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2-Mercaptobenzothiazole – Determination of 2-mercaptobenzothiazole in urine by LC-MS/MS

Biomonitoring Method – Translation of the German version from 2020

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Keywords

2-Mercaptobenzothiazole, MBT, vulcanisation accelerator, biomonitoring, urine, 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.

This method enables the selective detection of the vulcanisation accelerator 2-mercaptobenzothiazole (MBT) in urine. After adding a labelled internal standard (MBT-d_4), the samples are enzymatically hydrolysed to release free MBT from the conjugated MBT compounds. After online purification and enrichment, the analyte is separated from accompanying components by liquid chromatography and analysed using tandem mass spectrometry. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed.

1 Characteristics of the method

Matrix Urine

Analytical principle LC-MS/MS

Parameter and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
2-Mercaptobenzothiazole (MBT)	149-30-4	MBT	149-30-4
Zinc salt of 2-mercaptobenzothiazole	155-04-4		

Reliability data of the method

2-Mercaptobenzothiazole (MBT)

Within-day precision: Standard deviation (rel.) s_w = 2.3%, 1.9% or 1.6%

Prognostic range u = 5.1%, 4.2% or 3.6%

at a spiked concentration of 10 µg, 100 µg or 1000 µg MBT per litre urine and where n = 10 determinations

Day-to-day precision: Standard deviation (rel.) s_w = 4.3%, 5.8% or 3.4%

Prognostic range u = 9.6%, 13.0% or 7.6%

at a spiked concentration of 10 µg, 100 µg or 1000 µg MBT per litre urine and where n = 10 determinations

Accuracy: Recovery rate (rel.) r = 86%, 96% or 96%

at a nominal concentration of 10 µg, 100 µg or 1000 µg MBT per litre urine and where n = 10 determinations

Detection limit: 0.4 µg MBT per litre urine

Quantitation limit: 1.2 µg MBT per litre urine

2 General information on 2-mercaptobenzothiazole

2-Mercaptobenzothiazole (MBT) is an organic compound belonging to the group of heteroaromatics. Under standard conditions, the substance is present in the form of pale yellow crystals which are poorly soluble in water. The industrial production takes place by reaction of aniline, carbon disulphide and sulphur under high pressure at high temperatures of around 230 °C (Greim 1999; IARC 2018).

MBT is mainly used as a vulcanisation accelerator in tyre production and in the manufacture of technical rubber products. In addition, the zinc salt of MBT and sulfenamides, a compound class derived from MBT, are potential sources of MBT. Therefore, MBT can potentially be found in many articles of daily use, such as tyres, cables, rubber gloves, rubber bands and seals, as well as in drilling and cutting oils. It is also used as a reagent in quantitative wet chemical analysis of metals (IARC 2018).

2-Mercaptobenzothiazole occurs in two tautomeric forms, with the equilibrium lying on the side of 2-(3H) benzothiazolethione (NH form) (cf. Figure 1).

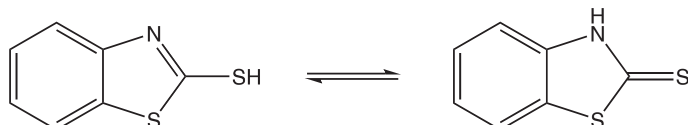

Fig. 1 Tautomeric structures of MBT

The Commission classified MBT as a Category 3B carcinogen and established a MAK value of 4 mg/m³ E. In addition, MBT was categorised in pregnancy risk Group C by the Commission and designated with 'Sh' (danger of sensitisation of the skin) (DFG 2019). The IARC (International Agency for Research on Cancer) classifies MBT as probably carcinogenic to humans (Group 2A) (IARC 2018). For details on the toxicological evaluation of MBT, please refer to the respective MAK Value Documentation and an IARC monograph (Greim 1999; IARC 2018).

In animal studies, MBT is rapidly absorbed after both oral and dermal administration and is excreted primarily in urine (IARC 2018). Metabolism studies in rats have shown that more than 90% of orally administered MBT is excreted in urine within four days. MBT is predominantly excreted in the form of conjugates (as glucuronide or sulphate) (Fukuoka et al. 1995; IARC 2018). Based on data from animal studies, the elimination half-life of MBT in urine is less than 8 hours (Greim 1999; IARC 2018).

The analytical method described herein has already been applied to a small collective of occupationally exposed persons (n = 4), in which MBT was detected in all urine samples (range from 567 µg/l to 6210 µg/l) (Gries et al. 2015).

Due to the wide range of applications of MBT in many articles of daily use and its possible release in the environment (e.g. tyre wear), the general population is also likely to be exposed to MBT. To determine possible background exposure, 40 individual urine samples from persons not occupationally exposed to MBT were analysed using the method described herein. The analysis was performed by the developers of this method as well as in the context of external verification. The results obtained are presented in Table 1. In addition, MBT was determined in urine samples of the general population using this method as part of the German Environmental Survey (GerES V, German Environmental Survey, Part V). To this end, a sub-sample of the GerES V collective, 516 urine samples from children and adolescents (3–17 years), was analysed. These results are also summarised in Table 1.

Tab. 1 MBT levels in urine samples from persons not occupationally exposed to MBT

Collective	Number	Number > LOQ[a)]	Median (range) [µg/l]	95th percentile [µg/l]	Reference
General population	40	1	< LOQ (< LOQ–10.8)	< LOQ	Gries et al. 2015
General population	40	13	< LOQ (< LOQ–7.9)	2.4	data obtained during external method verification
General population (3–17 years)	516	256	< LOQ (< LOQ–43.5)	4.8	Murawski et al. 2020

[a)] LOQ: limit of quantitation

3 General principles

This method enables the selective detection of the vulcanisation accelerator MBT in urine. After adding a labelled internal standard (MBT-d_4), the samples are enzymatically hydrolysed to release MBT from the conjugated MBT

compounds. After online purification and enrichment, the analyte is separated from accompanying components by liquid chromatography and analysed using tandem mass spectrometry. Calibration standards are prepared in pooled urine and processed in the same way as the samples to be analysed.

4 Equipment, chemicals and solutions

4.1 Equipment

- LC-MS/MS system: Waters Alliance LC coupled with Waters Quattro Ultima Tandem MS (e.g. Waters GmbH, Eschborn, Germany)
- Analytical column: Zorbax Eclipse XDB-C8 5 µm, 4.6 × 50 mm (e.g. Agilent Technologies Deutschland GmbH, Waldbronn, Germany, No. 946975-906)
- Pre-concentration column: Oasis HLB 25 µm, 2.1 × 20 mm (e.g. Waters GmbH, Eschborn, Germany, No. 186002036)
- Laboratory shaker (e.g. IKA Vibrax VXR, Eppendorf AG, Hamburg, Germany)
- Pasteur pipettes (e.g. transfer pipettes made of PE from Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Analytical balance (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Incubator (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- Various volumetric flasks and beakers (e.g. BRAND GMBH & Co. KG, Wertheim, Germany)
- Variably adjustable pipettes (e.g. Eppendorf AG, Hamburg, Germany)
- Multipette (e.g. Eppendorf AG, Hamburg, Germany)
- Disposable pipettes 3.5 ml (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- 1.5 mL glass vials with crimp caps (e.g. Waters GmbH, Eschborn, Germany, No. 186000327C)
- pH meter (e.g. Mettler-Toledo GmbH, Gießen, Germany)
- Urine collection container (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

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

- Acetonitrile, SupraSolv® (e.g. Merck KGaA, Darmstadt, Germany, No. 100017)
- Ultrapure water (e.g. Merck KGaA, Darmstadt, Germany, No. 116754)
- β-Glucuronidase (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 03707598001)
- Glacial acetic acid, 100% (e.g. Merck KGaA, Darmstadt, Germany, No. 100066)
- Formic acid, 98–100% (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. 56302)
- Ammonium acetate (e.g. Merck KGaA, Darmstadt, Germany, No. 101116)
- 2-Mercaptobenzothiazole, 97% (e.g. Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany, No. M3302)
- 2-Mercaptobenzothiazole-d_4 (MBT-d_4) (e.g. Toronto Research Chemicals, Toronto, Canada, No. B206652)

4.3 Solutions

- Ammonium acetate buffer (1 mol/l, pH 6.5)

  Exactly 38.5 g ammonium acetate are weighed into a 400 ml beaker and dissolved in approximately 250 ml ultrapure water. After the pH has been adjusted to 6.5 (using a pH meter) with glacial acetic acid, the solution is quantitatively transferred to a 500 ml volumetric flask. The flask is then made up to the mark with ultrapure water.

- Eluent B (1% aqueous formic acid)

  Approximately 700 ml ultrapure water are placed into a 1000 ml volumetric flask. Then, 10 ml formic acid are added and the solution is thoroughly mixed. The flask is then made up to the mark with ultrapure water.

The solutions are stable for at least one week when stored at room temperature.

4.4 Internal standard (ISTD)

- ISTD stock solution (1000 mg/l)

  10 mg MBT-d_4 are exactly weighed into a 10 ml volumetric flask and dissolved in acetonitrile. The flask is then made up to the mark with acetonitrile.

- ISTD spiking solution (10 mg/l)

  100 µl of the ISTD stock solution are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

The stock solution and the spiking solution of the internal standard are stable for about four weeks when stored in the refrigerator at 4 °C. Longer storage has been found to cause significant degradation (Gries et al. 2015).

4.5 Calibration standards

- MBT stock solution (1000 mg/l)

  10 mg MBT are weighed exactly into a 10 ml volumetric flask and dissolved in acetonitrile. The flask is then made up to the mark with acetonitrile.

- MBT spiking solution 1 (100 mg/l)

  1 ml of the MBT stock solution is pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- MBT spiking solution 2 (10 mg/l)

  100 µl of the MBT stock solution are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- MBT spiking solution 3 (1 mg/l)

  1 ml of MBT spiking solution 2 is pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- MBT spiking solution 4 (0.1 mg/l)

  100 µl of MBT spiking solution 2 are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

- MBT spiking solution 5 (0.01 mg/l)

  100 µl of MBT spiking solution 3 are pipetted into a 10 ml volumetric flask. The flask is then made up to the mark with acetonitrile.

The MBT solutions are stored in glass vials at 4 °C (in the refrigerator). Under these conditions, they are stable for about four weeks.

Calibration standards in the concentration range between 0.5 µg/l and 10 000 µg/l are prepared in pooled urine according to the pipetting scheme shown in Table 2. Unspiked pooled urine is included as a blank. The calibration standards are prepared afresh every working day. For application of the method for environmental medicine, calibration up to 50 µg/l is usually sufficient. The calibration standards are prepared and processed in the same way as the samples to be analysed.

Tab. 2 Pipetting scheme for the preparation of calibration standards used to determine MBT in urine

Calibration standard	MBT spiking solution	Volume of MBT spiking solution [µl]	Volume of pooled urine [µl]	MBT level [µg/l]
0	–	–	500	0
1	5	25	475	0.5
2	5	50	450	1
3	4	10	490	2
4	4	25	475	5
5	4	50	450	10
6	3	10	490	20
7	3	25	475	50
8	3	50	450	100
9	2	10	490	200
10	2	25	475	500
11	2	50	450	1000
12	1	10	490	2000
13	1	25	475	5000
14	1	50	450	10 000

5 Specimen collection and sample preparation

5.1 Specimen collection

The urine samples are collected in suitable plastic containers and stored at −20 °C until analysis.

5.2 Sample preparation

Prior to analysis, the urine samples are thawed at room temperature and mixed thoroughly. A 0.5 mL aliquot is taken and pipetted into a 1.5 ml vial. Then, 10 µl of the ISTD spiking solution and 1 ml of the ammonium acetate buffer (pH 6.5) are added. For hydrolysis, 5 µl of the enzyme β-glucuronidase are added, the sample is mixed thoroughly and then incubated for three hours in an incubator at 37 °C. Afterwards, an aliquot of the sample solution is directly injected into the LC-MS/MS system for analysis.

6 Operational parameters

Analysis was performed using a Waters Alliance HPLC system coupled with a Waters Quattro Ultima tandem mass spectrometer.

6.1 High performance liquid chromatography

Analytical column:	Zorbax Eclipse XDB-C8, 5 µm, 4.6 × 50 mm
Pre-concentration column:	Oasis HLB 25 µm, 2.1 × 20 mm
Separation principle:	Reversed phase
Eluent:	A: ultrapure water B: 1% aqueous formic acid C: acetonitrile
Column temperature:	30 °C
Injection volume:	100 µl
Flow rate:	0.2 ml/min, constant
Gradient program:	see Table 3
Column switching program:	0–3.5 min via pre-concentration column; 3.5–15 min via pre-concentration column and analytical column (see schematic representation of the column switching program in Figure 2)

Tab. 3 Gradient pump program

Time [min]	Eluent A [%]	Eluent B [%]	Eluent C [%]
0	70	10	20
4	70	10	20
5	0	10	90
8	0	10	90
8.5	70	10	20
12	70	10	20
15	70	10	20

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

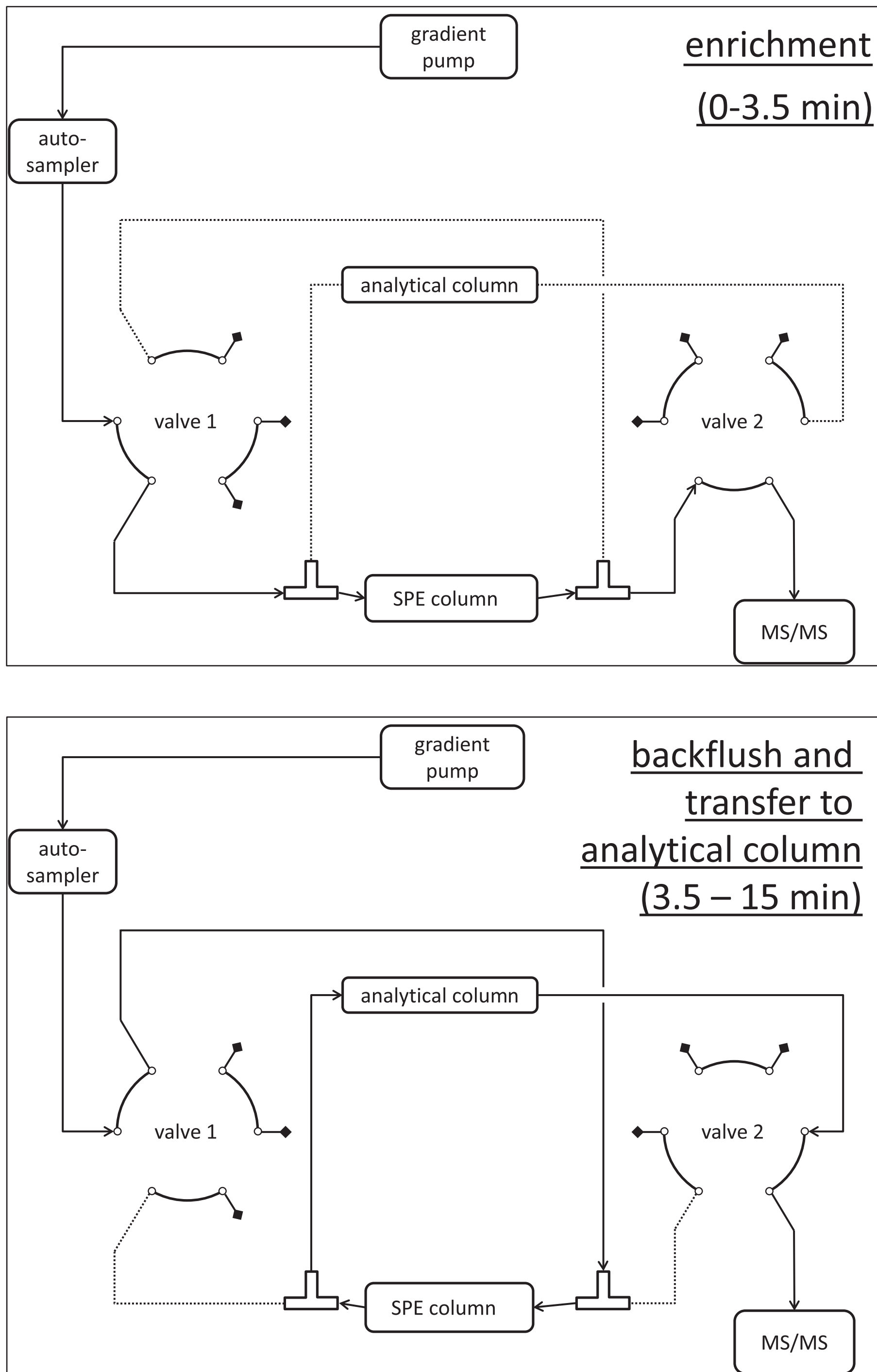

Fig. 2 Schematic representation of the column switching program

6.2 Tandem mass spectrometry

Ionisation mode:	Positive electrospray ionisation
Source temperature:	150 °C
Desolvation temperature:	300 °C
Cone gas flow:	100 l/h
Desolvation gas flow:	636 l/h
Dwell time:	0.2 s
Parameter-specific settings:	see Table 4

Tab. 4 Parameter-specific settings for the analyte MBT and the internal standard

Analyte	Retention time [min]	Precursor ion [*m/z*]	Product ion [*m/z*]
MBT	12.94	167.9	135.1 124.2 (qualifier)
MBT-d_4 (ISTD)	12.90	171.9	139.1 128.2 (qualifier)

All settings are instrument-specific and must be adjusted individually by the user. The parameters specified above are therefore intended as a rough guide only. Figures 3 and 4 show the product ion spectra of the analyte MBT and the ISTD.

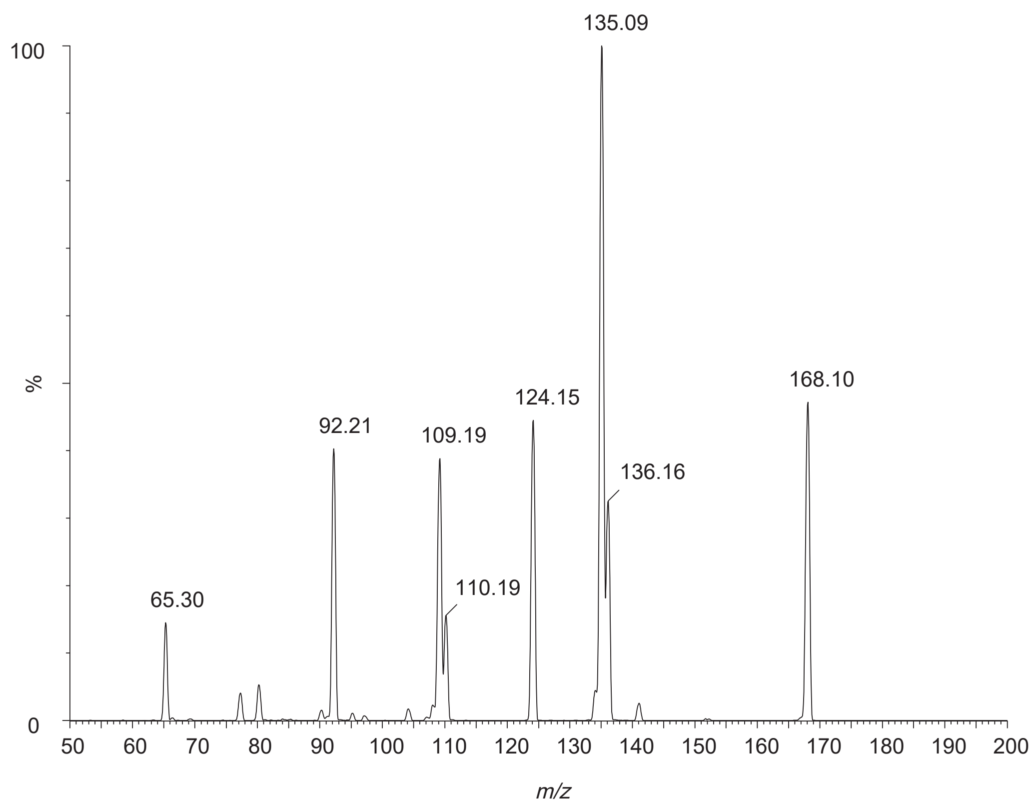

Fig. 3 Product ion spectrum of the analyte MBT

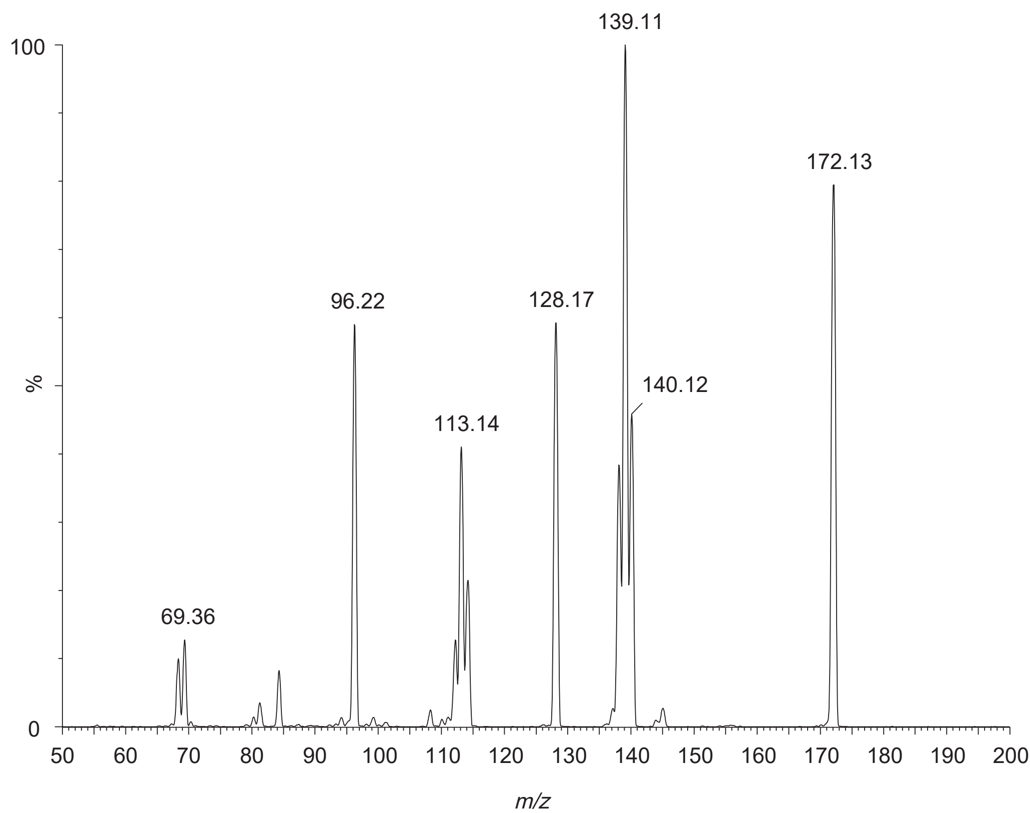

Fig. 4 Product ion spectrum of the internal standard MBT-d_4

7 Analytical determination

100 µl each of the samples prepared as described in Section 5 are injected into the LC-MS/MS system and transferred to the pre-concentration column. The analyte is then transferred from the pre-concentration column to the analytical column in backflush mode, where the actual analytical separation takes place. Identification of the analyte is based on the retention time and specific ion transitions. The retention times of the analyte and of the internal standard as well as the recorded ion transitions are listed in Table 4. The retention times given in Table 4 are intended as a rough guide only. Users must ensure proper separation performance of the HPLC column used influencing the resulting retention behaviour of the analyte. Figures 5 to 7 show, as an example, chromatograms of a reagent blank, of a native urine sample and of a urine sample spiked with MBT.

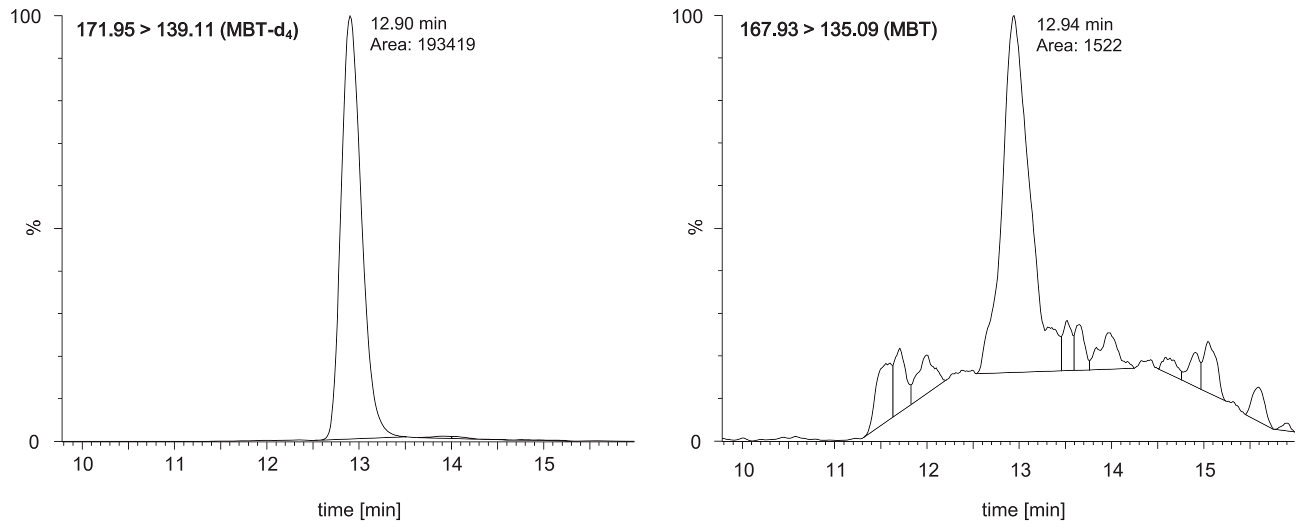

Fig. 5 Chromatogram of a reagent blank (MBT level < LOD)

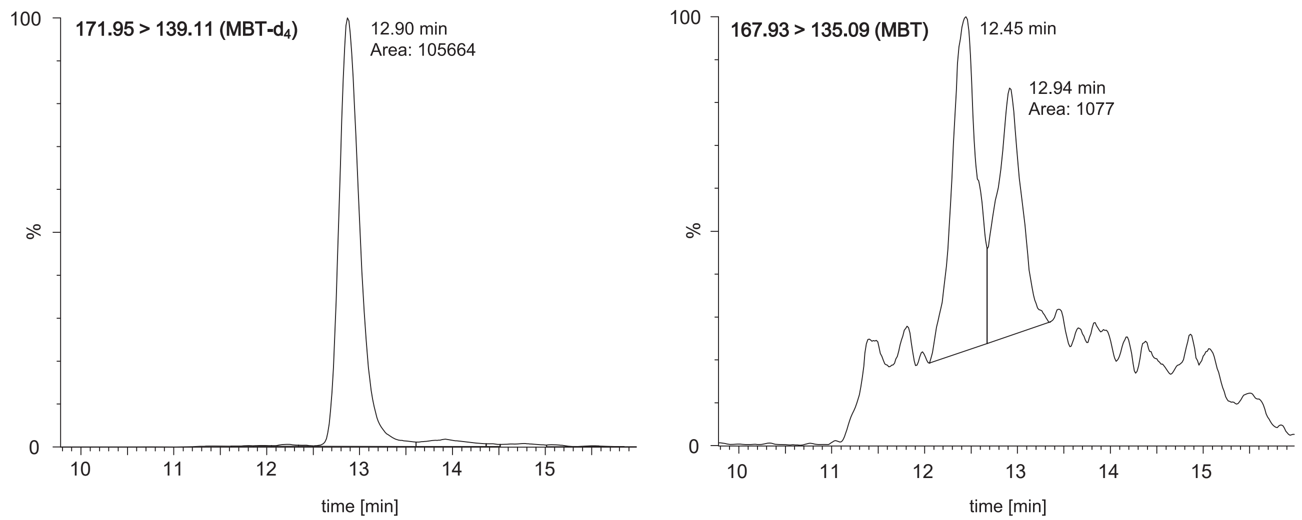

Fig. 6 Chromatogram of a native urine sample (MBT level < LOD)

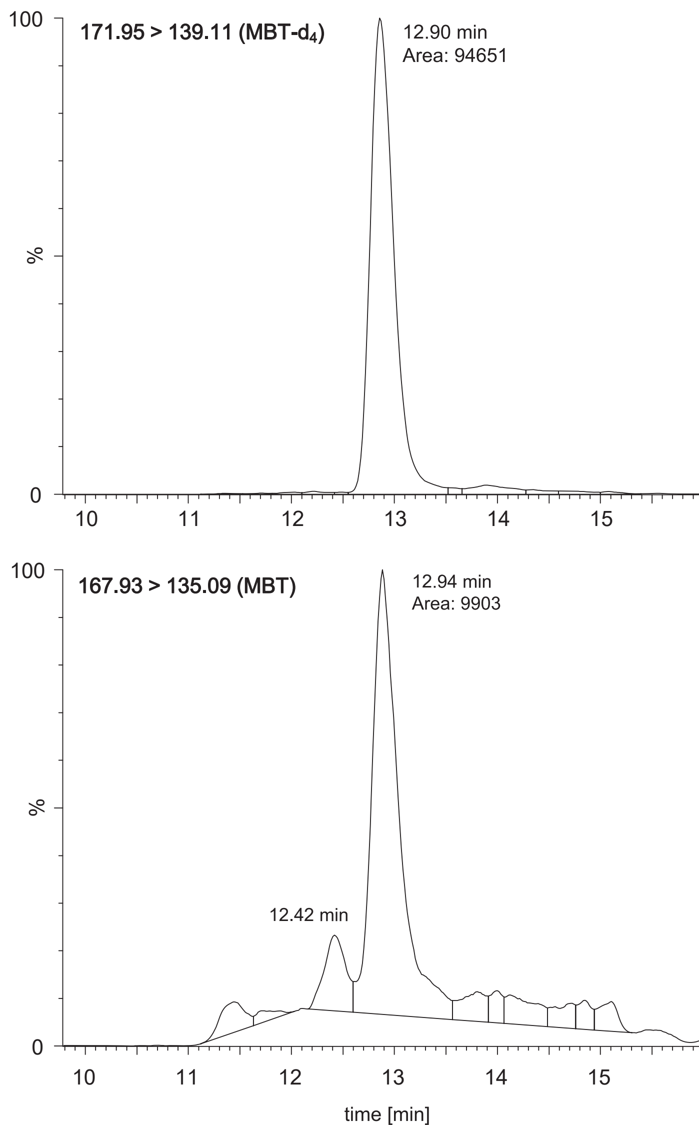

Fig. 7 Chromatogram of a urine sample spiked with 10 µg MBT/l

8 Calibration

The calibration standards described in Section 4.5 are prepared and processed in the same way as the samples (cf. Section 5.2) and analysed by LC-MS/MS (cf. Section 6). Calibration graphs are obtained by plotting the quotients of the peak areas of the analyte and of the internal standard against the spiked concentration of the respective calibration standards. The calibration graph is linear in the concentration range from the detection limit to 2000 µg/l. In the extended calibration range of up to 10 000 µg/l, it is advisable to use a quadratic regression. As an example, Figure 8 shows a calibration graph of the analyte MBT prepared in urine.

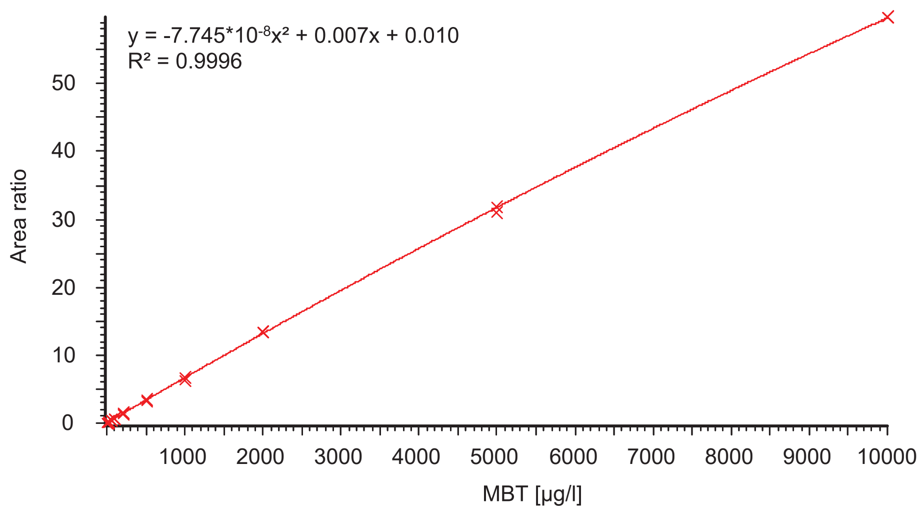

Fig. 8 Calibration graph for the determination of MBT in urine

9 Calculation of the analytical results

The determined peak area of MBT is divided by the peak area of the internal standard MBT-d_4. The quotient thus obtained is entered in the calibration function (cf. Section 8) to give the respective analyte concentration in µg/l urine. Any reagent blank values as well as background levels detected in the pooled urine samples used have to be subtracted from the analytical result. The analyte concentrations were calculated using the Waters MassLynx 4.1 software.

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 of the MAK-Collection for Occupational Health and Safety (Bader et al. 2010; Bundesärztekammer 2014). To check precision, at least three 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, spiking solutions of the analyte are added to pooled urine samples from persons not occupationally exposed to MBT, so that the concentration of the control material is within the relevant concentration range (e.g. 10 µg/l, 100 µg/l and 1000 µg/l). Aliquots of these samples are stored at −20 °C and are included in each analytical run as quality control samples. The nominal values and the tolerance ranges of the quality control materials are determined in a pre-analytical period (one analysis of the control materials each on 10 different days) (Bader et al. 2010).

11 Evaluation of the method

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

11.1 Precision

The control material described in Section 10 was used to determine within-day precision. The spiked pooled urine samples were processed (Section 5.2) and analysed (Section 6) ten times in parallel as described. The obtained within-day precision data are presented in Table 5.

Tab. 5 Within-day precision for the determination of MBT in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
MBT	10	2.3	5.1
	100	1.9	4.2
	1000	1.6	3.6

To determine day-to-day precision, the control material described in Section 10 was processed and analysed twice each on five different days. The obtained day-to-day precision data are presented in Table 6.

Tab. 6 Day-to-day precision for the determination of MBT in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
MBT	10	4.3	9.6
	100	5.8	13.0
	1000	3.4	7.6

11.2 Accuracy

The accuracy of the method was determined on the basis of the within-day precision data and the day-to-day precision data (cf. Section 11.1). The relative recovery rates thus obtained are presented in Tables 7 and 8.

Tab. 7 Mean relative recovery rates for the determination of MBT in urine, derived from the within-day precision data (n = 10)

Analyte	Spiked concentration [µg/l]	Mean relative recovery r [%]	Range [%]
MBT	10	87	85–91
	100	93	90–96
	1000	90	89–94

Tab. 8 Mean relative recovery rates for the determination of MBT in urine, derived from the day-to-day precision data (n = 10)

Analyte	Spiked concentration [µg/l]	Mean relative recovery r [%]	Range [%]
MBT	10	86	81–93
	100	96	93–112
	1000	96	91–102

To determine accuracy in different matrices, ten different urine samples were analysed (unspiked and spiked with 10 and 100 µg MBT per litre, respectively) to determine the influence of different urine matrices. The results obtained are presented in Table 9.

Tab. 9 Mean relative recovery rates for the determination of MBT in individual urine samples (n = 10)

Analyte	Spiked concentration [µg/l]	Mean relative recovery *r* [%]	Range [%]
MBT	10	84	76–105
	100	95	92–99

11.3 Hydrolysis

In order to test the analytical method using real urine samples and to assess the need for hydrolysis, urine samples from five subjects were analysed, four of whom had been occupationally exposed to MBT (samples 2–5). The results are presented in Table 10. Figures 9 and 10 show the chromatograms of a processed urine sample without hydrolysis and with hydrolysis.

Tab. 10 MBT levels in urine with and without hydrolysis (n = 5)

Sample	Creatinine [g/l]	MBT without hydrolysis [µg/l]	MBT with hydrolysis [µg/l]	Conjugation rate [%]
1	0.86	< LOQ	2.5	100
2	0.58	< LOQ	567	100
3	1.24	67	3840	98
4	1.99	73	5261	99
5	3.29	137	6210	98

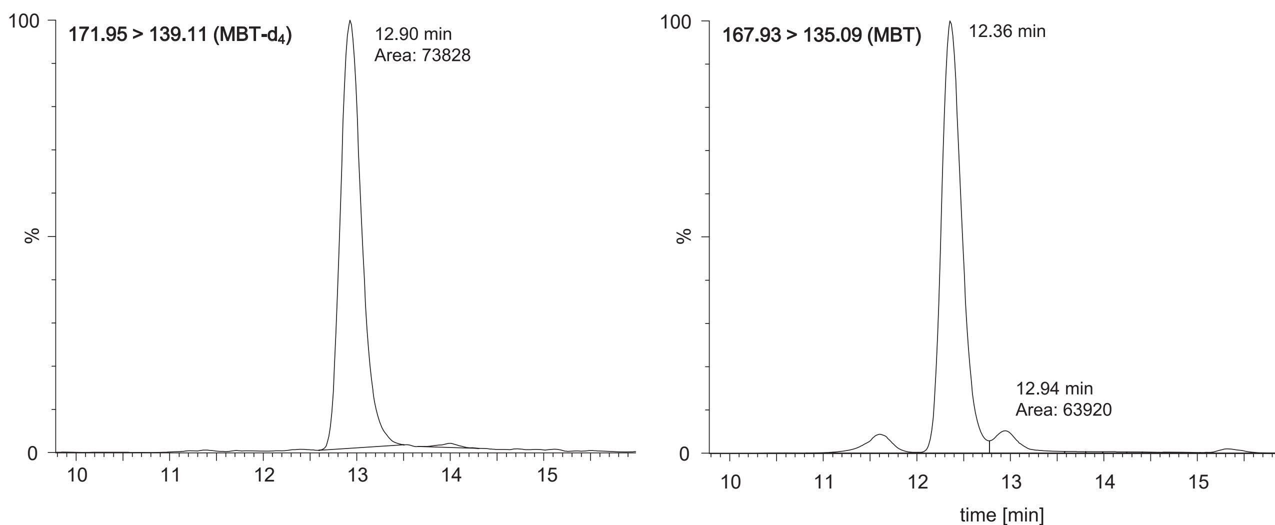

Fig. 9 Chromatogram of a native urine sample without enzymatic hydrolysis (determined MBT level of 67 µg/l)

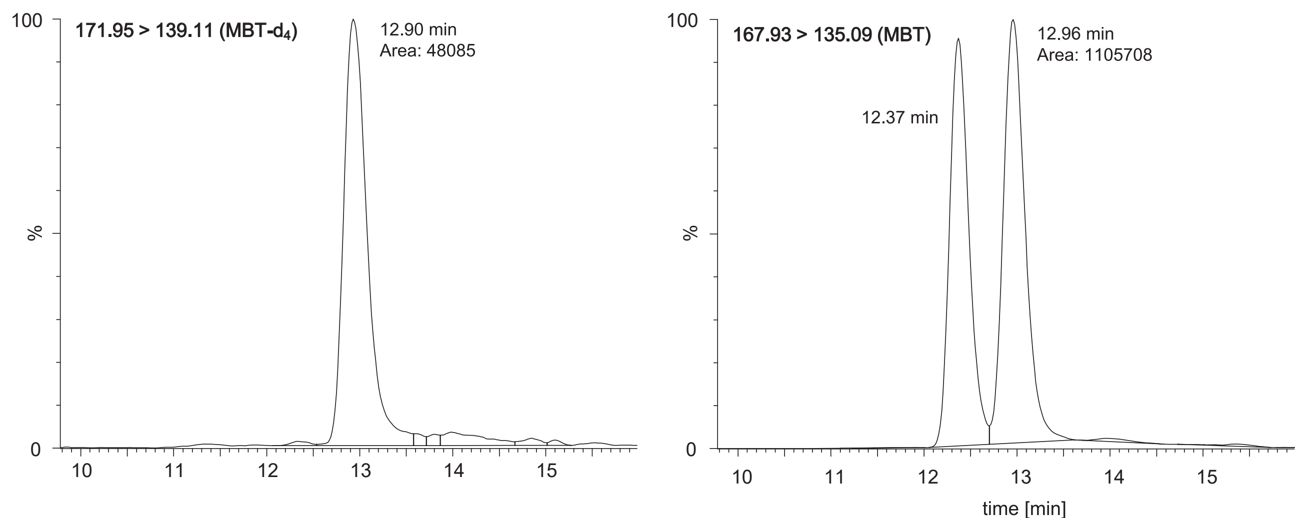

Fig. 10 Chromatogram of a native urine sample with enzymatic hydrolysis (determined MBT level of 3840 µg/l)

11.4 Limit of detection and limit of quantitation

The detection limit and the quantitation limit were determined according to the calibration curve method using the six lowest calibration levels. The values obtained are presented in Table 11.

Tab. 11 Limit of detection and limit of quantitation for the determination of MBT in urine

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
MBT	0.4	1.2

11.5 Sources of error

A reagent blank of approximately 1 µg MBT/l was detected, which remained constant throughout the entire method development and could be traced back to a contamination from the internal standard. All samples and calibration standards were blank-subtracted. Further blanks from the reagents or other laboratory equipment were not observed.

It should also be noted that MBT as a standard is not stable in solution. A degradation of approximately 20% within three months was observed. After nine months of storage, the peak intensity of the standard solution was only 40% compared to a freshly prepared standard solution, even though the standard was dissolved in acetonitrile and stored in the refrigerator at 4 °C in the dark. Thus, the MBT standard solution should be freshly prepared at least every four weeks.

In the course of external verification, the process-related analyte losses were estimated by analysing a standard solution once with and once without the pre-concentration column, revealing analyte losses on the pre-concentration column of approximately 10%.

As early as in the initial phase of LC-MS/MS method development, it was observed that the internal standard 2-mercaptobenzoxazole used for GC-MS analysis of MBT by Manninen et al. (1996) is not suitable for the presented method, as its deviating chemico-physical behaviour could not compensate the analysis-related variations when using LC-MS/MS analysis. However, the use of MBT-d_4 as an internal standard with similar fragmentation patterns

enables an excellent compensation of analytical variations, which is confirmed by the very good precision data. Tests with spiked individual urine samples also confirm the robustness of the analytical method.

12 Discussion of the method

In the initial phase of method development, attempts were made to isolate MBT from urine using liquid-liquid extraction. Due to the obtained absolute recovery rates of < 10%, this approach was not pursued any further.

As MBT is highly adsorptive due to its polar structure and weak acidity at the SH group, the use of external solid phase extraction could not significantly increase the absolute recovery rates. Due to the adsorptive properties of MBT, the entire analysis of the sample was performed in a single vial in order to minimise work-up related losses. The hydrolysed sample was enriched and purified online using a Waters Oasis HLB pre-concentration column. After sample backflush onto the Zorbax Eclipse HDB-C8 column, the sample was separated by liquid chromatography and analysed using tandem mass spectrometry.

Another step towards optimisation was the establishment of enzymatic hydrolysis. Compared to acid hydrolysis described in the literature (Manninen et al. 1996), it led to a fourfold higher hydrolysis rate (data not shown). Moreover, the analysis of urine samples from persons occupationally exposed to MBT showed that well over 90% of the MBT eliminated in urine is present in the form of conjugates (cf. Section 11.3). Hydrolysis is therefore essential in order to enable a valid determination of MBT in urine.

On the whole, this method enables the reliable quantitation of MBT in the urine of occupationally exposed persons. The studies on background levels of MBT in the urine of the general population (see Table 1) show that this method can also be used to determine the upper range of this background exposure to MBT.

Instruments used Analysis was performed using a Waters Alliance HPLC system coupled with a Waters Quattro Ultima tandem mass detector.

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