Monohydroxybutenyl-mercapturic acid (MHBMA) and dihydroxybutylmercapturic acid (DHBMA)

Application Determination in urine

Analytical principle High performance liquid chromatography/ tandem mass spectrometric detection (LC/MS/MS)

Summary

The procedure described here is suitable for the determination of monohydroxy-butenylmercapturic acid (MHBMA) and dihydroxybutylmercapturic acid (DHBMA), two main metabolites of 1,3-butadiene, in the urine of persons exposed to the substance at the workplace or in the environment. After acidification of the urine, MHBMA and DHBMA are separated from interfering components of the matrix by means of a solid phase extraction. Then the analytes are separated by high performance liquid chromatography and quantified by tandem mass spectrometric detection. Ionisation is achieved by negative APCI (atmospheric pressure chemical ionisation). Calibration is carried out with spiked pooled urine samples from non-smokers in the concentration range from 0.5 µg/L to 500 µg/L (MHBMA) or 50.0 µg/L to 1250 µg/L (DHBMA). Deuterated structural analogues of the pure substances ($[D_6]$-MHBMA and $[D_7]$-DHBMA) are used as internal standards to enable quantification.

Monohydroxybutenylmercapturic acid (MHBMA)

Within-series imprecision: Standard deviation (rel.) $s_w = 4.1\%$
Prognostic range $u = 10.5\%$
at a spiked concentration of 7.35 µg MHBMA per litre urine and where n = 5 determinations

Between-day imprecision: Standard deviation (rel.) $s_w = 7.5\%$
Prognostic range $u = 15.7\%$
at a spiked concentration of 37.64 µg MHBMA per litre urine and where n = 21 determinations

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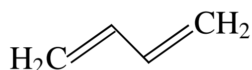
Accuracy:	Recovery rate and	$r = 93.7\%$ at 0.5 µg/L 95.2% at 2 µg/L
Quantitation limit:	2.73 µg MHBMA per litre urine	

Dihydroxybutylmercapturic acid (DHBMA)

Within-series imprecision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 496.5 µg DHBMA per litre urine and where $n = 5$ determinations	$s_w = 0.8\%$ $u = 2.1\%$
Between-day imprecision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 394.8 µg DHBMA per litre urine and where $n = 21$ determinations	$s_w = 5.7\%$ $u = 11.9\%$
Accuracy:	Recovery rate and	$r = 95.3\%$ at 50 µg/L 73.6% at 1000 µg/L
Quantitation limit:	75.9 µg DHBMA per litre urine	

1,3-Butadiene



1,3-Butadiene is one of the most important basic chemicals. It is used alone for the manufacture of rubber and as a copolymer together with styrene [1]. The annual production is more than 5 million tonnes worldwide. Automobile exhaust gases [2] and the burning of fossil fuels (especially in domestic heating) represent important sources of environmental and human exposure. A car emits approx. 6 mg of 1,3-butadiene for every kilometre it travels [1]. Heating exhaust gases contain 33 mg/m^3. The half-life of 1,3-butadiene in the external air is estimated as approx. 4 h [3]. The concentration in urban air is 1 to 20 µg/m^3, depending on the density of traffic [4, 5]. Tobacco smoke is an important source of 1,3-butadiene in the air in enclosed spaces. Concentrations of between 2.7 and 19 µg/m^3 have been reported for 1,3-butadiene in smoke-filled rooms [1, 6, 7]. It was determined that smokers inhale 16 to 75 µg of 1,3-butadiene per cigarette in the mainstream smoke [7]. Exposure to 1,3-butadiene in food (values in the lower ppb range) and drinking water is thought to play a subordinate role [5, 8].

The International Agency for Research on Cancer (IARC) has classified 1,3-butadiene as "probably carcinogenic in humans" (Group 2A) [1]. The Commission has

assigned 1,3-butadiene to Carcinogen Category 1 ("causing cancer in humans") [9, 10]. A comprehensive review of the toxicological aspects of 1,3-butadiene can be found in the MAK value documentation [10].

Figure 1 shows an overview of the metabolism of 1,3-butadiene [11]. The first step of the metabolism of 1,3-butadiene is oxidation to the corresponding epoxides (1,2-epoxy-3-butene and 1,2,3,4-diepoxybutane) and unsaturated aldehydes (3-butenal, crotonaldehyde). In principle, the reactive epoxides can react with the cellular macromolecules, such as proteins or DNA, to form adducts or can be metabolised to hydroxymetabolites by epoxide hydrolases. An important metabolic pathway of the reactive intermediate products of 1,3-butadiene is conjugation with glutathione (GSH) (which may proceed under the enzymatic control of glutathione-S-transferases (GST) or spontaneously). These conjugates are further transformed to mercapturic acids. To date, three mercapturic acids have been identified for 1,3-butadiene: firstly, monohydroxybutenylmercapturic acid (MHBMA, sometimes designated MII in the literature), which consists of an isomeric mixture of 1-hydroxy-2-(N-acetylcysteinyl)-3-butene and 1-(N-Acetylcysteinyl)-2-hydroxy-3-butene, secondly, dihydroxybutylmercap-

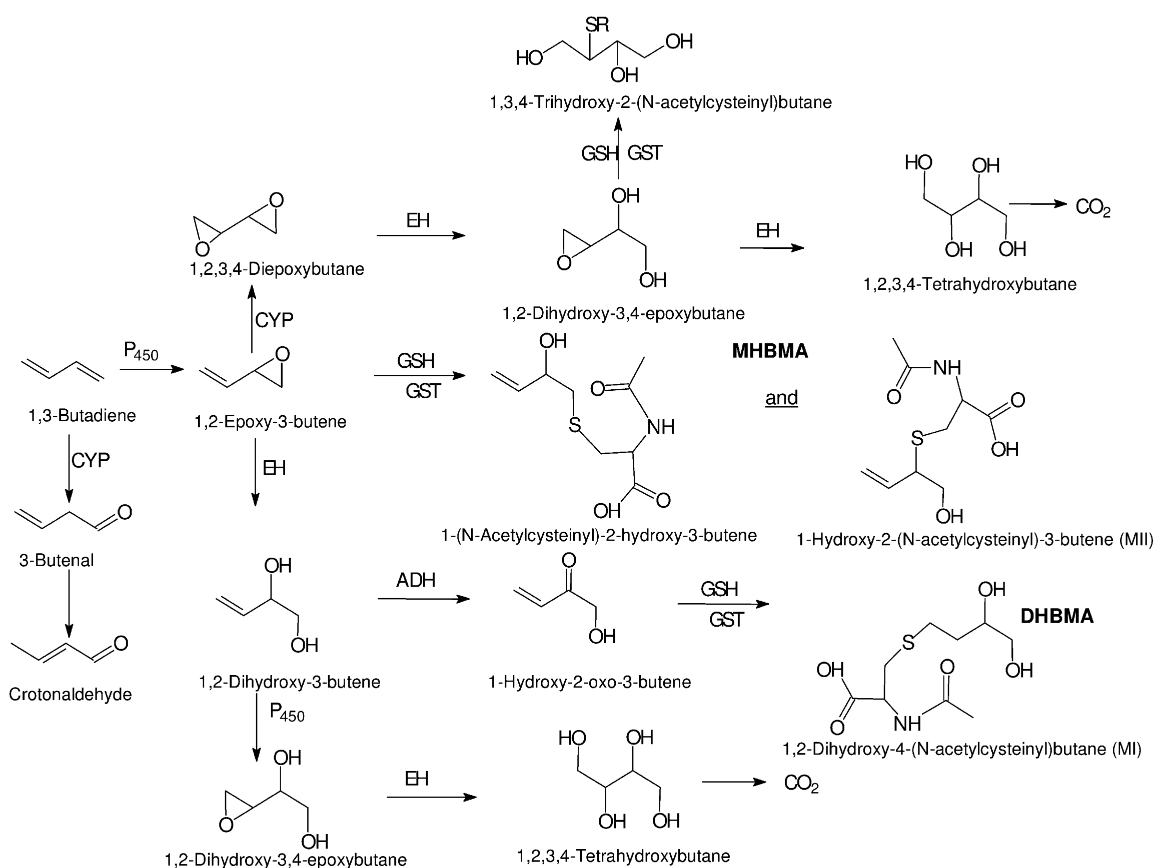


Fig. 1. Overview of the metabolism of 1,3-butadiene [11]. (SR = N-acetylcysteinyl group; GST = glutathione-S-transferase; GSH = glutathione; EH = epoxide hydrolase; CYP = cytochrome P_{450}, ADH = alcohol dehydrogenase)

turic acid (DHBMA or MI, systematic name 1,2-dihydroxy-4-(N-acetylcysteinyl)butane) and thirdly, 1,3,4-trihydroxy-2-(N-acetylcysteinyl)butane.
As a rule, the MHBMA and DHBMA metabolites are determined in urine for the purpose of biomonitoring. It can be assumed that DHBMA indicates the extent of the hydrolysis of 1,2-epoxy-3-butene to 1,2-dihydroxy-3-butene before the latter conjugates with GSH (probably after oxidation to unsaturated 1-hydroxy-2-oxo-3-butene). In contrast, MHBMA indicates the direct detoxification of 1,2-epoxy-3-butene by conjugation with GSH [12, 13]. The ratio of the metabolites in urine after exposure to 1,3-butadiene DHBMA/(DHBMA+MHBMA) is clearly dependent on the species and is approx. 0.9 for humans and monkeys [12–15], 0.25 to 0.5 for rats and 0.2 to 0.4 for mice [12, 14]. As 1,3-butadiene exposure increases the metabolic ratio decreases in various species [11, 14, 15].
Table 1 presents a brief overview of the MHBMA and DHBMA concentrations detected in investigations carried out in the fields of occupational and environmental medicine.

Table 1. MHBMA and DHBMA concentrations in the urine of persons after occupational and environmental exposure to 1,3-butadiene

Group	n	Concentration MHBMA [µg/L]	Concentration DHBMA [µg/L]	Reference
Investigations in occupational medicine				
Highly exposed workers	7	–	Mean 3200 ± 1600	[14]
Moderately exposed workers	3	–	Mean 1390 ± 550	[14]
Non-exposed Workers	10	–	Mean 630 ± 190	[14]
Control group	9	–	Mean 320 ± 70	[14]
Styrene-butadiene rubber workers	30	Range <0.1–962	Range 60 – 26207	[11]
1,3-Butadiene monomer workers	23	Range <0.1–44	Range 52–3522	[11]
Non-exposed control subjects	24	Range <0.1–8.2	Range 197–1211	[11]
Investigations in environmental medicine				
Smokers	10	Mean 86.4±14.0	Mean 644 ± 90	[15]
Non-smokers	10	Mean 12.5±1.0	Mean 459 ± 72	[15]

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1 General principles

After acidification of the urine, monohydroxybutenylmercapturic acid and dihydroxybutylmercapturic acid are separated from interfering components of the matrix by means of a solid phase extraction. Then the analytes are separated by high performance liquid chromatography and quantified by tandem mass spectrometric detection. Ionisation is achieved by means of negative APCI. Calibration is performed using calibration standard solutions that are prepared in pooled urine from non-smokers and are treated in the same manner as the samples to be analysed. Deuterated structural analogues of the pure substances ([D_6]-MHBMA and [D_7]-DHBMA) are used as internal standards for quantification.

2 Equipment, chemicals and solutions

2.1 Equipment

LC/MS/MS system consisting of a binary high-pressure pump, column oven with a switch valve, degasser, automatic thermostatically controlled liquid sampling device and a tandem mass spectrometric detector with APCI, as well as a PC system for data evaluation.

Compressor (e.g. Manglitz model 4000 KCT-401-100-M.H.)

Membrane air dryer (e.g. Whatman model 64-01)

Nitrogen generator (e.g. Whatman model 75-72)

HPLC column:
Atlantis dC_{18}, length: 150 mm; inner diameter: 4.6 mm; particle diameter: 3 µm (e.g. Waters) with C_{18} pre-column, length: 4 mm; inner diameter: 3 mm, and precolumn holder (e.g. Phenomenex)

Test-tube shaker (e.g. Vortex, Carl Roth)

Variably adjustable pipettes: 10 to 100 µL, 100 to 1000 µL, 1000 to 5000 µL (e.g. Eppendorf Varipettes)

Volumetric flasks: 10 mL, 50 mL, 100 mL, 250 mL, 1000 mL

Glass beakers: 20 mL, 150 mL

100 mL Measuring cylinders

4 mL Graduated glass bottles with screw cap (e.g. Zefa Laborservice)

25 mL Amber glass bottles

0.5 to 2 L Polyethylene bottles for collecting human urine samples

10 mL Polyethylene tubes for portioning

Rotation evaporator (e.g. Büchi Labortechnik Rotavapor R-134)

Vacuum pump (e.g. Büchi Labortechnik)

Solid phase extraction columns (e.g. Waters Oasis HLB, 500 mg, 6 mL, No. 186000115)

Solid phase extraction station (e.g. Separtis VacMaster 20 with collection tube rack and lid 121-2016 and Isolute PTFE needle and stopcock, No. 121-0001)

Vacuum centrifuge (e.g. Jouan Speedvac Concentrator)

Roller mixer

Filter system 0.22 µm (e.g. Millipore GS)

100 mL Microvials (e.g. from Agilent Technologies)

Pipettes: 20 mL, 50 mL, 100 mL (e.g. from Brand)

pH meter (e.g. Lab 850, Schott Instruments)

Gassing system with four 250 mL washing bottles

Device for evaporation under a stream of nitrogen (e.g. Reacti-Therm, Pearce)

2.2 Chemicals

All chemicals must be analytical grade (p.a.) or purer.

(R)/(S)-N-acetyl-S-(1-hydroxymethyl)-2-propenyl)-L-cysteine / (R)/(S)-N-acetyl-S-(2-hydroxy)-3-butenyl)-L-cysteine, MHBMA (e.g. Toronto Research Chemicals No. A179005)

(R)/(S)-N-acetyl-S-(1-hydroxymethyl)-2-propenyl)-L-cysteine-D_6/(R)/(S)-N-acetyl-S-(2-hydroxy)-3-butenyl)-L-cysteine-D_6, [D_6]-MHBMA (e.g. Toronto Research Chemicals No. A179007)

N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine, DHBMA (e.g. Toronto Research Chemicals No. A173710)

N-acetyl-S-(3,4-dihydroxybutyl)-L-cysteine-D_7, [D_7]-DHBMA (e.g. Toronto Research Chemicals No. A173712)

Hydrochloric acid, 32% (e.g. Roth, Order No. P 074.1)

Formic acid, p.a., 98 to 100% (e.g. Merck No. 1.00264.1000)

Ammonium acetate p.a. (e.g. Merck No. 1.01116.0500)

Ammonia 3.8, anhydrous (e.g. Linde No 4930795)

Nitrogen 5.0 (e.g. from Linde)

Deionised water (e.g. Seradest ultrapure water plant)

Methanol, HPLC grade (e.g. Promochem code 3041)

Ethyl acetate, trace analysis grade (e.g. Promochem code 1191.1)

Acetonitrile, HPLC grade (e.g. Merck No. 1.00030.9010)

2.3 Solutions

4 M Hydrochloric acid:
Approx. 100 mL water are placed in a 250 mL volumetric flask and a pipette is used to carefully add 100 mL hydrochloric acid (32%). After the concentrated hydrochloric acid has been pipetted, the pipette is rinsed several times with the contents of the flask to remove any residual hydrogen chloride gas from the body of the pipette. The flask is subsequently filled to its nominal volume with water. The solution is kept at room temperature and can be used over a period of 6 months.

1 M Hydrochloric acid:
Using a pipette, 50 mL hydrochloric acid (32%) are added to a 500 mL volumetric flask into which approx. 250 mL water has been previously placed. After the concentrated hydrochloric acid has been pipetted, the pipette is rinsed several times with the contents of the flask to remove any residual hydrogen chloride gas from the body of the pipette. The flask is subsequently filled to its nominal volume with water. The solution is kept in the refrigerator at 4 to 6 °C and can be used over a period of 6 months.

2% Formic acid pH 2 for HPLC:
Approx. 500 mL deionised water are placed in a 1000 mL volumetric flask and a pipette is used to add 20 mL formic acid. The flask is subsequently filled to its nominal volume with water. The solution is filtered through a 0.22 µm filter.
The solution is stored at 4 to 6 °C in the refrigerator. It is stable for 1 week.

Hydrochloric acid pH 2.0:
1 M hydrochloric acid is added drop by drop using a pipette to 1000 mL deionised water in a glass beaker, while the pH is constantly checked with a pH meter, until the desired pH value is reached. The solution is stored at room temperature in a sealable glass vessel. It is stable for 1 week.

Ethyl acetate (saturated with ammonia) with 20% MeOH (v/v):
A washing bottle filled with 250 mL ethyl acetate is integrated in second place into a gassing system that consists of four washing bottles. The first and third bottles are empty. The fourth bottle is two-thirds filled with water. All the bottles are secured by clamps. The first bottle is connected to the pressure valve of an ammonia bottle. The valve is opened for 3 min. 50 mL methanol are placed in a 250 mL volumetric flask and the flask is filled to its nominal volume with the freshly prepared ethyl acetate solution.

The solution is kept at room temperature and can be used over a period of 1 week.

0.5% Acetonitrile in hydrochloric acid pH 2.0:
0.5 mL Acetonitrile are placed in a 100 mL volumetric flask. The flask is subsequently filled to its nominal volume with hydrochloric acid pH 2.0. The solution is stored at room temperature. The solution is stable for 1 week.

2.4 Calibration standards

2.4.1 Internal standard

[D_6]-MHBMA stock solution:
10.0 mg of (R)/(S)-N-acetyl-S-(1-hydroxymethyl)-2-propenyl)-L-cysteine-D_6/(R)/(S)-N-acetyl-S-(2-hydroxy)-3-butenyl)-L-cysteine-D_6 are weighed exactly in a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (1 g/L).

[D_7]-DHBMA stock solution:
10.0 mg of N-acetyl-S-(3,4-dihydroxy-butyl)-L-cysteine-D_7 are weighed exactly in a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (1 g/L).

[D_6]-MHBMA working solution ISTD I:
100 µL of the [D_6]-MHBMA stock solution are pipetted into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (10 mg/L).

[D_7]-DHBMA working solution ISTD II:
100 µL of the [D_7]-DHBMA stock solution are pipetted into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (10 mg/L).

The stock solutions and working solutions of the internal standards are frozen in 25 mL amber glass bottles at –18 °C and are stable for at least 12 months under these conditions.

2.4.2 Calibration standards

MHBMA stock solution:
10.0 mg of (R)/(S)-N-acetyl-S-(1-hydroxymethyl)-2-propenyl)-L-cysteine / (R)/(S)-N-acetyl-S-(2-hydroxy)-3-butenyl)-L-cysteine are weighed exactly in a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (1 g/L).

DHBMA stock solution:
10.0 mg of N-acetyl-S-(3,4-dihydroxy-butyl)-L-cysteine are weighed exactly in a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (1 g/L).

MHBMA working solution I:
100 µL of the MHBMA stock solution are pipetted into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (10 mg/L).

MHBMA working solution II:
100 µL of MHBMA working solution I are pipetted into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (100 µg/L).

DHBMA working solution:
100 µL of the DHBMA stock solution are pipetted into a 10 mL volumetric flask. The flask is subsequently filled to its nominal volume with methanol (10 mg/L).

The stock solutions and working solutions of the calibration standards are frozen in 25 mL amber glass bottles at –18 °C and are stable for at least 12 months under these conditions.

The calibration standard solutions are prepared as follows:
Frozen pooled human urine is thawed overnight. As soon as it has reached room temperature, it is shaken vigorously and then centrifuged (10 °C, 2000 g, 10 min). A 20 mL aliquot of the supernatant liquid is placed in a glass beaker and the pH is adjusted initially to pH 3.0–2.5 by adding 4 M hydrochloric acid drop by drop using a pH meter while the liquid is stirred constantly, and then the pH is adjusted to exactly 2.0 using 1 M hydrochloric acid (see also Section 3.1). The volumes of MHBMA working solutions I and II, the DHBMA working solution and the solutions of the internal standards ISTD I and II shown in Table 2 are each pipetted into a 10 mL PE tube, and are then evaporated to dryness under a gentle stream of nitrogen without heating. Then 2 mL of the acidified pooled urine are pipetted into each tube and the contents are mixed on a roller mixer for at least 10 min in order to ensure complete dissolution of the standard substances in urine (see pipetting scheme in Table 2).

Table 2. Pipetting scheme for the preparation of the calibration standard solutions in human urine

Volume of the working solutions			Volume of the spiking solutions of the internal standards		Volume of the pooled urine (adjusted to pH 2.0)	Concentration of the calibration standards	
MHBMA		DHBMA	[D_6]-MHBMA	[D_7]-DHBMA		MHBMA	DHBMA
I [µL]	II [µL]	[µL]	ISTD I [µL]	ISTD II [µL]	[mL]	[µg/L]	[µg/L]
–	25	25	100	100	2	1.25	125
–	50	50	100	100	2	2.5	250
–	100	100	100	100	2	5.0	500
–	250	250	100	100	2	12.5	1250
10	–	500	100	100	2	50	2500
25	–	750	100	100	2	125	3750
100	–	–	100	100	2	500	–
250	–	–	100	100	2	1250	–

The resulting calibration standard solutions are processed as described in Section 3.1, whereby the working steps before the solid phase extraction are omitted for the calibration standard solutions, as they are used directly for the solid phase extraction.

3 Specimen collection and sample preparation

The polyethylene bottles used to collect the urine specimens are washed with a cleansing agent and rinsed with bidistilled water. The samples are divided into aliquots after collection (5 to 10 mL sample in 10 mL tubes) and they are then deep-frozen (–18 °C) until analysis is carried out. Stabilisation is unnecessary. The aliquots can be used for at least 6 months.

3.1 Sample preparation

The deep-frozen urine samples are thawed overnight. As soon as they have reached room temperature, they are shaken vigorously and then centrifuged (10 °C, 2000 g, 10 min). The supernatant liquid is cautiously withdrawn, transferred to a new 10 mL tube and the pH value is initially adjusted to 3.0–2.5 using 4 M hydrochloric acid while the contents are stirred continuously, and then the pH is adjusted to exactly 2.0 using 1 M hydrochloric acid. The electrode of the pH meter must be immersed in the sample tube during the entire pH adjustment. Adjustment of the pH value is regarded as complete when the pH value does not change over a period of at least 20 seconds without the contents being shaken. To perform a duplicate determination of human urine, 100 µL [D_6]-MHBMA working solution ISTD I (10 mg/L) and 100 µL [D_7]-DHBMA working solution ISTD II (10 mg/L) are each pipetted into a 10 mL PE tube, and are then evaporated to dryness under a gentle stream of nitrogen without heating. Then 2 mL of the centrifuged human urine are pipetted into each tube, and the contents are mixed on a roller mixer for at least 10 min in order to ensure complete dissolution of both internal standards in urine.
Then solid phase extraction is carried out on the Oasis HLB polymer material (500 mg cartridge, 6 mL). First the cartridges are conditioned with 2 × 6 mL ethyl acetate, 2 × 6 mL methanol and 2 × 6 mL dilute hydrochloric acid pH 2.0. After complete application of the samples at atmospheric pressure, the cartridges are washed first with 3 × 3 mL dilute hydrochloric acid pH 2.0, then with 6 mL of the solution of 0.5% acetonitrile in dilute hydrochloric acid pH 2.0, and then dried under vacuum (550 mbar, 3 min). Subsequently the cartridges are centrifuged (10 °C, 500 g, 10 min) and dried in a stream of nitrogen with the help of the drying lid. The pre-pressure at the nitrogen bottle is set at 2.0 bar for this purpose. When the aqueous phase has been completely compressed from the cartridge, drying is continued for 2 min.
Elution is carried out using the solution of 20% methanol in ethyl acetate saturated with ammonia (v/v) into a 4 mL vial. For this purpose 5 mL of the solution are applied and, after being allowed to act for 1 min, the solution is passed through under

slightly reduced pressure at 920 mbar so that drop-by-drop elution can be observed. In order to achieve complete elution the cartridges are subjected to a vacuum of 550 mbar for 2 min until complete dryness. The sample is evaporated to dryness using a vacuum centrifuge and then taken up in 100 µL of a 2% formic acid/methanol mixture 70 : 30 (v/v). The analytical determination is subsequently carried out by means of LC/MS/MS.
Figure 2 shows the sample processing in the form of a flowchart.

4 Operational parameters

The analytical measurements are performed with a combination of instruments comprising a HPLC system with a binary pump, column oven, degasser and autosampler, as well as a tandem quadrupole mass spectrometer with the possibility of negative APCI.

4.1 Operational parameters for high performance liquid chromatography

Separation column:	Material:	Steel
	Length:	150 mm
	Inner diameter:	4.6 mm
	Column packing:	Atlantis dC_{18}, 3 µm, 100 Å
Separation principle:	Reversed phase	
Temperature:	50 °C	
Detection:	Tandem mass spectrometric detector	
Mobile phase:	Eluent A:	2% Formic acid pH 2.0, aq.
	Eluent B:	Methanol
Gradient:	See Table 3	

Table 3. Gradient program of the binary pump

Time (min)	Eluent A vol. %	Eluent B vol. %
0.00	70	30
1.00	70	30
4.00	10	90
5.00	10	90
5.01	70	30
10.00	70	30

Stop time:	10 minutes		
Flow rate:	1 mL/min		
Autosampler:	Cooling:	10 °C	
	Injection volume:	10 µL	

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

4.2 Operational parameters for mass spectrometry

Settings for the ion source:

Ionisation mode:	APCI negative
Source temperature:	495 °C
Curtain gas pressure:	50 psi
Needle current (NC):	–2 µA
Nebulizer gas (GAS 1):	70 psi
Auxiliary/heater gas (GAS 2):	20 psi
Settings for the analyser:	See Table 4

Table 4. Analyser settings

Parameter	$[D_7]$-DHBMA	DHBMA		$[D_6]$-MHBMA	MHBMA	
Transition	257.2 → 128.0	250.0 → 121.0	250.0 → 75.0	238.2 → 109.0	232.0 → 103.0	232.0 → 73.0
Measuring time [ms]	200	200	200	200	200	200
DP [V]	–31	–56	–56	–26	–31	–31
FP [V]	–80	–190	–190	–60	–330	–330
EP [V]	9.5	8.0	8.0	10.0	6.0	6.0
CEP [V]	–8.0	–52	–52	–10	–10	–10
CXP [V]	–8	–6	–14	–8	–24	–8
CE [V]	–20	–22	–36	–12	–14	–34

DP = declustering potential, FP = focussing potential, EP = entrance potential, CEP = collision cell entrance potential, CXP = collision cell exit potential, CE = collision energy.

The measurement conditions listed here were established for the configuration of instruments used in this case and they must be optimised for the instruments of other manufacturers in accordance with their instructions.

5 Analytical determination

For quantitative determination, 10 μL of the sample solution are injected onto the separation column. Two quality control samples of different concentrations are included in each analytical series of 18 samples. A reagent blank value is determined every day. Each real sample is assayed in duplicate. If the resulting measurement values are outside the linear range of the calibration curve, the urine is diluted appropriately with deionised water (e.g. 1 : 10) before processing and measuring the samples anew. The ion transitions shown in Table 5 are recorded in the MRM mode of the tandem mass spectrometer (APCI negative mode).

Table 5. Retention times and detected ion transitions

Analyte	Retention time [min]	Ion transitions (MS/MS, APCI neg. mode)	
		Q 1	Q 3
[D_7]-DHBMA	2.19	257.2	128.0*
DHBMA	2.20	250.0	121.0* 75.0
[D_6]-MHBMA	2.97	238.2	109.0*
MHBMA	3.01	232.0	103.0* 73.0

The masses marked * are used for quantitative evaluation.

The retention times shown in Table 5 serve only as a guide. Users of the method must satisfy themselves of the separation power of the HPLC column they use and of the resulting retention behaviour of the substances.
Figures 3a and 3b show examples of the chromatograms of two urine samples (a smoker and a non-smoker).

6 Calibration

A matrix calibration was carried out using the control urine spiked as shown in Table 2 and processed as described in Section 3.1, and using the instrumental parameters listed in Sections 4.1 and 4.2. The calibration curve is plotted by linear regression of the area ratios of MHBMA/[D_6]-MHBMA or DHBMA/[D_7]-DHBMA as a function of the spiked concentrations of MHBMA or DHBMA, respectively. The concentration without spiking is subtracted from the values in each case. When a sample of 2 mL was used, a linear measurement range from 1.25 to 1250 μg/L (for MHBMA) and from 125 to 3750 μg/L (for DHBMA) was achieved with the analyti-

cal instruments described here. It is not necessary to plot a complete calibration graph for every analytical series. New calibration graphs should be plotted only if the quality assurance results indicate systematic deviations, changes have been made to the system, or no analysis has been carried out for a longer period of time.
Examples of linear calibration graphs are shown in Figure 4.

7 Calculation of the analytical result

The concentration of the sample in µg/L can be calculated from the calibration function ($y = mx + t$) according to the following equation:

Concentration of the sample [µ/L]=(y − t)/m

where y: Peak ratio: analyte/ISTD
t: Intercept with the y-axis of the calibration function after the blank value has been subtracted (see Section 6)
m: Slope of the calibration function

Any reagent blank values must be subtracted from the analytical results for the real samples. This calculation may also be carried out by the evaluation software of the LC/MS/MS system (e.g. "Analyst" software from Applied Biosystems), if appropriate. If a sample has been diluted before processing, the concentration calculated from the above equation must be multiplied by the dilution factor.

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) [16, 17] and in the special preliminary remarks to this series. Pooled urine samples from smokers and non-smokers that were prepared in the laboratory are included in the analysis to check the reproducibility (precision from day to day). The "expected value" and the tolerance range of this quality control material are determined in a preliminary study (precision in the series with $n = 5$). A reagent blank value is determined every day. Two control samples with 2 different concentrations (e.g. pooled smoker sample, pooled non-smoker sample) are included in each processing series of 18 real samples in each case in order to record system changes. One control sample is measured before the 18 real samples and the second control is measured after the 18 samples. One standard solution of the analytes is measured per 100 injections. This standard sample is equivalent to a mixture of the 4 individual standards, the concentration of the individual components being 2.5 mg/L. If the results for the quality control samples indicate systematic deviations, calibration as described in Section 6 must be carried out anew.

9 Evaluation of the method

9.1 Precision

The precision in the series and from day to day was determined using unspiked human urine. One sample of urine from a smoker and one from a non-smoker were processed and analysed 5 times as described in the previous sections to check the precision in the series. The precision from day to day was determined as described in the previous sections by processing and analysing one aliquot of a pooled urine sample from smokers and one from non-smokers once a day on 21 days over a period of 5 weeks.
The results are listed in Table 6.

Table 6. Precision for MHBMA and DHBMA determinations in human urine

	n	Concentration [µg/L]	Variation coefficient [%]	Prognostic range [%]
MHBMA				
Non-smokers				
In the series	5	7.35	4.1	10.5
From day to day	21	37.6	7.5	15.7
Smokers				
In the series	5	9.35	6.3	16.2
From day to day	21	18.4	13.4	27.9
DHBMA				
Non-smokers				
In the series	5	496.5	0.8	2.1
From day to day	21	394.8	5.7	11.9
Smokers				
In the series	6	238.1	1.0	2.6
From day to day	21	219.8	4.2	8.7

9.2 Accuracy

The accuracy was checked for human urine by means of experiments with spiked pooled urine samples from non-smokers. For this purpose 5 pooled samples were each spiked with 2.5 and 10 ng MHBMA in a batch of 5 mL. The spiked amounts of DHBMA were 250 and 5000 ng. These samples were subsequently processed and analysed in accordance with Sections 3 and 4. The results are shown in Table 7.

Table 7. Accuracy for MHBMA and DHBMA determinations in human urine (n=5, starting concentration; 4.27 µg/L and 476.4/L respectively)

Spiked Concentration	Relative recovery rate [%] (Variation coefficient [%])	
[µg/L]	MHBMA	DHBMA
0.5	93.7 (8.1)	–
2.0	95.2 (6.1)	–
50	–	95.3 (4.6)
1000	–	73.6 (2.9)

9.3 Detection limits

The detection and quantitation limits were calculated from the signal/background noise ratios from 10 pooled urine samples of non-smokers based on the following ratios:

$$\text{Detection limit } (\underline{X}*) = 3\,\sigma_{\text{blank}}$$

$$\text{Quantitation limit} \quad = 10\,\sigma_{\text{blank}}$$

whereby σ_{blank} is the standard deviation of the mean blank value at the retention time of the mercapturic acid in question.
The results are shown in Table 8.

Table 8. Detection and quantitation limits of MHBMA and DHBMA in human urine

Analyte	DL [µg/L]	QL [µg/L]
MHBMA	0.91	2.73
DHBMA	23.0	75.9

DL=Detection Limit, QL=Quantitation Limit.

9.4 Sources of error

The chromatographic separation of the analytes by the gradient program is not only necessary due to the differing polarity of the analytes, but above all it also serves to separate interfering matrix components. The use of the strongly acidic HPLC solvent mixture permits a relatively good retention on the C_{18} column with 3 µm particles, so that the peaks can be clearly assigned by comparing the peak forms and retention times of the native metabolites with those of the internal standards. The isomeric mixture of MHBMA is not completely separated by means of this chromatography

(see Figures 3 a and 3 b). This method evaluates the sum of the two MHBMA isomers.
High specificity is ensured by detection of daughter ions by means of LC/MS/MS. The deuterated standards and their retention times (immediately before the analytes) may also serve as further identification criteria.
The drying of the SPE cartridges before elution can be regarded as a critical step. Incomplete drying may lead to uncontrolled losses of the analytes. Therefore it is essential to ensure complete drying of the SPE cartridges.
Our experience to date shows that MHBMA and DHBMA dissolved in urine remain stable for at least 12 months when stored at –25 °C.

10 Discussion of the method

This newly developed method to determine the mercapturic acids of 1,3-butadiene permits rapid, highly selective and sensitive quantification of 2 mercapturic acids simultaneously by means of HPLC/APCI-MS/MS with a measurement duration of less than 10 minutes. Thus a method of monitoring these classical detoxification products has been made available that permits the throughput of a relatively high number of samples in a short time. Up to 60 samples per week can be assayed as duplicates on account of the simple sample preparation. The principle of the method is a simple solid phase extraction on a polymer material. The adsorption of the analytes on the polymer material can only be based on pπ or pπ* interactions (“normal” reversed phase chromatography). Therefore the analytes, due to their high polarity, should be relatively loosely and in particular non-specifically bound. The unchanging flow rates required for constant recovery can be achieved for manual solid phase extraction only by means of a vacuum controller. It was shown that the mercapturic acids have sufficient thermal stability for measurement with APCI. Temperatures of 500 °C in the ion source are necessary due to the high proportion of water in the eluent. Thus the analytes reach temperatures of approx. 200 °C for brief periods of time. However, no measurable fragmentation occurs in the source. Measurement with APCI has some advantages over the TIS (turbo ion spray) source, as relatively short run times of 10 min can be achieved at higher flow rates of 1000 µL/min and column diameters of 4.6 mm, and the signal/background noise ratio is distinctly improved with the use of APCI. However, the absolute intensity of the signals is slightly reduced.
Both the calibration and the determination of the detection and quantitation limits were carried out using urine from non-smokers. As no urine is available that is free from the 1,3-butadiene mercapturic acids, this inevitably leads to an over-estimation of the detection and quantitation limits if they are determined by the blank value method. These are more objectively evaluated by the signal/background noise ratio.
The method is linear over 3 orders of magnitude for all the analytes. The selectivity is confirmed by recording full-scan mass spectra as part of the “quantitative optimisation”.
In the course of the method development it was demonstrated that the amount of urine used has a definite influence on the recovery. When larger quantities of urine

are used, there is a distinct decline in the recovery, which indicates that binding sites of the cartridges are mainly occupied by matrix components and therefore are no longer available for retention of the analytes.

Instruments used:
HP 1100 HPLC system from Agilent Technologies with G1312 A, G1314 A, G1316 A, G1322 A and G1329 A modules and a tandem quadrupole mass spectrometer with an API 2000 APCI source, as well as the “Analyst” evaluation software from Applied Biosystems.

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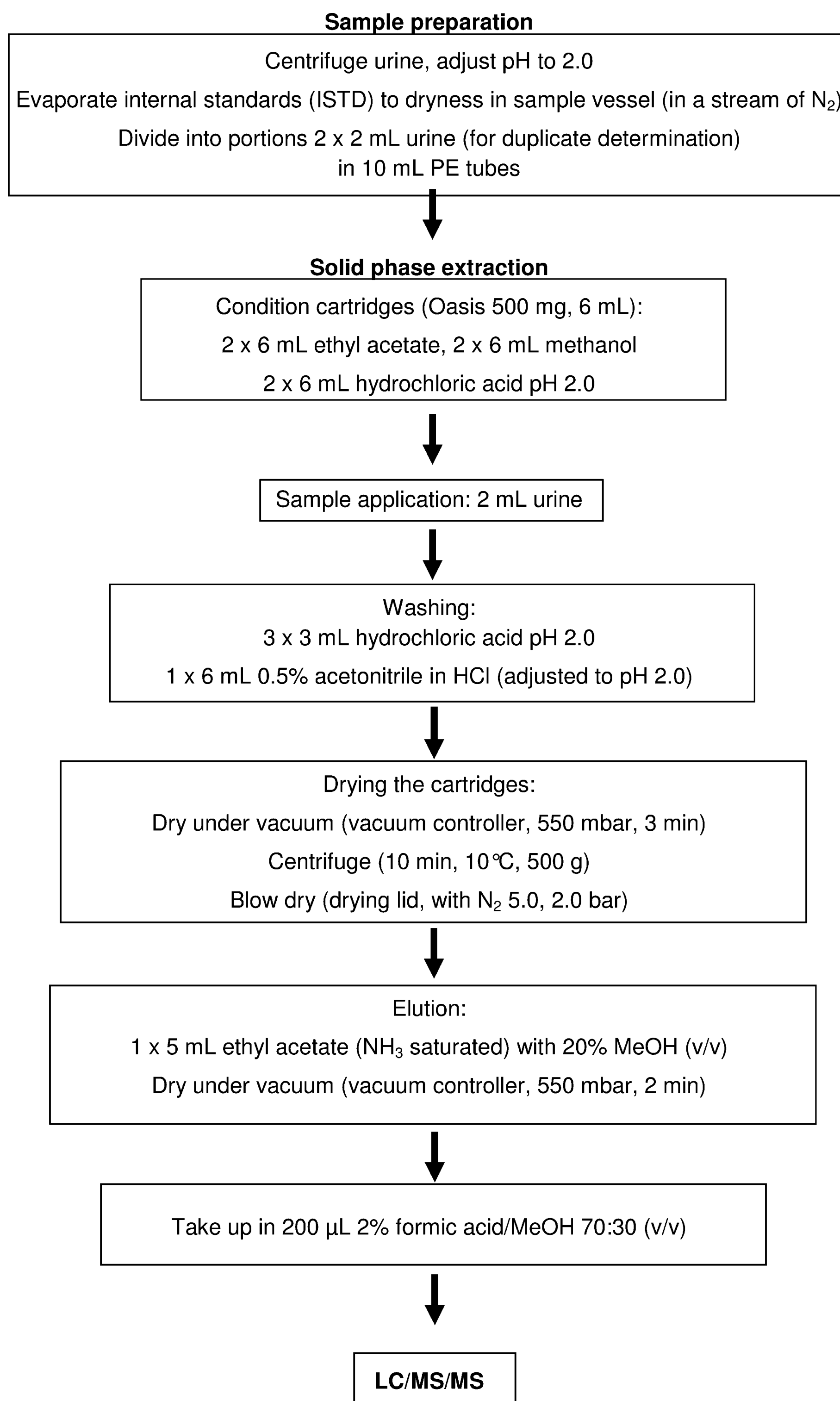
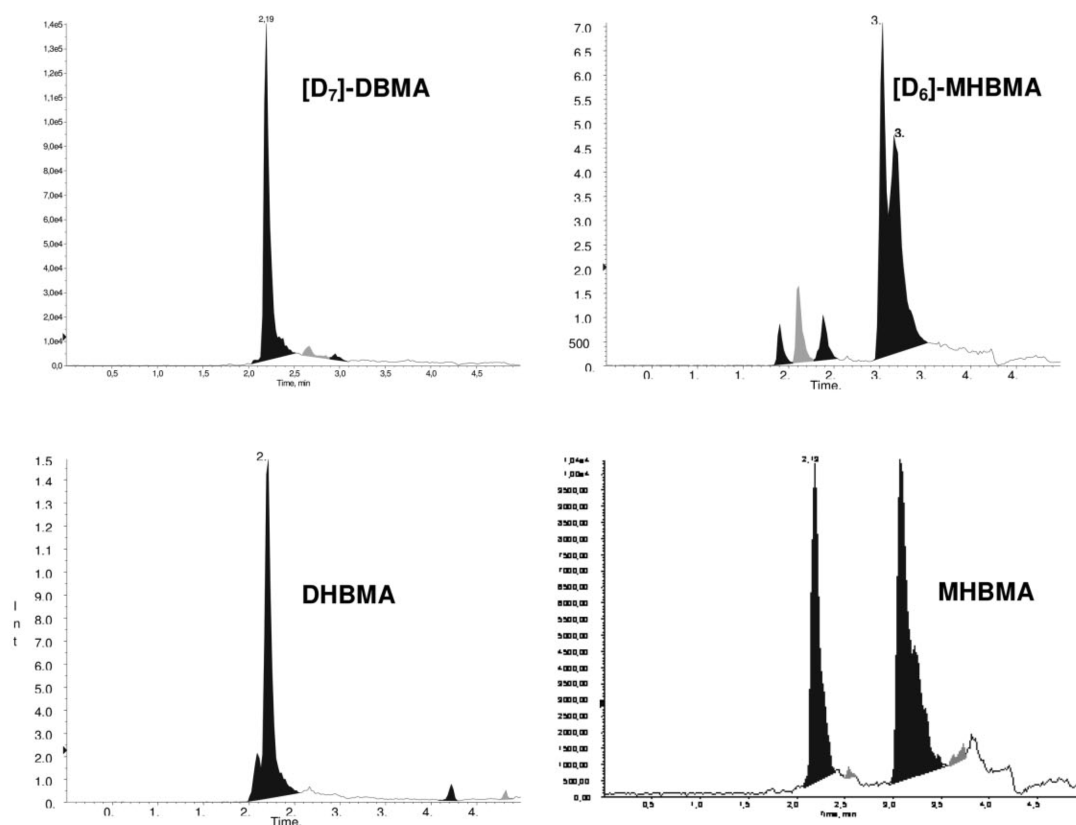


Fig. 2. Method flowchart

a



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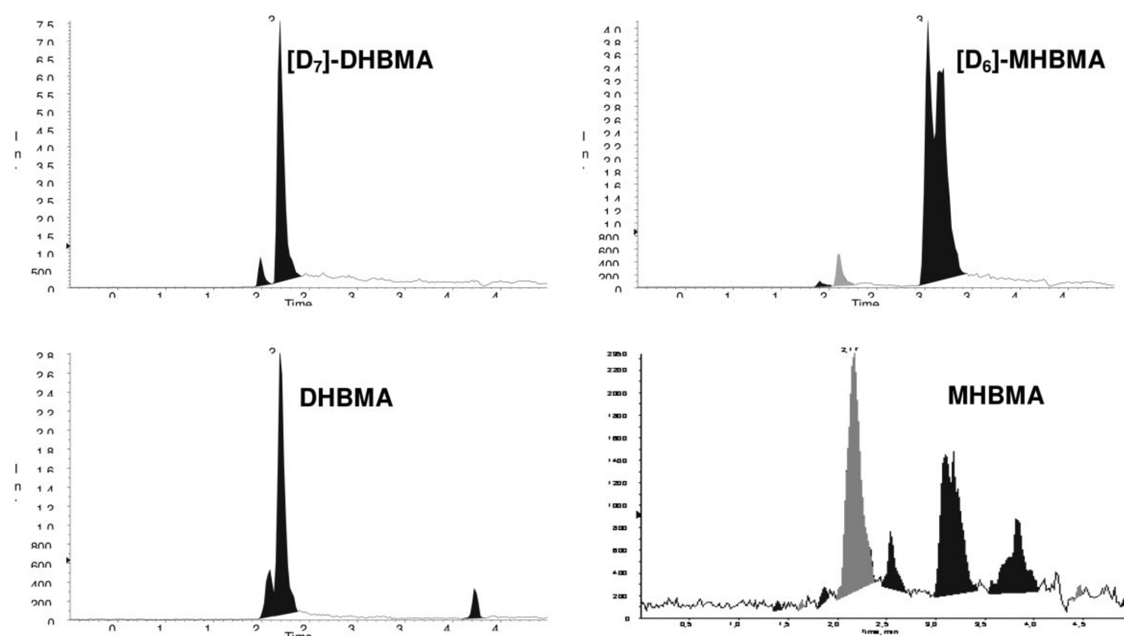

Fig. 3. Example chromatograms: **a** of a smoker: 11.43 µg/L MHBMA; 424.81 µg/L DHBMA. **b** of a non-smoker: 1.63 µg/L MHBMA; 112.3 µg/L DHBMA

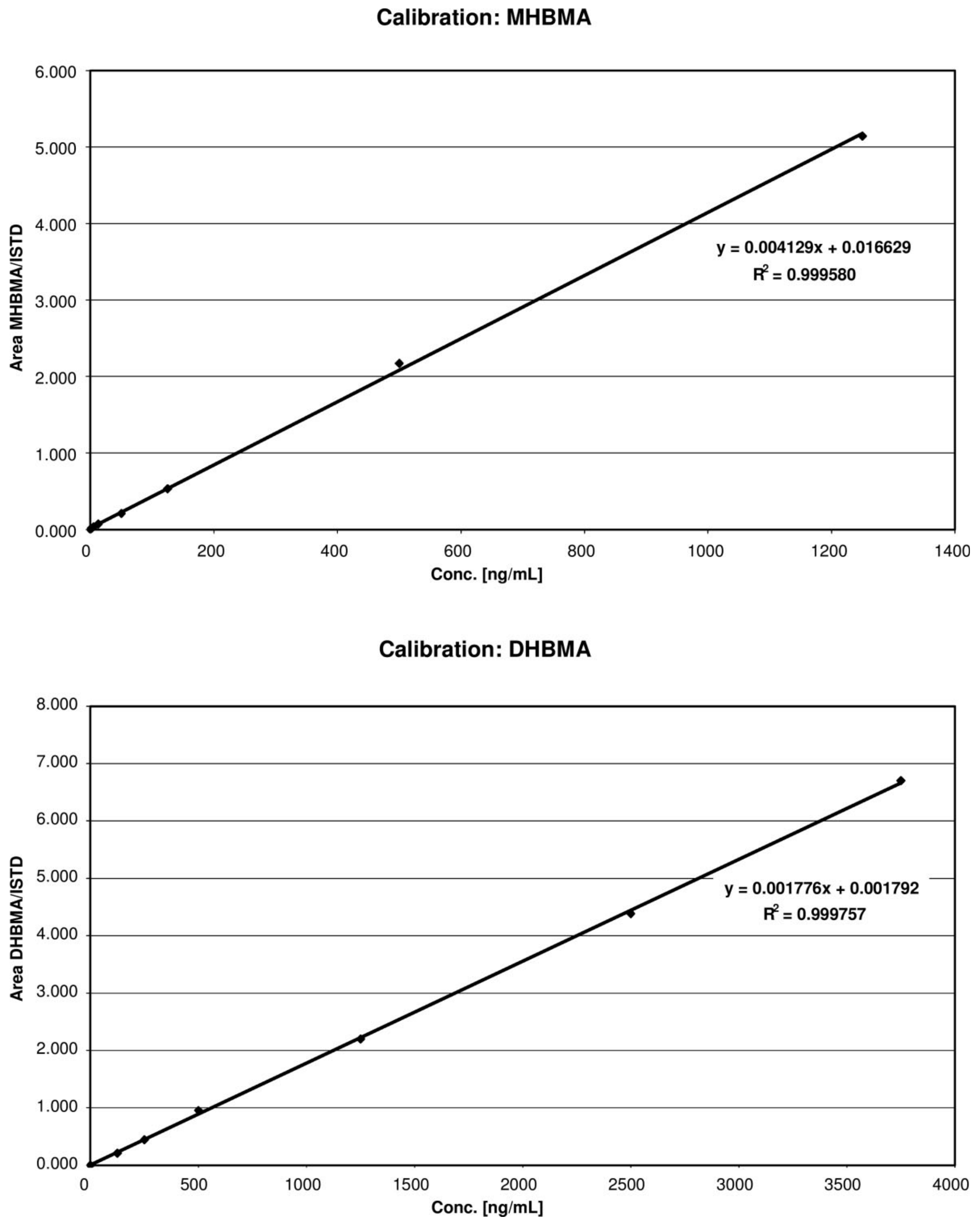

Fig. 4. Linear calibration graphs for MHBMA and DHBMA. A matrix calibration was carried out