Benzene, toluene, *o*-xylene, *m*-xylene, *p*-xylene, ethylbenzene, styrene, isopropylbenzene (cumene) – Determination of aromatic compounds in urine by dynamic headspace GC-MS

Biomonitoring Method – Translation of the German version from 2018

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Keywords: benzene; toluene; xylene; ethylbenzene; styrene; isopropylbenzene; cumene; biomonitoring; urine; headspace-GC-MS

Citation Note: Van Pul J, Roßbach B, Göen T, Hartwig A, MAK Commission. Benzene, toluene, *o*-xylene, *m*-xylene, *p*-xylene, ethylbenzene, styrene, isopropylbenzene (cumene) – Determination of aromatic compounds in urine by dynamic headspace-GC-MS. Biomonitoring Method – Translation of the German version from 2018. MAK Collect Occup Health Saf [Original edition. Weinheim: Wiley-VCH; 2018 Jul;3(3):1663-1686]. Corrected republication without content-related editing. Düsseldorf: German Medical Science; 2025. https://doi.org/10.34865/bi7143e2218a_w

Republished (online): 12 Dec 2025

Originally published by Wiley-VCH Verlag GmbH & Co. KGaA; https://doi.org/10.1002/3527600418.bi7143e2218a

Manuscript completed: 10 Oct 2013

Published (online): 26 Jul 2018

The commission established rules and measures to avoid conflicts of interest.

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Biomonitoring Methods

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DOI: 10.1002/3527600418.bi7143e2218a

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 verified the presented biomonitoring method.

The analytical method described hereinafter permits the simultaneous determination of the following unmetabolised aromatic compounds in urine: benzene, toluene, o-xylene, m-xylene, p-xylene, ethylbenzene, styrene and isopropylbenzene (cumene). Prior to the determination of the analytes by GC-MS, the analytes are extracted and enriched using ITEX (In Tube Extraction) or SPDE (Solid Phase Dynamic Extraction). To this end, the urine samples are incubated at 50 °C, the analytes extracted from the gas phase and then the enriched analytes are transferred to the gas chromatograph and analysed using mass spectrometry. Calibration standards are prepared in water and processed in the same way as the samples to be analysed. Deuterated benzene is used as internal standard.

The method was extensively validated and the reliability data were confirmed by an independent laboratory, which has established and cross-checked the whole procedure.

Keywords

benzene; toluene; o-xylene; m-xylene; p-xylene; ethylbenzene; styrene; iso-propylbenzene; cumene; aromatic compounds; urine; biomonitoring; Analyses in Biological Materials; SPDE; ITEX; headspace-gas chromatography mass spectrometry; HS-GC-MS

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Benzene, toluene, o-xylene, m-xylene, p-xylene, ethylbenzene, styrene, isopropylbenzene (cumene) – Determination of aromatic compounds in urine by dynamic headspace GC-MS

Matrix:	Urine
Hazardous substances:	Benzene, toluene, o-xylene, m-xylene, p-xylene, ethylbenzene, styrene, isopropylbenzene (cumene)
Analytical principle:	Dynamic headspace gas chromatography with mass spectrometric detection (HS-GC-MS)
Completed in:	October 2013

Overview of the parameters that can be determined with this method and the corresponding hazardous substances:

Hazardous substance	CAS	Parameter	CAS
Benzene	71-43-2	Benzene	71-43-2
Toluene	108-88-3	Toluene	108-88-3
o-Xylene	95-47-6	o-Xylene	95-47-6
m-Xylene	108-38-3	m-Xylene	108-38-3
p-Xylene	106-42-3	p-Xylene	106-42-3
Ethylbenzene	100-41-4	Ethylbenzene	100-41-4
Styrene	100-42-5	Styrene	100-42-5
Isopropylbenzene (cumene)	98-82-8	Isopropylbenzene (cumene)	98-82-8

Summary

The analytical method described hereinafter permits the simultaneous determination of the following unmetabolised aromatic compounds in urine: benzene, toluene, o-xylene, m-xylene, p-xylene, ethylbenzene, styrene and isopropylbenzene (cumene). Prior to the determination of the analytes by GC-MS, the analytes are

extracted and enriched using ITEX (In Tube Extraction) or SPDE (Solid Phase Dynamic Extraction). To this end, the urine samples are incubated at 50 °C, the analytes extracted from the gas phase and then the enriched analytes are transferred to the gas chromatograph and analysed using mass spectrometry. Calibration standards are prepared in water and processed in the same way as the samples to be analysed. Deuterated benzene is used as internal standard.

Reliability data of the method

Benzene

Within-day precision:	Standard deviation (rel.)	s_w = 4.0% or 1.9%
	Prognostic range	u = 9.5% or 4.5%
	at a spiked concentration of 0.15 µg or 1.5 µg benzene per litre urine and where n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 12.4% or 6.1%
	Prognostic range	u = 28.6% or 14.1%
	at a spiked concentration of 0.15 µg or 1.5 µg benzene per litre urine and where n = 9 determinations	
Accuracy:	Recovery rate (rel.)	r = 107% or 106%
	at a spiked concentration of 0.15 µg or 1.5 µg benzene per litre urine and where n = 8 determinations	
Detection limit:	0.007 µg benzene per litre urine	
Quantitation limit:	0.021 µg benzene per litre urine	

Toluene

Within-day precision:	Standard deviation (rel.)	s_w = 2.2% or 1.8%
	Prognostic range	u = 5.2% or 4.3%
	at a spiked concentration of 0.15 µg or 1.5 µg toluene per litre urine and where n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 21.8% or 2.6%
	Prognostic range	u = 50.3% or 6.0%
	at a spiked concentration of 0.15 µg or 1.5 µg toluene per litre urine and where n = 9 determinations	
Accuracy:	Recovery rate (rel.)	r = 110% or 107%
	at a spiked concentration of 0.15 µg or 1.5 µg toluene per litre urine and where n = 8 determinations	
Detection limit:	0.029 µg toluene per litre urine	
Quantitation limit:	0.087 µg toluene per litre urine	

o-Xylene

Within-day precision:	Standard deviation (rel.)	s_w = 2.3% or 1.6%
	Prognostic range	u = 5.4% or 3.8%
	at a spiked concentration of 0.15 µg or 1.5 µg o-xylene per litre urine and where n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 4.3% or 4.7%
	Prognostic range	u = 9.9% or 10.8%
	at a spiked concentration of 0.15 µg or 1.5 µg o-xylene per litre urine and where n = 9 determinations	
Accuracy:	Recovery rate (rel.)	r = 117% or 100%
	at a spiked concentration of 0.15 µg or 1.5 µg o-xylene per litre urine and where n = 8 determinations	
Detection limit	0.015 µg o-xylene per litre urine	
Quantitation limit:	0.045 µg o-xylene per litre urine	

m-Xylene

Within-day precision:	Standard deviation (rel.)	s_w = 2.6% or 1.5%
	Prognostic range	u = 6.1% or 3.6%
	at a spiked concentration of 0.15 µg or 1.5 µg m-xylene per litre urine and where n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 5.0% or 5.2%
	Prognostic range	u = 11.5% or 12.0%
	at a spiked concentration of 0.15 µg or 1.5 µg m-xylene per litre urine and where n = 9 determinations	
Accuracy:	Recovery rate (rel.)	r = 122% or 102%
	at a spiked concentration of 0.15 µg or 1.5 µg m-xylene per litre urine and where n = 8 determinations	
Detection limit:	0.011 µg m-xylene per litre urine	
Quantitation limit:	0.033 µg m-xylene per litre urine	

p-Xylene

Within-day precision:	Standard deviation (rel.)	s_w = 2.1% or 1.4%
	Prognostic range	u = 5.0% or 3.3%
	at a spiked concentration of 0.15 µg or 1.5 µg p-xylene per litre urine and where n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 4.9% or 5.8%
	Prognostic range	u = 11.3% or 13.4%
	at a spiked concentration of 0.15 µg or 1.5 µg p-xylene per litre urine and where n = 9 determinations	

Accuracy: Recovery rate (rel.) $r = 103\%$ or 99% at a spiked concentration of 0.15 µg or 1.5 µg p-xylene per litre urine and where n = 8 determinations

Detection limit: 0.011 µg p-xylene per litre urine

Quantitation limit: 0.033 µg p-xylene per litre urine

Ethylbenzene

Within-day precision: Standard deviation (rel.) $s_w = 2.2\%$ or 1.6%

Prognostic range $u = 5.2\%$ or 3.8%

at a spiked concentration of 0.15 µg or 1.5 µg ethylbenzene per litre urine and where n = 8 determinations

Day-to-day precision: Standard deviation (rel.) $s_w = 4.5\%$ or 5.2%

Prognostic range $u = 10.4\%$ or 12.0%

at a spiked concentration of 0.15 µg or 1.5 µg ethylbenzene per litre urine and where n = 9 determinations

Accuracy: Recovery rate (rel.) $r = 102\%$ or 101% at a spiked concentration of 0.15 µg or 1.5 µg ethylbenzene per litre urine and where n = 8 determinations

Detection limit: 0.010 µg ethylbenzene per litre urine

Quantitation limit: 0.030 µg ethylbenzene per litre urine

Styrene

Within-day precision: Standard deviation (rel.) $s_w = 3.1\%$ or 1.8%

Prognostic range $u = 7.3\%$ or 4.3%

at a spiked concentration of 0.15 µg or 1.5 µg styrene per litre urine and where n = 8 determinations

Day-to-day precision: Standard deviation (rel.) $s_w = 4.1\%$ or 5.9%

Prognostic range $u = 9.5\%$ or 13.6%

at a spiked concentration of 0.15 µg or 1.5 µg styrene per litre urine and where n = 9 determinations

Accuracy: Recovery rate (rel.) $r = 117\%$ or 97% at a spiked concentration of 0.15 µg or 1.5 µg styrene per litre urine and where n = 8 determinations

Detection limit: 0.014 µg styrene per litre urine

Quantitation limit: 0.042 µg styrene per litre urine

Isopropylbenzene (cumene)

Within-day precision: Standard deviation (rel.) s_w = 2.4% or 1.9%
Prognostic range u = 5.7% or 4.5%
at a spiked concentration of 0.15 µg or 1.5 µg cumene per litre urine and where n = 8 determinations

Day-to-day precision: Standard deviation (rel.) s_w = 5.6% or 4.7%
Prognostic range u = 12.9% or 10.8%
at a spiked concentration of 0.15 µg or 1.5 µg cumene per litre urine and where n = 9 determinations

Accuracy: Recovery rate (rel.) r = 98% or 100%
at a spiked concentration of 0.15 µg or 1.5 µg cumene per litre urine and where n = 8 determinations

Detection limit: 0.012 µg cumene per litre urine

Quantitation limit: 0.036 µg cumene per litre urine

General information on the hazardous substances. At the workplace, aromatic compounds are mostly used in solvent mixtures, so that persons subjected to aromatic solvent exposure are often exposed to a number of the aforementioned aromatic compounds at the same time. All aromatic compounds that can be determined by this method have been toxicologically evaluated by the Commission. For details on the toxicological evaluation, please refer to the particular MAK and BAT Value Documentations of the individual substances. Table 1 gives an overview of the individual classifications. Meanwhile, there are also assessment values for unmetabolised benzene and toluene in urine available (see Table 1). Figure 1 shows the structures of the analytes that can be determined by this method.

This method is used to analyse unmetabolised aromatic compounds in urine. It is thus predominantly suitable for detecting acute exposure that occurred a few hours or a few days before sampling.

Table 1 Classification of aromatic compounds by the Commission [DFG 2017][a].

Substance	Designation H or S	Carcinogen category	Germ cell mutagen category	Assessment value in urine	Maximum workplace conc. (MAK)
Benzene	H	1	3A	BAR: 0.3 µg/L EKA	–
Toluene	H	–	–	BAT: 75 µg/L	50 mL/m^3
Xylene (all isomers)	H	–	–	–	100 mL/m^3
Ethylbenzene	H	4	–	–	20 mL/m^3
Styrene	–	5	–	–	20 mL/m^3
Cumene	H	3B	–	–	10 mL/m^3

[a] BAT: biological tolerance value; BAR: biological reference value; EKA: exposure equivalent for carcinogenic substances

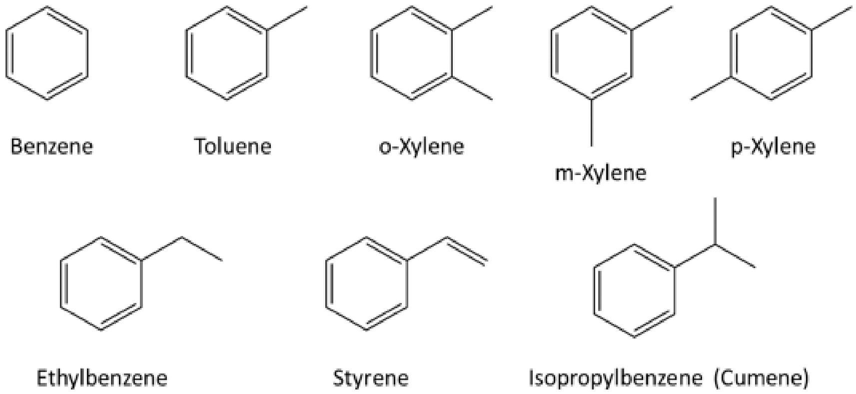

Figure 1 Chemical structures of the analytes.

Benzene occurs naturally as a component of crude oil. Other natural sources include gas emissions from volcanoes and forest fires. The major non-occupational exposure sources for benzene are tobacco smoke, refuelling of combustion engines and emissions from combustion engines [Arnold et al. 2013]. Benzene as a mono-constituent substance is produced and/or imported in the European Economic Area in quantities of 1 000 000 to 10 000 000 tonnes per year.

Benzene is used as an intermediate in the production of a wide range of chemical substances such as styrene, cumene and cyclohexane, which in turn are further used for the manufacture of plastics, various resins, nylon and other synthetic fibres. Benzene is also used in the manufacture of some types of rubbers, lubricants, dyes, detergents, drugs, and pesticides. Due to its carcinogenicity, benzene is no longer used as a solvent [Römpp 2017] and is subject to strict restrictions on use under the REACH Regulation (Annex XVII). Benzene is well resorbed after inhalation or oral exposure, accumulates in fatty tissues and can cross the placenta. Percutaneous resorption of benzene is also possible [Henschler 1988; 1992]. It is a well-known fact that approximately 50% of inhaled benzene are resorbed. Depending on the metabolic activity, on average 12% of the total amount of resorbed benzene is exhaled within a few hours. About 33% of resorbed benzene is excreted in urine, approximately 24% of it as conjugated phenol. Benzene metabolites in urine have long been used as reliable parameters to monitor occupational or environmental exposure to benzene [Carrieri et al. 2010; Ghittori et al. 1995; Pople et al. 2002; Weisel 2010]. Only about 0.2% of the benzene is excreted unchanged with the urine. Nevertheless, this parameter is highly suitable for biomonitoring of benzene, especially in the low-dose range [Campagna et al. 2014; Fustinoni et al. 2005]. The excretion of benzene and its metabolites in urine is completed within 24 to 48 h after the end of exposure.

Toluene is a fuel additive and is released into the environment via automobile exhaust emissions. Natural sources of toluene are volcanic eruptions and emissions from forest fires. In industry toluene is primarily used as a solvent for paints, resins, varnishes and adhesives as well as for the extraction of natural products. It is still an important starting material for numerous chemical syntheses [Greim 1996; Römpp 2017]. Apart from inhalation [Greim 1996], dermal absorption [Greim 1998] is one major route of exposure to toluene.

Technical xylene occurs as a mixture of the isomers o-xylene, m-xylene and p-xylene, which is why in practice there is often an exposure to all three isomers. Xylenes are used as solvents for oils, fats, resins, varnishes and paints as well as for degreasing of metals. Furthermore, o-xylene and p-xylene are starting materials for the production of phthalic anhydride and terephthalic acid [Arpe 2007; Römpp 2017]. Xylenes are released into the atmosphere as emissions from auto exhaust or other combustion processes. Inhalation is the main intake route, however, also dermal resorption of xylene is possible.

Ethylbenzene is produced by alkylating benzene with ethylene and is used both as a solvent or diluent and as a starting material for styrene synthesis [Arpe 2007; Römpp 2017]. It is readily resorbed through the lungs and, to a smaller extent, through the skin.

Styrene and cumene serve as starting materials in various industrial processes.

Styrene is primarily used in the production of polymers (polystyrene). The main route of exposure is inhalation. Dermal adsorption plays only a minor role [Henschler 1987].

Cumene is mainly used as an intermediate in the synthesis of acetone, phenol and α-methylstyrene. At the workplace inhalative and percutaneous adsorption are the main ways of exposure [Lehnert and Greim 2001].

In 2016 a biological reference value ("Biologischer Arbeitsstoff-Referenzwert": BAR) of 0.3 µg/L as well as an EKA-correlation (exposure equivalent for carcinogenic substances; "Expositionsäquivalent für krebserzeugende Arbeitsstoffe") (see Table 2) for benzene in urine was evaluated. For unchanged toluene in urine, the biological tolerance value ("Biologischer Arbeitsstoff-Toleranzwert": BAT) is 75 µg/L (cf. Table 1).

Table 2 Excerpt from the EKA correlation for benzene [DFG 2017].

Air Benzene [mg/m^3]	Urine Benzene [µg/L]
0.1	0.5*
0.2	0.8*
0.5	1.5
1.0	2.75
2.0	5.0
3.3	7.5
6.5	12.5

* derived for non-smokers

Some studies have been published measuring the levels of unmetabolised aromatic compounds in the urine of the general population or of occupationally exposed collectives (cf. Table 3). These studies mainly include data on unmetabolised benzene in urine.

According to Janasik et al. [2008], the elimination of unmetabolised aromatic compounds in urine follows a two-compartment model, with half-lives of 0.45 to 0.88 h in the first phase and of 6.7 to 19.2 h in the second phase. The urinary excretion rate in relation to the exposure dose is very low for all analysed aromatic compounds and is usually below 0.1% [Janasik et al. 2008].

Table 3 Levels of unmetabolised aromatic compounds in urine both in the general population and in occupationally exposed collectives.

Study	**Collective**	**Number of samples n**	**Level of the aromatic compound in urine, median (95th percentile) [µg/L]**				
			Benzene	**Toluene**	**o-Xylene**	**m-/p-Xylene**	**Ethylbenzene**
Basilicata et al. 2005	Workers[1]	11	0.21 (>3.0)				
Fustinoni et al. 2005	Workers[2], NS	46	0.34 (2.84)[a]				
	Workers[2], S	32	1.17 (5.11)[a]				
Manini et al. 2008	Workers[3], NS	80	0.16 (0.19)[b]	0.19 (0.22)[b]		0.35 (0.44)[b]	0.40 (0.47)[b]
	Workers[3], S	20	0.79 (1.92)[b]	0.27 (0.38)[b]		0.43 (0.62)[b]	0.46 (0.61)[b]
Fustinoni et al. 2010	General population, NS	65	0.09 (0.18)	0.38 (0.51)	0.04 (0.06)	0.12 (0.17)	0.07 (0.13)
	General population, S	43	0.44 (2.70)	0.44 (0.70)	0.04 (0.08)	0.13 (0.22)	0.07 (0.12)
Campagna et al. 2014	General population, NS	86	0.09 (0.31)				

NS = non-smokers, S = smokers; [1] Fuel depot workers, [2] Filling station attendants, [3] Traffic policemen, [a] maximum value, [b] 75th percentile

Contents

1 General principles

The analytical method described hereinafter permits the simultaneous determination of the following unmetabolised aromatic compounds in urine: benzene, toluene, ethylbenzene, o-xylene, m-xylene, p-xylene, styrene and isopropylbenzene (cumene). Prior to the determination of the analytes by GC-MS, the analytes are extracted and enriched using ITEX (In Tube Extraction) and SPDE (Solid Phase Dynamic Extraction), respectively. To this end, the urine samples are incubated at 50 °C, the analytes extracted from the gas phase and then the enriched analytes are transferred to the gas chromatograph and analysed using mass spectrometry. Calibration standards are prepared in water and processed in the same way as the samples to be analysed. Deuterated benzene is used as internal standard.

2 Equipment, chemicals and solutions

2.1 Equipment

- Gas chromatograph with mass selective detector and data processing system (e.g. Agilent 6890 with Agilent 5975 and Chemstation Software)
- Capillary gas chromatographic column: stationary phase: VF-WAXms, length: 60 m; inner diameter: 0.25 mm; film thickness: 0.5 µm (e.g. VF-WAXms, Agilent No. CP9223)
- Autosampler with dynamic headspace option, e.g. In Tube Extraction (ITEX) or Solid Phase Dynamic Extraction (SPDE) (e.g. CombiPal, CTC Analytics AG), optionally cold trap (e.g. JAS)
- Muffle furnace
- Analytical balance (e.g. Sartorius)
- 20 mL screw cap headspace vials (e.g. Supelco No. SU860097)
- Screw caps with silicone white/ PTFE red septum (e.g. LaPha Pack No. 18031578)
- Various volumetric flasks (e.g. Brand)
- Microlitre syringes
- Microlitre pipettes, adjustable between 10 µL and 100 µL as well as 100 µL and 1000 µL (e.g. Eppendorf Reference 10–100 µL and 100–1000 µL, respectively, Eppendorf, Germany)

2.2 Chemicals

Unless otherwise specified, all chemicals must be at least p.a. grade.

- Benzene (e.g. Merck No. 101783)
- Benzene-d_6 (e.g. Sigma-Aldrich No. 522104)
- Ethylbenzene (e.g. Merck No. 801372)
- Isopropylbenzene (Cumene) (e.g. Sigma-Aldrich No. 36698)
- Methanol (e.g. Merck Nr. 106011)
- Potassium carbonate (e.g. Merck No. 104924)
- Styrene (e.g. Sigma-Aldrich No. 45993)
- Toluene (e.g. Merck No. 100849)
- m-Xylene, 99% (e.g. Alfa Aesar No. L03788)
- o-Xylene, 99% (e.g. Alfa Aesar No. A11358)
- p-Xylene, 99% (e.g. Alfa Aesar No. A10534)
- Ultrapure water (e.g. Millipore Milli-Q)
- Helium 5.0 (e.g. Linde)
- Nitrogen 5.0 (e.g. Linde)
- Urine of non-smokers occupationally not exposed to aromatic compounds (pooled urine)

2.3 Internal standard (IS)

IS stock solution (95 mg/L). After placing approximately 50 mL methanol into a 100 mL volumetric flask, 10 µL benzene-d_6 are added. The flask is then filled up to the mark with methanol and the solution is mixed thoroughly.

IS spiking solution (9.5 mg/L). After placing approximately 10 mL methanol into a 20 mL volumetric flask, 2 mL of the IS stock solution are added. The flask is then filled up to the mark with methanol and the solution is mixed thoroughly.

The solutions can be stored in the refrigerator at 4 °C for at least six months.

2.4 Calibration standards

Stock solution. Approximately 50 mL methanol are placed in a 100 mL volumetric flask and spiked with 10 µL of each analyte. The flask is then filled up to the mark with methanol and the solution is mixed thoroughly. The analyte concentrations in the stock solution are as follows: 91 mg/L styrene, 88 mg/L each of benzene and o-xylene, 87 mg/L each of toluene, m-xylene, p-xylene and ethylbenzene as well as 86 mg/L cumene.

Spiking solution. 20 µL of the stock solution are pipetted into a 20 mL volumetric flask, which is then filled up to the mark with ethanol. The analyte concentrations in the spiking solution are as follows: 91 µg/L styrene, 88 µg/L each of benzene and o-xylene, 87 µg/L each of toluene, m-xylene, p-xylene and ethylbenzene as well as 86 µg/L cumene.

The solutions can be stored in the refrigerator at 4 °C for at least six months.

Table 4 Pipetting scheme for the preparation of calibration standards.

Calibration solution	Volume of the spiking solution [µL]	Analyte concentration in the calibration standard [µg/L]			
		Benzene, o-Xylene	Toluene, m-/p-Xylene, Ethylbenzene	Styrene	Cumene
0	–	0	0	0	0
1	5	0.88	0.87	0.91	0.86
2	10	1.8	1.7	1.8	1.7
3	20	3.5	3.5	3.6	3.4
4	40	7.0	7.0	7.3	6.9
5	60	10.6	10.4	10.9	10.3

Calibration solutions are prepared by placing 5 mL each of ultrapure water into 20 mL headspace vials, which were previously nitrogen-purged for approximately 30 s. Afterwards, 2 µL of the spiking solution of the internal standard are added. The vials are sealed and the volumes of the spiking solution listed in Table 4 are added to the water through the septum using a 25 µL dosing syringe (microliter syringe). The prepared calibration solutions are thoroughly mixed and can then be directly used for analysis.

3 Specimen collection and sample preparation

The collected urine samples are stored at −18 °C until analysis. Prior to analysis, the samples are thawed at room temperature and thoroughly mixed. For analysis, salt is added in excess to the urine samples. To this end, potassium carbonate is dried in a muffle furnace at 350 °C for three hours before use. Afterwards, the salt is cooled to room temperature in a desiccator under a stream of nitrogen for at least 30 min.

For analysis, exactly 5 g (± 0.1 g) of freshly dried potassium carbonate are weighed into a 20 mL headspace vial. The headspace vial containing the salt is thoroughly nitrogen-purged and directly afterwards, a 5 mL aliquot of the urine sample is added. The vial is immediately sealed and the solution thoroughly mixed, releasing the dissolution heat. After cooling to room temperature (approx. 30 min), 2 µL of the spiking solution of the internal standard are added through the septum.

The external verification of the method revealed that the saturation step involving potassium carbonate can be omitted (see Section 9.5).

4 Operational parameters

Analysis was performed using a gas chromatograph coupled to an autosampler for dynamic headspace extraction (ITEX or SPDE), a mass selective detector (MSD) and a data processing system.

4.1 Headspace autosampler

ITEX-Option

Incubation:	15 min at 50 °C
Sorbent:	Tenax TA
Syringe temperature:	110 °C
Extraction volume:	1000 µL
Extraction strokes:	20
Extraction flow rate:	50 µL/s
Desorption volume:	750 µL
Desorption temperature:	250 °C (GC injector)
Desorption flow rate:	20 µL/s
Trap cleaning:	10 min at 250 °C

SPDE-Option

Incubation:	24 min at 50 °C
Sorbent:	Polydimethylsiloxane with 10% activated carbon
Syringe temperature:	60 °C
Needle temperature:	−5 °C
Extraction volume:	1250 µL
Extraction strokes:	40
Extraction flow rate:	200 µL/s
Desorption gas:	Nitrogen
Desorption volume:	250 µL
Desorption temperature:	250 °C (GC injector)
Desorption flow rate:	50 µL/s
Trap cleaning:	24 min at 250 °C (nitrogen flushing)

4.2 Gas chromatography

Capillary column:	Stationary phase:	VF-WAXms
	Length:	60 m
	Inner diameter:	0.25 mm
	Film thickness:	0.5 µm
Detector:	MSD	
Temperatures:	Column:	Initial temperature 38 °C, 2 min hold, increase at a rate of 5 °C/min to 130 °C, then increase at a rate of 30 °C/min to 220 °C, 7 min at final temperature
	Injector:	250 °C
	Transfer line:	280 °C
Carrier gas:	Helium 5.0	
	Flow rate:	1.5 mL/min
Injection:	Split 1:10	

4.3 Mass spectrometry

Ionisation mode:	Electron impact ionisation (EI)
Ionisation energy:	70 eV
Source temperature:	230 °C
Quadrupole temperature:	150 °C
Dwell time:	50 ms
Detection mode:	Single Ion Monitoring (SIM)

All parameters serve as rough guidelines only and may have to be optimised in accordance with the manufacturer's specifications.

5 Analytical determination

For analytical determination of the urine samples prepared as described in Section 3, the samples are extracted by dynamic headspace extraction and the analytes are transferred into the GC-MS system. Identification of the analytes is based on retention times and characteristic ion traces. The time profiles of the ion traces shown in Table 5 are recorded in SIM mode. A quality control sample and a reagent blank value consisting of double-distilled water are included in each analytical run.

The retention times given in Table 5 are intended to be a rough guide only. Users of the method must ensure proper separation performance of the capillary column used influencing the resulting retention behaviour of the analytes. Figure 2 (in the Appendix) shows a GC-MS chromatogram of a standard solution.

6 Calibration

The calibration standards (see Section 2.4) are analysed in the same way as the urine samples according to Sections 4 and 5. Calibration graphs are obtained by plotting the ratio of analyte peak area to internal standard peak area against the spiked concentration. The calibration curves are linear for all analytes between the detection limit and the highest calibration point. Figure 3 (in the Appendix) shows examples of calibration curves of selected analytes.

Table 5 Retention times and detected ion traces of the analytes.

Analyte	**Retention time [min]**	**Ion trace [*m/z*]**	
		Quantifier	**Qualifier**
Benzene	9.55	78	77
Benzene-d_6	9.55	84	82
Toluene	12.40	91	92, 65
Ethylbenzene	15.00	91	106
p-Xylene	15.25	91	105, 106
m-Xylene	15.50	91	105, 106
Cumene	16.45	105	120
o-Xylene	16.90	91	105, 106
Styrene	19.05	104	78, 103

7 Calculation of the analytical results

The analyte concentration in the urine samples is calculated using the calibration function of the respective analytical run (Section 6). In order to determine the analyte concentration in a urine sample, the ratio of analyte peak area to internal standard peak area is determined and entered in the calibration function (Section 6). After any reagent blank values have been subtracted, the analyte concentration in µg/L is obtained.

8 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 of the MAK-Collection for Occupational Health and Safety Part IV: Biomonitoring Methods [Bundesärztekammer 2014; Bader et al. 2010]. To check precision, at least one quality control sample with a known and constant analyte concentration is analysed within each analytical run. As material for quality control is not commercially available, it must be self-prepared in the laboratory. To this end, 5 mL aliquots of pooled urine are pipetted in headspace vials and spiked with a standard solution containing all analytes. The concentration of the quality control material should lie within the relevant concentration range. After thorough mixing, the quality control material thus obtained is stored at −18 °C. The nominal value and the tolerance ranges of the quality control material are determined in a pre-analytical period [Bader et al. 2010]. The concentration level of the quality control material analysed within each run should lie within the tolerance ranges obtained.

9 Evaluation of the method

The reliability of the method was proven by comprehensive validation and by implementation and validation of the procedure in a second, independent laboratory.

9.1 Precision

Pooled urine was spiked with the standard solutions at two different concentrations and processed and analysed eight times each to determine within-day precision. The obtained within-day precision data are presented in Table 6.

Day-to-day precision was determined by processing and analysing urine samples spiked at the same concentration levels on nine different days. The obtained day-to-day precision data are given in Table 7.

Table 6 Within-day precision for the determination of aromatic compounds in urine (n = 8).

Analyte	Spiked concentration c = 0.15 µg/L		Spiked concentration c = 1.5 µg/L	
	Standard deviation (rel.) s_w [%]	Prognostic range u [%]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Benzene	4.0	9.5	1.9	4.5
Toluene	2.2	5.2	1.8	4.3
o-Xylene	2.3	5.4	1.6	3.8
m-Xylene	2.6	6.1	1.5	3.6
p-Xylene	2.1	5.0	1.4	3.3
Ethylbenzene	2.2	5.2	1.6	3.8
Styrene	3.1	7.3	1.8	4.3
Cumene	2.4	5.7	1.9	4.5

Table 7 Day-to-day precision for the determination of aromatic compounds in urine (n = 9).

Analyte	Spiked concentration c = 0.15 µg/L		Spiked concentration c = 1.5 µg/L	
	Standard deviation (rel.) s_w [%]	Prognostic range u [%]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Benzene	12.4	28.6	6.1	14.1
Toluene	21.8	50.3	2.6	6.0
o-Xylene	4.3	9.9	4.7	10.8
m-Xylene	5.0	11.5	5.2	12.0
p-Xylene	4.9	11.3	5.8	13.4
Ethylbenzene	4.5	10.4	5.2	12.0
Styrene	4.1	9.5	5.9	13.6
Cumene	5.6	12.9	4.7	10.8

9.2 Accuracy

Recovery tests were performed to determine the accuracy of the method. To this end, pooled urine was spiked with the aromatic compounds and analysed. Any background levels of the analytes in the pooled urine were subtracted from the result. The results are presented in Table 8.

Table 8 Relative recovery rates for the determination of aromatic compounds in urine (n = 8).

Analyte	Spiked concentration c = 0.15 µg/L		Spiked concentration c = 1.5 µg/L	
	Mean rel. recovery rate [%]	Range [%]	Mean rel. recovery rate [%]	Range [%]
Benzene	107	86–118	106	102–110
Toluene	110	106–117	107	103–110
o-Xylene	117	113–121	100	96–102
m-Xylene	122	116–127	102	98–103
p-Xylene	103	98–107	99	95–100
Ethylbenzene	102	99–107	101	97–103
Styrene	117	111–125	97	93–99
Cumene	98	94–102	100	96–103

Table 9 Precision and relative recovery rates for the determination of aromatic compounds in individual urine samples (n = 6).

Analyte	Spiked concentration c = 0.15 µg/L		Spiked concentration c = 1.5 µg/L	
	Mean rel. recovery rate [%]	Standard deviation (rel.) s_w [%]	Mean rel. recovery rate [%]	Standard deviation (rel.) s_w [%]
Benzene	103	13.4	90	7.3
Toluene	110	9.9	99	7.6
o-Xylene	101	3.1	94	6.8
m-Xylene	218	4.5	84	7.0
p-Xylene	99	4.8	94	6.1
Ethylbenzene	99	4.9	95	5.8
Styrene	92	15.3	98	7.2
Cumene	100	3.7	94	6.7

9.3 Matrix effects

In addition, the precision and accuracy of the method was verified in different urine samples. To this end, six individual urine samples (creatinine level 0.28 to 3.30 g/L) were analysed both unspiked and spiked with the analytes. The results are presented in Table 9.

Table 10 Comparison of the slopes of the calibration curves prepared in water and in pooled urine.

Analyte	Slope of the calibration curve in water	Slope of the calibration curve in pooled urine	Deviation [%]
Benzene	0.196	0.196	−0.4
Toluene	0.346	0.339	−2.2
o-Xylene	0.430	0.411	−4.5
m-Xylene	0.416	0.393	+5.5
p-Xylene	0.431	0.412	−4.6
Ethylbenzene	0.569	0.531	−6.6
Styrene	0.220	0.205	−7.0
Cumene	0.765	0.773	−1.1

Moreover, calibration curves prepared in water and in urine were compared. To this end, calibration standards were prepared both in water and in pooled urine (blank value, 0.1 to 5.0 µg/L), analysed and the slopes of the obtained calibration curves were compared. The results are presented in Table 10.

9.4 Limits of detection and limits of quantitation

The limits of detection and quantitation were calculated from the calibration curve (calibration standards in water) according to ISO 84661-11. The obtained limits of detection and limits of quantitation are presented in Table 11.

9.5 Sources of error

The determination of unmetabolised aromatic compounds in urine bears the risk of sample contamination by the equipment used, the laboratory air or impurities in the reagents used. To avoid or reduce contamination with the analytes, the laboratory vessels and equipment used should be systematically purged under a stream of dry nitrogen. Besides, only high-purity chemicals should be used. Basically, it is mandatory to include reagent blanks, which may have to be taken into account by means of correction factors. For instance, the internal standard benzene-d_6 always contains a small amount of non-deuterated benzene.

Moreover, while verifying the method it was noted that the potassium carbonate used for salting-out was contaminated with several of the analytes. Therefore, the method was verified without the salting-out step described above, leading to comparable and sometimes even better reliability criteria.

To avoid interferences by background levels in pooled urine, calibration can also be performed in water. A comparison of the calibration curves prepared in water and in pooled urine showed only minor deviations (cf. Section 9.3). However, the control material used for quality control should always be prepared in pooled urine.

Table 11 Detection and quantitation limits of the analytes according to ISO 84661-11.

Analyte	Limit of detection [µg/L]	Limit of quantitation [µg/L]
Benzene	0.007	0.021
Toluene	0.029	0.087
o-Xylene	0.015	0.045
m-Xylene	0.011	0.033
p-Xylene	0.011	0.033
Ethylbenzene	0.010	0.030
Styrene	0.014	0.042
Cumene	0.012	0.036

10 Discussion of the method

The method described above is suitable for the simultaneous quantification of eight unmetabolised aromatic compounds in urine. It permits sensitive determination of the analytes with limits of detection ranging between 0.01 and 0.03 µg/L. Besides, the method proved to be very precise for all analytes with relative standard deviations (within-day precision) below 5%. Although only one internal standard (benzene-d_6) was used, the method yielded good relative recovery rates between 97% and 107% for the individual analytes – at a spiked concentration of 1.5 µg/L. Excellent precision and recovery rates could also be achieved in individual urine samples.

Generally, the determination of unmetabolised aromatic compounds in urine can be considered to be very specific as physiological sources of these aromatic compounds have not been identified. Moreover, due to the very low limits of detection it is also possible to detect background exposure, for example to benzene. However, unlike the determination of metabolites, this case involves a certain risk of contamination (cf. Section 9.5) so that due diligence, in both specimen collection and sample preparation, and targeted quality control are imperative.

Biomonitoring of unmetabolised aromatic compounds in urine calls for a very sensitive and also reliable analytical method as on the one hand, the aromatic solvent levels both in the workplace air and in the ambient air have constantly decreased thanks to various measures and as on the other hand, the aromatic compounds are excreted unmetabolised to only a very limited extent.

The high sensitivity of the method is achieved, among other things, by using dynamic headspace extraction (ITEX or SPDE) to enrich the analytes. The ITEX technique takes advantage of a microtrap filled with adsorbent material (Tenax TA), which is placed in a headspace syringe between the barrel and the syringe needle. The heated syringe pumps the headspace gas through the cold trap. The loaded microtrap is then flash-heated and the analytes are desorbed into the GC injector [Jochmann et al. 2008; Rasanen et al. 2010].

An advantage of the ITEX technique (over the classic SPME technique) is that many parameters can be adjusted individually to achieve optimal results. Typical optimised parameters are the nature and amount of the salt used to salt out the analytes, the incubation temperature and time, the extraction volume characterised by the extraction strokes as well as the desorption conditions (such as desorption volume, temperature and flow rate).

Alternatively, SPDE can be used to enrich the analytes from urine. Also with this method, the headspace of the sample is brought into contact with a sorbent material. SPDE uses a temperature-controlled syringe equipped with a stainless steel needle that is coated on the inside with the extraction phase (e.g. polydimethylsiloxane with 10% activated carbon). The analytes are enriched by repeatedly drawing up and reinjecting aliquots of the headspace into the syringe. Optionally, the syringe needle can be cooled to facilitate the enrichment of the volatile substances. Subsequently, the enriched analytes are thermally desorbed from the extraction phase into the GC injector by injecting nitrogen as desorption gas.

Both enrichment methods led to comparable reliability criteria and thus prove the general suitability of both the ITEX and the SPDE technique.

Instruments used:

Gas chromatograph 6890 A with split/splitless injector, mass spectrometric detector MSD 5975 (Agilent, Santa-Clara, USA) and CombiPal autosampler with a dynamic headspace option (either In Tube Extraction (ITEX) or Solid Phase Dynamic Extraction (SPDE) (CTC Analytics AG)).

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12 Appendix

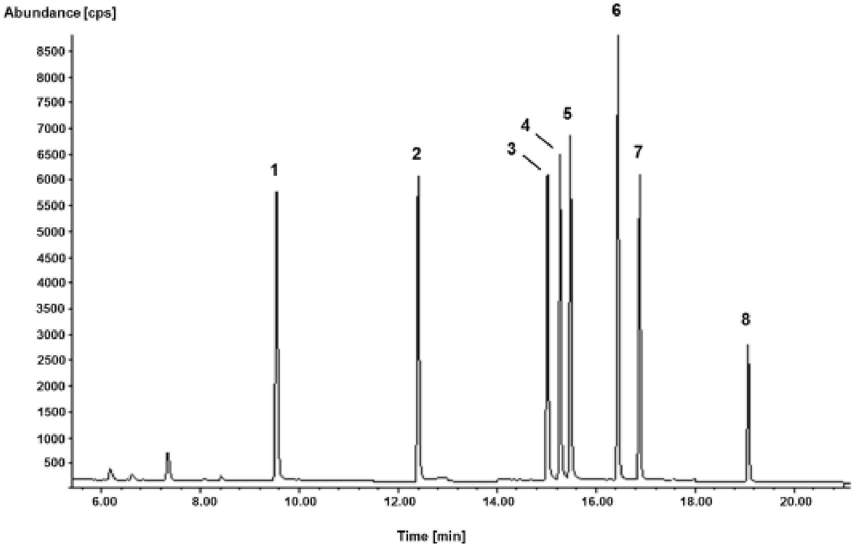

Figure 2 Chromatogram of an aqueous calibration standard with an analyte concentration of 1 µg/L each. (1 – benzene, 2 – toluene, 3 – ethylbenzene, 4 – p-xylene, 5 – m-xylene, 6 – isopropylbenzene, 7 – o-xylene, 8 – styrene).

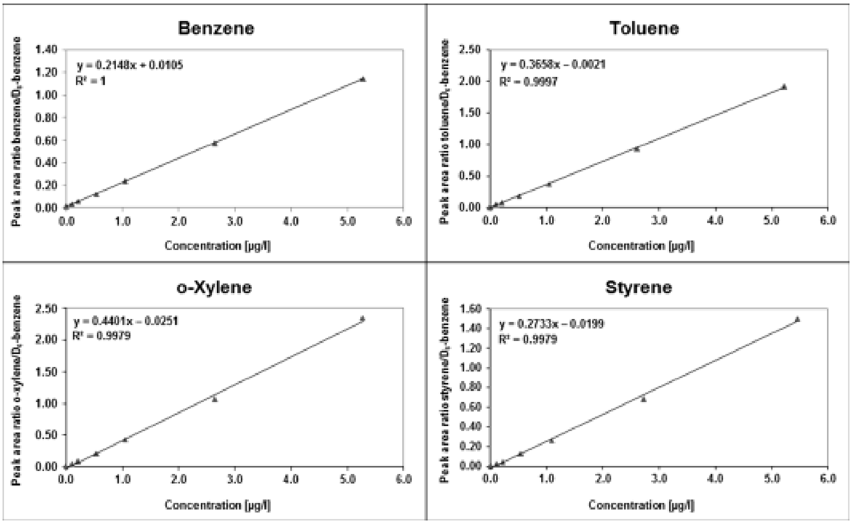

Figure 3 Examples of calibration curves for the analytes benzene, toluene, o-xylene and styrene in water.