Dimethylbenzoic acids – metabolites of trimethylbenzene

Application	Determination in urine
Analytical principle	Capillary gas chromatography/mass spectrometric detection (GC/MS)
Completed in	April 2008

Summary

The concentrations of the isomers 2,3-dimethylbenzoic acid, 2,4-dimethylbenzoic acid, 2,5-dimethylbenzoic acid, 2,6-dimethylbenzoic acid, 3,5-dimethylbenzoic acid as well as 3,4-dimethylbenzoic acid as the specific metabolites of trimethylbenzene can be determined in the urine of occupationally exposed persons by means of the GC/MS-SIM method described here.
The dimethylbenzoic acids are liberated from their conjugates by alkaline hydrolysis, and they are extracted in toluene after acidification of the sample. An aliquot of the toluene phase is subjected to derivatisation with N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA). The derivatised dimethylbenzoic acids are separated by gas chromatography and detected by mass spectrometry.
Calibration is performed using calibration standards that are prepared in pooled urine and are treated in the same manner as the samples to be analysed. 3-Methoxy-4-methylbenzoic acid is added to the urine samples as an internal standard.

2,3-Dimethylbenzoic acid (2,3-DMBA)

Intra-assay repeatability:	Standard deviation (rel.)	s_w = 6.0% or 4.0%
	Confidence interval	u = 14.2% or 9.4%
	at a spiked concentration of 20 or 120 mg per litre urine and where n = 8 determinations	
Inter-day repeatability:	Standard deviation (rel.)	s_w = 4.3% or 1.3%
	Confidence interval	u = 12.0% or 3.6%
	at a spiked concentration of 20 or 120 mg per litre urine and where n = 5 determinations	

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Accuracy:	Recovery rate r = 105% at a spiked concentration of 20 mg per litre urine, and determined in n = 5 individual urine samples
Detection limit:	Approx. 0.2 mg 2,3-DMBA per litre urine

2,4-Dimethylbenzoic acid (2,4-DMBA)

Intra-assay repeatability:	Standard deviation (rel.) s_w = 6.0% or 3.8% Confidence interval u = 14.2% or 9.0% at a spiked concentration of 20 or 120 mg per litre urine and where n = 8 determinations
Inter-day repeatability:	Standard deviation (rel.) s_w = 5.7% or 1.4% Confidence interval u = 15.8% or 3.9% at a spiked concentration of 20 or 120 mg per litre urine and where n = 5 determinations
Accuracy:	Recovery rate r = 93% at a spiked concentration of 20 mg per litre urine, and determined in n = 5 individual urine samples
Detection limit:	Approx. 0.2 mg 2,4-DMBA per litre urine

2,5-Dimethylbenzoic acid (2,5-DMBA)

Intra-assay repeatability:	Standard deviation (rel.) s_w = 6.4% or 4.1% Confidence interval u = 15.1% or 9.7% at a spiked concentration of 20 or 120 mg per litre urine and where n = 8 determinations
Inter-day repeatability:	Standard deviation (rel.) s_w = 5.0% or 2.9% Confidence interval u = 13.9% or 8.1% at a spiked concentration of 20 or 120 mg per litre urine and where n = 5 determinations
Accuracy:	Recovery rate r = 97% at a spiked concentration of 20 mg per litre urine, and determined in n = 5 individual urine samples
Detection limit:	Approx. 0.2 mg 2,5-DMBA per litre urine

2,6-Dimethylbenzoic acid (2,6-DMBA)

Intra-assay repeatability:	Standard deviation (rel.)	s_w = 5.7% or 4.0%
	Confidence interval	u = 13.5% or 9.4%
	at a spiked concentration of 20 or 120 mg per litre urine and where n = 8 determinations	
Inter-day repeatability:	Standard deviation (rel.)	s_w = 4.7% or 1.7%
	Confidence interval	u = 13.1% or 4.7%
	at a spiked concentration of 20 or 120 mg per litre urine and where n = 5 determinations	
Accuracy:	Recovery rate	r = 91%
	at a spiked concentration of 20 mg per litre urine, and determined in n = 5 individual urine samples	
Detection limit:	Approx. 0.2 mg 2,6-DMBA per litre urine	

3,5-Dimethylbenzoic acid (3,5-DMBA)

Intra-assay repeatability:	Standard deviation (rel.)	s_w = 6.8% or 4.1%
	Confidence interval	u = 16.0% or 9.7%
	at a spiked concentration of 20 or 120 mg per litre urine and where n = 8 determinations	
Inter-day repeatability:	Standard deviation (rel.)	s_w = 3.8% or 3.2%
	Confidence interval	u = 10.6% or 8.9%
	at a spiked concentration of 20 or 120 mg per litre urine and where n = 5 determinations	
Accuracy:	Recovery rate	r = 113%
	at a spiked concentration of 20 mg per litre urine, and determined in n = 5 individual urine samples	
Detection limit:	Approx. 0.2 mg 3,5-DMBA per litre urine	

3,4-Dimethylbenzoic acid (3,4-DMBA)

Intra-assay repeatability:	Standard deviation (rel.)	s_w = 6.2% or 5.7%
	Confidence interval	u = 14.6% or 13.5%
	at a spiked concentration of 20 or 120 mg 3,4-dimethylhippuric acid per litre urine (equivalent to 14.5 or 87 mg 3,4-DMBA per litre urine) and where n = 8 determinations	

Inter-day repeatability:	Standard deviation (rel.)	$s_w = 10.4\%$ or 17.8%
	Confidence interval	$u = 28.9\%$ or 49.5%
	at a spiked concentration of 20 or 120 mg 3,4-dimethylhippuric acid per litre urine (equivalent to 14.5 or 87 mg 3,4-DMBA per litre urine) and where n = 5 determinations	
Accuracy:	Recovery rate	$r = 106\%$
	at a spiked concentration of 20 mg per litre urine, and determined in n = 5 individual urine samples	
Detection limit:	Approx. 0.2 mg 3,4-DMBA per litre urine	

Dimethylbenzoic acids in urine as biomarkers for exposure to trimethylbenzene

Trimethylbenzene (TMB) is a natural component of petroleum. It occurs in fuel and in aromatic solvent mixtures. As a rule, a mixture of the isomers 1,2,3-trimethylbenzene (hemimellitene), 1,2,4-trimethylbenzene (pseudocumene) and 1,3,5-trimethylbenzene (mesitylene) is present, whereby the isomeric ratios are very variable. Solvent mixtures containing TMB are used above all in the paint, printing and plastics industries. Additional applications include the production of surface coatings, adhesives, rubber and as a solvent in the chemical industry. The TMB content differs, depending on its area of application [1].

Intake of TMB is chiefly by way of the lungs due to its high vapour pressure. Approximately 70% of the inhaled TMB quantity is retained in the lungs [2]; the penetration rate through the skin was determined as slight in *in vitro* tests [3]. The main metabolism pathway of TMB is by hydroxylation of one of the methyl groups and subsequent oxidation to dimethylbenzoic acid (DMBA). After conjugation with glycine, it is excreted in the urine as dimethylhippuric acid (DMHA), one of the main metabolites (see Figure 1). Animal studies have shown that the various TMB isomers are metabolised to different extents and excreted via the renal route [4]. Thus 78% of the oral dose of 1,3,5-TMB administered to rats is excreted as 3,5-DMHA, but in the case of 1,2,3-TMB only 17% is eliminated as glycine conjugates [1].

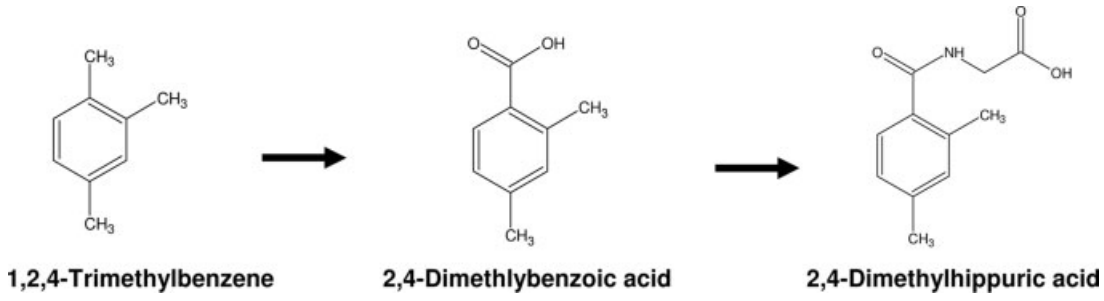

Fig. 1. Simplified metabolic scheme for trimethylbenzene.

The symptoms of TMB exposure are described as central nervous effects as well as headaches, dizziness and fatigue. A comprehensive review of the toxicological aspects of TMB can be found in the justification for the MAK value [5, 6]. The TMB isomers have been assigned a limit value at the workplace of 20 ppm (100 mg/m^3) both by the DFG [7] and by the *Ausschuss für Gefahrstoffe* (AGS) [Commission for Hazardous Substances] [8]. The excretion of the isomeric dimethylbenzoic acids in urine has proved to be suitable for biological monitoring, as experimental studies and field studies have shown [2, 9]. The MAK Commission has stipulated a BAT value of 400 mg/g creatinine for the sum of all the excreted dimethylbenzoic acids (i.e. after hydrolysis) [10].

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1 General principles

The dimethylbenzoic acids are liberated from their conjugates by alkaline hydrolysis, and they are extracted in toluene after acidification of the sample. An aliquot of the toluene phase is subjected to derivatisation with N-*tert*-butyldimethylsilyl-N-methyl-trifluoroacetamide (MTBSTFA). The derivatised dimethylbenzoic acids are separated by gas chromatography and detected by mass spectrometry.
Calibration is performed using calibration standards that are prepared in pooled urine and are treated in the same manner as the samples to be analysed. 3-Methoxy-4-methylbenzoic acid is added to the urine samples as an internal standard.

2 Equipment, chemicals and solutions

2.1 Equipment

Gas chromatograph with split/splitless injector, mass selective detector (MSD) and data processing system

Capillary gas chromatographic column:
Stationary phase: 95% dimethylpolysiloxane 5% diphenylpolysiloxane; length 30 m; inner diameter 0.25 mm; film thickness 0.25 µm (e.g. HP-5ms, Agilent J & W No. 19091S-433)

10 µL Syringe for gas chromatography, but the use of an autosampler is preferable

250 mL Glass beaker

100 mL Measuring cylinder

Magnetic stirrer with stirring rod

10, 20, and 100 mL Volumetric flasks (e.g. from Brand)

If required, ultrasonic bath

Variable microlitre pipettes with the appropriate tips for volumes between 10 and 5000 µL (e.g. from Eppendorf)

Hand dispenser with the appropriate tips for volumes between 20 and 2000 µL (e.g. Multipette from Eppendorf)

Sealable 0.5 mL reaction vessels (e.g. micro-test-tubes from Eppendorf)

Sealable plastic vessels for collecting urine (e.g. Sarstedt No. 77.577)

5 mL Screw-capped glass jars with screw caps and PTFE-coated inner septa (e.g. from Brand)

Drying cupboard (e.g. from Heraeus)

Vortex mixer (e.g. from Ikamag)

Laboratory centrifuge (e.g. Megafuge from Heraeus)

1.8 mL Crimp-capped vials with PTFE-coated septa and crimp caps as well as crimping tongs (e.g. from Macherey-Nagel)

Micro-inserts for the crimp-capped vials, usable volume 250 µL (e.g. from Macherey-Nagel)

2.2 Chemicals

If not otherwise specified, all the chemicals used must be at least p.a. grade.

Bidistilled water (e.g. Milli-Q water)

Sodium chloride (e.g. Merck No. 1.06498.0500)

Sulphuric acid (95 to 97%) (e.g. Merck No. 1.00731.1000)

Acetonitrile for gas chromatography (e.g. SupraSolv® from Merck, No. 1.00017.2500)

2,3-Dimethylbenzoic acid, 98% (e.g. Aldrich, No. 40726)

2,4-Dimethylbenzoic acid, 98% (e.g. Aldrich, No. 138169)

2,5-Dimethylbenzoic acid, 95% (e.g. Aldrich, No. S33612-086)

2,6-Dimethylbenzoic acid, 97% (e.g. Aldrich, No. 156906)

3,4-Dimethylbenzoic acid, 98% (e.g. Aldrich, No. D149403)

3,4-Dimethylhippuric acid (e.g. TCI, No. D2241)

3,5-Dimethylbenzoic acid, 99% (e.g. Aldrich, No. S37544-346)

3-Methoxy-4-methylbenzoic acid (e.g. Aldrich, No. M15052)

Toluene for organic trace analysis (e.g. Merck, No. 1.08388.2500)

N-*tert*-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) (e.g. Aldrich, No. 24205-5)

Helium 5.0 (e.g. from Linde)

2.3 Solutions

Sodium hydroxide (10 M):
Approx. 40 g of NaOH are weighed in a 250 mL glass beaker and dissolved in approx. 70 mL of bidistilled water while being stirred with a magnetic stirrer (caution: heat is generated). After cooling, the solution is transferred to a 100 mL volumetric

flask together with the bidistilled water used to rinse the glass beaker. The flask is subsequently filled to its nominal volume with bidistilled water.

Sulphuric acid (6 N):
Approx. 60 mL of bidistilled water are placed in a 100 mL volumetric flask. A pipette is used to cautiously transfer a total of 16.7 mL of concentrated sulphuric acid (caution: heat is generated). The flask is subsequently filled to its nominal volume with bidistilled water.

2.4 Calibration standards and quality control material

2.4.1 Internal standard (ISTD)

Working solution:
Approximately 10 mg of 3-methoxy-4-methylbenzoic acid are weighed exactly into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with acetonitrile. This working solution of the internal standard is stored in sealed vessels at –20 °C and is stable under these conditions for at least 6 months. The concentration of the working solution is 1 g/L.

2.4.2 Calibration standards

Starting solution of 2,3-dimethylbenzoic acid (2,3-DMBA):
Approximately 100 mg of 2,3-DMBA are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (c = 10 g/L). If necessary, the flask is treated for 10 minutes in an ultrasonic bath to ensure complete dissolution.

Starting solution of 2,4-dimethylbenzoic acid (2,4-DMBA):
Approximately 100 mg of 2,4-DMBA are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (c = 10 g/L). If necessary, the flask is treated for 10 minutes in an ultrasonic bath to ensure complete dissolution.

Starting solution of 2,5-dimethylbenzoic acid (2,5-DMBA):
Approximately 100 mg of 2,5-DMBA are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (c = 10 g/L). If necessary, the flask is treated for 10 minutes in an ultrasonic bath to ensure complete dissolution.

Starting solution of 2,6-dimethylbenzoic acid (2,6-DMBA):
Approximately 100 mg of 2,6-DMBA are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (c = 10 g/L). If necessary, the flask is treated for 10 minutes in an ultrasonic bath to ensure complete dissolution.

Starting solution of 3,4-dimethylbenzoic acid (3,4-DMBA):
Approximately 100 mg of 3,4-DMBA are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (c = 10 g/L). If necessary, the flask is treated for 10 minutes in an ultrasonic bath to ensure complete dissolution.

Starting solution of 3,5-dimethylbenzoic acid (3,5-DMBA):
Approximately 100 mg of 3,5-DMBA are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (c = 10 g/L). If necessary, the flask is treated for 10 minutes in an ultrasonic bath to ensure complete dissolution.

Spiking solution for the calibration standards:
1 mL each of the starting solutions of 2,3-DMBA, 2,4-DMBA, 2,5-DMBA, 2,6-DMBA, 3,4-DMBA and 3,5-DMBA are pipetted into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with bidistilled water (c = 1 g/L).

Calibration standard solutions in urine:
The calibration standard solutions are prepared in pooled urine from non-exposed persons in the general population. Pooled urine is prepared from spontaneous urine samples collected in suitable vessels. The samples are thoroughly mixed and stored at –20 °C until the standards are to be prepared. Calibration standards of the dimethylbenzoic acids are prepared from the spiking solution for calibration standards by dilution with pooled urine according to the pipetting scheme shown in Table 1. The dilutions are performed in 20 mL volumetric flasks and encompass a concentration range from 1 to 200 mg/L. The calibration standards are divided into 300 µL aliquots in suitable vessels (e.g. 0.5 mL reaction vessels) and stored in the deep-freezer. The pooled urine used for dilution is included as a blank.

Table 1. Pipetting scheme for the preparation of the calibration standard solutions in pooled urine.

Volume of spiking solution [µL]	Final volume of the reference standard [mL]	Concentration of calibration standard solution [mg/L]
0	20	0
20	20	1
200	20	10
500	20	25
1000	20	50
2000	20	100
4000	20	200

The starting solutions of the dimethylbenzoic acids, the spiking solution and the calibration standards are stable for at least 6 months without any loss of the analytes when stored at a temperature of –20 °C.

2.4.3 Quality control material

Stock solution of 3,4-dimethylhippuric acid (3,4-DMHA):
Approximately 10 mg of 3,4-dimethylhippuric acid are weighed exactly into a 10 mL volumetric flask. The flask is filled to its nominal volume with acetonitrile (c = 1 g/L).

Spiking solution for quality control material:
1 mL each of the starting solutions of 2,3-DMBA, 2,4-DMBA, 2,5-DMBA, 2,6-DMBA, and 3,5-DMBA (see Section 2.4.2) are pipetted into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with bidistilled water (c = 1 g/L).

Quality control material in urine:
Control material is prepared in urine from the spiking solution for quality control material and from the stock solution of 3,4-dimethylhippuric acid. For this purpose either pooled urine from non-exposed persons in the general population or a spontaneous urine sample from one non-exposed person can be used. Table 2 shows an example of a pipetting scheme for preparing quality control material at two different concentration levels.

Table 2. Pipetting scheme for the preparation of quality control material in urine.

Volume of the spiking solution [µL]	Volume of the stock solution of 3,4-DMHA [µL]	Final volume of the control urine [mL]	Concentration of the control urine [mg/L]
400	400	20	20
2400	2400	20	120

The quality control material is divided into 300 µL aliquots in suitable vessels (e.g. 0.5 mL reaction vessels). The stock solution of 3,4-DMHA, the spiking solution for the quality control material and the control urine aliquots are stored at –20 °C and are stable for at least 6 months under these conditions.

3 Specimen collection and sample preparation

The urine samples are collected in sealable plastic vessels and stored at –20 °C. The urine is stable for at least six months when stored in this manner.

3.1 Sample preparation

Before analysis, the samples are thawed at room temperature and thoroughly mixed. Then 250 µL of the sample are pipetted into a 5 mL screw-capped glass jar, and 20 µL of the working solution of the internal standard (3-methoxy-4-methylbenzoic

acid, 1 g/L in acetonitrile) and 250 µL of 10 M sodium hydroxide are added, and the mixture is swirled briefly. Subsequently the samples are hydrolysed for 120 min at 90 °C in the drying cupboard.
After the sample has cooled to room temperature, 1 mL of 6 N sulphuric acid is cautiously added by pipette and the sample is swirled briefly. For extraction 2 mL of toluene are added, the screw-capped glass jar is firmly sealed, and the sample is thoroughly mixed for 60 seconds on a vortex shaker. The phases are subsequently separated by centrifugation (10 min, 2700 rpm).
100 µL of the supernatant organic phase are transferred to a 1.8 mL crimp-capped vial. 20 µL N-*tert*-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) are added, then the solution is mixed by rinsing the glass wall several times and transferred to a micro-insert. The GC vial with the micro-insert is sealed. Then the sample is derivatised for 60 min at 90 °C in the drying cupboard.

4 Operational parameters

4.1 Operational parameters for gas chromatography

Capillary column:	Material:	Fused silica
	Stationary phase:	HP 5-ms
	Length:	30 m
	Inner diameter:	0.25 mm
	Film thickness:	0.25 µm
Temperatures:	Column:	Initial temperature 90 °C, 1 minute isothermal, then increase at a rate of 30 °C/min to 120 °C, 1 minute isothermal, then increase at a rate of 6 °C/ in to 210 °C, then increase at a rate of 35 °C/min until 280 °C, 5 min at the final temperature
	Injector:	280 °C
	Transfer line:	280 °C
Carrier gas:	Helium 5.0 at a constant flow rate of 1.2 mL/min	
Split:	Splitless, split on after 1 min	
Sample volume:	1 µL	

4.2 Operational parameters for mass spectrometry

Ionisation type:	Electron impact ionisation (EI)	
Ionisation energy:	70 eV	
Temperatures:	Quadrupole:	150 °C
	Source:	230 °C
Dwell time:	100 ms	
Electron multiplier:	2200 to 2400 V (optimised by autotuning)	

All other parameters must be optimised in accordance with the manufacturer's instructions.

5 Analytical determination

In order to analyse the urine samples processed as described in Section 3.1, 1 µL each of the derivatised extract is injected splitless into the GC/MS system by means of an airtight syringe or an autosampler. The analytes are identified by the retention times and the characteristic ion traces. At least one quality control sample is analysed with each analytical series.
The temporal profiles of the ion traces shown in Table 3 are recorded in the single ion monitoring (SIM) mode:

Table 3. Retention times and recorded masses.

Parameter	Retention time [min]	Quantifier ion [m/z]	Qualifier ion [m/z]
2,6-DMBA	11.46	207	133
2,5-DMBA	11.76	207	133
2,4-DMBA	12.24	207	133
3,5-DMBA	12.32	207	133
2,3-DMBA	12.55	207	133
3,4-DMBA	13.26	207	133
3-Methoxy-4-methylbenzoic acid (ISTD)	14.46	223	149

The retention times shown in Table 3 serve only as a guide. Users of the method must satisfy themselves of the separation power of the capillary column used and the resulting retention behaviour of the substances. Figure 2 shows a chromatogram of a processed standard in urine. Figure 3 gives an example of the mass spectrum of the derivatised 3,4-DMBA and of its fragmentation scheme.

6 Calibration

The calibration standards prepared in pooled urine (see Section 2.4.2) are processed in the same manner as the test material (as described in Section 3.1) and analysed by gas chromatography/mass spectrometry in accordance with Sections 4 and 5 in order to carry out external calibration. Then quotients of the peak areas of the derivatised dimethylbenzoic acid isomers and the internal standard are calculated, and the calibration curves are obtained by plotting the quotients versus the concentrations of the calibration standards. The gradient of the calibration curve and the intercept with the y-axis are calculated by linear regression.
The linear range of the calibration curve covers concentrations of the dimethylbenzoic acids from the detection limit (see Section 9.4) to 200 mg/L. Figure 4 gives examples of the calibration curves of the isomeric dimethylbenzoic acids in pooled urine.

7 Calculation of the analytical result

The dimethylbenzoic acid concentrations in the urine samples are calculated on the basis of external calibration curves as described in Section 6. Quotients are calculated in each case by dividing the peak area of the analyte by that of the internal standard. These quotients are used to read off the pertinent concentration of each dimethylbenzoic acid in mg per litre urine from the relevant calibration graph. Any reagent blank values and background exposure in the calibration material must be taken into consideration if necessary, but they have not been observed to date.

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] [11] and in the special preliminary remarks to this series.
In order to determine the precision of the method, at least one urine control sample containing a constant concentration of the dimethylbenzoic acids is analysed. As material for quality control is not commercially available, it must be prepared in the laboratory. For this purpose a defined quantity of the analytes is added to pooled urine or individual urine (see Section 2.4.3). The concentrations of this control material should lie within the expected concentration range. 3,4-DMHA instead of 3,4-DMBA was added to the control material to check the effectiveness of the hydrolysis carried out as part of the sample work-up.
The theoretical value and the tolerance ranges of this quality control material are ascertained in a pre-analytical period (one analysis of the control material on each of 15 different days) [11–13].

9 Evaluation of the method

9.1 Precision

To determine the intra-assay repeatability, pooled urine samples from occupationally non-exposed persons were spiked with the dimethylbenzoic acids (or 3,4-DMHA) to give concentrations of 20 mg/L and 120 mg/L in each case, then they were processed and analysed. Eight-fold analyses of these urine samples yielded the intra-assay repeatability values shown in Table 4.

Table 4. Intra-assay repeatability for the determination of dimethylbenzoic acids in urine (n = 8).

Parameter	Concentration [mg/L]	Standard deviation (rel) s_w [%]	Confidence interval u [%]
2,6-DMBA	20	5.7	13.5
	120	4.0	9.4
2,5-DMBA	20	6.4	15.1
	120	4.1	9.7
2,4-DMBA	20	6.0	14.2
	120	3.8	9.0
3,5-DMBA	20	6.8	16.0
	120	4.1	9.7
2,3-DMBA	20	6.0	14.2
	120	4.0	9.4
3,4-DMBA *	14,5	6.2	14.6
	87	5.7	13.5

* 20 or 120 mg 3,4-dimethylhippuric acid were added. Taking the molar ratio into account, this results in the stated spiked concentrations of 3,4-dimethylbenzoic acid.

In addition, the inter-day repeatability was determined. The material used was the same as for the determination of the intra-assay repeatability. These urine samples were processed and analysed on 5 different days. The precision results are shown in Table 5.

Table 5. Inter-day repeatability for the determination of dimethylbenzoic acids in urine (n=5).

Parameter	Concentration [mg/L]	Standard deviation (rel) s_w [%]	Confidence interval u [%]
2,6-DMBA	20	4.7	13.1
	120	1.7	4.7
2,5-DMBA	20	5.0	13.9
	120	2.9	8.1
2,4-DMBA	20	5.7	15.8
	120	1.4	3.9
3,5-DMBA	20	3.8	10.6
	120	3.2	8.9
2,3-DMBA	20	4.3	12.0
	120	1.3	3.6
3,4-DMBA *	14.5	10.4	28.9
	87	17.8	49.5

* 20 or 120 mg 3,4-dimethylhippuric acid were added. Taking the molar ratio into account, this results in the stated spiked concentrations of 3,4-dimethylbenzoic acid.

9.2 Accuracy

Recovery experiments were performed to check the accuracy of the method. For this purpose pooled urine from occupationally non-exposed persons was spiked with the dimethylbenzoic acids (or 3,4-DMHA) resulting in concentrations of 20 and 120 mg/L, divided into 8 aliquots, and analysed. The relative recovery rates given in Table 6 were obtained.

Table 6. Mean relative recovery rates for the dimethylbenzoic acids in a sample of pooled urine (n=8).

Parameter	Concentration [mg/L]	Mean relative recovery [%]	Range [%]
2,6-DMBA	20	95	88–102
	120	102	95–108
2,5-DMBA	20	98	89–105
	120	103	95–108
2,4-DMBA	20	95	87–100
	120	98	91–102
3,5-DMBA	20	109	99–119
	120	108	100–113
2,3-DMBA	20	100	91–105
	120	102	94–107
3,4-DMBA *	14.5	100	92–108
	87	106	94–114

* 20 or 120 mg 3,4-dimethylhippuric acid were added. Taking the molar ratio into account, this results in the stated spiked concentrations of 3,4-dimethylbenzoic acid.

As part of the investigation of matrix effects, the relative recovery was also measured in 5 urine samples from individual test persons with a creatinine content between 0.29 g/L and 1.56 g/L. The individual urine samples were spiked with 20 mg/L of the dimethylbenzoic acids for this purpose. The relative recovery rates are summarised in Table 7. No influence of the creatinine content on the relative recovery was found.

Table 7. Relative recovery rates of the dimethylbenzoic acids in five individual urine samples each spiked with 20 mg/L.

	Relative recovery rate [%]					
Individual urine sample	1	2	3	4	5	
Creatinine [g/L]	0.29	1.56	0.54	0.94	1.22	Mean value
2,6-DMBA	94	91	89	92	88	91
2,5-DMBA	100	96	96	98	94	97
2,4-DMBA	96	93	92	95	90	93
3,5-DMBA	117	112	113	114	109	113
2,3-DMBA	108	105	104	106	102	105
3,4-DMBA	109	106	106	107	104	106

To further investigate the matrix effects, a calibration series was prepared in water and also in pooled urine, and both were analysed as described in Sections 3, 4 and 5. Comparison of the calibration curves shows that only relatively slight matrix effects occur in the determination. The gradients of the regression curves are summarised in Table 8.

Table 8. Comparison of the gradients of the calibration curves of samples prepared in water and pooled urine.

Substance	Gradient in water	Gradient in pooled urine	Rel. deviation in water
2.6-DMBA	$Y = 0.0112 \cdot X$	$Y = 0.0119 \cdot X$	–5.9%
2,5-DMBA	$Y = 0.0110 \cdot X$	$Y = 0.0119 \cdot X$	–7.6%
2,4-DMBA	$Y = 0.0107 \cdot X$	$Y = 0.0116 \cdot X$	–7.8%
3,5-DMBA	$Y = 0.0107 \cdot X$	$Y = 0.0115 \cdot X$	–7.0%
2,3-DMBA	$Y = 0.0101 \cdot X$	$Y = 0.0109 \cdot X$	–7.3%
3,4-DMBA	$Y = 0.0095 \cdot X$	$Y = 0.0105 \cdot X$	–9.5%

9.3 Stability of the analytes – freezing/thawing stability

Three quality control samples (storage at –20 °C; spiked with 20 mg/L in each case) were subjected to three freezing and thawing cycles on three days and subsequently analysed to investigate the stability of the analytes after several freezing/thawing cycles.
The deviations from the mean values of the quality control chart were within the range of measurement inaccuracy. Therefore degradation of the analytes by repeated freezing and thawing was not observed.

9.4 Detection limits

Under the conditions for sample preparation and gas chromatographic/mass spectrometric analysis given here, the detection limit for all the dimethylbenzoic acids was about 0.2 mg per litre urine. The detection limit was estimated as three times the signal/background noise ratio in the vicinity of the analyte in each case.

9.5 Sources of error

A pre-condition for derivatisation using MTBSTFA is the absence of water. When the extraction with toluene was carried out, drying the phase with sodium sulphate was omitted for practical reasons. After centrifugation, it is essential to ensure that no water or proteins are carried over when 100 µL of the upper organic toluene phase is withdrawn. If emulsions occur after centrifugation, the sample should be treated briefly in the ultrasonic bath and centrifuged at a high rate again.
During the method development in the case of 3,4-dimethylhippuric acid it was observed that it is important to comply strictly with the hydrolysis conditions described here. The drying cupboard should already have reached the set temperature of 90 °C when the samples are placed in it, as otherwise insufficient hydrolysis to the dimethylbenzoic acids may result.
In the case of 2,4-DMBA an interference peak that proved impossible to separate from the analyte was observed in some individual urine samples. This can impede quantification in the lower concentration range (<0.5 mg/L) in some cases.

10 Discussion of the method

The analytical procedure presented here achieves reliable and specific determination of exposure to trimethylbenzene at the levels of relevance to occupational medicine. With regard to the hydrolysis, the method is based on the procedure presented by Kostrzewski et al. [2] and the extraction, derivatisation and GC conditions were optimised.

Assay of the relevant dimethylbenzoic acid metabolites is carried out after alkaline release of the analytes from their glycine conjugates; therefore the total quantity of the excreted dimethylbenzoic acids and dimethylhippuric acids is determined. The effectiveness of the alkaline hydrolysis was checked by addition of 3,4-dimethylhippuric acid to pooled urine as part of the validation. In this case somewhat greater scatter was found for the inter-day repeatability for 3,4-DMBA than for the other parameters. However, the excellent relative recovery rates of 100 or 106% when 20 or 120 mg of 3,4-dimethylhippuric acid are added confirm that the hydrolysis of the hippuric acids to the dimethylbenzoic acids is obviously almost complete, provided there is strict compliance with the hydrolysis conditions.

The choice of toluene as the extraction agent ensures a good extraction yield on the one hand and guarantees very pure extracts on the other, thus saving the inlet system of the gas chromatograph from unnecessary wear and tear. The extraction volumes were selected during development of the method to ensure that the linear working range was as large as possible (up to 200 mg/L urine) in order to enable urine samples of possibly highly exposed workers to be evaluated without further dilution steps. If necessary, the detection limit of the method can still be lowered considerably by increasing the volume of urine used (as well as the corresponding volumes of 10 M sodium hydroxide and 6 N sulphuric acid) without causing chromatographic interference. Derivatisation with MTBSTFA to determine the dimethylbenzoic acids has proved successful. The resulting derivatives are remarkable for their specific fragments with a high m/z ratio (see Figure 3), thus a high degree of specificity is given by simultaneous determination of two fragment ions.

It proved impossible to achieve a baseline separation of 2,4-DMBA and 3,5-DMBA under the given analytical conditions. The gas chromatographic temperature program was optimised to attain the most complete separation possible. However, as Figure 2 shows, the HP-5ms column used in this case achieves sufficient separation of the two isomers to permit integration of both peaks by the "perpendicular drop" method. In this context one examiner of the method recommends the use of a separation column of a different polarity as an alternative (DB 17-HT, 30 m×0.25 mm×0.15 µm; J&W Scientific), which permits improved separation of all the dimethylbenzoic acid isomers.

The procedure is notable for the simplicity of its sample work-up. The reliability criteria can be regarded as very good and the examiners of the method were able to reproduce them at their first attempt. The alkaline hydrolysis and the derivatisation require only 2 and 1 hours respectively, so experienced laboratory staff can process about 40 samples in a day.

To summarise, the method presented here is very suitable for investigation of the internal exposure of people who handle trimethylbenzene at their workplace.

Instruments used:

Gas chromatograph 6890 II with mass selective detector 5973, autosampler 7683 and data system from Hewlett-Packard

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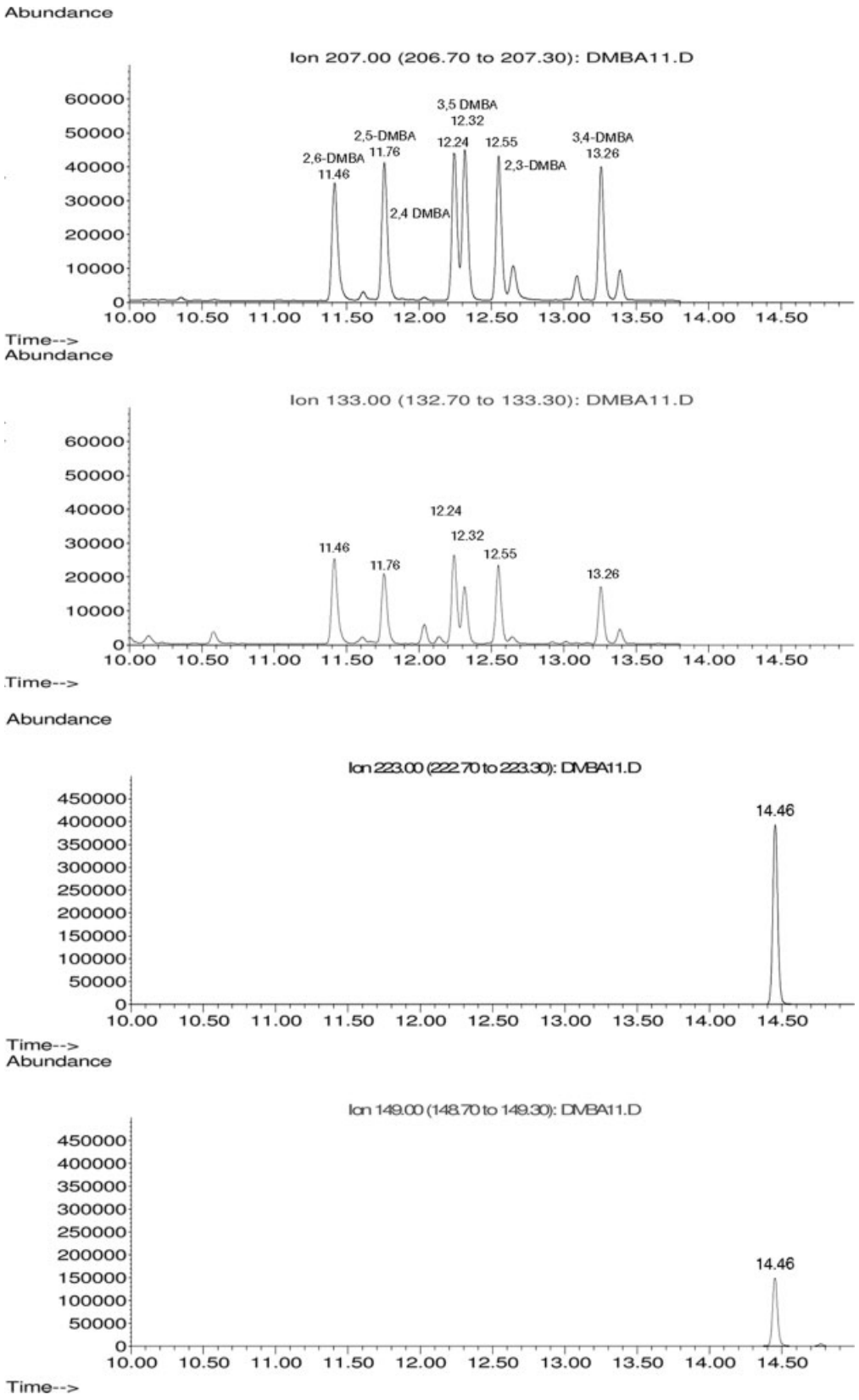

Fig. 2. GC/MS-SIM chromatogram of a worked-up standard in urine. Upper image: dimethylbenzoic acids with a spiked concentration of 10 mg per litre in each case (above: quantifier trace; below: qualifier trace). Lower image: internal standard, i.e. 3-methoxy-4-methylbenzoic acid.

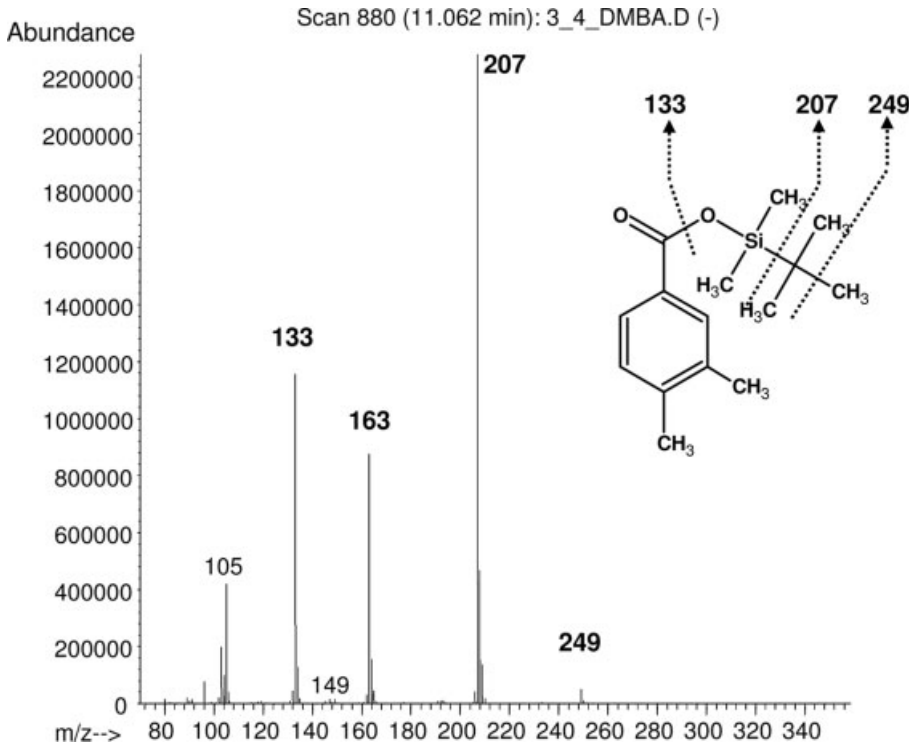

Fig. 3. EI mass spectrum and fragmentation scheme of the *tert*-butyl-dimethylsilyl derivative of 3,4-dimethylbenzoic acid.

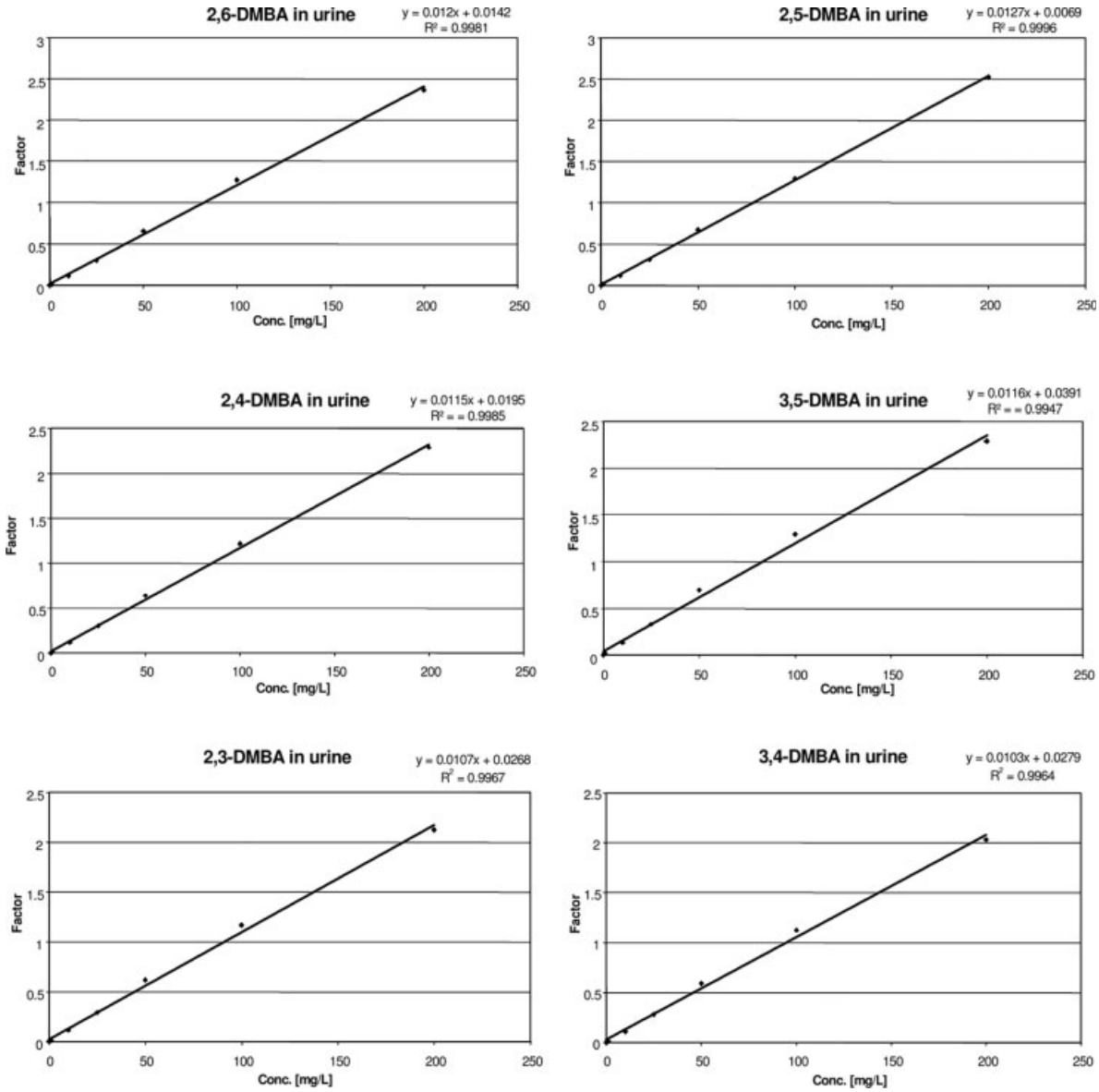

Fig 4. Calibration curves of the isomeric dimethylbenzoic acids in pooled urine.