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Benzene, benzyl chloride, and toluene – Determination of *S*-phenylmercapturic acid and *S*-benzylmercapturic acid in urine by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2021

Keywords

S-phenylmercapturic acid, SPMA, *S*-benzylmercapturic acid, SBMA, urine, biomonitoring, mercapturic acids, LC-MS/MS

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method.

The analytical method permits the quantification of the mercapturic acids of benzene (*S*-phenylmercapturic acid, SPMA), as well as of benzyl chloride or toluene (*S*-benzylmercapturic acid, SBMA), in the urine of potentially exposed individuals.

The mercapturic acids are selectively enriched online by high-performance liquid chromatography (HPLC) using restricted access material (RAM) and separated from matrix components. Afterwards, the analytes are transferred onto the analytical column for separation in backflush mode and subsequently detected by tandem-mass spectrometry. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed. As an internal standard, d_5-SPMA is added to the urine samples.

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1 Characteristics of the method

Matrix Urine

Analytical principle LC-MS/MS

Parameters and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
Benzene	71-43-2	*S*-Phenylmercapturic acid (SPMA)	4775-80-8
Benzyl chloride	100-44-7	*S*-Benzylmercapturic acid (SBMA)	19542-77-9
Toluene	108-88-3	*S*-Benzylmercapturic acid (SBMA)	19542-77-9

Reliability data

S-Phenylmercapturic acid (SPMA)

Within-day precision:	Standard deviation (rel.)	s_w = 10.4%, 5.8%, or 2.8%
	Prognostic range	u = 24.6%, 13.7%, or 6.6%
	at a spiked concentration of 0.3 µg, 20 µg, or 70 µg SPMA per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 12.2%, 7.3%, or 4.2%
	Prognostic range	u = 33.9%, 16.3%, or 9.4%
	at a spiked concentration of 0.3 µg, 20 µg, or 70 µg SPMA per litre of urine and n = 5, 11, or 11 determinations	
Accuracy:	Recovery rate (rel.)	r = 101% or 98%
	at a spiked concentration of 20 µg or 70 µg SPMA per litre of urine and n = 8 determinations	
Detection limit:	0.05 µg SPMA per litre of urine	
Quantitation limit:	0.15 µg SPMA per litre of urine	

S-Benzylmercapturic acid (SBMA)

Within-day precision:	Standard deviation (rel.)	s_w = 3.3% or 2.8%
	Prognostic range	u = 7.8% or 6.6%
	at a native concentration of 5.1 µg or a spiked concentration of 70 µg SBMA per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 10.0% or 2.8%
	Prognostic range	u = 22.3% or 6.2%
	at a native concentration of 5.1 µg or a spiked concentration of 70 µg SBMA per litre of urine and n = 11 determinations	
Accuracy:	Recovery rate (rel.)	r = 103%
	at a spiked concentration of 70 µg SBMA per litre of urine and n = 8 determinations	
Detection limit:	0.5 µg SBMA per litre of urine	
Quantitation limit:	1.5 µg SBMA per litre of urine	

2 General information on benzene, benzyl chloride, and toluene

Benzene

Benzene is a colourless, volatile, and flammable liquid with a characteristic odour. Formerly, it was used as an additive (anti-knock agent) to motor fuels. But nowadays, the benzene content in fuels has been limited to a maximum of one percent by volume (European Commission 2014). Benzene is used as a starting material for the synthesis of many benzene derivatives, such as ethyl benzene, cumene, aniline, nitrobenzene, styrene, and synthetic rubber. However, benzene is no longer used as a solvent because of its proven carcinogenic properties. The global annual production is estimated to be about 42 million tons (IARC 2018).

The primary route of occupational and environmental exposure to benzene occurs via inhalation. Dietary intake is also discussed as a possible source of benzene exposure for the general population (IARC 2018). In addition, dermal absorption of benzene through intact skin is possible. Thus, the substance is designated by the Commission with an "H" (danger from percutaneous absorption) (DFG 2019). Smokers are additionally exposed to benzene through the inhalation of tobacco smoke.

Benzene has been classified by the IARC (International Agency for Research on Cancer) and the Commission as a Category 1 carcinogen, i.e. it is known to be carcinogenic to humans (DFG 2019; IARC 2018). Moreover, benzene has been classified as a Category 3A germ cell mutagen. For details on the toxicological evaluation of benzene, please refer to the respective MAK Value Documentations (Henschler 1974, 1992).

A simplified metabolism scheme for benzene is shown in Figure 1. After inhalation exposure, benzene is readily absorbed. However, 16–50% of the dose are exhaled unchanged. The phenolic metabolites are excreted biphasically in urine with given half-lives of about 3–4 hours and 20 hours, respectively (Norpoth 1986). In addition to direct determination of urinary benzene, biomonitoring of persons occupationally exposed to benzene may preferably be carried out by the determination of benzene metabolites in urine. Both *t,t*-muconic acid and *S*-phenyl mercapturic acid are regarded as suitable biomarkers, with SPMA being the more specific parameter. For all three parameters,

there are both exposure equivalents for carcinogenic substances (EKA, *Expositionsäquivalente für krebserzeugende Arbeitsstoffe*) and biological reference values (BAR, *Biologischer Arbeitsstoff-Referenzwert*) available in the List of MAK and BAT Values (DFG 2019). The determination of benzene in blood, on the other hand, has not proved suitable due to its short half-life (Weistenhöfer and Drexler 2015). For details on the assessment values for benzene in biological materials, please refer to the respective BAT Value Documentations (Kraus et al. 2019; Norpoth 1986). In case of occupational benzene exposure, the SPMA excretion increases in the course of the work shift and reaches its peak at the end of the shift (Müller and Jeske 1996). The BAR for SPMA was set at 0.3 µg/g creatinine for non-smokers (DFG 2019).

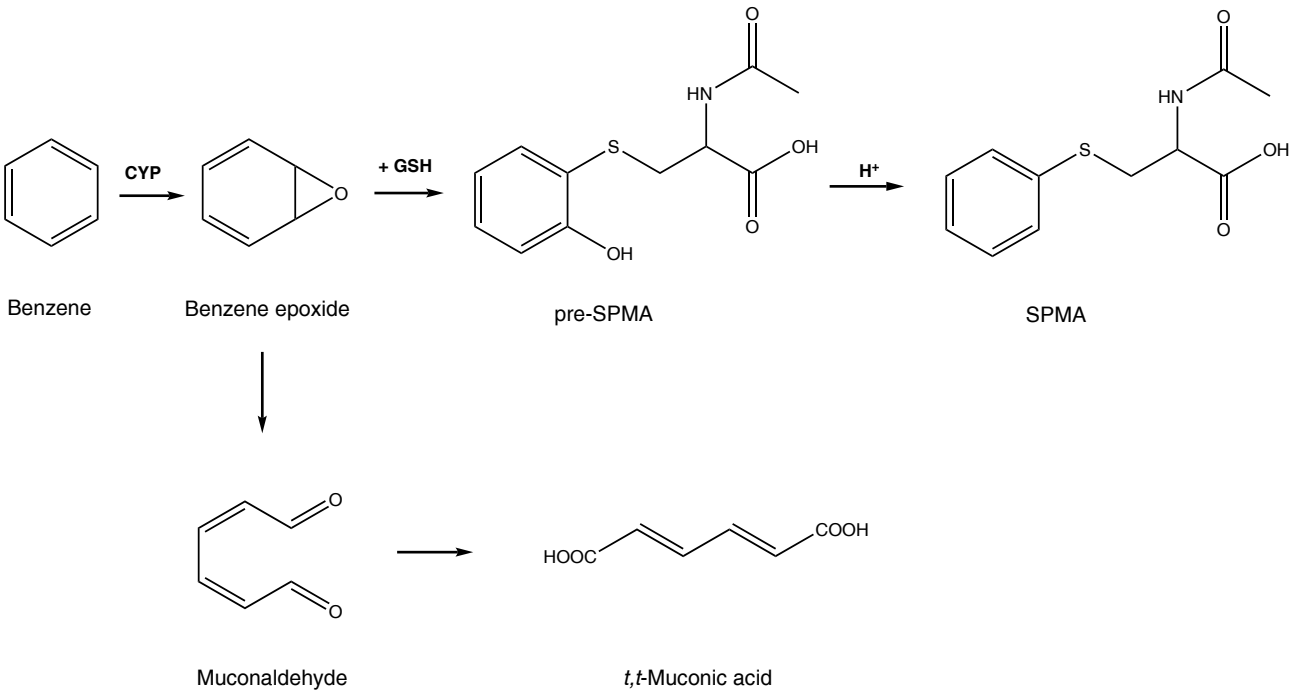


Fig. 1 Simplified metabolism scheme for benzene

Benzyl chloride

Benzyl chloride is a colourless liquid with a pungent odour. It is a typical intermediate in the chemical industry and used in the manufacture of benzyl alcohol, phthalic acid benzyl esters, benzylamine and its derivatives, among other products (IARC 1999 a).

Benzyl chloride has been classified by the Commission as a Category 2 carcinogen and has been designated with an "H" (danger from percutaneous absorption) as it readily penetrates the skin (DFG 2019). The IARC has also classified benzyl chloride as probably carcinogenic to humans (Group 2A) (IARC 1999 a). For a detailed overview of the toxicological effects of benzyl chloride, please refer to the respective MAK Value Documentations (Greim 2004; Henschler 1993). Due to its relatively high vapour pressure and ability to readily penetrate the skin, occupational contact with benzyl chloride is often linked with an inner exposure of workers (Lewalter et al. 2003). Following absorption, benzyl chloride is rapidly metabolised and mainly excreted renally with the urine. More than 65% of the dose is excreted in urine within 24 hours. The main metabolite was identified to be *S*-benzylmercapturic acid, with hippuric acid and benzylcysteine as minor metabolites. The oxidative degradation to the substituted benzoic

acid (or hippuric acid) proceeds to a significantly lesser extent than with the structurally related toluene (IARC 1999 a; Lewalter et al. 2003). Figure 2 shows a simplified metabolism scheme for benzyl chloride.

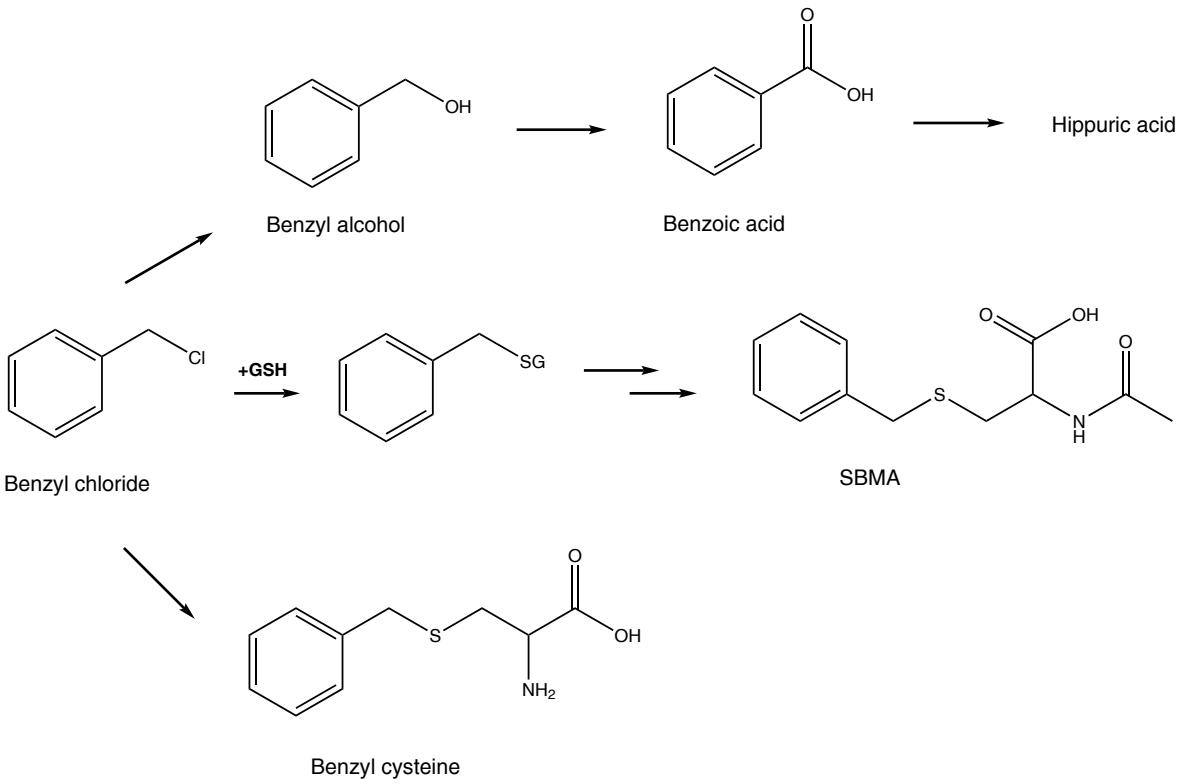


Fig. 2 Simplified metabolism scheme for benzyl chloride

Toluene

Toluene is a colourless, clear liquid with a benzene-like odour. It is used as a starting material for the manufacture of several industrial chemicals and is extensively used as a solvent. For instance, toluene is used for the production of benzene, diisocyanates, or explosives. It is used as a solvent in paints, varnishes, adhesives, and as a fuel additive for octane boosting (IARC 1999 b).

Exposure to toluene is possible in both occupational and environmental settings. For instance, toluene is formed during the incomplete combustion of organic matter and is thus present, for example, in cigarette smoke and exhaust fumes. The use of toluene in adhesives and spray paints intended for sale to the general public has meanwhile been restricted in the EU to a maximum toluene concentration of 0.1% (European Parliament and Council of the European Union 2006).

The Commission derived a MAK value of 50 ppm (190 mg/m^3) for toluene. Besides, toluene has been designated with an "H" as it readily penetrates the skin (DFG 2019). For details on the toxicological evaluation of toluene, please refer to the respective MAK Value Documentations (Greim 1996, 2002; Henschler 1986). The Commission recommends the determination of *o*-cresol in urine or the determination of toluene in blood or urine for the biomonitoring of toluene. BAT values were established for each of these parameters (DFG 2019).

In addition to the well-known biomarker *o*-cresol in urine, which is recommended by all national and international organisations for the biomonitoring of toluene exposure, SBMA has also been described as a metabolite of toluene

in solvent sniffers (Takahashi et al. 1993). In another study, SBMA was described as an even more suitable biomarker for occupational toluene exposure compared to hippuric acid and *o*-cresol (Inoue et al. 2004).

Figure 3 shows a metabolism scheme for toluene. It shows that benzyl alcohol is a metabolic precursor of SBMA. However, as benzyl alcohol is an approved ingredient in many cosmetic products, the dermal absorption of benzyl alcohol via cosmetics can be an additional factor affecting the urinary SBMA level. Therefore, the urinary excretion of SBMA seems to be a less suitable parameter for the determination of environmental exposure to toluene (Lovreglio et al. 2010). SBMA is also considered to be unsuitable as a parameter of occupational exposure to low concentration levels of toluene (Inoue et al. 2008).

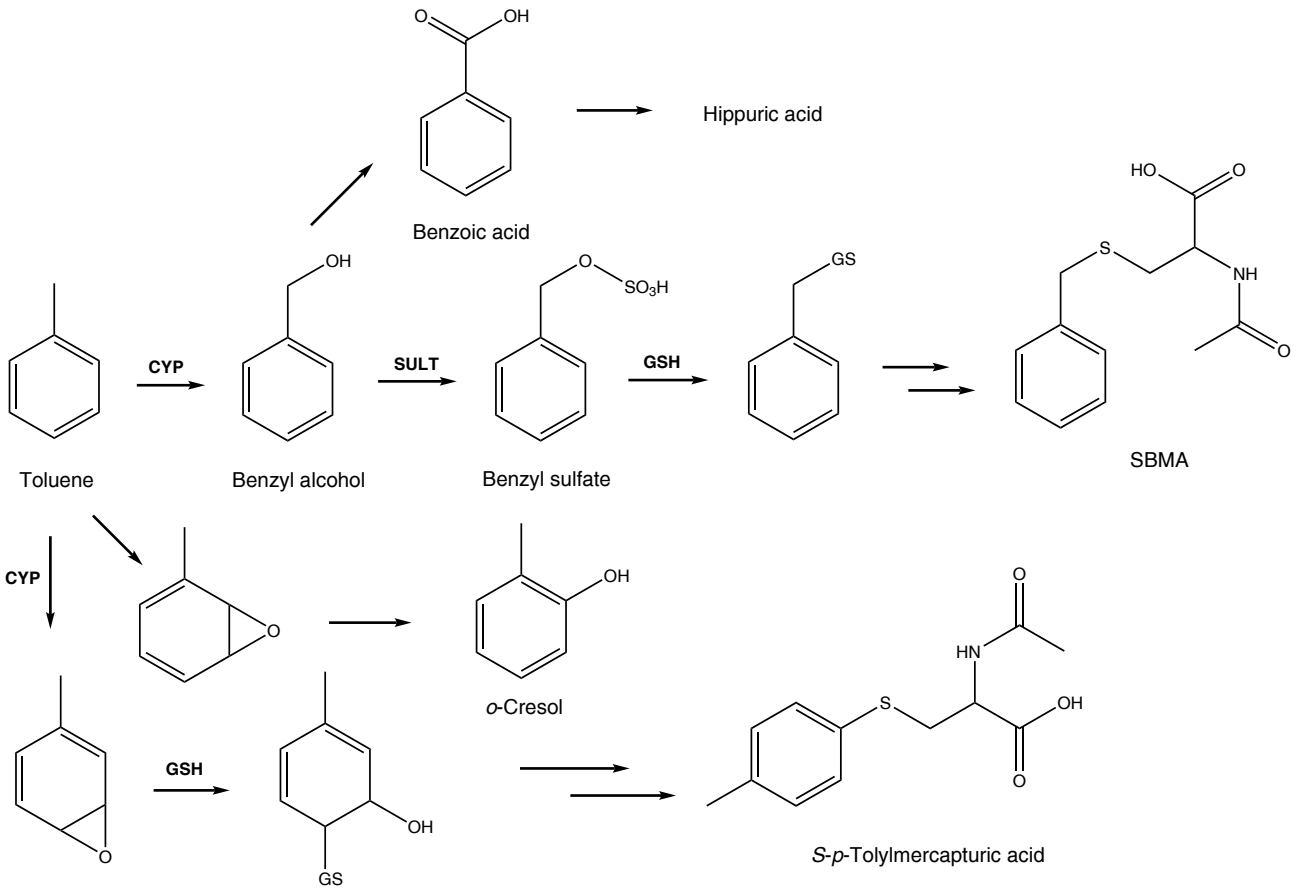


Fig. 3 Simplified metabolism scheme for toluene

Background levels of SPMA and SBMA

The method presented herein was used to analyse urine samples of both smokers and non-smokers from the general population. The classification as either smokers or non-smokers was based on urinary cotinine levels. The results of this study are summarised in Table 1.

Tab. 1 Urinary levels of SPMA and SBMA in the general population

Study group	SPMA [µg/l]	SBMA [µg/l]
	Median (range)	Median (range)
General population, Germany, non-smokers (n = 56)	0.13 (< LOD–0.29)	7.4 (0.50–77.4)
General population, Germany, smokers (n = 72)	1.3 (0.17–12.7)	11.2 (0.90–51.2)

LOD: limit of detection

3 General principles

The procedure enables the determination of the mercapturic acids of benzene (*S*-phenyl mercapturic acid, SPMA) as well as of benzyl chloride and toluene (*S*-benzyl mercapturic acid, SBMA) in the urine of potentially exposed persons.

The mercapturic acids are separated from matrix components by online-enrichment using high-performance liquid chromatography (HPLC) with a Restricted Access Material (RAM) phase. Afterwards, the analytes are transferred for separation onto the analytical column in backflush mode and subsequently detected by tandem-mass spectrometry. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed. As an internal standard (ISTD), d_5-SPMA is added to the urine samples.

4 Equipment, chemicals, and solutions

4.1 Equipment

- HPLC system (e.g. HP 1100, Agilent Technologies Deutschland GmbH, Waldbronn, Germany) consisting of a quaternary gradient pump, an isocratic pump, an eluent degasser, a column thermostat, an injection valve, a 100-µl sample loop, a pump head for 100 µl, an autosampler, a six-port valve, a tandem-mass spectrometric detector (e.g. API 3000, AB Sciex Germany GmbH, Darmstadt, Germany) as well as a PC system for data evaluation
- HPLC column: Luna C8 (2), length: 150 mm; inner diameter: 4.6 mm; particle size: 3 µm (e.g. Phenomenex Ltd., Aschaffenburg, Germany, No. 00F-4248-E0)
- Precolumn: C8, length: 4 mm; inner diameter: 3 mm; particle size: 3 µm (e.g. Phenomenex Ltd., Aschaffenburg, Germany, No. AJ0-4290)
- RAM phase: LiChrospher RP-8 ADS; length: 25 mm; inner diameter: 4 mm; particle diameter: 25 µm (e.g. Merck KGaA, Darmstadt, Germany, No. 1.50209.001)
- Precolumn filter: Supelco Replacement Frits 3 mm, 0.5 µm (e.g. Merck KGaA, Darmstadt, Germany, No. 57677)
- pH meter (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Magnetic stirrer (e.g. H+P Labortechnik AG, Oberschleißheim, Germany)
- 1.8-ml vials with screw-caps and PTFE septa (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)

- 5-ml amber glass vials with screw-caps and PTFE septa (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- Microlitre pipettes with adjustable volume between 10 µl and 100 µl as well as between 100 µl and 1000 µl (e.g. Eppendorf AG, Hamburg, Germany)
- Various volumetric flasks and beakers (e.g. BRAND GmbH + CO KG, Wertheim, Germany)
- Folded filters (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- Urine-collection containers (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be at least *pro analysi* grade.

- *S*-Phenylmercapturic acid, SPMA (e.g. Toronto Research Chemicals Inc., Toronto, Canada, No. P335600)
- d_5-*S*-Phenylmercapturic acid, d_5-SPMA (e.g. Toronto Research Chemicals Inc., Toronto, Canada, No. P335605)
- *S*-Benzylmercapturic acid, SBMA (e.g. TCI Deutschland GmbH, Eschborn, Germany, No. A0730)
- Formic acid, 100% (e.g. Merck KGaA, Darmstadt, Germany, No. 111670)
- Hydrochloric acid, 37% (e.g. Merck KGaA, Darmstadt, Germany, No. 100317)
- Acetonitrile (e.g. VWR International GmbH, Darmstadt, Germany, No. 10629112)
- Methanol (e.g. Merck KGaA, Darmstadt, Germany, No. 113351)
- Ultra-pure water (e.g. Millipore® water, Merck KGaA, Darmstadt, Germany)

4.3 Solutions

- 1% Aqueous formic acid

  1 ml formic acid is pipetted into a 100-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.

  When stored in the refrigerator at 4 °C, the solution is stable for approximately one month.

- Mobile phase A (0.05% aqueous formic acid, pH 2.5)

  50 ml of the 1% aqueous formic acid are placed into a 1000-ml volumetric flask. The flask is then filled with approximately 900 ml of ultra-pure water and the pH value is adjusted to pH 2.5 (using a pH meter) by adding drops of concentrated formic acid and stirring the solution using a magnetic stirrer. The volumetric flask is subsequently made up to the mark with ultra-pure water.

  When stored at room temperature in an amber glass bottle, the solution is stable for approximately two weeks.

- Mobile phase C (mobile phase A : acetonitrile, 95 : 5, v/v)

  25 ml acetonitrile are placed into a 500-ml volumetric flask. The flask is then made up to the mark with mobile phase A.

  When stored at room temperature in an amber glass bottle, the solution is stable for approximately two weeks.

4.4 Internal standard (ISTD)

- Stock solution d_5-SPMA (1 g/l)

  1 mg of d_5-SPMA (total quantity purchased) is dissolved in 1 ml of methanol in the original container (1.8-ml screw-cap vial) and transferred to a 5-ml amber screw-cap vial.

- Working solution d_5-SPMA (10 mg/l)

  100 µl of the d_5-SPMA stock solution are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with 1% aqueous formic acid.

- Spiking solution d_5-SPMA (1 mg/l)

  1 ml of the d_5-SPMA working solution is pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with 1% aqueous formic acid.

The ISTD solutions are stored in amber screw-cap vials at −20 °C and are stable for at least six months under these conditions.

4.5 Calibration standards

- Stock solution SPMA (1 g/l)

  10 mg of the SPMA standard are weighed exactly into a 10-ml volumetric flask, which is then made up to the mark with methanol.

- Stock solution SBMA (1 g/l)

  10 mg of the SBMA standard are weighed exactly into a 10-ml volumetric flask, which is then made up to the mark with methanol.

- Spiking solution 1 (5 mg/l)

  50 µl of each stock solution of SPMA and SBMA are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with 1% aqueous formic acid.

- Spiking solution 2 (0.5 mg/l)

  1 ml of spiking solution 1 is pipetted into a 10-ml volumetric flask, which is then made up to the mark with 1% aqueous formic acid.

The solutions are stored in amber screw-cap vials at −20 °C. Under these conditions, they are stable for at least six months.

The calibration standards are prepared in acidified, filtered pooled urine obtained from individuals with no known exposure to benzene, benzyl chloride, or toluene. In order to keep the background levels of the analysed mercapturic acids in the pooled urine used as low as possible, only urine samples from non-smokers should be used. To prepare the pooled urine, spot urine samples are collected in a suitable container, thoroughly mixed, and stored at −20 °C until preparation of the standards and control material. After thawing, the pooled urine is passed through a folded filter in order to remove precipitated proteins. Following this step, the urine is acidified using concentrated hydrochloric acid to an acid concentration level of approximately 1%.

This pooled urine is used to prepare calibration standards in a concentration range between 0.5 and 200 µg/l, according to the pipetting scheme shown in Table 2. Unspiked pooled urine is included as blank value.

Tab. 2 Pipetting scheme for the preparation of calibration standards for the determination of SPMA and SBMA in urine

Calibration standard	Spiking solution 1 [µl]	Spiking solution 2 [µl]	Final volume [ml]	Analyte level [µg/l]
1	–	25	25	0.5
2	–	100	25	2
3	25	–	25	5
4	125	–	25	25
5	250	–	25	50
6	500	–	25	100
7	1000	–	25	200

The calibration standards are aliquoted into quantities of 1 ml each and stored in 1.8-ml vials at −20 °C. Under these conditions, they are stable for approximately six months.

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine samples are collected in sealable plastic containers and acidified with concentrated hydrochloric acid (1 ml concentrated hydrochloric acid per 100-ml urine sample). The pH value of the samples should be ≤ 1. If necessary, the pH value can be adjusted by further addition of concentrated hydrochloric acid. The samples are then frozen at −20 °C. Under these conditions, the urine samples can be stored without analyte loss for at least six months.

5.2 Sample preparation

Prior to analysis, the samples are thawed at room temperature and thoroughly mixed. 1 ml of each sample is transferred into a separate 1.8-ml vial and 20 µl of the ISTD spiking solution are added. The samples are then mixed thoroughly by shaking. Particularly high-protein samples are centrifuged at 800 × *g* for five minutes, followed by transfer of the supernatant into a new 1.8-ml screw-cap vial. The samples can then be directly injected into the LC-MS/MS system for analysis.

6 Operational parameters

Analysis was performed using an HPLC system with two pumps coupled with a tandem-mass spectrometer.

6.1 High-performance liquid chromatography

Analytical column:	Phenomenex Luna C8 (2), 150 mm × 4.6 mm × 3 µm
Pre-concentration column:	LiChrospher RP-8 ADS, 25 mm × 4 mm × 25 µm
Separation principle:	Reversed phase
Mobile phase:	A: 0.05% aqueous formic acid (pH 2.5) B: 100% acetonitrile C: mobile phase A : acetonitrile, 95 : 5 (v/v)

Flow rate:	0.3 ml/min
Column temperature:	35 °C, isothermal
Injection volume:	100 µl
Isocratic pump:	100% mobile phase C
Gradient pump:	Gradient program see Table 3

Tab. 3 Program of the gradient pump

Time [min]	Mobile phase A [Vol.-%]	Mobile phase B [Vol.-%]
0	60	40
6.2	60	40
10.2	0	100
16.2	0	100
19.2	60	40
22.2	60	40

All other parameters have to be optimised in accordance with the manufacturer's specifications.

6.2 Tandem mass spectrometry

All ion source settings and MRM parameters are instrument-specific and must be adjusted individually by the user. The specified parameters are therefore intended as a rough guide only.

Ionisation mode:	negative electrospray ionisation (ESI–)
Source temperature:	450 °C
Detection mode:	MRM (Multiple Reaction Monitoring)
Curtain gas:	6 psi
Nebuliser gas:	6 psi
Ion-spray voltage:	–4500 V
Dwell time:	300 ms
Parameter-specific settings:	see Table 4

Tab. 4 Parameter-specific settings and retention times for the determination of SPMA and SBMA in urine (DP: declustering potential, CE: collision energy)

Analyte/ISTD	Retention time [min]	Ion transitions [*m/z*]		DP [V]	CE [V]
		Parent-ion	Product-ion		
d_5-SPMA	14.22	243	114	–16	–14
SPMA	14.23	238	109	–71	–16
SBMA	14.53	252	123	–31	–20

6.3 Six-port valve

A six-port valve controlled by the autosampler is used to enable analyte enrichment on the RAM phase followed by the transfer of the analytes onto the analytical column in backflush mode. The schematic diagram for the six-port valve is shown in Figure 4 and the column-switching program used is given in Table 5. The specified switching times were assessed for the system used in the course of method development and may have to be optimised for systems of other manufacturers.

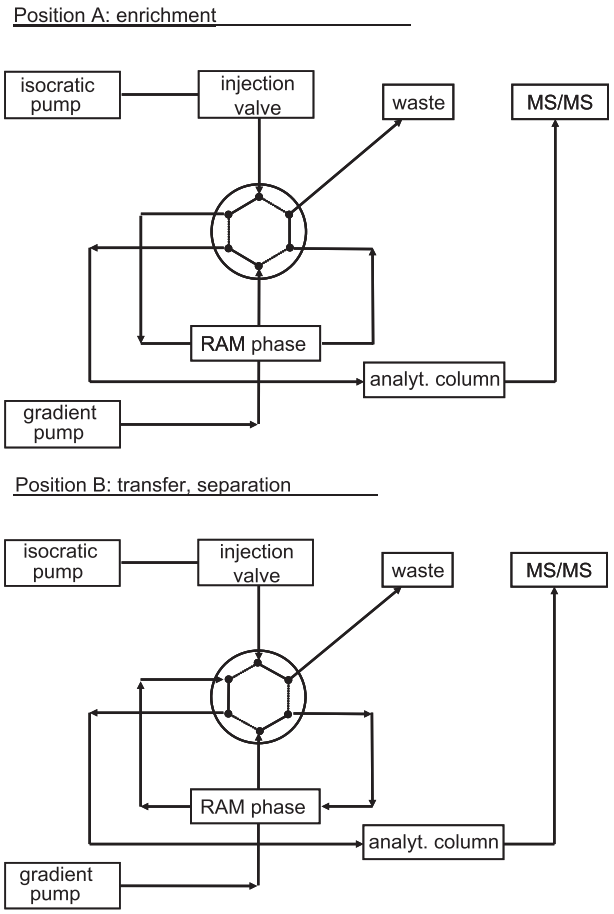


Fig. 4 Schematic diagram illustrating the column-switching procedure

Tab. 5 Switching program of the six-port valve

Time [min]	Switching position	Description
0–3.8	A	Enrichment of the analytes on the RAM phase using the isocratic pump, equilibration of the analytical column
3.8–4.8	B	Transfer of the analytes onto the analytical column using the gradient pump, chromatographic separation of the analytes
4.8–22.2	A	Reconditioning of the RAM phase using the isocratic pump, chromatographic separation of the analytes using the gradient pump (up to 19.2 min), then equilibration of both columns

7 Analytical determination

100 µl of each of the urine samples prepared according to Section 5.2 are injected into the LC-MS/MS system. Identification of the analytes is based on retention times and specific ion transitions. The temporal profiles of the ion transitions shown in Table 4 are recorded in the MRM mode of the tandem-mass spectrometer (negative electrospray ionisation).

The retention times given in Table 4 are intended as a rough guide only. Users of the method must ensure proper separation performance of the HPLC column used influencing the resulting retention behaviour of the analytes. Figures 5 a and 5 b show chromatograms of a processed urine sample from a non-smoker with no known exposure to benzene, benzyl chloride, or toluene. Figure 6 shows the product-ion spectra of SPMA and SBMA with the postulated fragment structures.

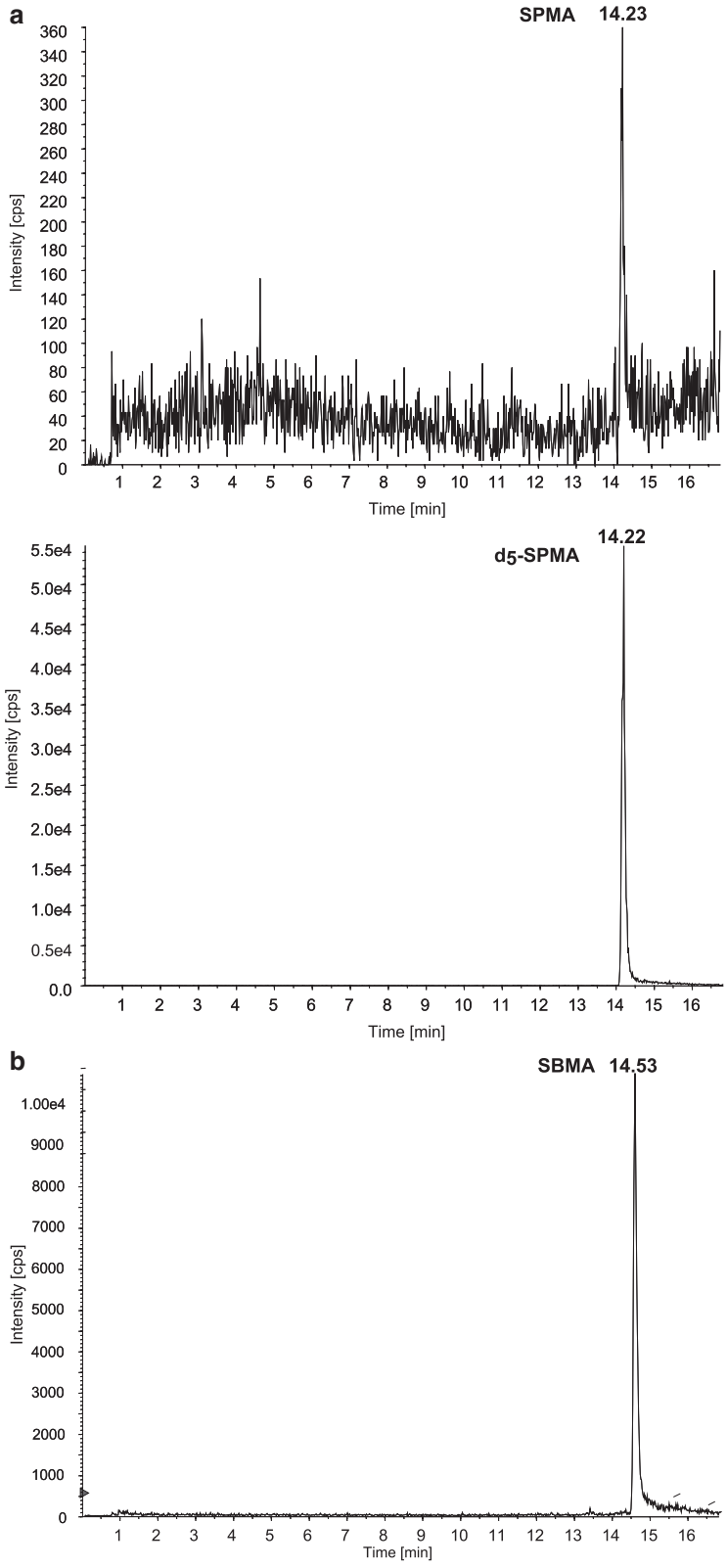


Fig. 5 **a** Chromatogram of a processed non-smoker's urine sample (creatinine level: 0.58 g/l) with a determined SPMA level of 0.11 µg/l. **b** Chromatogram of a processed non-smoker's urine sample (creatinine level: 0.58 g/l) with a determined SBMA level of 3.4 µg/l

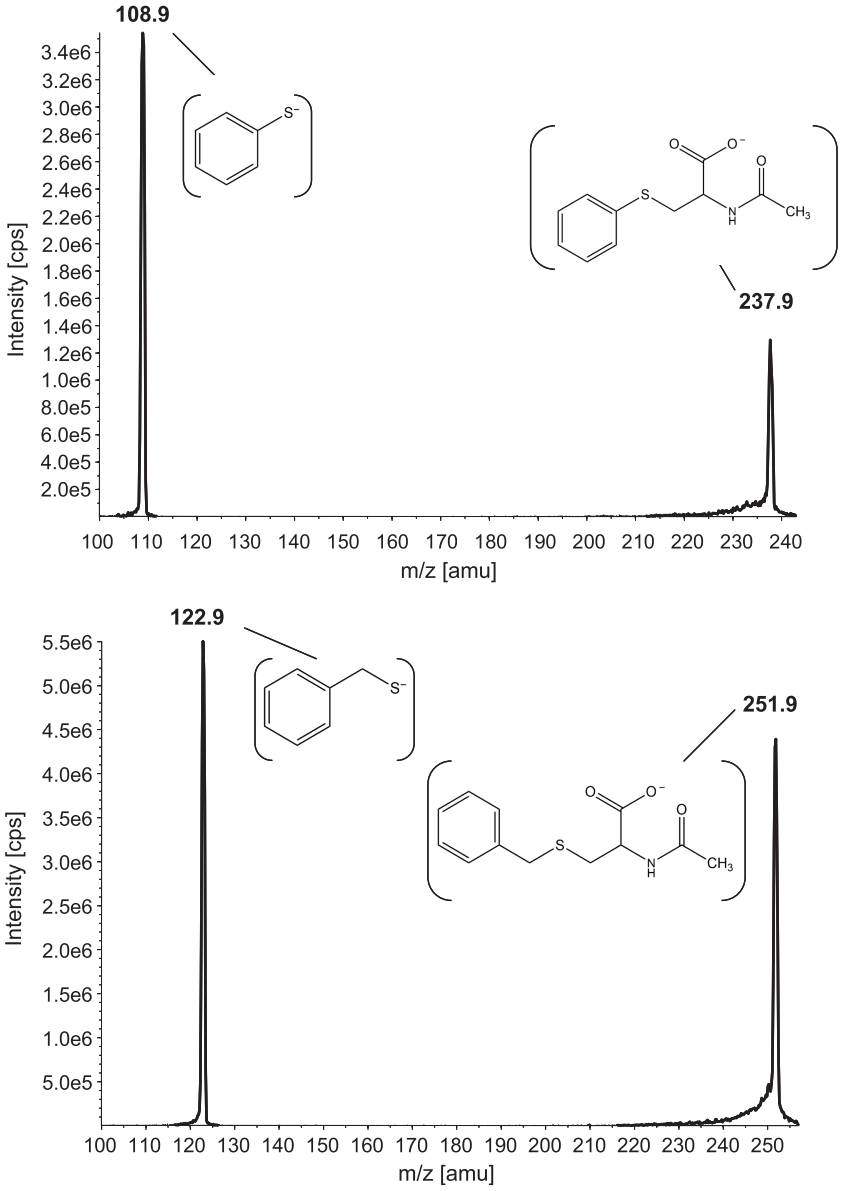
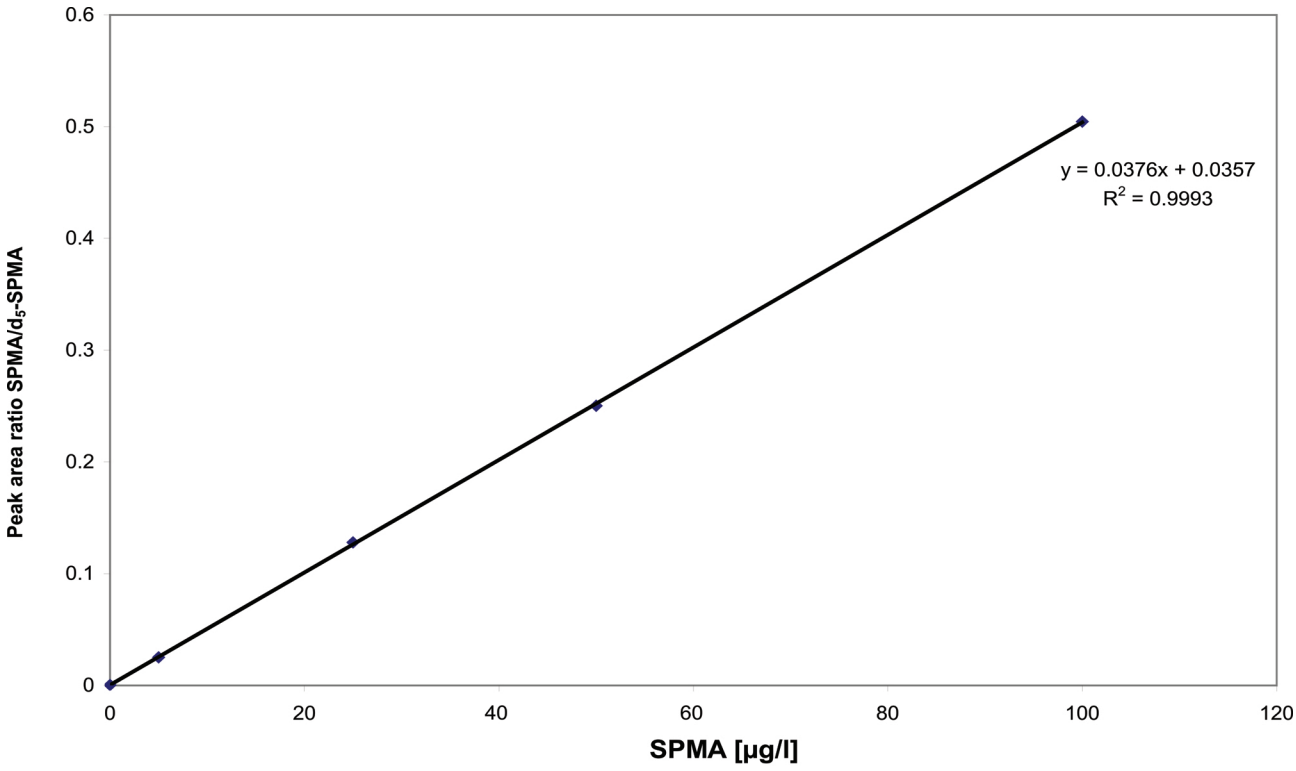


Fig. 6 Product-ion spectra of SPMA and SBMA with postulated fragment ions

8 Calibration

The calibration standards described in Section 4.5 are prepared and processed in the same way as the samples (cf. Section 5) and analysed using LC-MS/MS (cf. Section 6). Calibration curves are obtained by plotting the peak area ratios of the respective analyte and the isotope-labelled internal standard against the concentrations of the calibration standards. The internal standard d_5-SPMA is also used for SBMA. Under the analytical conditions described, the calibration curves are linear in the concentration range from 0.5 to 200 µg/l. If the determined analyte levels exceed the linear range of the calibration function, the urine samples are diluted with ultra-pure water, e.g. at a ratio of 1 : 10, reprocessed, and newly analysed. Figure 7 shows representative calibration curves of SPMA and SBMA in pooled urine.



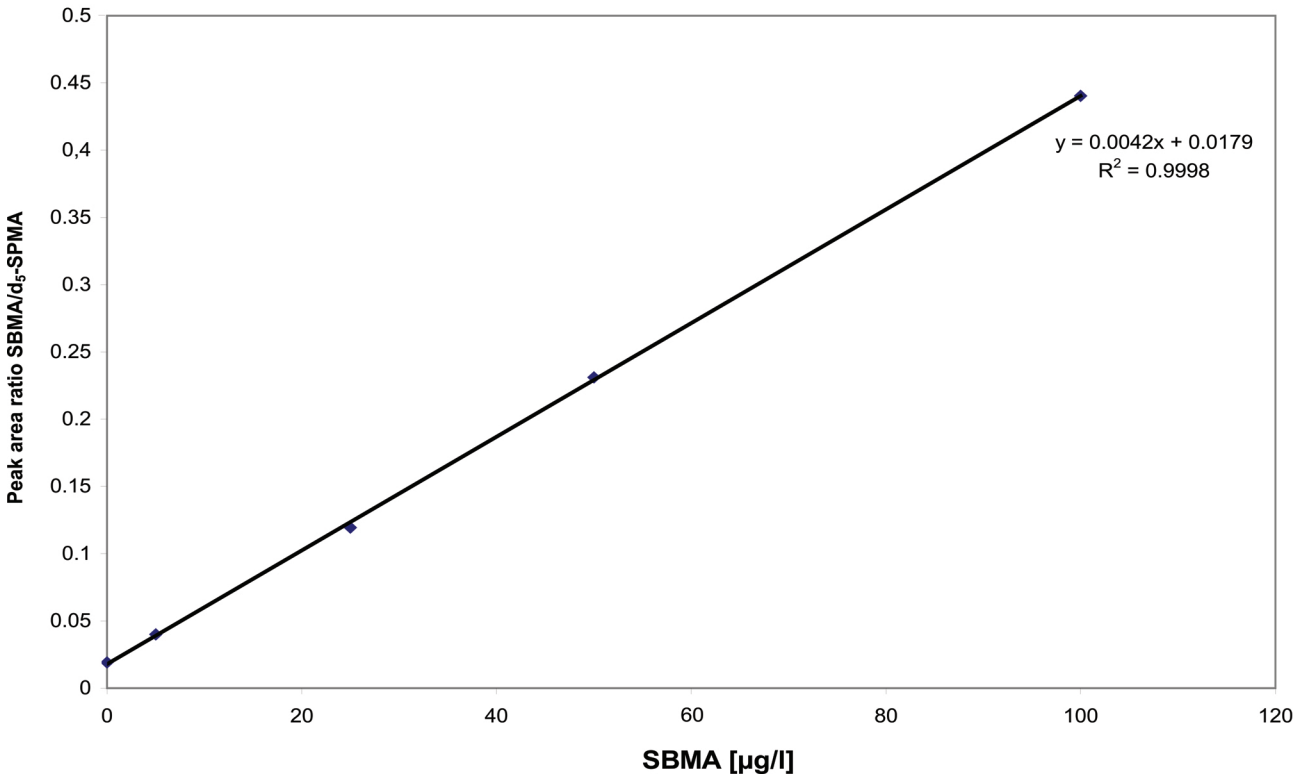


Fig. 7 Calibration curves of SPMA and SBMA in pooled urine (creatinine level: 0.52 g/l)

9 Calculation of the analytical results

The analyte concentrations in the urine samples are calculated using the calibration functions of the respective analytical run (Section 8). To calculate the analyte concentrations in a urine sample, the peak area of the respective analyte is divided by the peak area of the internal standard. The quotients thus obtained are entered in the calibration functions established according to Section 8 to give the respective analyte concentration in µg/l. Any reagent blank values have to be subtracted from the analytical results.

10 Standardisation and quality-control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014). To check precision, at least two quality-control samples with known analyte concentrations are analysed within each analytical run. As material for quality control is not commercially available, it must be prepared in the laboratory. To this end, the standard solutions of the analytes are added to pooled urine at two different concentration levels, resulting in analyte levels of the control material within the relevant concentration range. Aliquots of these samples are stored at −20 °C and are included in each analytical run as quality-control samples. The nominal values and tolerance ranges of the respective quality-control materials are determined in a pre-analytical period (one analysis of each control material on ten different days) (Bader et al. 2010). In addition to the quality-control samples, a reagent blank (ultra-pure water instead of urine) is also included in each analytical run.

11 Evaluation of the method

The reliability of the method was verified by comprehensive validation as well as by implementation and replication of the method in a second, independent laboratory.

11.1 Precision

Within-day precision was determined using pooled urine from non-smokers, which was spiked with SPMA at three different concentration levels. For SBMA, the native pooled urine was used as well as pooled urine spiked with 70 µg of SBMA per litre. These samples were processed eight times in parallel and analysed. The obtained precision data are presented in Table 6.

Tab. 6 Within-day precision for the determination of SPMA and SBMA in urine (n = 8)

Analyte	Mean concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
SPMA	0.43	10.4	24.6
	20.2	5.8	13.7
	68.4	2.8	6.6
SBMA	5.1[a)]	3.3	7.8
	77.2	2.8	6.6

[a)] native concentration/background level

To determine day-to-day precision, the same sample material was used as for determining within-day precision. The samples were processed on eleven or five days (for the lowest spiked SPMA concentration) and analysed. The obtained day-to-day precision data are presented in Table 7.

Tab. 7 Day-to-day precision for the determination of SPMA and SBMA in urine (n = 11 or 5)

Analyte	Mean concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
SPMA	0.45	12.2	33.9
	20.1	7.3	16.3
	71.9	4.2	9.4
SBMA	5.2[a)]	10.0	22.3
	73.5	2.8	6.2

[a)] native concentration/background level

11.2 Accuracy

Recovery experiments were performed to determine the accuracy of the method. To this end, the within-day precision data was used. Background levels were subtracted and the relative recovery rates thus obtained are presented in Table 8.

Tab. 8 Mean relative recovery rates for the determination of SPMA and SBMA in urine (n = 8)

Analyte	Spiked concentration [µg/l]	Mean relative recovery r [%]	Range [%]
SPMA	20	101	94–109
	70	98	94–103
SBMA	70	103	100–107

In addition, relative recovery rates were also determined in five individual urine samples with creatinine levels ranging between 0.34 g/l and 1.38 g/l. To this end, the individual urine samples were spiked with 5 µg/l of each analyte. The relative recovery rates determined in these samples are presented in Table 9.

Tab. 9 Relative recovery rates for the determination of SPMA and SBMA in individual urine samples

Sample	Creatinine level [g/l]	Spiked concentration [µg/l]	Relative recovery rate r [%]	
			SPMA	SBMA
1	0.34	5	88	91
2	0.67	5	116	138
3	0.99	5	108	106
4	1.11	5	102	59
5	1.38	5	127	107

External quality-control for the determination of SPMA was assured by successful participation in the ring trials No. 39 and 40 carried out by the German Society of Occupational and Environmental Medicine (*Deutsche Gesellschaft für Arbeitsmedizin und Umweltsmedizin; DGAUM*) under the German External Quality Assessment Scheme (G-EQUAS). The results obtained using the method described herein are presented in Table 10.

Tab. 10 G-EQUAS results for the determination of SPMA in urine

G-EQUAS No.	Determined level [µg/l]	Nominal value [µg/l]	Deviation [%]
39 A	15.5	13.6	14.0
39 B	41.0	34.0	20.6
40 A	16.6	15.5	7.1
40 B	29.0	27.4	5.8

11.3 Stability of the analytes

In order to investigate the stability of the analytes after several freeze-thaw cycles, three quality-control samples, each spiked with 70 µg analyte per litre, were thawed and then refrozen at −20 °C on three days. Afterwards, the samples were processed and analysed.

The measured values of these samples were within the tolerance ranges of the quality-control material. Thus, degradation of the analytes by triplicate freezing and thawing was not observed.

11.4 Limits of detection and limits of quantitation

Under the specified conditions of sample preparation and LC-MS/MS analysis, the limits of detection and quantitation shown in Table 11 were obtained. The detection limit was estimated on the basis of a signal-to-noise ratio of 3 : 1, while the quantitation limit was estimated on the basis of a signal-to-noise ratio of 9 : 1.

Tab. 11 Limits of detection and quantitation for the determination of SPMA and SBMA in urine

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
SPMA	0.05	0.15
SBMA	0.5	1.5

11.5 Sources of error

Depending on the individual matrix of the urine samples to be analysed, ionisation of the analytes may be suppressed (quenching) to some extent. However, purification of the samples using the RAM phase results in a very good separation of interfering matrix components. Nevertheless, quenching was observed in a few cases with high-protein samples, which resulted in somewhat higher quantitation limits than specified in Section 11.4.

After the injection of calibration standards containing high analyte levels, carryover effects may occur. In this case, appropriate measures should be taken to avoid carryover of the analytes. If necessary, a water blank has to be injected after the injection of standards or samples with high analyte levels. Interference peaks or reagent blanks have not been observed so far.

12 Discussion of the method

This method is based on an analytical method developed by Gonzalez-Reche et al. (2003) for the determination of the dimethylphenyl mercapturic acids of xylene and was optimised in its present form for the determination of SPMA and SBMA (Schettgen et al. 2008).

The method permits the simultaneous, sensitive, and specific quantitation of the mercapturic acids of benzene, benzyl chloride, and toluene in urine, enabling detection of both workers' occupational exposure and the background levels in the general population.

This method has a decisive advantage in that it requires little effort for sample preparation, making it ideal for the analysis of larger series of samples in occupational or environmental health studies. Matrix components are separated effectively during RAM purification over a period of about 4 minutes, and a good retention of the mercapturic acids is achieved even when using an isocratic eluent with 5% acetonitrile.

During the metabolism of benzene epoxide, a metabolic precursor of SPMA is formed, the "pre-SPMA" (cf. Figure 1), which is also excreted in urine and whose existence has already been postulated by Paci et al. (2007). Studies conducted by the author and external verifier of this method were able to determine this pre-SPMA in the urine of workers exposed to benzene. In these studies, pre-SPMA reacted quantitatively to SPMA if the pH value of the urine sample was adjusted to ~ 1 (Sterz et al. 2010). Further analyses during external verification showed that drastic hydrolysis conditions resulted in significantly higher SPMA levels of unknown origin.

To ensure a reproducible and comparable analysis, the described pH adjustment with concentrated hydrochloric acid to a value of pH ≤ 1 is also recommended in another method for the determination of SPMA in urine already published by the Commission (Müller and Jeske 1996). Using this procedure of pH adjustment, urine samples of workers exposed to benzene were analysed in parallel during method development and external verification, and highly consistent results were obtained.

The reliability data of the method show that matrix effects are effectively compensated for by the use of the isotope-labelled internal standard d_5-SPMA. However, by now, an isotope-labelled internal standard for SBMA (d_3-SBMA) is also commercially available (e. g. Toronto Research Chemicals Inc., Toronto, Canada, No. A168427).

Instruments used 1100 Series HPLC system with integrated autosampler by Agilent Technologies Deutschland GmbH, Waldbronn, Germany; API 3000 tandem-mass spectrometric detector by AB Sciex Germany GmbH, Darmstadt, Germany; six-port valve by VICI AG International, Schenkon, Switzerland; evaluation software Analyst Version 1.3 by AB Sciex Germany GmbH, Darmstadt, Germany.

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (https://www.dfg.de/en/dfg_profile/statutory_bodies/senate/health_hazards/conflicts_interest/index.html) ensure that the content and conclusions of the publication are strictly science-based.

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