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Fast determination of urinary *S*-phenylmercapturic acid (*S*-PMA) and *S*-benzylmercapturic acid (*S*-BMA) by column-switching liquid chromatography–tandem mass spectrometry

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Abstract

Benzene and toluene are important industrial chemicals and ubiquitous environmental pollutants. The urinary mercapturic acids of benzene and toluene, *S*-phenylmercapturic acid (*S*-PMA) and *S*-benzylmercapturic acids (*S*-BMA) are specific biomarkers for the determination of low-level exposures. We have developed and validated a fast, specific and very sensitive method for the simultaneous determination of *S*-PMA and *S*-BMA in human urine using an automated multidimensional LC–MS–MS-method that requires no additional sample preparation. Analytes are stripped from urinary matrix by online extraction on a restricted access material, transferred to the analytical column and subsequently determined by tandem mass spectrometry using isotopically labelled *S*-PMA as internal standard. The lower limit of quantification (LLOQ) for both analytes was 0.05 μg/L urine and sufficient to quantify the background exposure of the general population. Precision within series and between series for *S*-PMA and *S*-BMA ranged from 1.0% to 12.2%, accuracy was 108% and 100%, respectively. We applied the method on spot urine samples of 30 subjects of the general population with no known exposure to benzene or toluene. Median levels (range) for *S*-PMA and *S*-BMA in non-smokers ($n = 15$) were 0.14 μg/L (<0.05–0.26 μg/L) and 8.2 (1.6–77.4 μg/L), respectively. In smokers ($n = 15$), median levels for *S*-PMA and *S*-BMA were 1.22 μg/L (0.17–5.75 μg/L) and 11.5 μg/L (0.9–51.2 μg/L), respectively. Due to its automation, our method is well suited for application in large environmental studies.

Keywords: Biological monitoring; Benzene; Toluene; Mercapturic acids; Environmental exposure

1. Introduction

Benzene and toluene are important industrial solvents and natural components of petroleum and gasoline. Toluene is also used in many commercial products such as paints, varnishes and adhesives. Benzene is well known for its carcinogenic properties and is classified as a group 1 carcinogen by both the Deutsche Forschungsgemeinschaft (DFG) [1], and the International Agency for Research on Cancer (IARC) [2]. Exposure to toluene has several effects on the central nervous system [3–5] and has been reported to have effects on reproductive outcome in humans [6,7]. The DFG has noted that both substances are able to penetrate the skin, therefore, biological monitoring is an indispensable tool for protecting the health of workers handling these compounds [1].

Besides possible exposure at the workplace, benzene and toluene are also ubiquitous environmental pollutants and have long been identified as components of tobacco smoke [8–10]. Thus, industrial emissions and traffic exhaust originating from the burning of fossil fuels as well as personal smoking habits or exposure to environmental tobacco smoke (ETS) contribute to the exposure of the general population. In German households, indoor air levels for benzene have been reported to range from 2.2 to 5.9 μg/m^3, while the indoor air levels of toluene were 10-fold higher, ranging from 22.6 to 61 μg/m^3 [11].

Consequently, the determination of the internal exposure of humans to the known carcinogen benzene as well as toluene has been an important issue for several decades, both in occupational and environmental medicine. For biological monitoring

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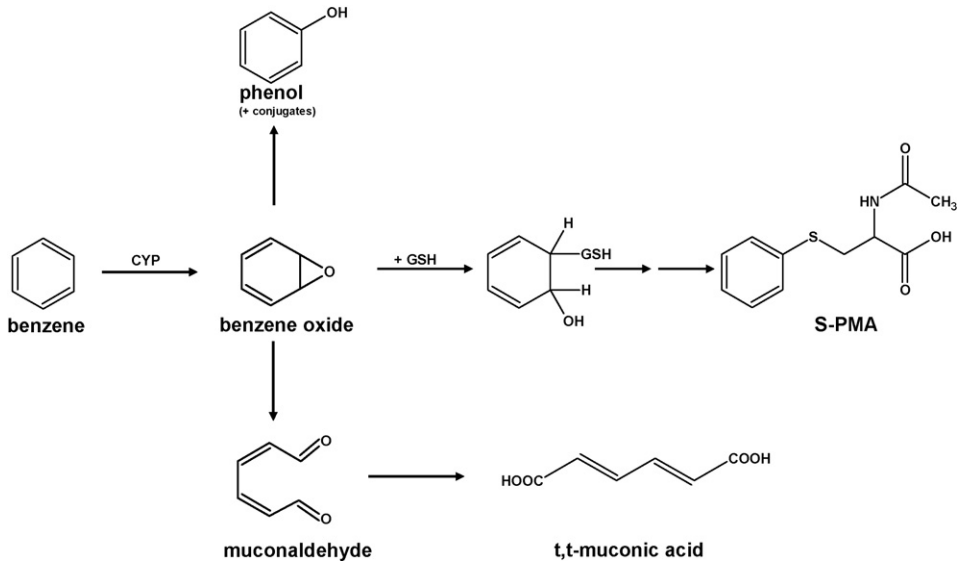

Fig. 1. Metabolic pathways of benzene. CYP, cytochrome P 450 and GSH, glutathione.

of benzene and toluene, a vast number of methods have been published so far [12–20].

S-phenylmercapturic acid (*S*-PMA) and *t*,*t*-muconic acid are both specific urinary metabolites of benzene and are recommended for a biological monitoring of benzene by the DFG [1] and the BEI-committee [21]. However, since the excretion of *t*,*t*-muconic acid might be influenced by the uptake of sorbic acid (a widely used food preservative), *S*-PMA is considered to be a more specific biomarker for low-level exposures to benzene [22,23]. *S*-phenylmercapturic acid is formed after conjugation of the highly reactive benzene oxide with glutathione. Dietary sources that would lead to the formation of urinary *S*-PMA are not known up to now. Thus, no other sources for excretion of *S*-PMA than exposure to benzene have been reported so far.

S-benzylmercapturic acid (*S*-BMA) has been identified as a metabolite of toluene by Takahashi et al. in 1993 [24]. Its use as a biomarker of toluene exposure has increased since then [25–27] and – similar to *S*-phenylmercapturic acid – its determination has been proposed to be more specific at low-level-exposures than the "classic" urinary biomarkers *o*-cresol or hippuric acid [28]. *S*-BMA is formed after *o*-sulfonation of benzyl alcohol by sulfotransferases and subsequent conjugation of the sulphate ester with glutathione, as reported for various xenobiotics like, e.g. chrysene [29,30]. *S*-*p*-tolylmercapturic acid, a minor urinary metabolite of toluene originating from metabolic activation at the aromatic ring after subsequent conjugation with glutathione, has also been used as a highly specific biomarker of toluene exposure at the workplace [31]. A short metabolism scheme for benzene and toluene considering all metabolites mentioned is shown in Figs. 1 and 2.

Methods published so far for the determination of urinary mercapturic acids of benzene and toluene involve mainly LC–MS–MS [26,27,32,33,34], LC–MS [35] or GC–MS after derivatisation of the mercapturic acids with carcinogenic diazomethane [15,31]. However, most of these methods determine only one metabolite [15,23,27,28,31,32,33,35]. Moreover, many methods require extensive manual sample preparation like liquid–liquid-extraction with ethyl acetate [15,31] or solid-phase extraction with anion-exchange cartridges [26,32]. Other methods suffer from the lack of a labelled internal standard [15,26,31] or an internal standard at all [34]. Moreover, the GC–MS-methods do not show the sensitivity needed for the investigation of environmental exposures to benzene, as their limit of detection for *S*-PMA was only 1 μg/L urine [15,31]. A fully automated method for the sensitive determination of both metabolites has been lacking so far.

Consequently, it was our aim to develop an accurate, fast and highly sensitive method for the simultaneous determination of *S*-PMA and *S*-BMA that does not require extensive manual sample preparation steps. The use of a restricted-access-material-phase in combination with column-switching allows for a fully automated clean-up from matrix components and thus results in excellent sensitivity and short run times. As a first application, we applied the newly developed method to spot urine samples of 30 subjects of the general population (15 non-smokers, 15 smokers) with no known exposure to benzene or toluene. With our method and future results we hope to provide insights into the actual internal exposure of the general population to benzene and toluene and identify possible confounders that contribute to the individual body burden like for example exposure to traffic exhaust, passive smoking or indoor VOC-sources.

2. Experimental

2.1. Chemicals

S-PMA and *S*-BMA were purchased from TCI (Tokyo, Japan). The deuterium-labelled analogue of *S*-PMA as internal standard, D_5-*S*-PMA was obtained from TRC (Toronto, Canada). Formic acid (100%, extra pure), acetonitrile and water (all of HPLC-grade) were purchased from Merck, Darmstadt, Germany.

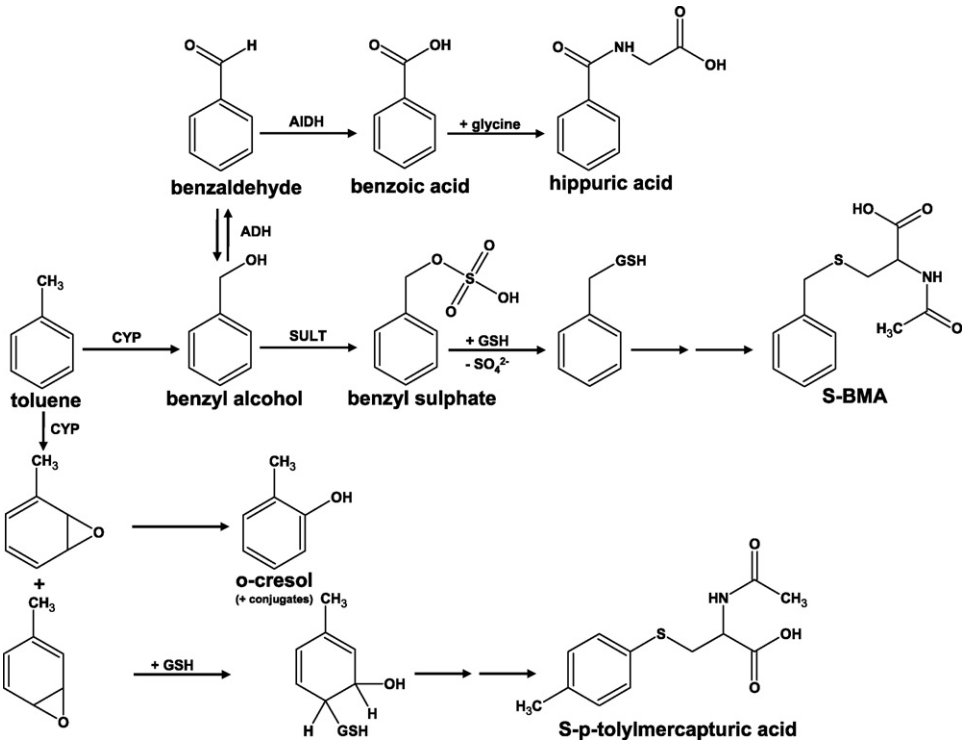

Fig. 2. Metabolic pathways of toluene. CYP, cytochrome P 450; SULT, sulfotransferases; ADH, alcohol dehydrogenase; AlDH, aldehyde dehydrogenase and GSH, glutathione.

2.2. Standard preparation

Stock solutions of the analytes were prepared by dissolving 10 mg of *S*-PMA and *S*-BMA each in 10 ml of methanol, respectively (1000 mg/L). These stock solutions were stored at −20 °C in a Teflon-capped brown glass vial. Aliquots of 50 μl of these stock solutions were placed in a 10-ml glass volumetric flask and diluted to the mark with 0.1% aqueous formic acid (5 mg/L). This solution served as the working solution for the preparation of urinary standards.

The stock solution for the internal standard was prepared by dissolving 1 mg of D_5-*S*-PMA in 1 ml of methanol (1000 mg/L). This stock solution was stored at −20 °C in a Teflon-capped brown 1.8-ml glass screw cap vial. A 100 μl-aliquot of this stock solution was placed in a 10-ml glass volumetric flask and diluted to the mark with 0.1% aqueous formic acid (working solution, 10 mg/L).

2.3. Sample preparation

Frozen urine samples were allowed to equilibrate to room temperature. The samples were shaken and 1-ml aliquots were then transferred to 1.8-ml glass screw-cap vials. Then 20 μl of formic acid (100%) and 20 μl of the D_5-*S*-PMA working solution (10 mg/L) were added to the samples. The samples were vortex mixed and centrifuged at 800 *g* for 5 min. When necessary, 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 [36].

2.4. Calibration procedure and quality control

From the working solution of the analytes (5 mg/L), 6 calibration standards were prepared by spiking pooled urine obtained from three non-smoking persons of the general population. These spot urine samples were pooled, frozen at −20 °C, thawed and filtered by a fluted filter before use. The urine was spiked with concentrations in the range from 2 to 250 μg/L. The pooled urine was used as a blank. Additionally, a blank value consisting of water was included in every analytical series. The standards were processed as described in Section 2.3. Linear calibration curves were obtained by plotting the quotients of the peak areas of *S*-PMA and *S*-BMA to the peak areas of the internal standard D_5-*S*-PMA as a function of the concentrations spiked. These graphs were used to ascertain the unknown concentrations of the mercapturic acids in urine samples.

As there was no quality control material commercially available at higher volumes, one had to be prepared in the laboratory. For the low-concentration and medium-concentration quality control material we spiked pooled urine with 0.3 and 20 μg of *S*-PMA per litre. These materials were not spiked with *S*-BMA and contained only the natural background levels of 7.3 and 5.0 μg/L, respectively. For the high-concentration quality control material we spiked pooled urine with 70 μg of each *S*-

Table 1
Analysis program of the gradient pump

Program step	Time (min)	Eluent A (%)	Eluent B (%)	Flow-rate (ml/min)	Position of six-port-valve	Analysis step
1	0	60	40	0.3	A	RAM-charging
2	3.8	60	40	0.3	B	Transfer
3	4.8	60	40	0.3	A	
4	6.2	60	40	0.3	A	Separation
5	10.2	0	100	0.3	A	
6	16.2	0	100	0.3	A	Washing
7	19.2	60	40	0.3	A	
8	22.2	60	40	0.3	A	Reconditioning

The second pump (isocratic pump) continuously pumps the mobile phase (0.05% aqueous formic acid, pH 2.5/acetonitrile 95/5, v/v) for the RAM-charging/fractionation step at a flow rate of 0.3 ml/min. Solvent A: 0.05% aqueous formic acid, pH 2.5; solvent B: acetonitrile.

PMA and *S*-BMA per litre. Due to the existing background levels of *S*-BMA in the urine used, this resulted in a concentration of ~75 μg/L *S*-BMA. The pools were divided into aliquots and stored at −20 °C. For quality assurance, one low-, one medium- and one high-concentration control sample was included in each analytical series.

Within-day repeatability was determined by analysing the low-, medium- and high-concentration quality control urine eight times in a row. Between-day repeatability was determined by analysing the medium- and high concentration quality control samples on eight different days and the low-concentration quality control samples on five different days. Furthermore, accuracy was determined using five spiked individual urine samples with creatinine contents ranging from 0.34 to 1.38 g/L. The level of *S*-PMA and *S*-BMA in the non-spiked subjects varied greatly from subject to subject. Spiked specimens (spiked concentration 5 μg/L for both analytes) were analysed before and after the addition of mercapturic acids.

2.5. LC–MS–MS analysis

2.5.1. Liquid chromatography

Liquid chromatography was carried out on an Agilent 1100 Series HPLC apparatus (auto sampler G 1313A, binary gradient pump G 1312A, vacuum degasser G 1379A) and an additional isocratic Agilent G 1310A pump. The latter was used to load the sample (100 μl) on a RAM (restricted access material) phase, a LiChrospher® RP-8 ADS (25 μm) 24 mm × 4 mm RAM from Merck (Darmstadt, Germany) using a solution of 0.05% aqueous formic acid (adjusted to pH 2.5 with formic acid) and acetonitrile (95:5, v/v) as the mobile phase and a flow rate of 0.3 ml/min. After this clean-up and enrichment step, the analytes were transferred to a reversed-phase HPLC column (Luna C 8 (2) 150 mm × 4.6 mm, 3 μm particle size from Phenomenex, Aschaffenburg, Germany) in backflush mode through a six-port valve (Valco Systems, Houston, Texas, USA) time-controlled by the autosampler and a pump gradient described in Table 1. Fig. 3 shows the backflush arrangement. All steps were controlled by Analyst 1.3.2 Software from Perkin-Elmer except the isocratic pump. A guard column (Luna C 8 (2), 4 mm × 3 mm, Phenomenex) was placed in front of the analytical column to extend its lifespan.

2.5.2. Mass spectrometry

The tandem mass spectrometric detection was performed on a Sciex API 3000 LC–MS–MS system in ESI-negative mode. The ion-source conditions were identical for all analytes with an electrospray needle voltage of – 4500 V in the negative ion mode. Nitrogen as nebulizer and turbo heater gas (450 °C) was set at a pressure of 65 psi. The curtain gas was set to 58 psi. The collision gas (nitrogen) for the MS–MS-mode was set to a flow of four instrument units.

Continuous flow injection (10 μl/min) of standard solutions (100 μg/L) in methanol for the analytes and the internal standard

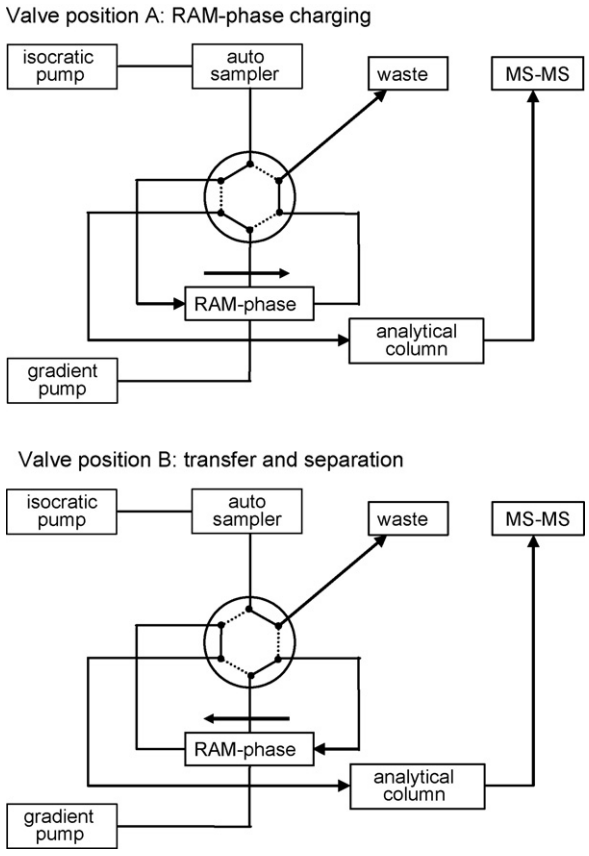

Fig. 3. Two-column HPLC system with backflush arrangement for the analyte transfer step in position B of the six-port-valve. The six-port-valve is time-controlled automatically by a signal given by the autosampler of the system.

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

Analyte	Retention time (min)	Parent ion (Q1)	Daughter ion (Q3)	DP	FP	EP	CE	CXP
S-PMA	14.23	238	108.9	−16	−60	−10	−14	−3
D_5-*S*-PMA (ISTD)	14.22	243	113.9	−71	−180	−10	−16	−5
S-BMA	14.53	252	123	−31	−70	−10	−20	−7

DP, declustering potential; FP, focusing potential; EP, entrance potential; CE, collision energy; CXP, collision exit potential. All potentials given in voltage (V).

were used to establish the optimum MS–MS-conditions for each analyte using a syringe pump (Model '22', Harvard Apparatus, Massachusetts, USA). The operating parameters in the multiple-reaction-mode (MRM) were as follows: resolution of Q1 and Q3 was set to "unit", settling time 5 ms, MR pause 5 ms and scan time 250 ms. Analyte specific parameters are shown in Table 2.

2.6. Study subjects

For a pilot study we investigated 30 spot urine samples from subjects of the general population with no known occupational exposure to benzene and toluene (no laboratory staff). The subjects were asked about their personal smoking behaviour and smoking status was additionally verified by urinary cotinine using a specific GC–MS-method [37]. Fifteen subjects (5 male, 10 female, with a median age of 24 years, ranging from 23 to 34 years of age) reported to be non-smokers and had urinary cotinine levels ranging from 1 to 16 μg/L. Fifteen subjects (10 male, 5 female with median age of 30 years, ranging from 23 to 56 years of age) reported to be smokers with daily cigarette consumptions of 2–22 cig./day and urinary cotinine levels ranging from 77 to 2790 μg/L. All subjects gave informed written consent about the donation of urine.

3. Results and discussion

3.1. General remarks

Method development was led by two considerations: avoidance of extensive manual clean-up and concentration steps that could deteriorate method performance and fast run-times that allow efficient use of the LC–MS–MS system.

In order to meet these requirements, we applied state-of-the-art on-line enrichment and column-switching techniques and combined them with common tandem mass spectrometric detection. The application of column-switching techniques in bioanalytical chemistry has increased in the last years. Successful applications were used for the determination of mercapturic acids of xylene [38], various phthalate metabolites [39,40] or environmental phenols [41].

The applied RAM-phase very effectively extracted the analytes from urinary matrix and the subsequent transfer and separation on the analytical column with highly specific ESI-negative-MS–MS-detection allowed an interference-free determination of both analytes up to the ng/L-range. Because of the limited number of manual steps in sample preparation we minimised not only the input of manpower but also possible sources of error.

3.2. Liquid chromatography

During sample preparation for liquid chromatography, thawed urine samples were centrifuged in order to precipitate proteins and particles. This procedure was necessary as we could not apply a guard column in front of the RAM-phase due to the applied backflush-technique and associated adsorption effects during transfer to the analytical column. As precipitated proteins could be considerable in some urine specimens, we decided to introduce this step to extent the lifetime of the RAM-phase. As the precision and accuracy data in individual urine specimens show, this procedure was proven not to have an influence on method performance (cf. Section 3.5.1). The acidification step with formic acid was checked with different urine specimens and resulted in pH-levels of 2–2.5, so that further addition of buffers was not necessary.

The LiChrospher® ADS-8 material used is based on alkyl-diol-silica material. This material's retention characteristics are based mainly on its ability to exclude macromolecules, like proteins, with a molecular weight >15 kDa that cannot enter the pores of the material and its reversed-phase-mechanisms, when interacting with the (inner) C-8-phase. The analytes showed good retention on the ADS-8-column as tests with direct connection of the ADS-8-column with the MS–MS-system after injection of an aqueous standard showed. Within method development, we also tried to use an ADS-18 column, which showed a slightly worse performance, probably due to the comparatively high polarity of the analytes. In contrast, an ADS-4 column showed no retention for the analytes and turned out to be unsuitable for our purpose.

For the LC–LC column-switching technique, we had to take into account several considerations: the solvent of the isocratic pump used for the loading of the sample onto the ADS-8-column should be sufficiently strong to elute disturbing matrix compounds from this column and thus result in good clean-up, but weak enough to ensure retention of the analytes. Tests with different solvent compositions showed that a mixture of 0.05% aqueous formic acid (pH 2.5) and acetonitrile (95:5, v/v) represents the optimum compromise between these needs and results in an enrichment and clean-up time of 4 min. In contrast, starting conditions of the gradient pump for the backflush transfer of the analytes to the analytical column had to be optimised under different conditions: the solvent should be strong enough to ensure rapid and complete elution of the analytes from the ADS-8-column, but weak enough to guarantee a focusing of the analytes and good resolution on the analytical column. These requirements were best met by starting the gradient with 60% of 0.05% aqueous formic acid (pH 2.5, solvent A) and 40%

acetonitrile. Under these conditions, transfer is performed fast (time: 1 min) and the RAM-phase is washed again and ready to be reconditioned by the isocratic pump.

The chromatographic separation on the analytical column was optimised with regard to fast run times: after focussing the analytes on the column with 40% acetonitrile, the solvent strength was rapidly increased within 4 min to 100% acetonitrile. After washing of the analytical column and subsequent reconditioning, a whole chromatographic run lasts no longer than 22.2 min. The analyte peaks are very sharp and without interferences, even in urine samples with high creatinine contents.

3.3. Mass spectrometry

ESI-negative mode has already been successfully applied for the determination of *S*-PMA [26,32–35] and *S*-BMA [26] and various other mercapturic acids in urine [38,42,43]. Other authors applied APCI in negative mode for the determination of mercapturic acids of 1,3-butadiene or acroleine [44,45]. As we got excellent limits of detection without elaborate optimisation of related APCI parameters, we decided to use the more robust and stable ESI-negative mode for quantification.

Compound-specific mass spectrometric parameters were optimised for each compound automatically by the Quantitative Optimisation Wizard of the Sciex Analyst^{TM} software. The source specific parameters were optimised manually during tuning of the parameters and later analysis. The ESI-negative product ion mass spectra with tentative fragment structures for *S*-PMA and *S*-BMA are shown in Fig. 4. The chosen parent ions for the MS–MS-fragmentation of the analytes were $[M-H]^-$. The MRM-parameters are illustrated in Table 2. The identity of each analyte was confirmed using the specific parent–daughter ion transition in combination with retention time as well as – in the case of *S*-PMA – matching the retention time with the deuterated internal standard.

For quantification, the peak area ratio of the analytes to the D_5-labelled internal standard was used. No unlabelled isotope fragments were observed in the labelled standard. To check for a possible D–H isotope exchange interfering with the determination of *S*-PMA, an aqueous standard was spiked with D_5-*S*-PMA at a concentration of 500 μg/L and processed as described in Section 2.3. No unlabelled *S*-PMA could be detected in this sample, proving that the addition of internal standard to urine as described in Section 2.3. (200 μg/L) does not produce any false positive results.

3.4. Calibration graphs

The calibration graphs were linear for *S*-PMA and *S*-BMA in the range from LLOQ – 250 μg/L. In both cases, the linear correlation coefficients of the calibration graphs were all higher than $r = 0.999$. This comparatively wide working range of the method was chosen in order to also cover the excretion range of persons with a possible occupational exposure.

There was a background excretion for both analytes detectable in the unspiked pooled urine used for the calibration curves (*S*-PMA: ~0.08 μg/L; *S*-BMA: ~3.8 μg/L). However, as only urine from non-smoking persons was used for the pooled urine with the described low levels, this did not influence the accurate quantification of the analytes at environmental levels.

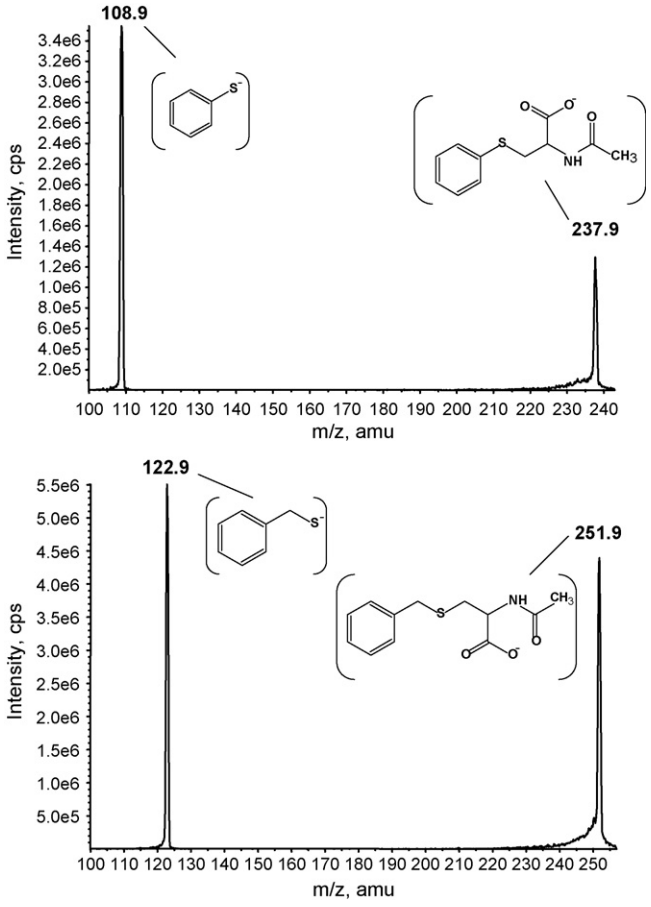

Fig. 4. ESI-negative product-ion mass spectra of *S*-PMA and *S*-BMA with the predicted structures of the fragments.

3.5. Reliability of the method

3.5.1. Precision and accuracy

We have checked the accuracy of our method by special recovery experiments that have proven to be very effective in evaluating performance of an analytical method. For this purpose, five different urine specimens were spiked with the analytes at a concentration of 5 μg/L. The urine specimens were selected to reflect a different composition as indicated by urinary creatinine content (range: 0.34–1.38 g/L). Good accuracy results under these conditions showed that the different biological matrix had no influence on the analytical result. For that experiment, mean relative recovery for *S*-PMA and *S*-BMA was determined to be 108% and 100%, respectively. Therefore, accuracy under these conditions can be regarded as excellent, which is mainly due to the use of a labelled internal standard. Urinary creatinine content was not observed to have an influence on recovery of the analytes.

In order to determine the within-day repeatability, low-, medium- and high-concentration urinary quality control samples were analysed eight times in a row. The quality control samples

Table 3
Quality control data and limits of detection for urinary *S*-PMA and *S*-BMA

Analyte	Precision									Accuracy		LLOQ (μg/L)
	Q_{low}			Q_{medium}			Q_{high}			Relative recovery in individual urine (%)		
	Conc. (μg/L)	RSD (%), intra-day ($n=8$)	RSD (%), inter-day ($n=5$)	Conc. (μg/L)	RSD (%), intra-day ($n=8$)	RSD (%), inter-day ($n=8$)	Conc. (μg/L)	RSD (%), intra-day ($n=8$)	RSD (%), inter-day ($n=8$)	Mean	Range	
S-PMA	0.43	10.4	12.2	20.2	5.8	5.4	68.4	2.8	3.4	108	88–125	0.05
S-BMA	7.30	3.6	5.3	5.0	3.3	5.0	74.2	2.8	1.0	100	75–138	0.05

RSD, relative standard deviation; LLOQ, lower limit of quantification.

Q_{low} and Q_{medium} were spiked with 0.3 and 20 μg/L *S*-PMA and contained only the natural background levels of *S*-BMA in the urine used for quality control purposes (7.3 and 5.0 μg/L, respectively), and the quality control sample Q_{high} was spiked with 70 μg/L of each analyte. For Q_{low} and Q_{medium}, relative standard deviations were 10.4% and 5.8% for *S*-PMA and 3.6% and 3.3% for *S*-BMA, while for Q_{high}, relative standard deviations were 2.8% for both analytes.

The relative standard deviation of the between-day repeatability was determined for Q_{low} on five and for Q_{medium} and Q_{high} on eight different days and was 12.2% and 5.3% for Q_{low}, 5.4% and 5.0% for Q_{medium} and 3.4% and 1.0% for Q_{high}, demonstrating very good repeatability of our method. The data are summarised in Table 3.

3.5.2. Freeze–thaw stability of the analytes

In order to check for the freeze–thaw-stability of the analytes, three high-concentration quality control samples were subjected to three freeze–thaw cycles on three different days. After that, the samples were analysed as described in Section 2.3 and the results were compared to the results obtained for the between-day repeatability. No significant deviation from the mean value of the between-day repeatability was observed. Consequently, a decomposition of the analytes in urine during several freeze–thaw-cycles could not be confirmed.

3.5.3. External quality control

The accuracy of our new method was assured by successful participation at an interlaboratory comparison program organised in Germany for the determination of *S*-PMA and various other occupationally relevant metabolites in human urine (www.g-equas.de). Our results for *S*-PMA corresponded well with the theoretical values of the spiked urine samples and the results of other participating laboratories ($n=9$). This is another proof of the high level of accuracy of the method presented.

3.5.4. Limit of detection

The limit of detection was estimated to be 0.02 μg/L for both analytes (based on a signal-to-noise ratio of three for the registered ion transitions) and the lower limit of quantification (LLOQ) was 0.05 μg/L. However, as the background levels of *S*-PMA and *S*-BMA in human urine turned out to be considerably higher, this LOD has never been reached in practice.

Table 4
Results of our study measuring *S*-PMA and *S*-BMA in non-smoking ($n=15$) and smoking persons ($n=15$) of the general population not knowingly exposed to benzene or toluene and comparison with the results of previous studies analysing these metabolites in groups of the general population

Reference	Group		*S*-PMA (μg/g creatinine)	*S*-BMA (μg/g creatinine)
This study	Non-smokers ($n=15$)*	Median	0.12	4.9
		Range	<0.05–0.42	2.4–81.4
	Smokers ($n=15$)*	Median	1.31	8.0
		Range	0.24–3.33	1.7–31.2
Melikian et al. [32]	Non-smokers ($n=18$)	Median	3.6	–
		Range	1.0–19.6	–
	Smokers ($n=28$)	Median	5.8	–
		Range	<LOD – 33.4	–
Rolle-Kampczyk et al. [34]	Children with no exposure to passive smoke	Mean	2.44	37.97
		SD	3.10	35.18
	Children with exposure to passive smoke	Mean	3.32	44.40
		SD	3.06	84.60

* Urinary cotinine was determined to unequivocally confirm smoking status of the persons: non-smokers had cotinine values ranging from 1 to 16 μg/L; smokers ranged from 77 to 2790 μg/L.

3.6. Sources of error

For urinary *S*-PMA, in some cases ionisation was slightly suppressed as observed by reduced peak areas of the labelled internal standard, resulting in a 2–3-fold loss of sensitivity. This was probably due to co-eluting substances of the urinary matrix. As quantification was based on isotope dilution, this variation in absolute signal intensity did not significantly deteriorate accuracy, as shown by the experiments in individual urines (cf. Section 3.5.1).

The whole system works fully automated and showed no severe deterioration in sensitivity or increases in column pressure so far. Solely the pre-column of the analytical column was replaced after ~200 injections. Despite the direct injection of 100 μl of urine into the system, contamination of the mass spectrometric detector was minimal, indicating very efficient clean-up. Light contamination of the curtain plate of the API-3000 after 100 injections can be cleaned using a lint-free paper and methanol.

3.7. Results of biological monitoring

The results of biomonitoring for the 15 non-smoking and 15 smoking subjects of the general population are summarised in Table 4 on a creatinine basis. *S*-PMA and *S*-BMA were detectable in 96% and 100% of all urine samples. Fig. 5 shows a chromatogram of a processed spot urine sample that is representative for a non-smoking person. It also illustrates the high sensitivity of our method.

A remarkable issue of these preliminary results is that quantification of the individual internal exposure to benzene due to environmental exposure sources was possible in almost all subjects that were confirmed to be non-smokers. This distinguishes our results from most earlier studies, where the LOD of the method usually only allowed for the reliable quantification of *S*-PMA in smoking subjects or persons with occupational exposure. A comparison of our results with the results of two previous studies measuring these metabolites in non-exposed groups of the general population is also included in Table 4. As can be seen, our results are remarkably lower than those of former studies. Methodical differences can be discussed as a reason for these discrepancies, e.g. the use of a non-processed, non-matrix based calibration [32] or the lack of any internal standard [34].

As expected, smokers showed an excretion of *S*-PMA that is approximately 10-fold higher than that of non-smokers (median: 0.12 μg/g creatinine vs. 1.31 μg/g creatinine). A significant relationship between urinary cotinine as specific marker of tobacco consumption and *S*-PMA could be observed (Pearsson, $R = 0.73$, $p < 0.001$), indicating smoking to be the main source of environmental benzene exposure in active smokers. A significant elevation in *S*-PMA-excretion has already been observed in children exposed to passive smoke as reported by Rolle-Kampczyk et al. [34].

Surprisingly, excretion of the proposed toluene metabolite *S*-BMA was not significantly elevated in smoking subjects as compared to non-smokers (Mann–Whitney-test, $p = 0.52$).

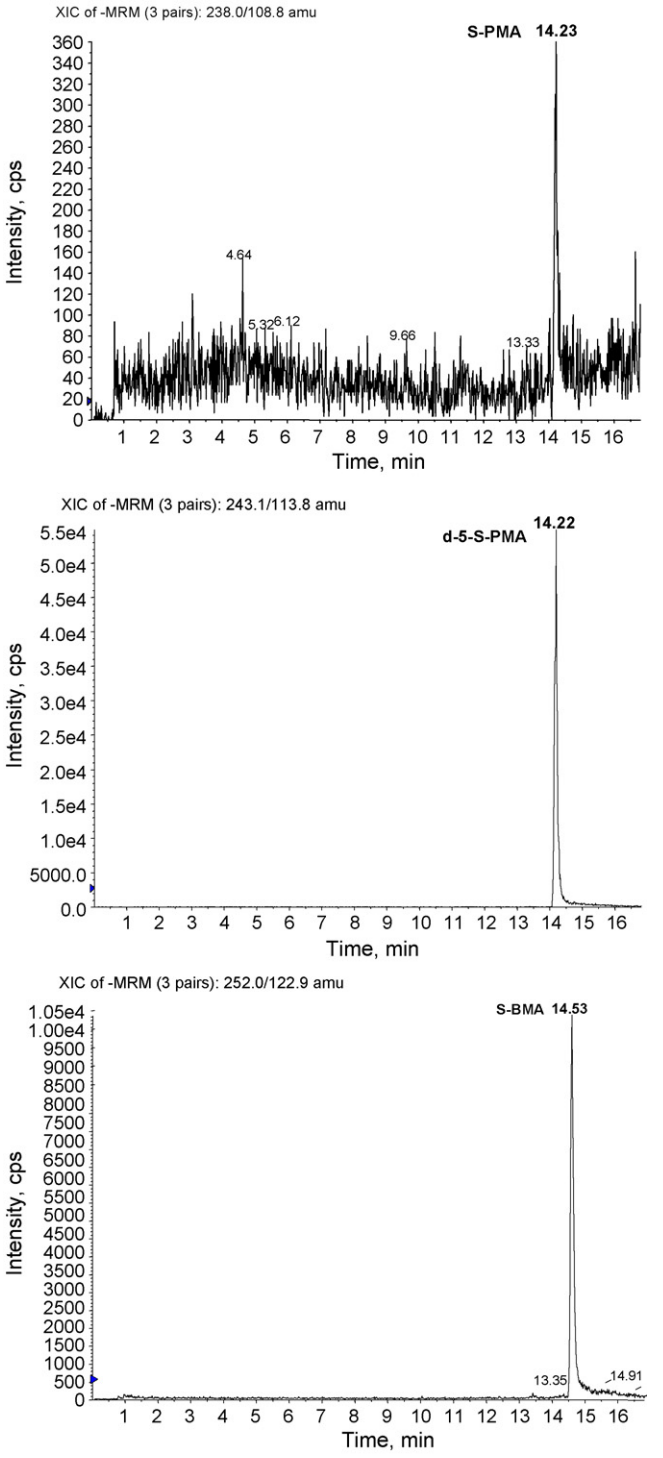

Fig. 5. Chromatogram of a processed urine sample of a non-smoking person of the general population not knowingly exposed to benzene or toluene (creatinine: 0.6 g/L). Concentrations for *S*-PMA and *S*-BMA were 0.11 and 3.4 μg/L, respectively.

According to the levels of benzene and toluene reported in cigarette mainstream smoke [10] (benzene: 3.2–61.7 μg/cigarette; toluene: 5.4–81.8 μg/cigarette), a similar distinction between non-smokers and active smokers as for the benzene metabolite *S*-PMA would have been expected. The reason for this discrepancy remains to be clarified: on the one hand, it might be speculated

that other environmental sources of toluene are contributing in such a way to the individual exposure that smoking is only a minor factor.

On the other hand, these results could indicate that the specifity of *S*-BMA excretion as a biomarker of toluene exposure is insufficient for the determination of environmental exposures and possibly other metabolic routes might in part also lead to the formation (and excretion) of *S*-BMA. As *S*-BMA is also formed during metabolism of benzyl alcohol (see Fig. 2), which is a widespread constituent of cosmetic products like shampoo, creams, after-shave lotions or fragrances, it is a reasonable assumption that the use of these products and uptake of benzyl alcohol by skin penetration also influences the excretion of *S*-BMA. As we did not explicitly ask our study subjects about the recent use of specific cosmetic products, we cannot finally analyse this effect. However, the use of *S*-BMA as a biomarker of toluene exposure in environmental settings [34] should be critically revised.

4. Conclusions

We have developed a reliable, accurate, fast and easy procedure for the determination of urinary *S*-phenylmercapturic acid and *S*-benzylmercapturic acid as biomarkers of individual exposure to benzene and toluene. To our knowledge, this method is one of the most sensitive methods published so far and does not require manual work-up procedures. Moreover, the use of a labelled internal standard guarantees high accuracy of the results. The data determined for precision and accuracy were very good and no interfering effect of the urinary matrix on the result was observed. The automated on-line clean-up turned out to be very efficient, allowing interference-free quantification down to levels of 0.05 μg/L urine.

The comparatively short run-time of 23 min allows analysis of more than 60 samples per day, assuring efficient use of LC–MS–MS capabilities. Consequently, this method is perfectly suited for the determination of individual benzene and toluene exposure in large studies. Moreover, due to its high sensitivity, it also allows to objectify the impact of indoor VOC-sources like environmental tobacco smoke (ETS) or printer and copier machines on individual benzene burden. Thus, our method can serve as a powerful tool for the estimation of internal exposure to these substances.

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