbed dose, making them very important biomarkers of exposure [9], especially concerning a potential carcinogenic effect of the substance. The extent of this mercapturic acid metabolic pathway of 1,3-DCB in humans is currently unknown. In rat experiments, the mercapturic acids were reported to be the main metabolites of 1,3-DCB [10], while the corresponding mercapturic acids were only minor human metabolites after exposure to the isomer 1,2-dichlorobenzene (1,2-DCB) [11].

Therefore, we were aimed to develop a highly sensitive and specific method based on liquid chromatography tandem mass spectrometry (LC-MS/MS) for the quantification of two isomeric mercapturic acids derived from 1,3-DCB, namely 2,4-dichlorophenylmercapturic acid and 3,5-dichlorophenylmercapturic acid or shortly 24CPhMA and 35CPhMA according to the recently published harmonised naming system for VOC-metabolites [12]. Besides the custom-synthesized mercapturic acids as reference material, we also made use of custom-synthesized D_3 – labelled internal standards in order to achieve accurate results. According to our knowledge, these mercapturic acids have not yet been quantified in human urine.

In a pilot study, we applied the newly developed method to post-shift urine samples of 16 workers (11 m, 5 f) of the silicone rubber industry with exposure to 1,3-DCB, which served as a proof for the successful applicability of this method.

2. Experimental

2.1. Reagents and standards

2,4-dichlorophenylmercapturic acid (N-acetyl-S-2,4-dichlorophenyl-cysteine, 24CPhMA) as well as 3,5-dichlorophenylmercapturic acid (N-acetyl-S-3,5-dichlorophenyl-cysteine, 35CPhMA) were synthesized with an analytical purity > 98 %. Two deuterium-labelled analoga of these

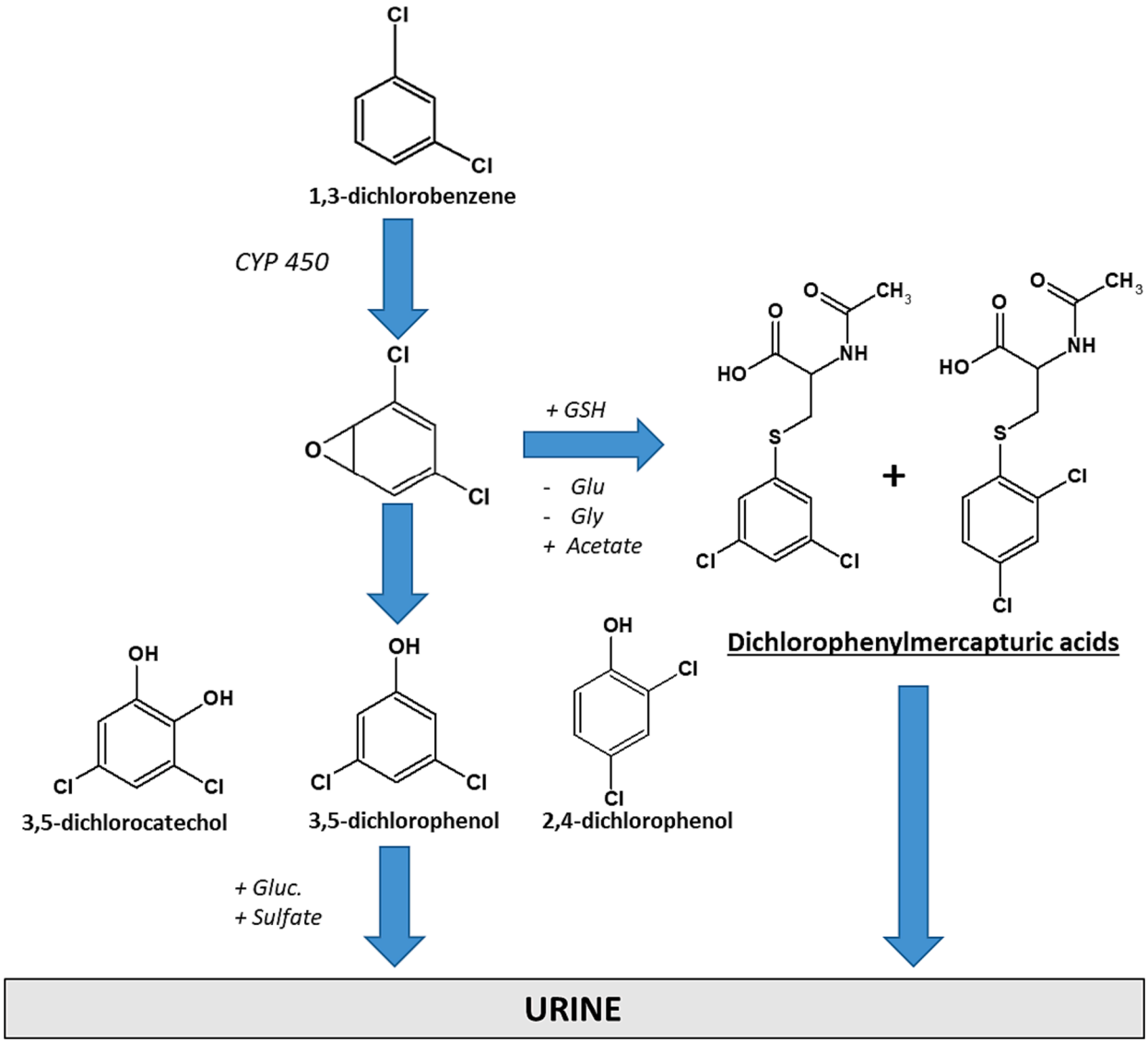

Fig. 1. Simplified metabolism scheme of 1,3-DCB, showing the formation of dichlorophenylmercapturic acids.

mercapturic acids (D_3-labelled at the N-acetyl-moiety) were also prepared from N-([D_3]acetyl)cysteine with a purity of > 98 % and an isotopic purity > 99 %. The identification of the synthesis products was verified and proven by ^{1}H NMR spectra. The purity of the reference substances was controlled by HPLC-UV. Information on the synthesis of standards and the complete characterization of the standards is available in the supplemental files (Fig. S1-8).

Formic acid (100 %), hydrochloric acid (fuming, 37 %) and water for chromatography (LiChrosolv®) were supplied by Merck (Darmstadt, Germany). Acetonitrile (LC/MS grade) and methanol (LC/MS grade) was purchased from J.T. Baker (Germany). Ammonium formate (>99 %) was purchased from Fluka (Buchs, Switzerland).

2.2. Standard preparation

Two stock solutions were prepared by dissolving 10 mg of 24CPhMA and 35CPhMA each in 10 mL of methanol (1 mg mL^{-1}). 10 μL of these stock solutions each were placed in a 10 mL volumetric flask and diluted to the mark with methanol to form the spiking solution 1 of CPhMAs (1 μg mL^{-1}). 1000 μL of this spiking solution 1 were then placed in a 10 mL volumetric flask and diluted to the mark with methanol to form the spiking solution 2 (100 ng mL^{-1}).

Two stock solutions of the internal standards were prepared by dissolving 10 mg of D_3-24CPhMA and D_3-35CPhMA each in 10 mL of methanol (1 mg mL^{-1}). 10 μL-of these stock solutions each were transferred in a 10-mL glass volumetric flask and diluted to the mark with 0.1 % aqueous formic acid to form the combined working solution of the internal standards (1 μg mL^{-1} of D_3-CPhMAs). All solutions were stored at – 20 °C in Teflon-capped brown glass vials or bottles.

2.3. Sample preparation

Frozen urine samples were allowed to equilibrate to room temperature. The samples were vigorously shaken and 500-μL aliquots were then transferred to 1.8-mL glass screw-cap vials. Then, 10 μL of concentrated hydrochloric acid (fuming, 37 %) were added to the samples and they were left at room temperature for 3 h for hydrolysis of potential premercapturic acids. 10 μL of the working solution of the internal standards (D_3-CPhMAs, 1 μg mL^{-1}) were added to the samples and 500 μL of 0.1 M ammonium formate buffer (pH 2.5). The samples were vortex mixed and centrifuged at 1500 *g* for 10 min. When necessary (due to protein precipitation), the supernatant was transferred to a new 1.8-mL glass screw-cap vial. A 100-μL aliquot was then injected into the LC-MS/MS system for quantitative analysis. Urinary creatinine concentrations were determined photometrically according to Larsen using a 96-well-plate photometer [13].

2.4. Calibration procedure and quality control

From the two spiking solutions of CPhMAs, we prepared 7 calibration standards by spiking pooled urine (creatinine: 0.61 mg mL^{-1}) of laboratory personnel with CPhMA-concentrations ranging between 0.2 ng mL^{-1} – 20 ng mL^{-1}. The pooled urine was used as a blank. These standards were aliquoted to 500 μL in 1.8-mL glass screw-cap vials, stored at – 20 °C and used for calibration. Additionally, a blank value consisting of water was included in every analytical series. The standards were processed as described above. Calibration curves were obtained by plotting the quotients of the peak areas of the analytes to the peak areas of its corresponding labelled internal standard versus the concentrations spiked. These graphs were used to ascertain the unknown concentrations of CPhMAs in urine samples. The calibration graphs were linear in the ranges described for calibration with correlation coefficients higher than $r = 0.998$. CPhMAs were below LOD in the water blank and unspiked pooled urine used for the calibration curves. A chromatogram of the blank value and blank pooled urine is shown in the Supplemental Files to this manuscript (Figs. S 11 and S 12).

As quality control material we spiked a spot urine sample of a non-smoker (creatinine: 0.85 mg mL^{-1}) with concentrations of 0.6 and 3 ng mL^{-1} of CPhMAs, respectively. There was also no background excretion of CPhMAs detectable in this spot urine sample. The prepared quality control material was also divided into aliquots and stored at – 20 °C. For quality assurance, both quality control samples were included in each analytical series.

Within-day repeatability was determined by the analysis of both quality control urines six times in a row. Between-day repeatability was determined by analysing the quality control urines on ten different days. In order to determine potential matrix effects and accuracy we used seven spiked individual urine samples with creatinine contents ranging from 0.46 to 2.96 mg mL^{-1}. Spiked specimens (spiked concentration: 2 ng mL^{-1} CPhMAs) were analysed before and after the addition of CPhMAs and accuracy was calculated by the difference vs. spiked concentration. To further investigate the influence of matrix on the result, we also prepared aqueous calibration standards, processed these standards together with the urinary calibration standards and compared the slopes and linearity of both calibrations.

2.5. LC-ESI-MS/MS analysis

We performed liquid chromatography on a system consisting of two Agilent 1260 Infinity II Series HPLC pumps (G7112B) with integrated degasser and an Agilent 1260 Autosampler (G7167A) connected to a six-port-valve (Valco Systems, Houston, Texas, USA). All steps of the system were controlled by Analyst 1.7 Software from AB Sciex.

100 μL of the processed sample were injected onto a restricted access material (RAM) phase, a LiChrospher® RP-18 ADS (25 μm) 24 x 4 mm RAM from Merck (Darmstadt, Germany) using a mixture of water (adjusted to pH 2.5 with formic acid, Eluent A) and 0.1 % formic acid in acetonitrile (Eluent B) as the mobile phase and a flow rate of 0.4 mL/min. After this clean-up and enrichment step, the analytes were transferred to a reversed-phase HPLC column (Luna C 18 (2) 150 x 4.6 mm, 3 μm particle size from Phenomenex, Aschaffenburg, Germany) by the six-port-valve in backflush mode. Chromatography of CPhMAs was performed using water (adjusted to pH 2.5 with formic acid) as mobile phase A and 0.1 % formic acid in methanol as solvent C at a constant flow rate of 0.4 mL/min. The programmed gradient for both HPLC pumps and the switching times of the six-port-valve are described in Table 1. We used a precolumn filter (0.5 μm, Supelco) and a guard column (Luna C 18 (2), 4 × 3 mm, Phenomenex) in front of the analytical column to extend its lifespan. The temperature of the analytical column was kept at 35 °C using a column thermostat.

The tandem mass spectrometric detection was performed on a Sciex API 4500 LC-MS/MS system (Sciex, Darmstadt, Germany) in ESI-negative mode. We used an electrospray needle voltage of – 4500 V and nitrogen as nebulizer and turbo heater gas (500 °C) was set to a pressure of 65 psi. Nitrogen as curtain gas and collision gas was set to 40 instrument units and 10 instrument units, respectively. The mass spectrometric conditions were optimised using continuous flow injection of standards and internal standards (10 ng mL^{-1} in methanol) using the syringe pump of the instrument. Retention time (RT), collision energies (CE) and collision cell exit potentials (CXP) for the analytes and internal standards are summarized in Table 2. The declustering and entrance potentials for all analytes were − 50 and −10 V, respectively. Temperature of the ion source and needle voltage was optimised by injecting an aqueous standard (10 ng mL^{-1}) twice using the final method under different conditions (ion source temperature: 350 – 600 °C; needle voltage: − 3000 - −4500 V). The mass spectrometer operated in the scheduled multiple-reaction-mode (sMRM) with a “unit” resolution of Q1 and Q3, a target cycle time across sMRMs of 1 s and a pause between mass ranges of 5 ms. The six-port valve of the instrument was used to control the flow in the mass spectrometer, so that only the fraction of interest (14.3 – 16 min, cf. Table 2) from the LC-system is transferred to the mass spectrometer to avoid unnecessary contamination.

Table 1
Analysis program of the gradient pumps and switching times of the six-port-valve.

Time (min)	Valve position	Pump 1			Pump 2			Analysis step
		Eluent A (%)	Eluent B (%)	Flow-rate (ml/min)	Eluent A (%)	Eluent C (%)	Flow-rate (ml/min)	
0	A	80	20	0.4	30	70	0.4	*clean-up*
5	B	80	20	0.4	30	70	0.4	*Transfer*
6.5	A	80	20	0.4	30	70	0.4	*Separation and washing of RAM C 18*
7	A	80	20	0.4	30	70	0.4	
10	A	0	100	0.4	0	100	0.4	
15	A	0	100	0.4	0	100	0.4	
16	A	80	20	0.4	30	70	0.4	*Reconditioning*
22	A	80	20	0.4	30	70	0.4	

Eluent A: water, adjusted to pH 2.5 with formic acid; Eluent B: 0.1% formic acid in acetonitrile; Eluent C: 0.1 % formic acid in methanol.

Table 2
Retention times and MRM-parameters for the selected parent and daughter ion combinations of the analytes.

Analyte	Retention time (min)	Precursor ion (Q 1)	Fragment ion (Q 3)	DP	CE	CXP
24CPhMA $Cl^{35}Cl^{35}$	14.86	305.7	176.8	−50	– 16	– 11
24CPhMA $Cl^{35}Cl^{37}$	14.86	307.8	178.8	−50	– 16	– 13
D_3-24CPhMA $Cl^{35}Cl^{37}$	14.84	310.8	178.8	−50	– 18	– 13
35CPhMA $Cl^{35}Cl^{35}$	15.37	305.7	176.8	−50	– 16	– 11
35CPhMA $Cl^{35}Cl^{37}$	15.37	307.8	178.8	−50	– 16	– 13
D_3-35CPhMA $Cl^{35}Cl^{37}$	15.36	310.8	178.8	−50	– 18	– 13

DP, declustering potential (V); CE, collision energy (V); CXP, collision exit potential (V).

2.6. Study subjects

For our pilot study we investigated post-shift spot urine samples obtained from 16 workers (11 m, 5f) of the silicone industry with confirmed exposure to 1,3-DCB that were previously investigated for 3,5-dichlorocatechol (3,5-DCC) and 2,4- and 3,5-dichlorophenol (2,4-DCP and 3,5-DCP) as main urinary metabolites of 1,3-DCB using LC-MS/MS [3]. All urine samples were collected anonymously and stored at –20 °C until analysis. The age of the persons ranged from 22 to 62 years (median: 36 years). Creatinine content of the urine samples ranged from 0.22 mg mL^{-1} – 2.81 mg mL^{-1} (median: 1.30 mg mL^{-1}). The collection of urine samples for this study was approved by the Ethics Committee of the University hospital of the RWTH Aachen (EK 111/20).

3. Results

3.1. Optimization of the method

This method is based on previously published methods of our working group on the determination of urinary mercapturic acids [14,15] and was adapted with regard to solvent composition and switching times to guarantee a maximum of clean-up on the RAM-phase as well as good sensitivity for the CPhMAs. With the announcement of discontinuation of the online-RAM-phases by Merck a few years ago, our group has secured a stock of several units of these unique and robust columns that are perfectly suited for bioanalytical purposes due to its ability to exclude macromolecules (>15 kDa) combined with its reversed-phase mechanism. The RAM-C18 phase we used allowed good retention of the analytes and tolerated a solvent strength of 20 % acetonitrile for efficient clean-up from urinary matrix for 5 min at a flow rate of 0.4 mL/min. After transfer and separation on the analytical column, the tandem mass spectrometric determination of both CPhMAs was free of chromatographic interferences, even in high-creatinine urine samples.

The analytical column chosen (Phenomenex C18(2), 150 x 4.6 mm, 3 μm, 100 Å) showed the best performance in combination with the column-switching technique and provided sharp peaks of both CPhMAs and internal standards as well as a good resolution. Other columns used during method development (e.g. Phenomenex Luna C8(2) (150 x 4.6 mm, 3 μm, 100 A; Phenomenex Kinetex C8 (150 x 4.6 mm, 2.6 μm, 100 A) or Phenomenex Kinetex F5 (150 x 4.6 mm, 2.6 μm, 100 A)) showed a worse performance either regarding peak shape or sufficient resolution of both isomers. The use of large diameter-columns is necessary in order to avoid overloading of the column during transfer of the analytes to the analytical columns. Moreover, the high backpressure of small-diameter columns might damage the RAM-phases during transfer that have an upper pressure limit of ~ 250 bar.

After the transfer, the analytes are first focussed on the analytical column for approximately 2 min (from 5 to 7 min, see Table 1) before the gradient starts. Subsequent to the washing of the analytical column as well as the RAM-phase and the reconditioning, a whole chromatographic run lasts no longer than 22 min total and illustrates the potential of automated online-SPE as shown before [16–20]. Fig. 2 shows a chromatogram of the processed urine of a person with exposure to 1,3-DCB (see *2.6 Study subjects*).

The source-specific parameters for tandem-mass spectrometric detection were optimised manually. Source temperature was optimised by repeated processing of an aqueous standard of CPhMAs (10 ng mL^{-1}) twice at different temperature settings in steps of 50 °C starting from 300 °C to 600 °C. The instrument settings for curtain gas and collision gas were also optimised manually (in steps of 2 instrument settings) using repeated processings of this aqueous standard at the optimised source temperature. Furthermore, needle voltage was also optimised in steps of −500 V from – 3000 V to −4500 V.

The selected precursor ions at the first quadrupole for both CPhMAs and their deuterium-labelled analogues was [M−H]. For quantification, we used the transition for the chlorine isotopes Cl^{35} Cl^{35} and we used the transition for $Cl^{35}Cl^{37}$ as qualifier. The product ion fragments consist of the corresponding specific dichlorothioyl moieties with no further fragmentation ions. Exemplarily, the product ion mass spectra for 24CPhMA and D_3-CPhMA together with the structures of the fragments for the transition are shown in Fig. 3.

Analyte peaks were confirmed both by the specific precursor-product ion combinations and the ratio of qualifier and quantifier as well as by matching the retention time with the labelled internal standard. No unlabelled isotope fragments were observed in the labelled standards at the spiked concentration.

The limit of detection for CPhMAs was determined according to DIN 32 645 using a calibration curve prepared in spot urine (creatinine: 0.52 mg mL^{-1}). To do so, ten equidistant calibration points between 0.1 and 1 ng mL^{-1} were prepared and processed in triplicate. The error of the axis intercept as proposed by DIN 32 645 revealed an LOD of app. 0.03

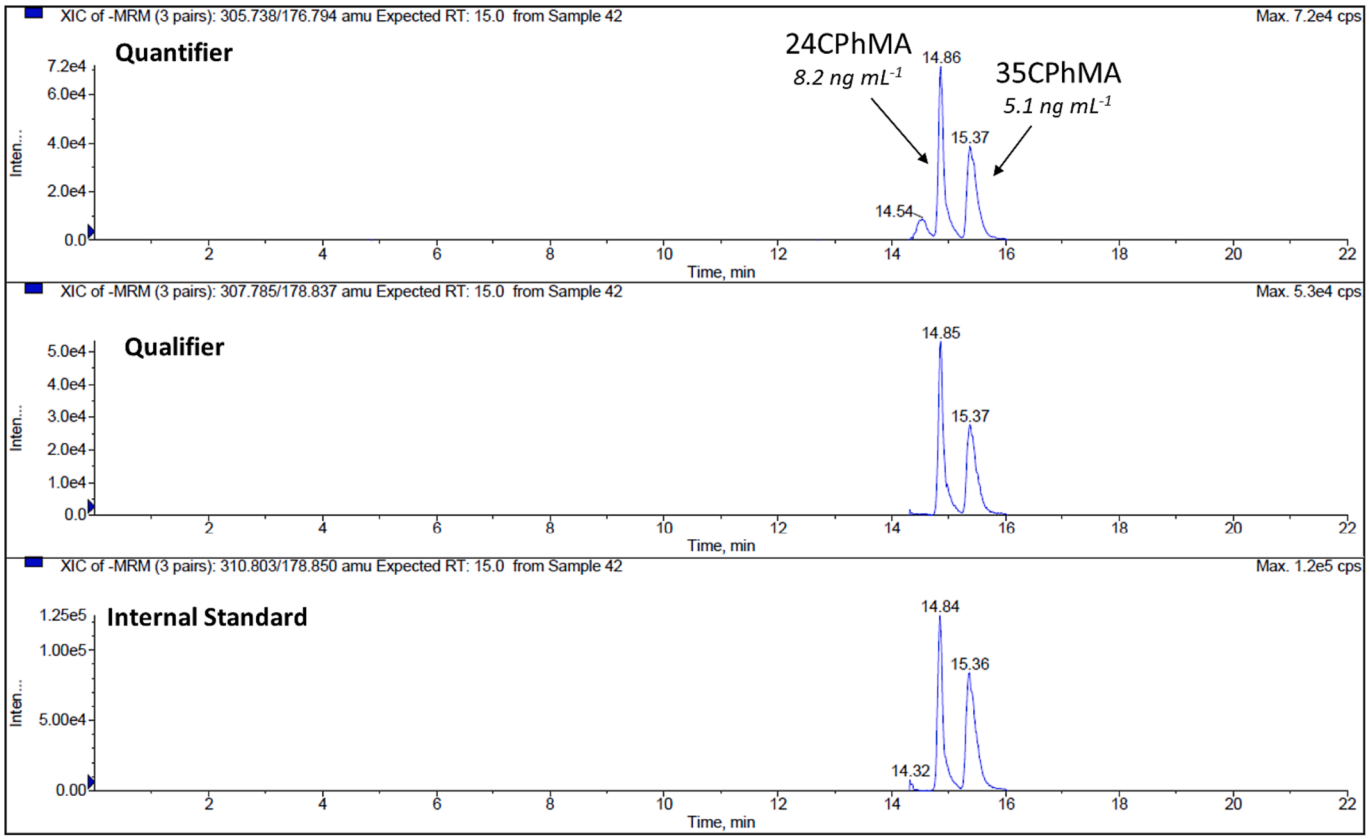

Fig. 2. LC/MS/MS-chromatogram of a processed urine sample of a worker (33 yrs, m) with a creatinine content of 1.12 mg mL^{-1} and concentrations of 8.2 and 5.1 ng mL^{-1} for 24CPhMA and 35CPhMA, respectively.

ng mL^{-1} for both analytes. The limit of quantification (LOQ) for the analytes was defined to be 3 times the LOD (0.1 ng mL^{-1}). The corresponding calibration curves can be found in the supplements to this manuscript (Figure S9).

3.2. Pre-treatment of urine samples

Previous reports on the determination of aromatic mercapturic acids like S-phenylmercapturic acid (PhMA) describe the presence of a pre-mercapturic acid that is converted to the mercapturic acid by acidification of the urine [21]. Consequently, the Deutsche Forschungsgemeinschaft recommends the addition of 1 vol% concentrated hydrochloric acid (37 %) after sample collection for a biomonitoring of benzene exposure to ensure a pH < 1 to convert the pre-mercapturic acid to PhMA [22]. Thus, we evaluated the pre-treatment of urine samples in the validation of our method by analysing two urine samples of persons exposed to 1,3-DCB (see 2.6 Study subjects) using different pre-treatments. 500 µL of these samples were analysed untreated (without addition of acid), after addition of 500 µL 100 mM ammonium formate buffer (pH 2.5) and after addition of 10 µL concentrated hydrochloric acid (37 %, fuming) for 1 h, 2 h and 4 h at room temperature. Finally both samples were also analysed after addition of 10 µL concentrated hydrochloric acid (37 %, fuming) and heating to 80 °C for 4 h. The results of this experiment are shown in Fig. 4.

A lowering of pH increased the CPhMA-concentration remarkably only in sample 1 with an increase of untreated < buffer pH 2.5 < hydrochloric acid. However, the concentrations do not markedly increase between 1 and 4 h of HCl-treatment. In sample 2, the lowering of pH does not have a significant effect, indicating obviously no presence of pre-mercapturic acids.

Interestingly, the heating of the acidified samples markedly increased the concentration only for 24CPhMA in both samples by about 50 %. In previous reports [23], extremely strong hydrolysis conditions (e.g. concentrated sulphuric acid) also led to an excessive increase of PhMA-concentrations in urine samples of benzene-exposed workers with unclear origin, thus suggesting a pre-treatment of urine samples to pH < 1 as advisable condition [21,22]. Therefore, we decided to add 10 µL of concentrated hydrochloric acid (37 %, fuming) for 3 h at room temperature as best compromise in accordance with the recommendations of the DFG [22].

3.3. Reliability of the method

We determined the repeatability of our method using a spontaneous urine sample of a person of the general population (creatinine: 0.85 mg mL^{-1}) spiked with CPhMA concentrations of 0.6 (Q_{06}) and 3 ng mL^{-1} urine (Q_3), respectively. These concentrations were chosen to reflect the expected concentrations in exposed persons in order to give a realistic insight in method performance under real conditions. For within-day repeatability, these quality control samples were analysed six times in a row with relative standard deviations ranging between 1.8 and 3.2 % and accuracies between 99.4 and 109.9 %. Between-day repeatability was determined analysing this material in ten different batches and ranged between 1.7 and 4.3 % with an accuracy of 100.1 – 102 %, demonstrating very good repeatability of our method. These data are summarised in Table 3.

To give deeper insights into the performance of our method under real-world conditions, we have determined accuracy in seven different urine specimens with varying creatinine content (0.46 – 2.96 mg mL^{-1}) reflecting a wide range of urine compositions. These urine samples were analysed unspiked and spiked a concentration of 2 ng mL^{-1} of both CPhMAs. The results of this experiment are summarised in Table 4. No CPhMAs were determined above LOD in the unspiked urine samples. Accuracy in the different spiked urinary specimen ranged from 101.2 to

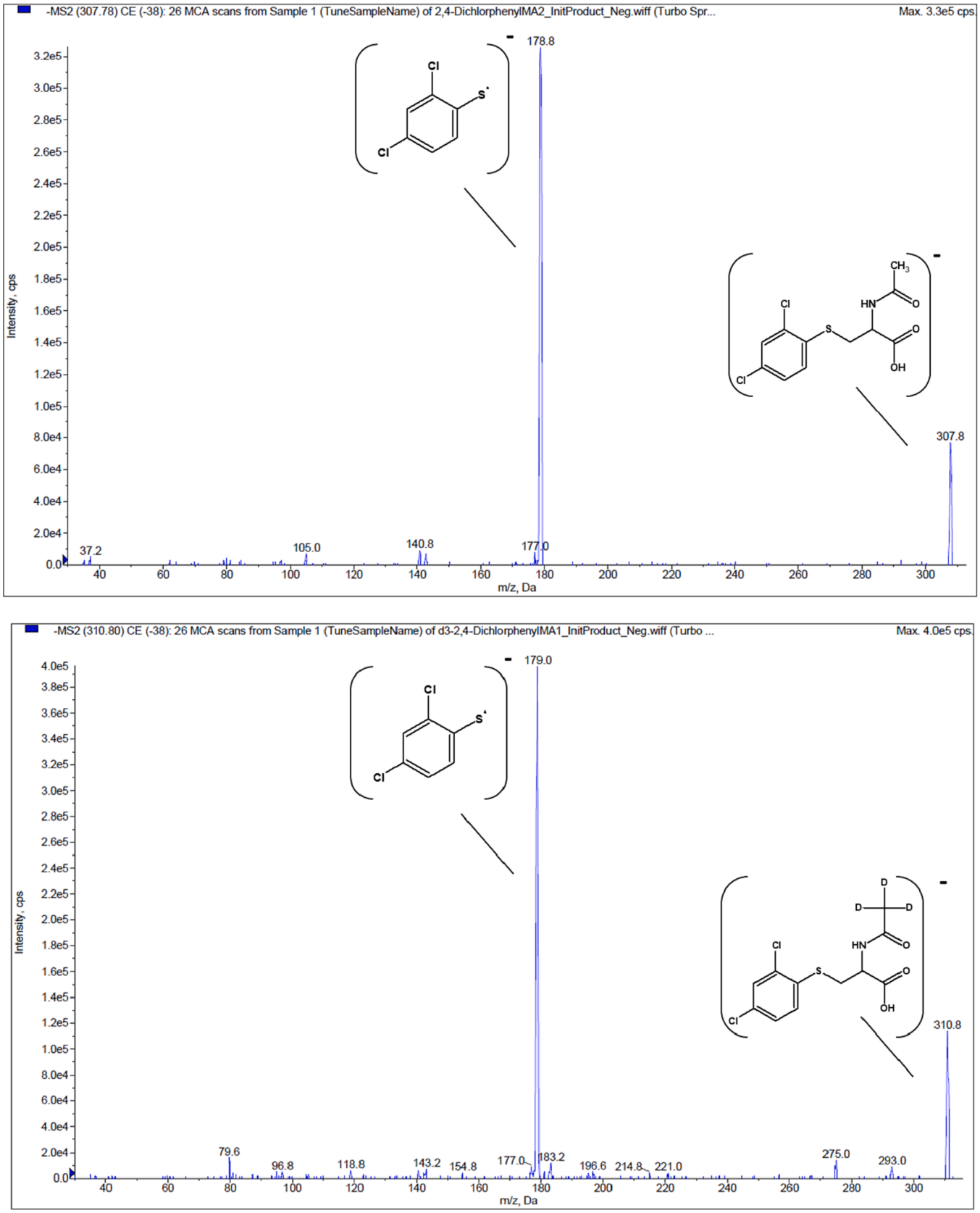

Fig. 3. ESI-negative product-ion mass spectra of 24CPhMA and D_3-24CPhMA with the predicted structures of the fragments. Note that the masses are shown for the $Cl^{35}Cl^{37}$-isotopes of both compounds.

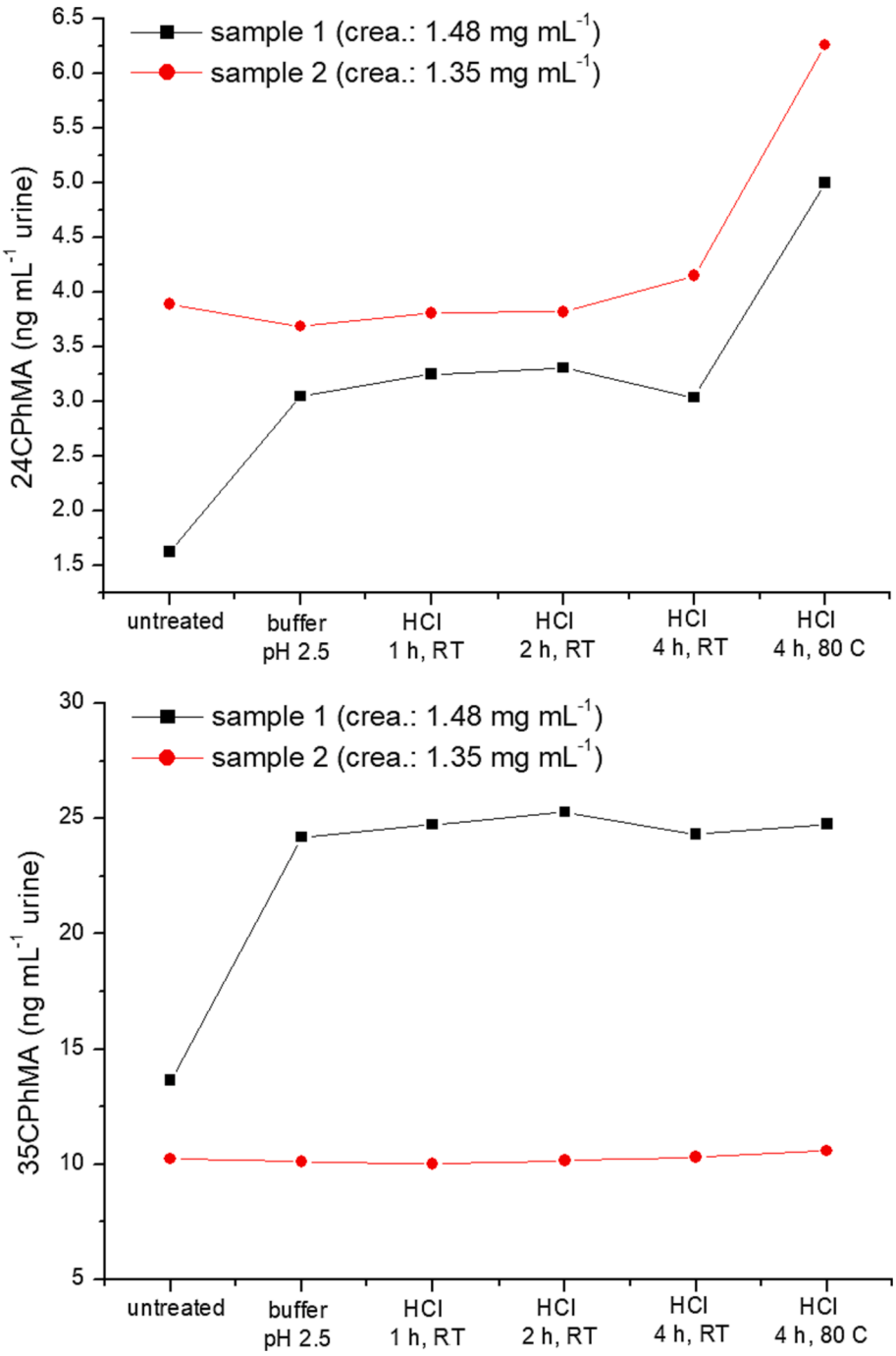

Fig. 4. Effect of different pre-treatment methods on concentrations of CPhMAs in two urine samples of persons exposed to 1,3-DCB.

Table 3
Data for precision and accuracy for the determination of CPhMAs in urine as well as stability data.

Analyte		Q_{06}		Q_3		Short-term stability at room temperature (n = 3)		Freeze-thaw-samples (n = 3)	
		Intradays (n = 6)	Interdays (n = 10)	Intradays (n = 6)	Interdays (n = 10)	Q_{06}	Q_3	Q_{06}	Q_3
24CPhMA	c (ng mL^{-1} urine)	0.60	0.60	3.07	3.04	0.58	3.05	0.62	3.09
	RSD (%)	1.8	4.3	1.9	1.7	2.8	3.7	1.7	1.6
	Accuracy (%)	99.4	100.7	102.3	100.6	96.7	101.7	103.1	103.0
35CPhMA	c (ng mL^{-1} urine)	0.66	0.61	3.02	3.00	0.61	3.08	0.63	3.13
	RSD (%)	3.2	3.2	1.9	2.6	3.1	1.5	1.8	0.3
	Accuracy (%)	109.9	102.0	101.2	100.1	102.1	102.7	105.5	104.3

Table 4
Accuracy experiment for the determination of CPhMAs in seven different urine samples and effect of urinary matrix on signal intensity.

Urine	Creatinine (mg mL^{-1})	Analyte	Blank value (ng mL^{-1})	Spiked conc. (ng mL^{-1})	Conc. Found (ng mL^{-1})	Accuracy (%)	effect on signal (%)
1	0.46	24CPhMA	< 0.2	2	2.05	102.6	− 1
		35CPhMA	< 0.2	2	2.03	101.6	− 6
2	1.05	24CPhMA	< 0.2	2	2.06	103.1	− 1
		35CPhMA	< 0.2	2	2.13	106.7	− 13
3	1.30	24CPhMA	< 0.2	2	2.07	103.6	− 34
		35CPhMA	< 0.2	2	2.10	104.9	− 32
4	1.70	24CPhMA	< 0.2	2	2.09	104.4	− 39
		35CPhMA	< 0.2	2	2.03	101.4	− 44
5	2.08	24CPhMA	< 0.2	2	2.02	101.2	− 62
		35CPhMA	< 0.2	2	2.09	104.7	− 33
6	2.41	24CPhMA	< 0.2	2	2.13	106.6	− 41
		35CPhMA	< 0.2	2	2.05	102.3	− 51
7	2.96	24CPhMA	< 0.2	2	2.05	102.4	− 59
		35CPhMA	< 0.2	2	2.13	106.4	− 57

106.7 % (mean: 103.4 % for 24CPhMA and 104.0 % for 35CPhMA). We also used these data to evaluate the effect of urinary matrix on signal intensity by comparing the mean peak areas of the internal standards in the different urine samples with the mean peak areas of the internal standards in an aqueous blank sample analysed in triplicate. Signal quenching was clearly influenced by urine composition as reflected by the creatinine content and can reach up to 62 % in some urine samples (mean: 34 % for both analytes). This quenching effect was not always equally distributed for both analytes and illustrates the need for individual isotopically labelled internal standards. As shown by the good accuracy under these conditions, the internal standards are able to efficiently compensate these effects in the mass spectrometer. Likewise, a comparison of aqueous with urinary calibrations shows identical slopes for both calibration curves, indicating that urinary matrix does not have an effect on the result. The comparison of aqueous and urinary calibration is shown in the Supplemental File to this manuscript (Figure S10).

3.4. Stability of CPhMAs

To investigate the stability of the CPhMAs, we subjected three samples of both quality control materials to three freeze–thaw cycles on three different days and analysed them afterwards as described. Furthermore, three samples of the quality controls were left at room temperature for 24 h and analysed thereafter to investigate the short-term stability. These results were compared to the results of the within-day and between-day repeatability and show no significant deviation from these values, proving sufficient stability of the analytes under real-world conditions. The frozen quality control samples were stable over 12 months.The results of these experiments are also included in Table 3.

3.5. Results of biological monitoring

The results of human biomonitoring for CPhMAs in urine of the 16 workers of the silicone rubber industry with exposure to 1,3-DCB are summarised in Table 5. The results for the main urinary metabolites of 1,3-DCB (3,5-dichlorocatechol (35DCC), 2,4-dichlorophenol (24DCP) and 3,5-dichlorophenol (3,5DCP), see Fig. 1) in these samples using our previously described LC-MS/MS-method [3] are also included in this Table for overview.

24CPhMA could be quantified in 15 of the 16 investigated urine samples (93.8 %), whereas 35CPhMA was quantified in all urines of the workers, illustrating the good applicability of our method for human biomonitoring of workers exposed to 1,3-DCB. Overall, 35CPhMA was excreted in significantly higher amounts compared to 24CPhMA with a median of 1.64 vs. 3.98 ng mL^{-1}, respectively.

Table 5
Results of biological monitoring of CPhMAs in urine of 16 workers with occupational exposure to 1,3-DCB. For comparison, the levels of the main urinary metabolites of 1,3-DCB are also included in the table (see Fig. 1).

	24CPhMA (ng mL^{-1} urine) [ng mg^{-1} creatinine]	**35CPhMA** (ng mL^{-1} urine) [ng mg^{-1} creatinine]	**35DCC** (ng mL^{-1} urine) [ng mg^{-1} creatinine]	**24DCP** (ng mL^{-1} urine) [ng mg^{-1} creatinine]	**35DCP** (ng mL^{-1} urine) [ng mg^{-1} creatinine]
n > LOQ	15	16	16	16	16
Median	1.64 [1.18]	3.98 [3.83]	3,530 [3,349]	1,117 [1,137]	68 [64]
Min.	< 0.1 [< 0.1]	0.36 [0.28]	30 [18]	101 [72]	5.8 [3.6]
Max.	8.2 [16.1]	24.1 [22.6]	22,914 [24,577]	10,124 [10,432]	499 [466]

35DCC: 3,5-dichlorocatechol; 24DCP: 2,4-dichlorophenol; 35DCP: 3,5-dichlorophenol.

As visible from Table 4, CPhMAs are only excreted at levels approximately 3 orders of magnitude lower compared to the main metabolites 35DCC and 24DCP. In contrast to the good correlations between the main metabolites, the individual levels of both mercapturic acids do not correlate well, as some persons excrete higher levels of 24CPhMA, while others showed higher levels of 35CPhMA. Furthermore, while the levels of 35CPhMA correlate very well with its phenolic analogue 35DCP, the relationship between the levels of 24CPhMA and its phenolic counterpart 24DCP is only moderate. The reason for these variances is currently unclear, different urinary kinetics of the CPhMAs as well as an influence of potential polymorphisms in GSH-transferases can be speculated as explanation. Fig. 5 shows the relationship between the CPhMAs and the discussed urinary metabolites of 1,3-DCB in these persons.

4. Discussion

We have developed a reliable, accurate and automated procedure for the determination of two isomeric mercapturic acids (24CPhMA and 35CPhMA) as urinary biomarkers of the toxicologically relevant absorbed dose of 1,3-DCB that is formed during decomposition of an initiator used in the production of silicone rubber. Authentic analytical standards and labelled internal standards were custom synthesized and fully characterised.

The applied on-line clean-up using a Restricted-Access-Material (RAM) phase based on alkyl-diol-silica turned out to be very effective, allowing us to achieve a LOQ of both CPhMAs down to levels of 0.1 ng mL^{-1} urine with a mean quenching effect on the signal in individual urinary matrices of 34 %. The whole system is fully automated and needs

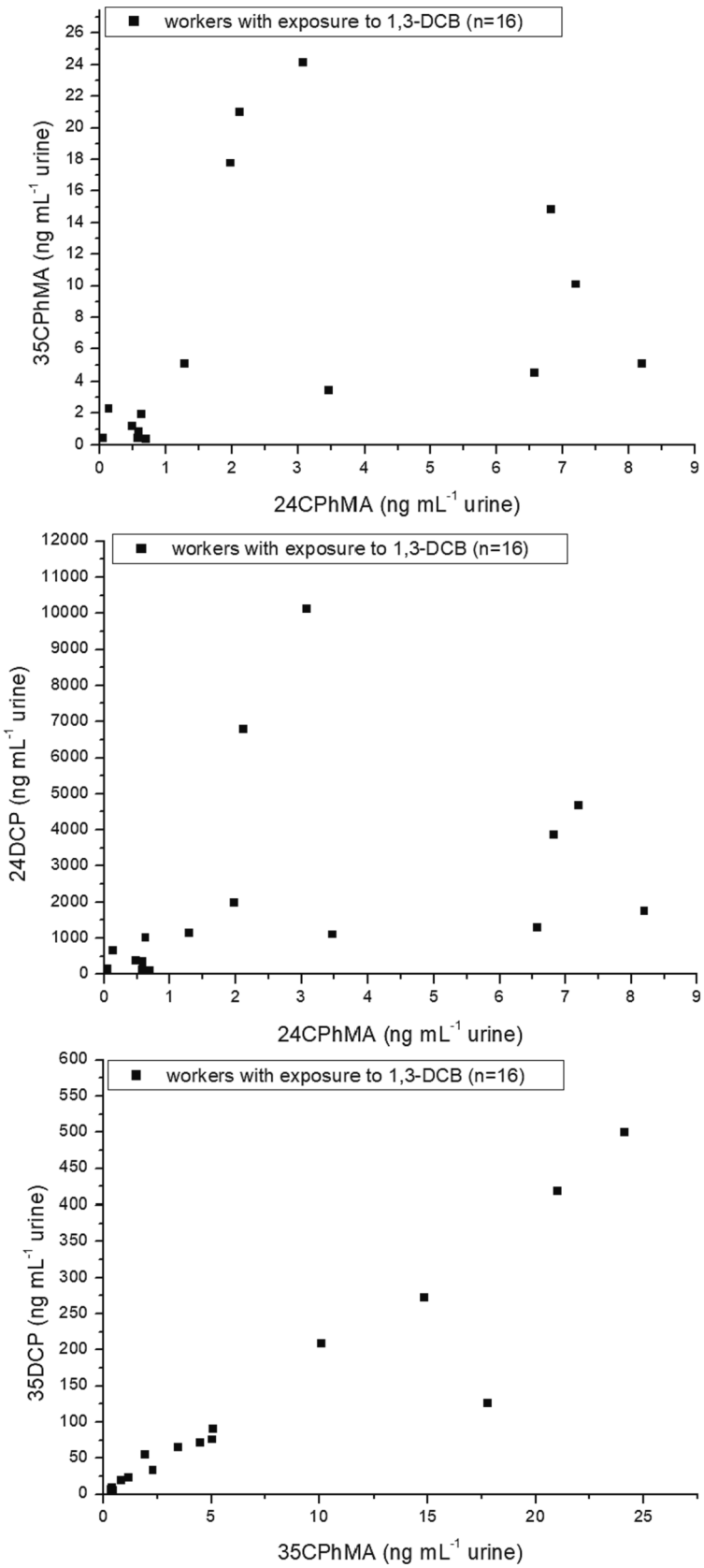

Fig. 5. Relationship between the two isomeric CPhMAs in urine samples of workers with occupational exposure to 1,3-DCB (n = 16) as well as CPhMAs and the phenolic main metabolites of 1,3-DCB.

minimal manual sample preparation. The total run time of 22 min might allow analysis of more than 50 urine samples per day including calibration, blank and quality controls. Provided that appropriate analytical standards are available (or synthesized), the present method can easily be transferred to the quantification of further urinary dichlorophenyl mercapturic acids of the occupationally relevant isomers 1,4- and 1,2-dichlorobenzene [11].

Our method was validated according to the FDA guidelines for bioanalytical method validation [24] and achieved excellent figures of merit with respect to precision and accuracy. We have taken great care to validate the method under most realistic conditions concerning the choice of concentrations applied. Pre-diagnostic treatment of urine samples was evaluated using native samples and harmonised with respect to previous recommendations on acidification of urine samples for the determination of mercapturic acids.

The achieved LOQ of 0.1 ng mL^{-1} for both analytes turned out to be sufficient for human biomonitoring of workers with exposure to 1,3-DCB, as both CPhMAs were quantifiable in 15 of 16 urine samples of exposed workers from the silicone industry. It turned out that the mercapturic acids of 1,3-DCB are only a small fraction of its total urinary metabolites with levels 3 orders of magnitude lower compared to the main metabolites. This is in line with various other mercapturic acids of aromatic compounds, e.g. benzene, where urinary PhMA also represents only 0.11 % of the inhaled fraction [25] or styrene, where ring-oxidised mercapturic acids were found to represent only 3.5 x 10^{-4} % of the absorbed dose of styrene in humans [26]. Regarding the variable relationship found between both CPhMAs in urine of exposed workers and the observed correlations between the other main metabolites in urine, further research regarding excretion kinetics as well as the potential influence of polymorphisms on CPhMA-formation is needed.

5. Conclusion

The method presented here is to our knowledge the first method published so far to quantify the mercapturic acids of 1,3-DCB in human urine. We attached great importance on the thorough validation of our method according to international guidelines.

Due to its automation and high sensitivity, our method is perfectly suited for the determination of CPhMAs as biomarkers of the toxicologically relevant absorbed dose of 1,3-DCB in occupational studies, as proven by the application of our method to urine samples of workers of the silicone rubber industry with occupational exposure to 1,3-DCB. Despite the very low metabolic share of CPhMAs, their formation clearly indicates that ring oxidation is a toxicologically relevant metabolic pathway. Future additional studies on occupationally exposed workers as well as experimentally exposed volunteers will provide further insights into the formation and excretion of CPhMAs as biomarkers of 1,3-DCB.

CRediT authorship contribution statement

T. Schettgen: . **V. Belov:** Formal analysis, Investigation, Methodology. **T. Kraus:** Conceptualization, Project administration, Supervision. **P. Ziegler:** Funding acquisition, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2024.124034.

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