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3-(4′-Methylbenzylidene)camphor (4-MBC) – Determination of 3-(4′-carboxybenzylidene)-camphor and 3-(4′-carboxybenzylidene)-hydroxycamphor in urine by UPLC-MS/MS

Biomonitoring Method – Translation of the German version from 2024

Keywords

3-(4′-methylbenzylidene)-camphor; 3-(4′-carboxy-benzylidene)camphor; 3-(4′-carboxybenzylidene)-hydroxycamphor; biomonitoring; urine; UPLC-MS/MS

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Abstract

The working group "Analyses in Biological Materials" of the German Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area (MAK Commission) developed and verified the presented biomonitoring method. The aim of this method is the selective and sensitive quantitation of the two main metabolites of the UVB filter 3-(4′-methylbenzylidene)camphor (4-MBC), 3-(4′-carboxybenzylidene)-camphor (cx-MBC) and 3-(4′-carboxybenzylidene)hydroxycamphor (cx-MBC-OH), in urine. After adding deuterated internal standards (cx-MBC-d_4 and cx-MBC-OH-d_4), the samples are enzymatically hydrolysed. After extraction and enrichment by online SPE, the analytes are separated by liquid chromatography and analysed by tandem mass spectrometry. Good precision data with standard deviations below 5.5% for cx-MBC and below 6.5% for cx-MBC-OH, as well as good accuracy data with mean relative recoveries in the range of 90–110% for cx-MBC and 89–106% for cx-MBC-OH, show that the method provides reliable and accurate analytical results. The method is both selective and sensitive, and the limits of quantitation of 0.15 µg/l (cx-MBC) and 0.3 µg/l (cx-MBH-OH) are sufficient to determine background exposure in the general population.

1 Characteristics of the method

Matrix	Urine
Analytical principle	Ultra-high-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS)

Parameters and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
3-(4′-Methylbenzylidene)camphor (4-MBC)	36861-47-9	3-(4′-Carboxybenzylidene) camphor (cx-MBC)	68801-01-4
		3-(4′-Carboxybenzylidene) hydroxycamphor (cx-MBC-OH)	915796-58-6

Reliability criteria

3-(4′-Carboxybenzylidene)camphor (cx-MBC)

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.5 µg, 1.0 µg, 10 µg, or 100 µg cx-MBC per litre of urine and n = 6 or 10 determinations	s_w = 4.1%, 3.3%, 2.8%, or 2.2% u = 10.6%, 7.3%, 6.3%, or 4.8%
Accuracy:	Recovery (rel.) at a spiked concentration of 0.5 µg, 1.0 µg, 10 µg, or 100 µg cx-MBC per litre of urine and n = 6 or 10 determinations	r = 99%, 90%, 98%, or 110%
Limit of detection:	0.05 µg cx-MBC per litre of urine	
Limit of quantitation:	0.15 µg cx-MBC per litre of urine	

3-(4′-Carboxybenzylidene)hydroxycamphor (cx-MBC-OH)

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.5 µg, 1.0 µg, 10 µg, or 100 µg cx-MBC-OH per litre of urine and n = 6 or 10 determinations	s_w = 6.2%, 6.1%, 4.0%, or 1.4% u = 15.9%, 13.7%, 8.9%, or 3.2%
Accuracy:	Recovery (rel.) at a spiked concentration of 0.5 µg, 1.0 µg, 10 µg, or 100 µg cx-MBC-OH per litre of urine and n = 6 or 10 determinations	r = 89%, 89%, 92%, or 106%
Limit of detection:	0.1 µg cx-MBC-OH per litre of urine	
Limit of quantitation:	0.3 µg cx-MBC-OH per litre of urine	

2 General information on 3-(4′-methylbenzylidene)camphor

3-(4′-Methylbenzylidene)camphor (4-MBC, 3-(4′-methylbenzylidene)bornan-2-one, enzacamene, (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one) is a white, crystalline powder with a melting point of 66–69 °C which exhibits a weak, camphorous odour. It is practically insoluble in water and slightly soluble in ethanol or isopropanol (SCCS 2022). 4-MBC is produced in or imported into the EU in quantities of 10 to 1000 tonnes per year

and is thereby categorised as a "low production-volume chemical" (HBM 2016). The chemical structure of 4-MBC is presented in Figure 1.

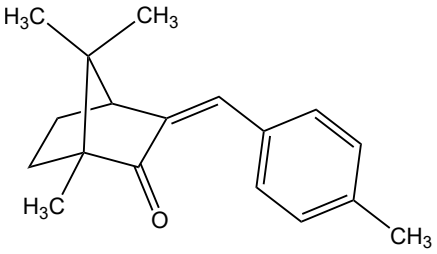


Fig. 1 Structure of 4-MBC

4-MBC is a UVB filter in cosmetic products, especially in sunscreens, whereby a maximum content of 4% is permitted (European Parliament and European Council 2009). Cosmetic products containing the substance may no longer be sold in the EU from May 2026 (European Commission 2024).

Exposure to 4-MBC primarily takes place via the use of cosmetic products, although the market share of 4-MBC-containing products is on the decline (Hauri et al. 2003; Kerr 2011; Manová et al. 2013; Poiger et al. 2004). The general population may be further exposed via the consumption of 4-MBC-contaminated fish (Balmer et al. 2005; Cunha et al. 2018) or via contaminated dusts (Ao et al. 2018; Negreira et al. 2009).

Information on the absorption and metabolism of 4-MBC are available from both animal studies and studies on humans. In rats, 3-(4′-carboxybenzylidene)camphor (cx-MBC) and 3-(4′-carboxybenzylidene)hydroxycamphor (cx-MBC-OH) were identified as main metabolites in urine after oral application (Völkel et al. 2006). The same main metabolites were detected in humans and rats after dermal application of 4-MBC (Schauer et al. 2006). In rat urine, both metabolites are present as bound to glucuronide, whereby the glucuronidated proportion of cx-MBC is higher than that of cx-MBC-OH (Völkel et al. 2006). The ratio of cx-MBC to cx-MBC-OH in human urine is about 1 : 4 (Schauer et al. 2006). The systemic availability of 4-MBC, which was investigated in various studies after dermal application, was found to be 0.29–0.74% (Schauer et al. 2006) or 1.9% (SCCNFP 2004; SCCP 2006).

In a first step, absorbed 4-MBC is hydroxylated by cytochrome P450 (CYP450), forming the intermediate benzyl alcohol 3-(4′-hydroxymethylbenzylidene)camphor. Via alcohol dehydrogenase and aldehyde dehydrogenase, this metabolite is further oxidised to cx-MBC; it can then be hydroxylated by CYP450 to form cx-MBC-OH (see Figure 2).

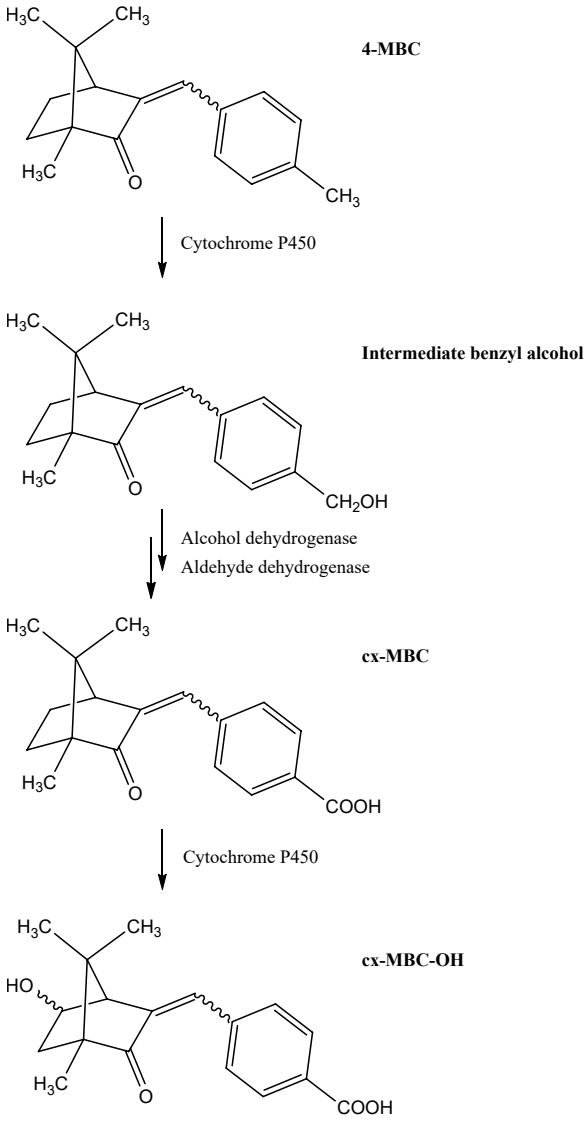


Fig. 2 Metabolism of 4-MBC according to Schauer et al. (2006)

The European Chemicals Agency (ECHA) sees sufficient evidence that 4-MBC is an endocrine disruptor and influences both the thyroid and oestrogen systems. At the EU level, 4-MBC has been classified as a "substance of very high concern" (SVHC) (ECHA 2021; SCCS 2022). The Federal German Environment Agency has derived HBM-I values for the sum of cx-MBC and cx-MBC-OH of 470 µg/l urine for adults and of 310 µg/l urine for children (HBM 2016).

To investigate possible exposure of the general population to 4-MBC, various studies have been carried out. Table 1 shows representative concentrations of cx-MBC and cx-MBC-OH in the urine of the German general population; these were largely below the respective quantitation limit of the method. Only the samples from the years 1995, 2005, and 2010, measured in the study by Schmidtkunz et al. (2023), showed slightly higher concentrations of the 4-MBC metabolites in young adults, although the analysed time trend of exposure of young adults in the years 1995, 2005, 2010, 2015, and 2019 showed a significant decrease in the maximum cx-MBC and cx-MBC-OH concentrations in urine, which is probably due to less frequent use of 4-MBC in cosmetic products. The highest concentration was measured in a sample from 2005 and was 16.2 µg cx-MBC/l (Schmidtkunz et al. 2023).

Tab. 1 cx-MBC und cx-MBC-OH concentrations in the urine of the general population in Germany

Study collective (number of persons)	cx-MBC [µg/l]		cx-MBC-OH [µg/l]		References
	Mean	Range	Mean	Range	
Children and teenagers (447)	< LOQ (0.15)	< LOQ–12.7	< LOQ (0.30)	all < LOQ (0.30)	Murawski et al. 2021
Adults (40)	< LOQ (0.15)	all < LOQ (0.15)	< LOQ (0.3)	all < LOQ (0.3)	Leng and Gries 2017
Young adults (250)	0.155[a)]	< LOQ (0.15)–16.2	< LOQ (0.30)	< LOQ (0.30)–2.96	Schmidtkunz et al. 2023

LOQ: Limit of quantitation
[a)] Geometric mean

3 General principles

The aim of the method hereby presented is the selective and sensitive measurement of both main metabolites of the UVB filter 4-MBC, cx-MBC and cx-MBC-OH, in urine. After adding the deuterated internal standards (cx-MBC-d_4 and cx-MBC-OH-d_4), the samples are subject to enzymatic hydrolysis with β-glucuronidase in order to cleave potential conjugates. After extraction and enrichment of the samples by online SPE, the analytes are separated from matrix components by liquid chromatography and quantified by tandem mass spectrometry. Quantitation limits of 0.15 µg/l (cx-MBC) and 0.3 µg/l (cx-MBC-OH) are achieved.

4 Equipment, chemicals, and solutions

4.1 Equipment

- UPLC-MS/MS system (e.g. Waters Acquity coupled to a Waters Xevo-TQS tandem mass spectrometer, Waters GmbH, Eschborn, Germany)
- Enrichment column (e.g. X Bridge C8 Direct Connect HP, 10 µm, 2.1 × 30 mm, No. 186005233, Waters GmbH, Eschborn, Germany)
- Analytical column (e.g. Acquity UPLC HSS C18, 1.8 µm, 2.1 × 150 mm, No. 186003534, Waters GmbH, Eschborn, Germany)
- Incubator (e.g. MMM Medcenter Einrichtungen GmbH, Planegg, Germany)
- Laboratory centrifuge (e.g. Heraeus Deutschland GmbH & Co. KG, Hanau, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Shaker (e.g. Vibrax VXR, IKA-Werke GmbH & Co. KG, Staufen, Germany)
- pH meter (e.g. SevenEasy™, Mettler-Toledo GmbH, Gießen, Germany)
- pH paper Universal 0–11 (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)
- 250-ml and 400-ml glass beakers (e.g. VWR International GmbH, Darmstadt, Germany)
- 10-ml, 100-ml, and 500-ml volumetric flasks (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- Multipette® with matching Combitips® (e.g. Eppendorf AG, Hamburg, Germany)
- Single-channel pipettes with variable volumes of 1–10 µl and 10–100 µl with matching pipette tips (e.g. Eppendorf Research®, Eppendorf AG, Hamburg, Germany)
- 3.5-ml transfer pipettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- 96-well plates with 2-ml square wells (e.g. No. 186002482, Waters GmbH, Eschborn, Germany)
- Silicone sealing mats for 96-well plates (e.g. No. 60180-M123, Thermo Fisher Scientific GmbH, Dreieich, Germany)
- 1.5-ml threaded bottles with screw caps (e.g. MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany)

- Screw-top urine cups (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

Unless otherwise specified, all chemicals must be a minimum of *pro analysi* grade.

- Acetonitrile, SupraSolv® (e.g. No. 100017, Merck KGaA, Darmstadt, Germany)
- Formic acid (e.g. No. 56302, Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany)
- Ammonium acetate (e.g. No. 101116, Merck KGaA, Darmstadt, Germany)
- Glacial acetic acid, SupraPur® (e.g. No. 100066, Merck KGaA, Darmstadt, Germany)
- *β*-Glucuronidase from *E. coli K12,* ≥ 140 U/mg protein (e.g. No. 03708446103, Roche Diagnostics Deutschland GmbH, Mannheim, Germany)
- Tetrahydrofuran, LiChrosolv® (e.g. No. 108101, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Milli-Q® IQ 7000 Direct water-purification system, Merck KGaA, Darmstadt, Germany)
- Nitrogen 5.0 (Air Liquide Deutschland GmbH, Düssedorf, Germany)
- Argon 5.0 (Linde GmbH, Pullach, Germany)

4.3 Standards

- 3-(4-Carboxybenzylidene)camphor, > 95% (custom synthesis, Institute for Thin Film Technology and Microsensors e.V., Teltow, Germany)
- 3-(4-Carboxybenzylidene)camphor-ring-d_4, > 95% (custom synthesis, Institute for Thin Film Technology and Microsensors e.V., Teltow, Germany)
- 3-(4-Carboxybenzylidene)-6-hydroxycamphor, > 95% (custom synthesis, Institute for Thin Film Technology and Microsensors e.V., Teltow, Germany)
- 3-(4-Carboxybenzylidene)-6-hydroxycamphor-ring-d_4, > 95% (custom synthesis, Institute for Thin Film Technology and Microsensors e.V., Teltow, Germany)

4.4 Solutions

- Ammonium acetate buffer (1 mol/l; pH 6.4)
  38.5 g Ammonium acetate are weighed into a 400-ml glass beaker and dissolved in about 250 ml ultra-pure water. After using glacial acetic acid to adjust the pH to pH 6.4 (pH meter), the solution is quantitatively transferred into a 500-ml volumetric flask, which is then made up to the mark with ultra-pure water.

The ammonium acetate buffer is stored at room temperature and is stable for at least seven days.

- Eluent A (0.1% formic acid in water)
  In a 1000-ml volumetric flask, 1 ml of formic acid is placed. The volumetric flask is subsequently made up to the mark with ultra-pure water.
- Eluent B (0.1% formic acid in acetonitrile)
  In a 1000-ml volumetric flask, 1 ml of formic acid is placed. The volumetric flask is subsequently made up to the mark with acetonitrile.
- *β*-Glucuronidase (1 : 1 dilution (v/v))
  One part ultra-pure water is added to one part *β*-glucuronidase from *E. coli K12.*

4.5 Internal standards (ISTDs)

- cx-MBC-d_4 stock solution (1000 mg/l)
  10 mg cx-MBC-d_4 are weighed into a 10-ml volumetric flask and dissolved in tetrahydrofuran. The volumetric flask is then made up to the mark with tetrahydrofuran.
- cx-MBC-OH-d_4 stock solution (1000 mg/l)
  10 mg cx-MBC-OH-d_4 are weighed into a 10-ml volumetric flask and dissolved in tetrahydrofuran. The volumetric flask is then made up to the mark with tetrahydrofuran.
- ISTD working solution (10 mg/l)
  100 µl of each ISTD stock solution are pipetted into a 10-ml volumetric flask. The volumetric flask is then made up to the mark with acetonitrile.
- ISTD spiking solution (0.25 mg/l)
  25 µl of the ISTD working solution are pipetted into a 1.5-ml threaded bottle with screw cap containing 975 µl acetonitrile.

The stock solutions of the ISTDs are stored at –20 °C and are stable for at least six months. The spiking solution and the working solution can be stored at 4 °C for six months.

4.6 Calibration standards

- cx-MBC stock solution (1000 mg/l)
  10 mg cx-MBC are weighed into a 10-ml volumetric flask and dissolved in tetrahydrofuran. The volumetric flask is then made up to the mark with tetrahydrofuran.
- cx-MBC-OH stock solution (1000 mg/l)
  10 mg cx-MBC-OH are weighed into a 10-ml volumetric flask and dissolved in tetrahydrofuran. The volumetric flask is then made up to the mark with tetrahydrofuran.
- Spiking solution I (10 mg/l)
  100 µl of each stock solution are pipetted into a 10-ml volumetric flask. The volumetric flask is then made up to the mark with acetonitrile.
- Spiking solution II (1 mg/l)
  1 ml of spiking solution I are pipetted into a 10-ml volumetric flask. The volumetric flask is then made up to the mark with acetonitrile.
- Spiking solution III (0.1 mg/l)
  100 µl of spiking solution I are pipetted into a 10-ml volumetric flask. The volumetric flask is then made up to the mark with acetonitrile.
- Spiking solution IV (0.01 mg/l)
  100 µl of spiking solution II are pipetted into a 10-ml volumetric flask. The volumetric flask is then made up to the mark with acetonitrile.
- Spiking solution V (0.001 mg/l)
  100 µl of spiking solution III are pipetted into a 10-ml volumetric flask. The volumetric flask is then made up to the mark with acetonitrile.

The stock solutions are stored at –20 °C and are stable under these conditions for at least six months. The spiking solutions can be stored at 4 °C for six months.

The calibration standards are prepared in 1.5-ml threaded bottles with screw caps, according to the pipetting scheme given in Table 2, using pooled urine. Pooled urine is prepared by collecting and mixing urine samples from persons not exposed to 4-MBC. The calibration range may need to be adjusted to the expected concentrations in the real samples.

The calibration standards are processed analogously to the samples to be analysed (see Section 5.2).

Tab. 2 Pipetting scheme for the preparation of calibration standards for the determination of cx-MBC and cx-MBC-OH in urine

Calibration standard	Spiking solution	Spiking solution [µl]	Pooled urine [µl]	Analyte concentration [µg/l]
00[a)]	–	–	ad 500	0
0	–	–		0
1	V	25		0.05
2	IV	5		0.1
3	IV	10		0.2
4	IV	25		0.5
5	III	5		1
6	III	10		2
7	III	25		5
8	II	5		10
9	II	10		20
10	II	25		50
11	I	5		100
12	I	10		200

[a)] The calibration standard 00 is processed without addition of ISTD.

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in screw-top urine cups and stored at 4 °C until processing. If sample preparation is not possible within three days, the urine samples should be stored at –20 °C.

5.2 Sample preparation

The samples are brought to room temperature and mixed well. 0.5 ml of the urine sample are pipetted into a well of the 96-well plate. Subsequently, 20 µl of the ISTD spiking solution (concentration in the sample: 10 µg/l) and 1 ml of the ammonium acetate buffer are added by pipetting. After adding 10 µl of 1 : 1-diluted β-glucuronidase, the sample is incubated for 3 h at 37 °C. Subsequently, 50 µl of concentrated formic acid are added to the sample. 50 µl of the samples thus processed are injected into the analytical system.

The verifiers of the method, who performed the sample preparation in 1.5-ml threaded bottles with screw caps, observed that some samples exhibited turbidity after the addition of formic acid. These samples were centrifuged at 3000 rpm for 10 min, and the supernatant was used for analysis.

6 Operational parameters

Analytical determination was carried out on a device configuration comprised of a UPLC system coupled to a tandem mass spectrometer (UPLC-MS/MS).

6.1 Ultra-high-performance liquid chromatography

Enrichment column: X Bridge C8 Direct Connect HP, 10 µm, 2.1 × 30 mm

Analytical column: Acquity UPLC HSS C18, 1.8 µm, 2.1 × 150 mm

Eluent: A: Ultra-pure water + 0.1% formic acid
B: Acetonitrile + 0.1% formic acid

Stop time: 10 min

Column-oven temperature: 40 °C

Injection volume: 50 µl

Gradient programme: see Tables 3 and 4

Column-switching: see Table 5

Tab. 3 Gradient programme of the enrichment column for the determination of cx-MBC and cx-MBC-OH in urine

Time [min]	Eluent A [%]	Eluent B [%]	Flow rate [ml/min]
0.0	75	25	1.0
1.0	75	25	1.0
1.5	0	100	1.0
5.0	0	100	1.0
5.5	75	25	1.0
10.0	75	25	1.0

Tab. 4 Gradient programme of the analytical column for the determination of cx-MBC and cx-MBC-OH in urine

Time [min]	Eluent A [%]	Eluent B [%]	Flow rate [ml/min]
0	50	50	0.35
1.0	50	50	0.35
5.0	5	95	0.35
7.0	5	95	0.35
7.5	50	50	0.35
10.0	50	50	0.35

Tab. 5 Valve-switching times for online SPE-UPLC-MS/MS

Time [min]	Valve position	Description
0.0–0.5	1	Loading of SPE column, equilibration of UPLC column
0.5–5.5	2	Elution of analytes from the SPE column in backflush mode, separation of the analytes on the UHPLC column
5.5–10.0	1	Equilibration of SPE column, equilibration of UPLC column following the separation

As part of the system used by the verifying laboratory, an isocratic pump (Shimadzu LC-20AD) was used to load and rinse the enrichment column. In order to enable switching between the two mobile phases, an upstream solenoid valve was used. However, the use of this valve could not enable the mixing of mobile phases A and B, such that a corresponding mobile phase A1 was mixed in advance (25% acetonitrile in water with 0.1% formic acid). Table 6 provides an overview of the operational parameters used for the enrichment column in the verifying laboratory.

Tab. 6 Enrichment-column parameters for the determination of cx-MBC and cx-MBC-OH in urine (external method verification)

Time [min]	Eluent A1 [%]	Eluent B [%]	Flow rate [ml/min]
0	100	0	1.0
1.25	0	100	1.0
5.25	100	0	1.0

6.2 Tandem mass spectrometry

Ionisation:	Electrospray, negative (ESI–)
Capillary voltage:	1.3 kV
Source offset:	10 V
Source temperature:	150 °C
Desolvation temperature:	550 °C
Cone-gas flow:	150 l/h
Desolvation-gas flow:	800 l/h
Collision-gas flow:	0.15 ml/h
Nebuliser-gas flow:	6.0 bar
Desolvation, cone, and nebuliser gas:	Nitrogen
Collision gas:	Argon
Parameter-specific settings:	see Tables 7 and 8

The instrument-specific parameters must be ascertained and adjusted by the user for the individual MS/MS system used. The instrument-specific parameters given in this section have been determined and optimised for the system used during method development (Waters Xevo-TQS tandem mass spectrometer, Waters GmbH, Eschborn, Germany).

Two mass transitions were selected for cx-MBC, whereas three were selected for cx-MBC-OH. Of these mass transitions, one served the purpose of quantification (quantifier) and the other or others served the purpose of confirmation (qualifier). The corresponding mass transitions were used for the ISTDs. The selected transitions are summarised in Table 7 alongside the retention times and other MRM parameters.

The retention times given below can only serve as a point of reference. The user of the method must ensure the separation performance of the LC column and the resulting retention behaviour of the substances.

Tab. 7 Retention times, mass transitions, and MS/MS parameters

Substance	Retention time [min]	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time [s]	Cone voltage [V]	Collision energy [eV]	Delay [s]
cx-MBC-OH	2.98	299.10	240.10	0.030	61.0	22.0	Auto
		299.10	255.05[a)]	0.030	61.0	18.0	Auto
		299.10	199.15[a)]	0.030	61.0	20.0	Auto
cx-MBC-OH-d_4	2.97	303.15	244.10	0.030	51.0	22.0	Auto
		303.15	259.10[a)]	0.030	51.0	18.0	Auto
		303.15	203.15[a)]	0.030	51.0	22.0	Auto
cx-MBC	4.17	283.15	239.05	0.055	63.0	18.0	Auto
		283.15	130.15[a)]	0.055	63.0	20.0	Auto
cx-MBC-d_4	4.15	287.15	243.10	0.055	63.0	18.0	Auto
		287.15	134.20[a)]	0.055	63.0	18.0	Auto

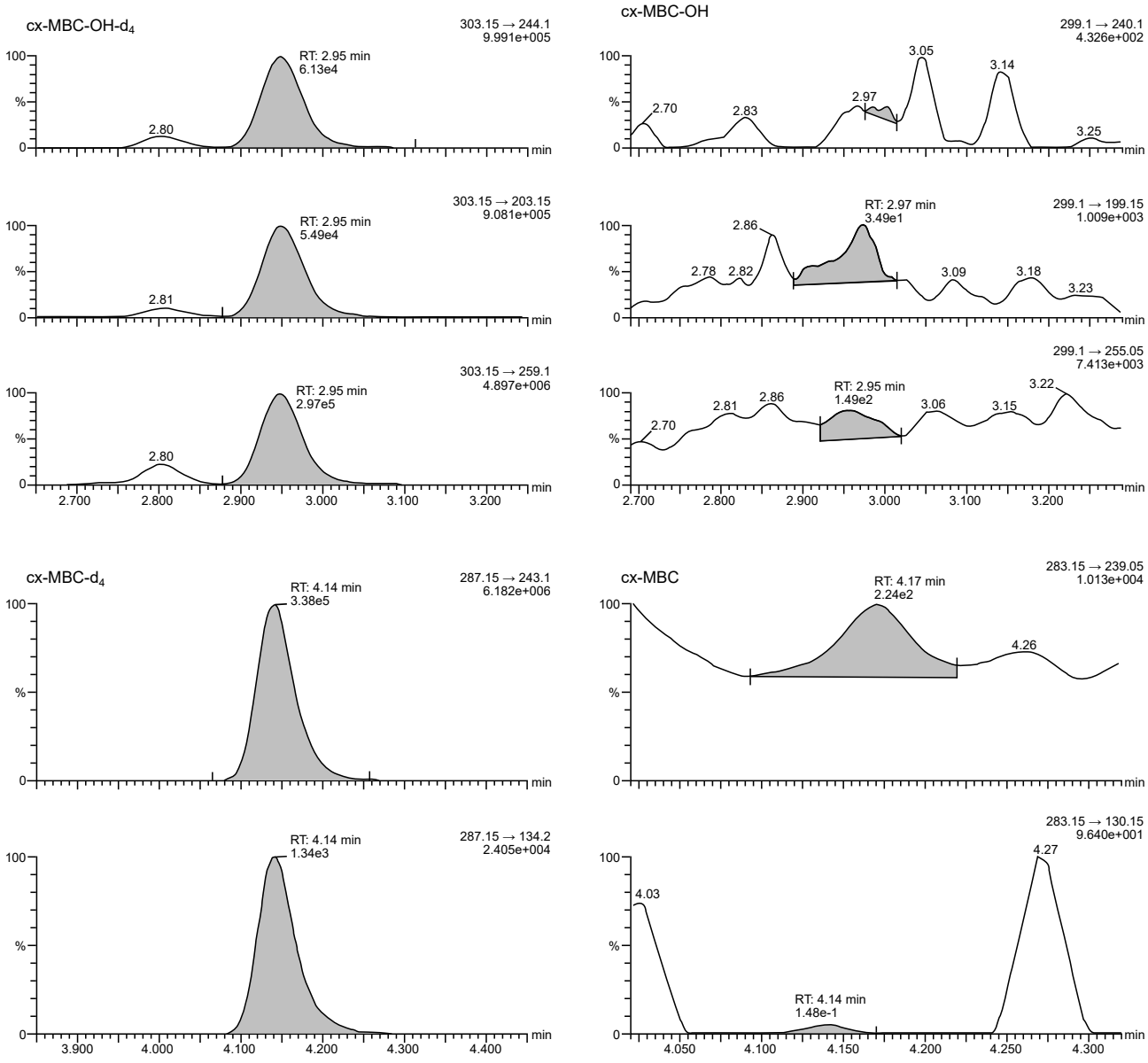
[a)] Qualifier

Using a SCIEX QTRAP 6500+ MS/MS system, the verifying laboratory could not confirm the qualifier transitions for cx-MBC and its ISTD cx-MBC-d_4. The mass transitions indicated in the original method could not be detected (*m/z* 283 ⟶ 130 and *m/z* 287 ⟶ 134).

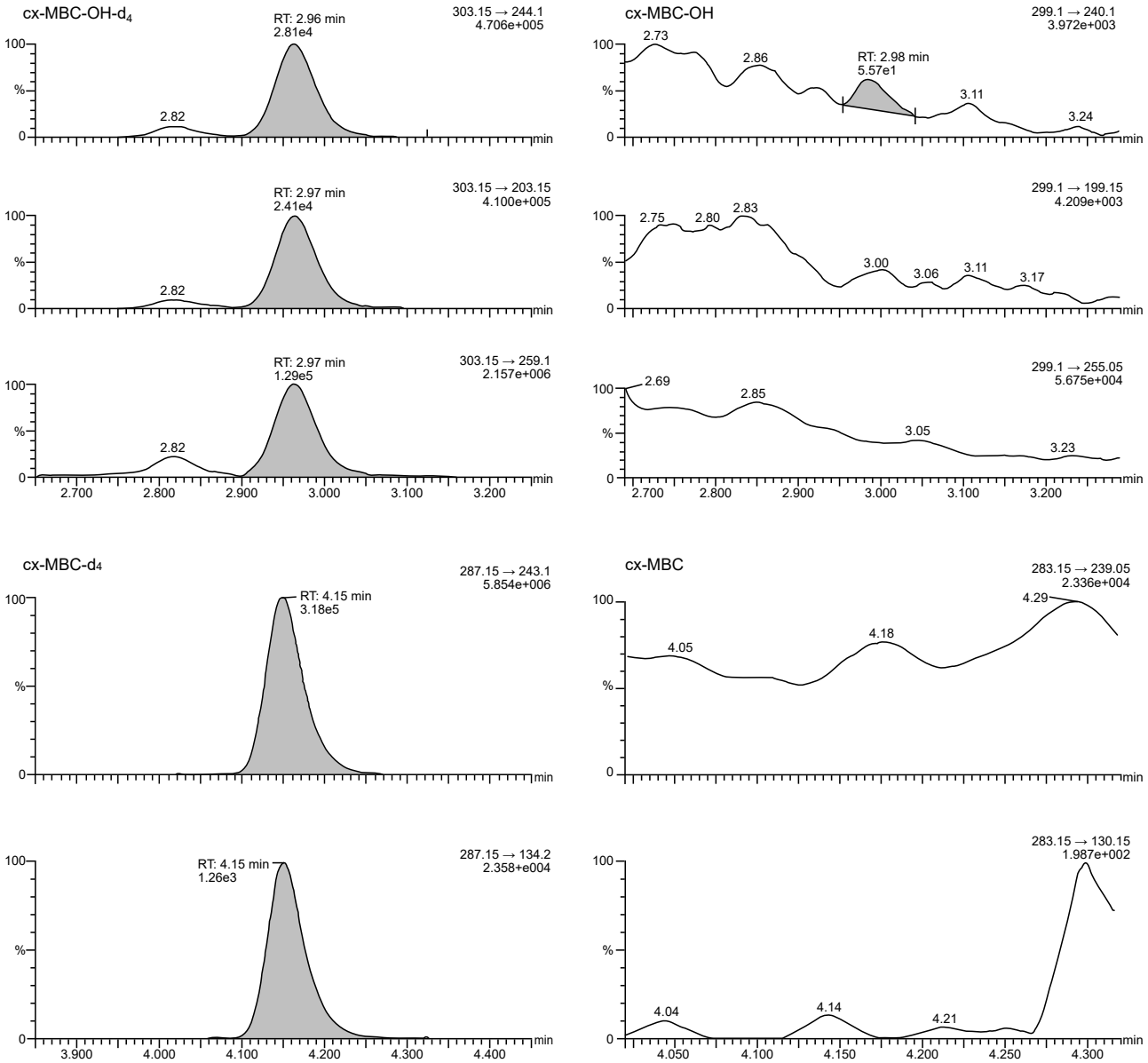
7 Analytical determination

The operational parameters given in Section 6 are set, and 50 µl of the analytical sample prepared according to Section 5.2 are injected into the LC-MS/MS system. The injected sample is thereby enriched and purified on the pre-column (X Bridge C8 Direct Connect HP; enrichment column). After 0.5 minutes, elution is carried out by backflushing on the analytical column. Subsequently, analytical separation is performed on the Acquity UPLC-HSS C18. The analytes are identified by their retention times and the transitions of the substances in question, and the qualifier mass transitions are used as an optional identification parameter. Figure 3 shows representative chromatograms of a) a reagent blank value, b) a native urine sample, and c) a urine sample spiked with 1.0 µg cx-MBC or cx-MBC-OH/l.

a)


cx-MBC-OH-d4
303.15 → 244.1
9.991e+005
RT: 2.95 min
6.13e4
2.80
cx-MBC-OH
299.1 → 240.1
4.326e+002
2.70
2.83
2.97
3.05
3.14
3.25
303.15 → 203.15
9.081e+005
RT: 2.95 min
5.49e4
2.81
RT: 2.97 min
3.49e1
299.1 → 199.15
1.009e+003
2.78
2.82
2.86
3.09
3.18
3.23
303.15 → 259.1
4.897e+006
RT: 2.95 min
2.97e5
2.80
299.1 → 255.05
7.413e+003
RT: 2.95 min
1.49e2
2.70
2.81
2.86
3.06
3.15
3.22
cx-MBC-d4
287.15 → 243.1
6.182e+006
RT: 4.14 min
3.38e5
cx-MBC
283.15 → 239.05
1.013e+004
RT: 4.17 min
2.24e2
4.26
287.15 → 134.2
2.405e+004
RT: 4.14 min
1.34e3
283.15 → 130.15
9.640e+001
4.27
4.03
RT: 4.14 min
1.48e-1
100
%
0
min
2.700
2.800
2.900
3.000
3.100
3.200
3.900
4.000
4.100
4.200
4.300
4.400
4.050
4.150
4.250

b)


cx-MBC-OH-d4
RT: 2.96 min
2.81e4
303.15 → 244.1
4.706e+005
2.82
cx-MBC-OH
299.1 → 240.1
3.972e+003
2.73
2.86
RT: 2.98 min
5.57e1
3.11
3.24
RT: 2.97 min
2.41e4
303.15 → 203.15
4.100e+005
2.82
299.1 → 199.15
4.209e+003
2.75
2.80
2.83
3.00
3.06
3.11
3.17
RT: 2.97 min
1.29e5
303.15 → 259.1
2.157e+006
2.82
299.1 → 255.05
5.675e+004
2.69
2.85
3.05
3.23
cx-MBC-d4
RT: 4.15 min
3.18e5
287.15 → 243.1
5.854e+006
cx-MBC
283.15 → 239.05
2.336e+004
4.05
4.18
4.29
RT: 4.15 min
1.26e3
287.15 → 134.2
2.358+e004
283.15 → 130.15
1.987e+002
4.04
4.14
4.21
%
min

c)

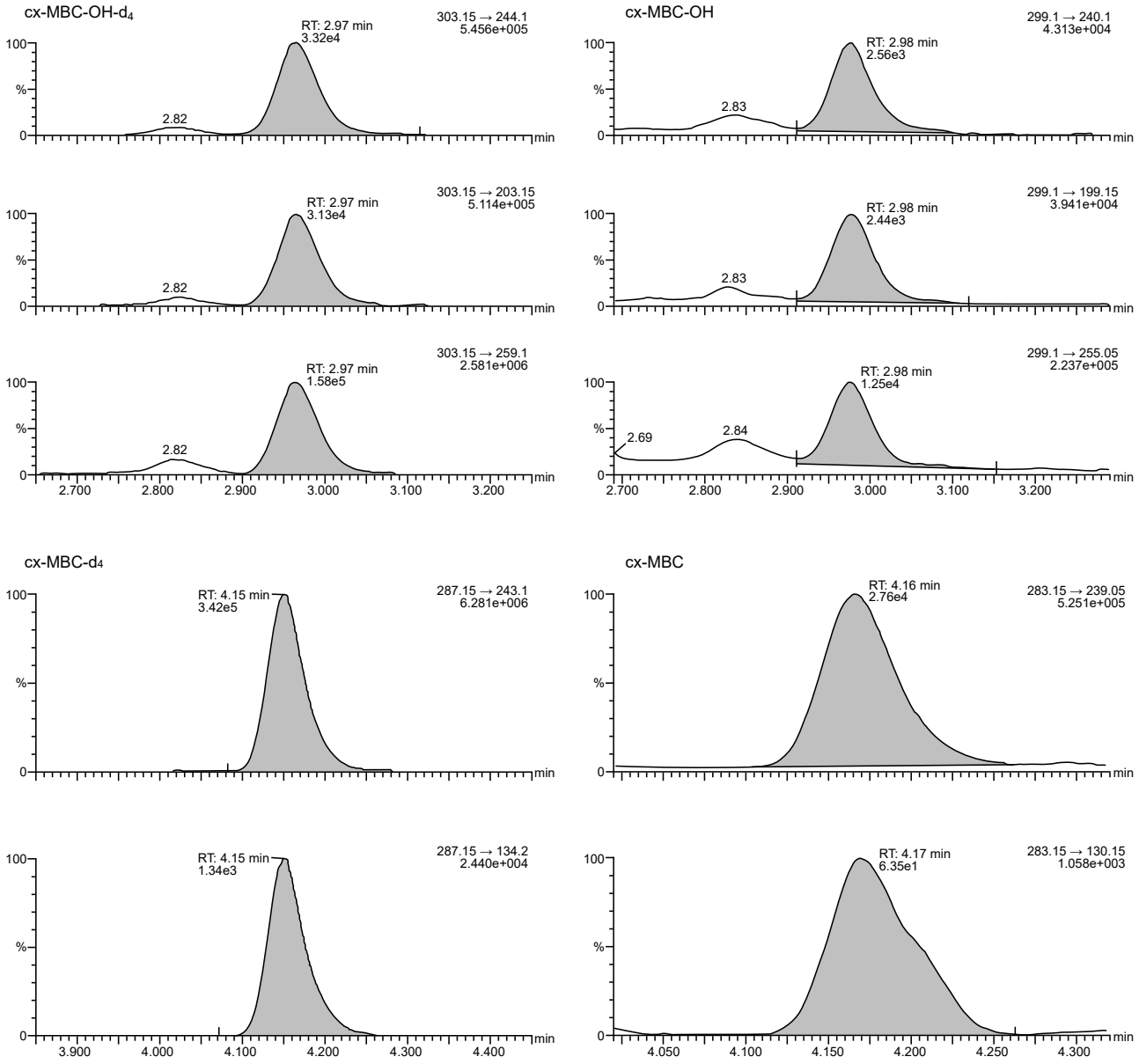


Fig. 3 Chromatograms of a) a reagent blank value, b) a native urine sample, and c) a urine sample spiked with 1.0 µg cx-MBC or cx-MBC-OH/l

8 Calibration

The calibration solutions are prepared as described in Section 4.6, processed analogously to the urine samples (see Section 5.2), and analysed. The calibration curves are generated by plotting the quotients of the peak areas of the analytes and the corresponding ISTDs against the spiked concentrations of the analytes. If blank values arise, these must be accounted for by subtraction from all measurement points.

Using the aforementioned analytical instrument, a linear measurement range for both analytes was determined from the detection limit up to 200 µg/l. Figure 4 shows representative calibration curves for the determination of cx-MBC and cx-MBC-OH in urine in a concentration range up to 20 µg/l.

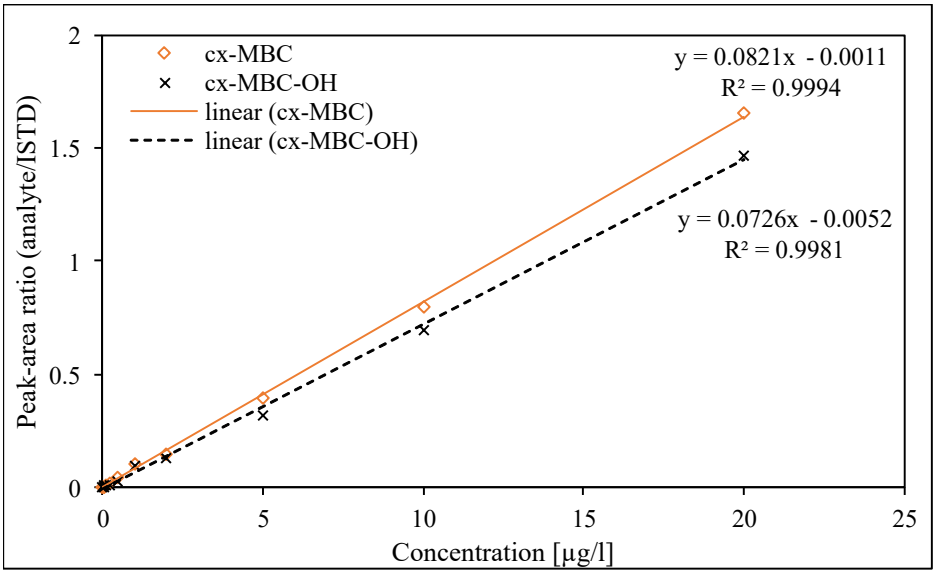


Fig. 4 Calibration curves for the determination of cx-MBC or cx-MBC-OH in urine

9 Calculation of the analytical results

The peak areas determined for the 4-MBC metabolites are divided by the peak areas of the corresponding deuterated ISTD. The quotient thus obtained is used to calculate the analyte concentration in µg/l using the calibration function. Any blank values which arise must be subtracted from the analytical results.

During method development, analyte concentrations were calculated using the MassLynx 4.1 software from Waters GmbH, Eschborn, Germany.

10 Standardisation and quality control

Quality assurance of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter published by the Commission (Bader et al. 2010; Bundesärztekammer 2014).

For quality assurance of the individual analytical runs, at least three urines with known analyte concentrations are processed and analysed parallel to the samples. Since no control materials are currently commercially available for 4-MBC metabolites, these must be prepared in the in-house laboratory. To this end, pooled urine from persons not occupationally exposed to 4-MBC is used and spiked with cx-MBC and cx-MBC-OH at concentrations of 1.0 µg, 10 µg, or 100 µg per litre, for example.

At the same time, at least three blank values are included in each analytical run in order to recognise any potential interferences from reagents, matrix components, or the ISTDs.

11 Evaluation of the method

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

11.1 Within-day precision

The determination of within-day precision was carried out using pooled urine. Ten samples, each of 0.5 ml urine, were each spiked at analyte concentrations of 0.5 µg, 1.0 µg, 10 µg, or 100 µg cx-MBC and cx-MBC-OH per litre urine. The samples were processed as described above (Section 5.2) and analysed (Section 7). The precision data thus obtained are given in Table 8.

Tab. 8 Within-day precision for the determination of cx-MBC and cx-MBC-OH in urine (n = 6 or 10)

Analyte	Spiked concentration [µg/l]	Number of repeated measurements n	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
cx-MBC	0.5[a)]	6	4.1	10.6
	1.0	10	3.3	7.3
	10	10	2.8	6.3
	100	10	2.2	4.8
cx-MBC-OH	0.5[a)]	6	6.2	15.9
	1.0	10	6.1	13.7
	10	10	4.0	8.9
	100	10	1.4	3.2

[a)] These data were collected by the verifiers of the method.

11.2 Accuracy

Relative recovery

The mean relative recovery was calculated from the within-day precision data. The recoveries thus obtained are summarised in Table 9.

Tab. 9 Relative recoveries for the determination of cx-MBC and cx-MBC-OH in urine (n = 6 or 10)

Analyte	Spiked concentration [µg/l]	Number of repeated measurements n	Recovery (rel.) r [%]	Range [%]
cx-MBC	0.5[a)]	6	99	–
	1.0	10	90	85–93
	10	10	98	95–105
	100	10	110	105–113
cx-MBC-OH	0.5[a)]	6	89	–
	1.0	10	89	84–100
	10	10	92	87–99
	100	10	106	104–108

[a)] These data were collected by the verifiers of the method.

11.3 Matrix effects

Comparison of absolute peak areas

The verifiers of the method estimated the influence of the urine matrix on the signal intensities. To this end, both urine and ultra-pure water were spiked with both analytes at two concentrations (0.5 µg/l and 100 µg/l). These samples were processed six (urine) or three (water) times and measured, and the peak areas of the analytes and the corresponding ISTDs were compared. The results of these measurements are presented in Table 10.

Tab. 10 Influence of the urine matrix on the peak areas of the analytes and ISTD (n = 6); the peak areas of the samples prepared in ultra-pure water (n = 3) were set at 100%

Substance	Spiked concentration [µg/l]	Ratio of peak area in urine/peak area in water [%]
cx-MBC	0.5	77.4
	100	79.5
cx-MBC-d_4	0.5	89.4
	100	84.6
cx-MBC-OH	0.5	59.0
	100	41.0
cx-MBC-OH-d_4	0.5	58.5
	100	48.9

Influence of various urine matrices

The developers of the method tested the influence of various urine matrices on precision and recovery. To this end, the method developers spiked each of ten native urine samples (creatinine concentrations of 0.40–3.17 g/l) with 1.0 µg, 10 µg or 100 µg cx-MBC and cx-MBC-OH per litre of urine. The urine samples were subsequently processed as described above and analysed.

The measured concentrations and the corresponding standard deviations are summarised in Table 11. It is clear from Table 11 that the individual urine matrix does not have any influence on the precision and accuracy of the analytical results.

Tab. 11 Influence of various urine matrices on the determination of cx-MBC and cx-MBC-OH in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Measured concentration [µg/l]	Standard deviation (rel.) s_w [%]	Recovery r (range) [%]
cx-MBC	1.0	0.91	5.3	91 (84.7–99.8)
	10	9.61	4.0	96.1 (90.3–105)
	100	108	3.6	108 (99.5–112)
cx-MBC-OH	1.0	0.97	10.6	97 (84.5–109)
	10	9.11	7.8	91.1 (82.8–105)
	100	105	4.8	105 (98.7–117)

11.4 Absolute recovery

Since, in the method herein described, sample extraction and enrichment took place primarily via automated online SPE, the determination of absolute recovery by spiking samples before and after sample preparation did not appear appropriate.

11.5 Limits of detection and quantitation

At an analyte concentration of 0.1 µg per litre of urine, the signal-to-noise ratio was found to be 1 : 25 for cx-MBC and 1 : 10 for cx-MBC-OH. The determination of robust, routinely suitable detection and quantification limits was performed using the six lowest measurement points of the calibration curve based on the calibration-curve method according to DIN 32645 (DIN 2008). The values calculated from the standard deviation of the method s_{x0} (detection limit = 4 × s_{x0}) are given in Table 12 and represent the limits of detection and quantitation for routine analysis.

Tab. 12 Limits of detection and quantitation for the determination of cx-MBC und cx-MBC-OH in urine

Analyte	from the standard deviation of the method s_{x0}	
	Detection limit [µg/l]	Quantitation limit [µg/l]
cx-MBC	0.05	0.15
cx-MBC-OH	0.1	0.3

11.6 Sources of error

The verifiers of the method first tested two columns which were available in their laboratory. A Strata C8 On-Line Extraction Column (2.1 × 20 mm, Phenomenex Ltd. Deutschland, Aschaffenburg, Germany) was used as the enrichment column, and an Acquity UPLC HSS C18 (1.8 µm, 2.1 × 100 mm, Waters GmbH, Eschborn, Germany) was used as the separation column. The specifications of both columns largely corresponded to those of the columns used in the original method, although they were shorter (the enrichment column was 20 mm rather than 30 mm, and the separation column was 100 mm rather than 150 mm). For both analytes, the retention times obtained with this combination of columns was about 0.5 min shorter than those indicated for the columns used in method development. Furthermore, interfering matrix compounds could not be sufficiently seperated, such that interferences from the urine disturbed the MRM analysis of both metabolites, which negatively impacted method performance. The detection and quantitation limits were therefore four times higher than those later determined with the original columns.

When using different tandem mass spectrometers, the mass transitions may vary in intensity, which may require an adjustment to the method. During external method verification, the qualifier transitions for cx-MBC and its internal standard, cx-MBC-d_4, could not be confirmed when using a different mass spectrometer, whereby these qualifier transitions already showed relatively low intensities during method development.

For both analytes, no relevant interferences were identified in the reagent blank. The verifiers of the method however observed matrix-related interferences in the urine blank-value samples (calibration standard 00 (without the addition of analyte or ISTD) and calibration standard 0 (without analyte but with added ISTD)). Interfering peaks, originating from the urine, were observed in pooled urine in close proximity to the retention times of the actual analyte peaks on the MRM traces of both cx-MBC and cx-MBC-OH; these peaks could only be partially chromatographically separated. If these interferences are not reliably separated by chromatography, this can result in significantly higher quantitation limits.

By comparing spiked urines with spiked ultra-pure water, a matrix effect was primarily determined for the metabolite cx-MBC-OH; this effect led to a reduced signal intensity. Since this matrix effect, however, occurred consistently in all individual urines and affected both the signal of the analyte and the corresponding deuterated ISTD, the effect does not influence the accuracy and precision of the analytical results.

It is further necessary to note that the efficiency of the enzymatic cleavage could not be tested as no conjugated reference substances were available.

12 Discussion of the method

The analytical method hereby presented was developed in order to quantify both main metabolites of 4-MBC, cx-MBC and cx-MBC-OH, in urine, thereby enabling the estimation of exposure of the general population to 4-MBC, a UVB filter in sunscreens and other cosmetic products. The method allows for a rapid, selective, and sensitive determination of cx-MBC and cx-MBC-OH in urine. Applying the newest instrumental developments, a robust UPLC-MS/MS method was developed which uses a column-switching technique for the extraction and chromatographic separation of the analytes in one measurement cycle.

Due to insufficient separation performance of the available chromatographic columns, tests using an HPLC-MS/MS system were not successful. The use of a UPLC device enabled, due to high pressure and new, more compact separation materials, a significantly better chromatographic resolution.

The use of structurally identical, deuterated internal standards, in combination with the high chromatographic resolution described above, enabled the compensation of previously frequently observed analytical variations, which is confirmed by the excellent precision data. At analyte concentrations of 0.5 µg/l, 1.0 µg/l, 10 µg/l, and 100 µg/l, relative standard deviations of 2.2–4.1% for cx-MBC and 1.4–6.2% for cx-MBC-OH were ascertained as within-day precision data. Spiking tests were performed rather than determining day-to-day precision. The results of the spiking tests in 10 individual urines underline the robustness of the analytical method, as the relative standard deviations were considerably less than 20%, even at a spiking level of 0.5 µg/l. In this respect, the spiking tests likewise confirm the calculated quantitation limits of 0.15 µg/l for cx-MBC and 0.3 µg/l for cx-MBC-OH.

When applying the method to urine samples from the Environmental Specimen Bank, the parameter cx-MBC could be quantified in 30% of samples and the parameter cx-MBC-OH in 6.4% of samples (Schmidtkunz et al. 2023), so that the method appears to be suitable for identifying exposed persons in the general population and quantifying their exposure to 4-MBC.

The proposed HBM-I value–for the sum of both metabolites in urine–of 470 µg/l for adults and 310 µg/l for children makes it clear that the detection sensitivity of the presented analytical method is very well-suited to reliably detect critically high exposures.

Instruments used UPLC-MS/MS system (Waters Acquity coupled to Waters Xevo-TQS tandem mass spectrometer, Waters GmbH, Eschborn, Germany) (method developers); Shimadzu LC system coupled to a SCIEX QTRAP 6500+ MS/MS (AB Sciex Germany GmbH, Darmstadt, Germany) (method verifiers)

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (www.dfg.de/mak/conflicts_interest) ensure that the content and conclusions of the publication are strictly science-based.

References

Ao J, Yuan T, Gu J, Ma Y, Shen Z, Tian Y, Shi R, Zhou W, Zhang J (2018) Organic UV filters in indoor dust and human urine: a study of characteristics, sources, associations and human exposure. Sci Total Environ 640–641: 1157–1164. https://doi.org/10.1016/j.scitotenv.2018.05.367

Bader M, Barr D, Göen T, Schaller KH, Scherer G, Angerer J (2010) Reliability criteria for analytical methods. Biomonitoring Method, 2010. In: Angerer J, Hartwig A, editors. The MAK-Collection for Occupational Health and Safety. Part IV: Biomonitoring Methods. Volume 12. Weinheim: Wiley-VCH. p. 55–101. Also available from https://doi.org/10.1002/3527600418.bireliabe0012

Balmer ME, Buser H-R, Müller MD, Poiger T (2005) Occurrence of some organic UV filters in wastewater, in surface waters, and in fish from Swiss Lakes. Environ Sci Technol 39(4): 953–962. https://doi.org/10.1021/es040055r

Bundesärztekammer (2014) Richtlinie der Bundesärztekammer zur Qualitätssicherung laboratoriumsmedizinischer Untersuchungen. Dtsch Ärztebl 111(38): A1583–A1618

Cunha SC, Trabalón L, Jacobs S, Castro M, Fernandez-Tejedor M, Granby K, Verbeke W, Kwadijk C, Ferrari F, Robbens J, Sioen I, Pocurull E, Marques A, Fernandes JO, Domingo JL (2018) UV-filters and musk fragrances in seafood commercialized in Europe Union: occurrence, risk and exposure assessment. Environ Res 161: 399–408. https://doi.org/10.1016/j.envres.2017.11.015

DIN (Deutsches Institut für Normung), editor (2008) DIN 32645:2008-11. Chemische Analytik - Nachweis-, Erfassungs- und Bestimmungsgrenze unter Wiederholbedingungen - Begriffe, Verfahren, Auswertung. Berlin: Beuth. https://doi.org/10.31030/1465413

ECHA (European Chemicals Agency) (2021) Member State Committee support document for identification of (±)-1,7,7-trimethyl-3-[(4-methylphenyl)methylene]bicyclo[2.2.1]heptan-2-one covering any of the individual isomers and/or combinations thereof (4-MBC) as a substance of very high concern because of its endocrine disrupting properties (article 57(f) – human health). Adopted on 29 November 2021. Helsinki: ECHA. https://echa.europa.eu/documents/10162/6a6d37ea-30ea-5cc5-74f7-6c1f6e678fc0, accessed 01 Jul 2024

European Commission (2024) Commission Regulation (EU) 2024/996 of 3 April 2024 amending Regulation (EC) No 1223/2009 of the European Parliament and of the Council as regards the use of Vitamin A, Alpha-Arbutin and Arbutin and certain substances with potential endocrine disrupting properties in cosmetic products. OJ L (996): 1–8

European Parliament, European Council (2009) Regulation (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products (recast). OJ L (342): 59–209

Hauri U, Lütolf B, Hohl C (2003) Determination of organic sunscreen filters in cosmetics with HPLC/DAD. Mitt Lebensmittelunters Hyg 94(1): 80–92

HBM-Kommission (Kommission "Human-Biomonitoring" des Umweltbundesamts) (2016) Stoffmonographie für 3-(4-Methylbenzyliden)-kampfer (4-MBC) – HBM-Werte für die Summe der Metaboliten 3-(4-Carboxybenzyliden)-kampfer (3-4CBC) und 3-(4-Carboxybenzyliden)-6-Hydroxykampfer (3-4CBHC) im Urin von Erwachsenen und Kindern. Stellungnahme der Kommission „Human-Biomonitoring" des Umweltbundesamtes. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz 59(1): 132–150. https://doi.org/10.1007/s00103-015-2272-9

Kerr AC (2011) A survey of the availability of sunscreen filters in the UK. Clin Exp Dermatol 36(5): 541–543. https://doi.org/10.1111/j.1365-2230.2010.04007.x

Leng G, Gries W (2017) New specific and sensitive biomonitoring methods for chemicals of emerging health relevance. Int J Hyg Environ Health 220(2 Pt A): 113–122. https://doi.org/10.1016/j.ijheh.2016.09.014

Manová E, von Goetz N, Hauri U, Bogdal C, Hungerbühler K (2013) Organic UV filters in personal care products in Switzerland: a survey of occurrence and concentrations. Int J Hyg Environ Health 216(4): 508–514. https://doi.org/10.1016/j.ijheh.2012.08.003

Murawski A, Schmied-Tobies MIH, Rucic E, Schmidtkunz C, Küpper K, Leng G, Eckert E, Kuhlmann L, Göen T, Daniels A, Schwedler G, Kolossa-Gehring M (2021) Metabolites of 4-methylbenzylidene camphor (4-MBC), butylated hydroxytoluene (BHT), and tris(2-ethylhexyl) trimellitate (TOTM) in urine of children and adolescents in Germany – human biomonitoring results of the German Environmental Survey GerES V (2014–2017). Environ Res 192: 110345. https://doi.org/10.1016/j.envres.2020.110345

Negreira N, Rodríguez I, Rubí E, Cela R (2009) Determination of selected UV filters in indoor dust by matrix solid-phase dispersion and gas chromatography-tandem mass spectrometry. J Chromatogr A 1216(31): 5895–5902. https://doi.org/10.1016/j.chroma.2009.06.020

Poiger T, Buser H-R, Balmer ME, Bergqvist P-A, Müller MD (2004) Occurrence of UV filter compounds from sunscreens in surface waters: regional mass balance in two Swiss lakes. Chemosphere 55(7): 951–963. https://doi.org/10.1016/j.chemosphere.2004.01.012

SCCNFP (Scientific Committee on Cosmetic Products and Non-food Products intended for Consumers) (2004) Opinion concerning 4-methylbenzylidene camphor COLIPA n° S60. SCCNFP/0779/04. Brussels: European Commission. https://ec.europa.eu/health/ph_risk/committees/sccp/documents/out282_en.pdf, accessed 27 Jun 2024

SCCP (Scientific Committee on Consumer Products) (2006) Opinion on 4-methylbenzylidene camphor COLIPA N° S60. SCCP/1042/06. Brussels: European Commission. https://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_075.pdf, accessed 10 Jul 2023

SCCS (Scientific Committee on Consumer Safety) (2022) Scientific opinion on 4-methylbenzylidene camphor (4-MBC), preliminary version of 22 December 2021, final version of 29 April 2022. SCCS/1640/21. Luxembourg: Publications Office of the European Union. https://doi.org/10.2875/915957

Schauer UMD, Völkel W, Heusener A, Colnot T, Broschard TH, von Landenberg F, Dekant W (2006) Kinetics of 3-(4-methylbenzylidene)camphor in rats and humans after dermal application. Toxicol Appl Pharmacol 216(2): 339–346. https://doi.org/10.1016/j.taap.2006.05.011

Schmidtkunz C, Küpper K, Weber T, Leng G, Kolossa-Gehring M (2023) A time trend of urinary 4-methylbenzylidene camphor metabolites in young adults from Germany. Environ Res 228: 115833. https://doi.org/10.1016/j.envres.2023.115833

Völkel W, Colnot T, Schauer UMD, Broschard TH, Dekant W (2006) Toxicokinetics and biotransformation of 3-(4-methylbenzylidene)camphor in rats after oral administration. Toxicol Appl Pharmacol 216(2): 331–338. https://doi.org/10.1016/j.taap.2006.05.012