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Lysmeral – Determination of hydroxylysmerylic acid, 4-*tert*-butylbenzoic acid, *tert*-butylhippuric acid, lysmerylic acid, and lysmerol in urine by UPLC-MS/MS

Biomonitoring Method – Translation of the German version from 2021

Keywords

lysmeral; lysmeral metabolites; fragrance; biomonitoring; urine; UPLC-MS/MS; ESI–

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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.

With the procedure described below, the following lysmeral metabolites are determined in urine: hydroxylysmerylic acid, 4-*tert*-butylbenzoic acid (TBBA), *tert*-butylhippuric acid (TBHA), lysmerylic acid, and lysmerol. Following the addition of the deuterated internal standards (TBBA-d_{13}, lysmerylic acid-d_8, and lysmerol-d_8) to 1 ml of urine, an enzymatic cleavage is carried out using β-glucuronidase. The samples are then subjected to liquid-liquid extraction with dichloromethane. The extracts are evaporated to dryness and derivatised with 3-nitrophthalic anhydride. The analysis is performed by UPLC-MS/MS following negative electrospray ionisation (ESI–).

1 Characteristics of the method

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

Parameters and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
Lysmeral	80-54-6	Hydroxylysmerylic acid	1897559-05-5
		4-*tert*-Butylbenzoic acid (TBBA)	98-73-7
		tert-Butylhippuric acid (TBHA)	87015-91-6
		Lysmerylic acid	66735-04-4
		Lysmerol	56107-04-1

Reliability data

Hydroxylysmerylic acid

Within-day precision:	Standard deviation (rel.)	s_w = 6.9%, 4.7%, or 4.9%
	Prognostic range	u = 19.2%, 13.1%, or 13.6%
	at spiked concentrations of 0.4 µg, 0.8 µg, or 4.0 µg per litre of urine and n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 13.7%, 10.5%, or 9.7%
	Prognostic range	u = 35.2%, 27.0%, or 24.9%
	at spiked concentrations of 0.4 µg, 0.8 µg, or 4.0 µg per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 88.9%, 88.4%, or 89.9%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 5 determinations	
Detection limit:	0.15 µg per litre of urine	
Quantitation limit:	0.45 µg per litre of urine	

4-*tert*-Butylbenzoic acid (TBBA)

Within-day precision:	Standard deviation (rel.)	s_w = 4.1%, 1.3%, or 0.8%
	Prognostic range	u = 11.4%, 3.61%, or 2.22%
	at spiked concentrations of 0.6 µg, 8.0 µg, or 85.0 µg per litre of urine and n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 8.1%, 1.3%, or 0.8%
	Prognostic range	u = 20.8%, 3.34%, or 2.06%
	at spiked concentrations of 0.6 µg, 8.0 µg, or 85.0 µg per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 92.6%, 96.8%, or 98.1%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 5 determinations	
Detection limit:	0.14 µg per litre of urine	

Quantitation limit: 0.42 µg per litre of urine

tert-Butylhippuric acid (TBHA)

Within-day precision:	Standard deviation (rel.)	s_w = 7.9%, 8.5% or 6.8%
	Prognostic range	u = 21.9%, 23.9%, or 18.9%
	at spiked concentrations of 0.8 µg , 1.0 µg, or 14.5 µg per litre of urine and n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 23.6%, 48.7%, or 17.8%
	Prognostic range	u = 60.7%, 125%, or 45.8%
	at spiked concentrations of 0.8 µg, 1.0 µg, or 14.5 µg per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 107%, 110%, or 110%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 5 determinations	
Detection limit:	0.13 µg per litre of urine	
Quantitation limit:	0.39 µg per litre of urine	

Lysmerylic acid

Within-day precision:	Standard deviation (rel.)	s_w = 6.8%, 2.1%, or 3.0%
	Prognostic range	u = 18.9%, 5.8%, or 8.3%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 5.8%, 5.2%, or 6.2%
	Prognostic range	u = 14.9%, 13.4%, or 15.9%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 103%, 102%, or 101%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 5 determinations	
Detection limit:	0.12 µg per litre of urine	
Quantitation limit:	0.36 µg per litre of urine	

Lysmerol

Within-day precision:	Standard deviation (rel.)	s_w = 4.6%, 3.9%, or 2.0%
	Prognostic range	u = 12.8%, 10.8%, or 5.5%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 16.5%, 4.2%, or 9.9%
	Prognostic range	u = 42.4%, 10.8%, or 25.5%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 92.9%, 98.7%, or 113%
	at spiked concentrations of 0.5 µg, 1.0 µg, or 10.0 µg per litre of urine and n = 5 determinations	

Detection limit: 0.035 µg per litre of urine

Quantitation limit: 0.10 µg per litre of urine

2 General information on lysmeral

Lysmeral (lilial, 2-(4-*tert*-butylbenzyl)propionaldehyde) is a synthetic fragrance with a lily-of-the-valley scent which is widely used in cosmetics and cleaners, as well as hygiene and household products (Pluym et al. 2016). The chemical structure of lysmeral is depicted in Figure 1. Because lysmeral can have a skin-sensitising effect in humans (Uter et al. 2013), in Europe, the use of lysmeral in cosmetics must be listed in the ingredients when its concentration exceeds 0.001% in leave-on products or 0.01% in rinse-off products (Heisterberg et al. 2011).

Important metabolites as well as their toxicokinetics and metabolic conversion factors were investigated in a metabolism study with five volunteers within the framework of the cooperative project between Germany's Federal Ministry of the Environment, Nature Conservation and Nuclear Safety (*Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit*; BMU) and the German Chemical Industry Association (*Verband der chemischen Industrie*; VCI), which aimed to develop a biomonitoring method for the determination of lysmeral exposure in the general population (Scherer et al. 2017). Four metabolites of lysmeral (hydroxylysmerylic acid, *tert*-butylbenzoic acid (TBBA), lysmerylic acid, and lysmerol) were detected in urine, accounting for a total of 16.5% of the single applied dose of lysmeral. Quantifying lysmeral metabolites in the urine of 40 volunteers from the general population, Scherer et al. (2017) estimated the median uptake of lysmeral to be about 140–220 µg per day. The metabolism scheme for lysmeral shown in Figure 1 was derived from this data as well as from *in vitro* studies with diverse animal species.

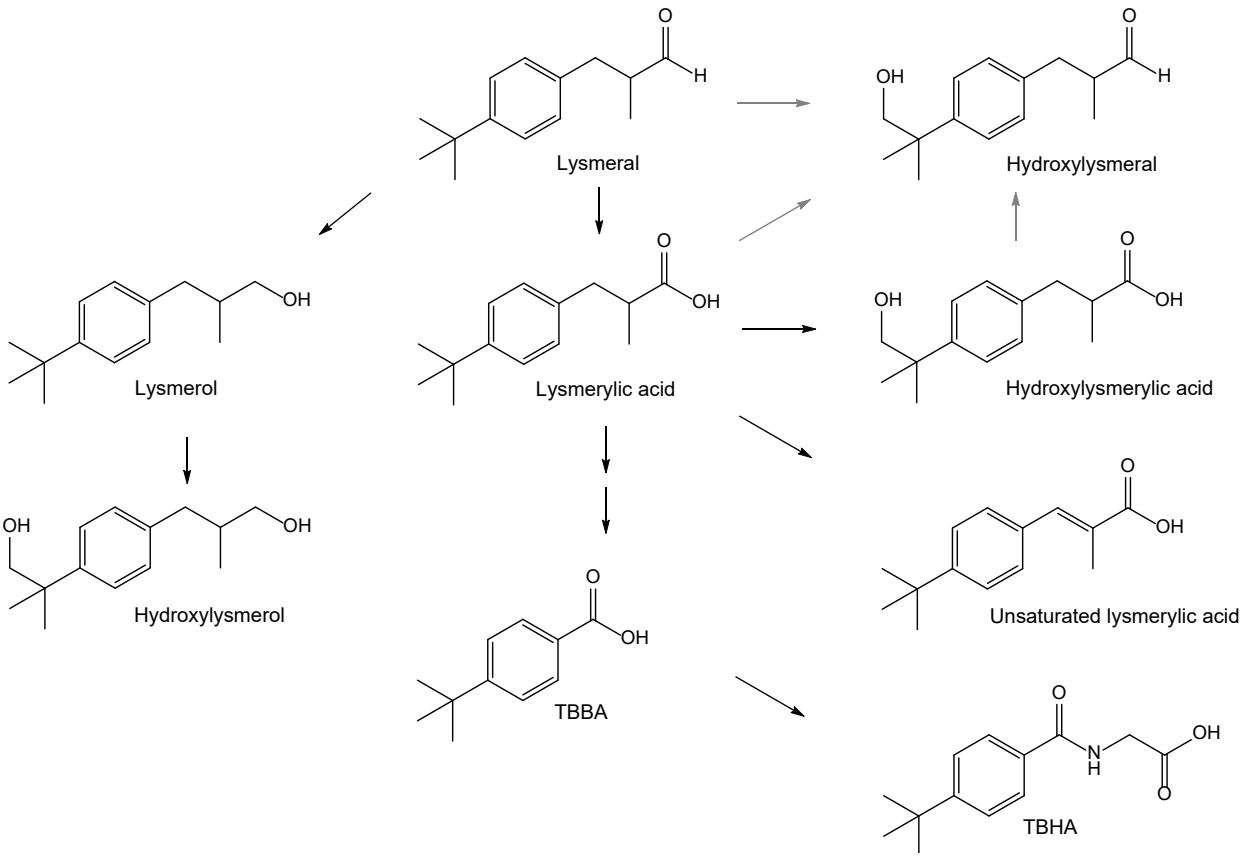

Fig. 1 Hypothetical scheme for the lysmeral metabolism in animals and humans (according to Scherer et al. 2017). Most of the metabolites were deduced from rodents (rats, mice) and in vitro studies (hepatocytes and microsomes from rodents and humans). Black arrows indicate that there is experimental evidence for this pathway. Grey arrows indicate that this pathway is hypothetical and not very likely

Hydroxylysmerylic acid, lysmerylic acid, and lysmerol represent suitable and specific biomarkers for lysmeral exposure. TBBA and TBHA can also arise from other relatively widespread precursors (e.g., *tert*-butyltoluene) (Scherer et al. 2017). However, due to the high detection rate and good correlation between TBBA and the other metabolites, TBBA can also be considered a specific biomarker (Scherer et al. 2021). In population studies in Germany, the main metabolites TBBA and lysmerol were detected in almost all urine samples. Hydroxylysmerylic acid and lysmerylic acid were detected in about 30–40% of the samples, with TBBA found in significantly higher concentrations than the other metabolites (Murawski et al. 2020; Scherer et al. 2021).

3 General principles

With the procedure described below, the following lysmeral metabolites are determined in urine: hydroxylysmerylic acid, *tert*-butylbenzoic acid (TBBA), *tert*-butylhippuric acid (TBHA), lysmerylic acid, and lysmerol. After addition of the deuterated internal standards (TBBA-d_{13}, lysmerylic acid-d_8, and lysmerol-d_8) to 1 ml of urine, an enzymatic cleavage is carried out using β-glucuronidase, followed by liquid-liquid extraction with dichloromethane. The extracts are evaporated to dryness and derivatised with 3-nitrophthalic anhydride. The analysis is performed by UPLC-MS/MS following negative electrospray ionisation (ESI–).

4 Equipment, chemicals, and solutions

4.1 Equipment

- UPLC system comprised of a Sample Manager (SM-FTN), an I-Class Binary Solvent Manager, Column Manager, and a Sample Organiser (e.g. Acquity UPLC I-Class System, Waters GmbH, Eschborn, Germany)
- HPLC column: Acquity UPLC BEH C18 1.7 µm, 2.1 mm × 100 mm (e.g. Waters GmbH, Eschborn, Germany)
- Tandem mass spectrometer (e.g. Xevo TQ-S Tandem Quadrupole, Waters GmbH, Eschborn, Germany)
- Incubator with a shaker (e.g. Incucell 111 with Shaker GFL 3005, MMM Medcenter GmbH, Planegg, Germany)
- Nitrogen generator (e.g. CMC Instruments, Eschborn, Germany)
- Centrifuge (e.g. Rotixa KS, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- SpeedVac concentrator (e.g. Jouan GmbH, Unterhaching, Germany)
- Tube roller (e.g. Stuart Equipment, Cole-Parmer, Stone, United Kingdom)
- Multi-tube vortexer (e.g. VWR International GmbH, Darmstadt, Germany)
- pH Meter (e.g. Type CG 842, Schott AG, Mainz, Germany)
- 1-ml, 2-ml, 10-ml, 100-ml, and 1000-ml volumetric flasks (e.g. Schott AG, Mainz, Germany)
- 100-ml and 1000-ml graduated cylinders (e.g. Schott AG, Mainz, Germany)
- 4-ml sample vials with screw caps (e.g. BGB Analytik Vertrieb GmbH, Lörrach, Germany)
- Autosampler vials (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- Multipette® (e.g. Eppendorf AG, Hamburg, Germany)
- Variably adjustable microlitre pipettes with matching tips (1–10 µl, 10–100 µl, and 100–1000 µl) (e.g. Eppendorf AG, Hamburg, Germany)
- Urine-collection containers (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

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

- Ammonium acetate (e.g. No. 012406, Biosolve BV, Valkenswaard, Netherlands)
- Ammonium hydroxide solution ≥ 25% in water (e.g. No. 15650920, Fisher Scientific GmbH, Schwerte, Germany)
- Dichloromethane (e.g. No. DRE-C12424500, LGC Standards GmbH, Wesel, Germany)
- Disodium hydrogen phosphate (e.g. No. 106586, Merck KGaA, Darmstadt, Germany)
- Ethyl acetate (e.g. No. 270989, Merck KGaA, Darmstadt, Germany)
- *β*-Glucuronidase, Type IX-A from *E. coli*, lyophilised powder, 500,000 units (e.g. No. G7396-500KU, Merck KGaA, Darmstadt, Germany)
- Methanol (e.g. No. 136806, Biosolve BV, Valkenswaard, Netherlands)
- 3-Nitrophthalic anhydride (e.g. No. 820903, Merck KGaA, Darmstadt, Germany)
- *ortho*-Phosphoric acid 85% (e.g. No. 1.00573, Merck KGaA, Darmstadt, Germany)
- Potassium dihydrogen phosphate (e.g. No. 104873, Merck KGaA, Darmstadt, Germany)
- Water (e.g. No. REAH2O25AG, LGC Standards GmbH, Wesel, Germany)

4.3 Reference materials

- Hydroxylysmerylic acid (4-(2-hydroxy-1,1-dimethylethyl)-α-methylbenzenepropionic acid) (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- TBBA (4-*tert*-butylbenzoic acid) (e.g. No. 820238, Merck KGaA, Darmstadt, Germany)
- TBBA-d_{13} (e.g. No. D-7697, C/D/N Isotopes Inc., Pointe-Claire, Canada)
- TBHA ([(4-*tert*-butylbenzoyl)amino]acetic acid) (e.g. No. OTV000050, Merck KGaA, Darmstadt, Germany)
- Lysmerylic acid (3-(4-*tert*-butylphenyl)-2-methylpropionic acid) (e.g. No. JH-7214, Combi-Blocks Inc., San Diego, USA)
- Lysmerylic acid-d_8 (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- Lysmerol (3-(4-*tert*-butylphenyl)-2-methyl-1-propanol) (e.g. custom synthesis, Otava Chemicals, Vaughan, Canada)
- Lysmerol-d_8 (e.g. custom synthesis, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)

4.4 Solutions

- *ortho*-Phosphoric acid (20%)
  70 ml of ultra-pure water are filled into a 100-ml volumetric flask and 23.5 ml of *ortho*-phosphoric acid (85%) are added. The flask is made up to the mark with ultra-pure water.
- Methanol : water (1 : 1, v/v)
  50 ml of methanol are filled into a 100-ml volumetric flask, which is then made up to the mark with ultra-pure water.

- Ammonium acetate solution (5 mmol/l)
  358.41 mg of ammonium acetate are weighed into a 1000-ml volumetric flask and dissolved in ultra-pure water. The flask is then made up to the mark with ultra-pure water.
- Ammonium hydroxide solution (0.025%, v/v)
  90 ml of ultra-pure water are filled into a 100-ml volumetric flask and 25 µl of ammonium hydroxide solution are added. The flask is then made up to the mark with ultra-pure water.
- Ammonium acetate solution (5 mmol/l) + ammonium hydroxide solution (0.025%), pH 9.2
  Using 0.025% ammonium hydroxide solution, the ammonium acetate solution (5 mmol/l) is adjusted to a pH value of pH = 9.2.
- Potassium dihydrogen phosphate solution (0.67 mol/l)
  90.73 g of potassium dihydrogen phosphate are weighed into a 1000-ml volumetric flask and dissolved in ultra-pure water. The flask is then made up to the mark with ultra-pure water.
- Disodium hydrogen phosphate solution (0.67 mol/l)
  94.64 g of disodium hydrogen phosphate are weighed into a 1000-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.
- Phosphate buffer (0.67 mol/l, pH 6.4)
  Using the potassium dihydrogen phosphate solution (0.67 mol/l), one litre of the disodium hydrogen phosphate solution (0.67 mol/l) is adjusted to a pH value of pH = 6.4.

The aqueous solutions are stored in the refrigerator (4–8 °C) and are stable under these conditions for at least three months.

- 3-Nitrophthalic anhydride in ethyl acetate (100 mg/l)
  25 mg of 3-nitrophthalic anhydride are dissolved in 250 ml of ethyl acetate. The solution is stored in the refrigerator (4–8 °C). No data on stability are available.
- β-Glucuronidase (150 U/µl)
  The total amount of the enzyme is dissolved in 3324 µl of phosphate buffer (pH 6.4). The solution is stored at –20 °C and is stable under these conditions for at least one month.

4.5 Internal standards (ISTDs)

- ISTD stock solutions (1 g/l)
  10 mg of TBBA-d_{13}, lysmerylic acid-d_8, or lysmerol-d_8 are each weighed exactly into a 10-ml volumetric flask and dissolved in methanol. The flasks are then made up to the mark with methanol.
- ISTD working solutions (100 mg/l)
  100 µl of each of the ISTD stock solutions are each pipetted into a 1-ml volumetric flask. The flasks are then made up to the mark with methanol.
- ISTD spiking solution (1 mg/l)
  100 µl of each of the ISTD working solutions are pipetted into a 10-ml volumetric flask. The volumetric flask is then made up to the mark with ultra-pure water.

The ISTD stock and working solutions, as well as the ISTD spiking solution, are stored at –20 °C. No data on stability are available.

4.6 Calibration standards

- Stock solutions (1 g/l)
  10 mg of hydroxylysmerylic acid, TBBA, TBHA, lysmerylic acid, or lysmerol are each weighed exactly into a 10-ml volumetric flask and dissolved in methanol. The flasks are then made up to the mark with methanol.
- Working solutions (100 mg/l)
  100 µl of each of the stock solutions are each pipetted into a 1-ml volumetric flask. The flasks are then made up to the mark with methanol.
- Spiking solution I (10 mg/l)
  100 µl of each of the working solutions are pipetted into a 1-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.
- Spiking solution II (2 mg/l)
  200 µl of spiking solution I are pipetted into a 1-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.
- Spiking solution III (100 µg/l)
  100 µl of spiking solution II are pipetted into a 2-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.

All stock, working, and spiking solutions of the analytes are stored at –20 °C. No data on stability are available.

Calibration standards are prepared by spiking pooled urine with spiking solutions I–III according to the pipetting scheme given in Table 1. A calibration range of 0.2–500 µg/l is used for the analytes hydroxylysmerylic acid, TBBA, and lysmerol; for TBHA and lysmerylic acid, a range of 0.2–100 µg/l is used.

Tab. 1 Pipetting scheme for the preparation of calibration standards for the determination of lysmeral metabolites in urine

Calibration standard	Spiking solution	Volume of spiking solution [µl]	Volume of pooled urine [µl]	Analyte concentration [µg/l]
00	–	–	1000	0 (without ISTD)
0	–	–	1000	0
1	III	2	998	0.2
2	III	5	995	0.5
3	III	10	990	1
4	III	20	980	2
5	III	50	950	5
6	II	5	995	10
7	II	25	975	50
8	II	50	950	100
9	I	50	950	500

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in sealable polypropylene containers and stored at –20 °C until sample preparation.

5.2 Sample preparation

The urine samples are brought to room temperature and thoroughly mixed. Of each sample, 1 ml is pipetted into a 4-ml sample vial and mixed with 10 µl of the ISTD spiking solution, 1 ml of phosphate buffer (pH 6.4), and 10 µl of β-glucuronidase (150 U/µl). The samples are mixed and shaken overnight in an incubator at 37 °C (about 100 rpm).

50 µl of 20% *ortho*-phosphoric acid and 1.5 ml of dichloromethane are pipetted to the samples. The samples are then mixed for 30 min on a tube roller and subsequently centrifuged for 15 min at 1860 × *g*. The dichloromethane phase is pipetted into a new sample vial and evaporated to dryness in the SpeedVac without heat or gas inlet. The residue is redissolved in 1 ml of the derivatisation solution (3-nitrophthalic anhydride in ethyl acetate) and the sample is then incubated for 30 min at 80 °C. After cooling to room temperature, the solution is again evaporated to dryness. Resuspension of the residue is performed in 100 µl of methanol/water (1 : 1, v/v).

6 Operational parameters

6.1 Ultra high-pressure liquid chromatography

Separation column:	Acquity UPLC BEH C18 1.7 µm, 2.1 mm × 100 mm
Column temperature:	30 °C
Autosampler:	10 °C
Injection volume:	10 µl
Eluent:	A: Methanol B: Ammonium acetate (0.5 mM) + ammonium hydroxide (0.025%), pH 9.2
Flow rate:	0.35 ml/min
Runtime:	13 min

The gradient program is given in Table 2. All other parameters must be optimised according to the specifications of the individual manufacturer.

Tab. 2 Gradient program for the determination of lysmeral metabolites in urine

Time [min]	Eluent A [%]	Eluent B [%]
0	20	80
4	45	55
10	65	35
11	100	0
13	20	80

6.2 Tandem mass spectrometry

Ionisation:	Negative electrospray ionisation (ESI–)
Ion-source temperature:	150 °C
Desolvation temperature:	600 °C
Cone gas flow:	148 l/h
Desolvation gas flow:	798 l/h

Collision gas flow: 8.4 ml/h

Parameter-specific settings: See Table 3

The instrument-specific parameters must be ascertained and adjusted by the user for the individual tandem mass-spectrometric system used. The parameters given in this section have been identified and optimised for the device configuration used during method development.

Tab. 3 Retention times and parameter-specific settings for the determination of lysmeral metabolites in urine

Analyte or ISTD	Retention time [min]	Mass transition [*m/z*]	Status	Measurement time [ms]	Cone [V]	Collision energy [V]
Hydroxylysmerylic acid	4.48	235.0 ⟶ 205.1	Quantifier	43	2	14
		428.2 ⟶ 384.1	Qualifier	43	12	16
TBBA-d_{13}	5.17	190.2 ⟶ 146.2	ISTD	51	48	14
TBBA	5.27	177.0 ⟶ 133.0	Quantifier	51	44	14
TBHA	5.49	234.1 ⟶ 133.1	Quantifier	43	52	20
		234.1 ⟶ 190.1	Qualifier	43	52	14
Lysmeryic acid-d_8	8.11	227.0 ⟶ 227.0[a)]	ISTD	43	60	14
Lysmerylic acid	8.19	219.1 ⟶ 219.1[a)]	Quantifier	43	60	14
Lysmerol-d_8	11.92	406.0 ⟶ 239.0	ISTD	80	4	14
Lysmerol	11.92	398.2 ⟶ 231.1	Quantifier	80	4	14
		398.2 ⟶ 166.0	Qualifier	80	4	14

[a)] Since no suitable mass transition was found for this compound, identical ions were chosen as parent and daughter ions.

7 Analytical determination

10 µl of the processed urine sample (see Section 5.2) are injected into the UPLC-MS/MS system. Identification of the analytes is carried out using their specific ion transitions and retention times. The retention times given in Table 3 are intended only as a point of reference. Users must ensure proper separation performance of the column used influencing the resulting retention behaviour of the analytes. A representative chromatogram of a urine sample spiked with the individual analytes is depicted in Figure 2.

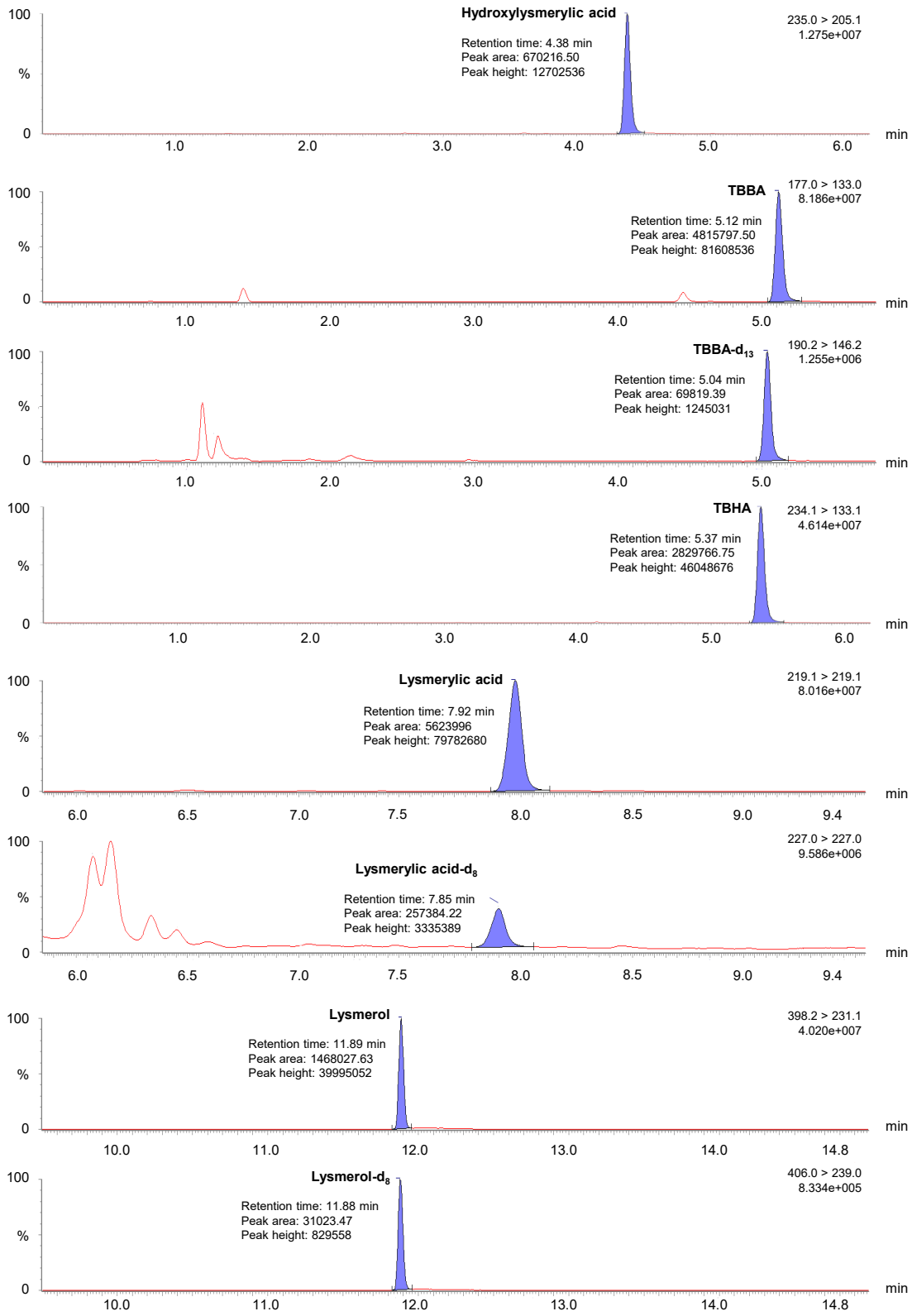

Fig. 2 MRM chromatogram of a urine sample spiked with hydroxylysmerylic acid (100 µg/l), TBBA (500 µg/l), TBHA (500 µg/l), lysmerylic acid (100 µg/l), and lysmerol (100 µg/l)

8 Calibration

Calibration standards are prepared as specified in Section 4.5, processed analogously to the urine samples (see Section 5.2), and analysed. The calibration curve is constructed by plotting the peak area ratio of the analyte to the deuterated internal standard against the spiked concentration. TBBA-d_{13} is used as an internal standard for the analytes hydroxylysmerylic acid, TBBA, and TBHA. The calibration graphs are linear in the concentration range from 0.2–100 µg/l and 0.2–500 µg/l, respectively. Figure 3 shows representative calibration curves for the individual analytes.

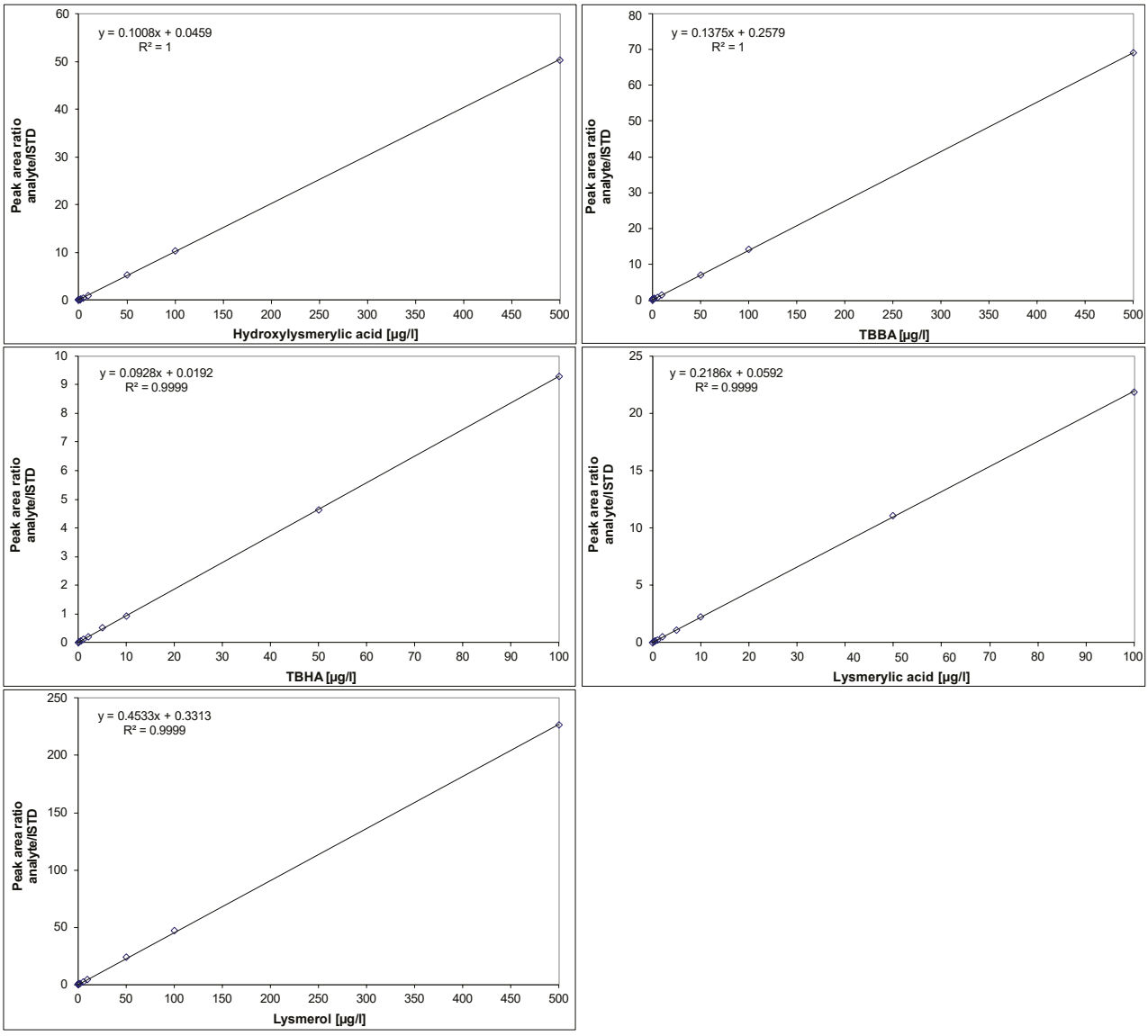

Fig. 3 Calibration curves for the determination of lysmeral metabolites in urine

9 Calculation of the analytical results

In order to calculate the analyte concentration in a urine sample, the peak area of the analyte is divided by the peak area of the corresponding ISTD. Using the calibration function corresponding to the analyte and analytical run in question (cf. Section 8), the quotient thus calculated can be used to determine analyte concentration in µg/l of urine.

The area ratio of the unspiked calibration standard is subtracted from the remaining calibration standards and the regression curve is forced through the origin of the coordinate system. If the measured result lies above the calibration range, the corresponding sample is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

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

To check precision, at least three quality control samples with low, medium, and high concentrations of the analytes are included in each analytical run. As control material is not commercially available, it must be prepared in the laboratory by spiking pooled urine with standard solutions of the analytes. The analyte concentrations in the quality control materials should be within the relevant concentration range (e.g. 0.5 µg/l, 1 µg/l, and 10 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 per control material on ten different days) (Bader et al. 2010).

11 Evaluation of the method

The reliability of this method was confirmed by comprehensive validation as well as by replication and verification in a second, independent laboratory. During method development, validation was performed in accordance with the criteria indicated in a general chapter published by the Commission (Bader et al. 2010) as well as guidelines issued by the U.S. Food and Drug Administration (FDA 2018).

11.1 Precision

Within-day precision

The determination of the within-day precision was performed at a low, a medium and a high concentration level. For the determination of the within-day precision, the samples were each processed five times in one day. The results are summarised in Table 4.

Tab. 4 Within-day precision for the determination of lysmeral metabolites in urine (n = 5)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Hydroxylysmerylic acid	0.4	6.9	19.2
	0.8	4.7	13.1
	4.0	4.9	13.6
TBBA	0.6	4.1	11.4
	8.0	1.3	3.6
	85.0	0.8	2.2
TBHA	0.8	7.9	21.9
	1.0	8.5	23.9
	14.5	6.8	18.9
Lysmerylic acid	0.5	6.8	18.9
	1.0	2.1	5.8
	10.0	3.0	8.3

Tab. 4 *(continued)*

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Lysmerol	0.5	4.6	12.8
	1.0	3.9	10.8
	10.0	2.0	5.6

Day-to-day precision

Day-to-day precision was also determined at a low, a medium, and a high concentration level. For the determination of day-to-day precision, the spiked urine samples were each investigated once per day on six different days over a timeframe of about three weeks. The precision data calculated are presented in Table 5.

Tab. 5 Day-to-day precision for the determination of lysmeral metabolites in urine (n = 6)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
Hydroxylysmerylic acid	0.4	13.7	35.2
	0.8	10.5	27.0
	4.0	9.7	24.9
TBBA	0.6	8.1	20.8
	8.0	1.3	3.3
	85.0	0.8	2.1
TBHA	0.8	23.6	60.7
	1.0	48.7	125
	14.5	17.8	45.8
Lysmerylic acid	0.5	5.8	14.9
	1.0	5.2	13.4
	10.0	6.2	15.9
Lysmerol	0.5	16.5	42.4
	1.0	4.2	10.8
	10.0	9.9	25.5

11.2 Accuracy

In order to ascertain the accuracy of the method, analyte-free pooled urine was used. The urine was spiked with three different analyte concentrations (0.5 µg/l, 1 µg/l, or 10 µg/l). For each concentration level, five determinations were performed. The mean relative recovery rates thus obtained are given in Table 6.

Tab. 6 Mean relative recovery rates for the determination of lysmeral metabolites in urine (n = 5)

Analyte	Spiked concentration [µg/l]	Mean rel. recovery rate r [%]
Hydroxylysmerylic acid	0.5	88.9
	1.0	88.4
	10.0	89.9
TBBA	0.5	92.6
	1.0	96.8
	10.0	98.1
TBHA	0.5	107
	1.0	110
	10.0	110
Lysmerylic acid	0.5	103
	1.0	102
	10.0	101
Lysmerol	0.5	92.9
	1.0	98.7
	10.0	113

11.3 Absolute recovery

The determination of absolute recovery served towards estimating process-related losses. For this process, the urine was spiked with three different analyte concentrations (0.5 µg/l, 1 µg/l, or 10 µg/l). These samples were investigated six times. The calculation of absolute recovery was carried out by comparison with reference samples. As reference samples, the same urine matrix was used, however, this urine was spiked with the three different analyte concentrations after processing. The reference samples were investigated three times. The absolute recovery rates thus obtained are given in Table 7.

Tab. 7 Absolute recovery rates for the determination of lysmeral metabolites in urine (n = 6)

Analyte	Spiked concentration [µg/l]	Absolute recovery rate *r* [%]
Hydroxylysmerylic acid	0.5	85.5
	1.0	80.1
	10.0	80.4
TBBA	0.5	96.7
	1.0	103
	10.0	99.5
TBHA	0.5	104
	1.0	87.4
	10.0	104
Lysmerylic acid	0.5	86.3
	1.0	86.8
	10.0	80.6
Lysmerol	0.5	74.1
	1.0	72.2
	10.0	72.5

11.4 Matrix effects

Matrix effects, which arise in LC-MS/MS measurements, were investigated using three individual urine samples. The urine samples were processed without spiking and, only immediately prior to the actual LC-MS/MS analysis, were spiked with the analytes at low as well as high concentrations and with the internal standards. Pure solvent was spiked with the same analyte concentrations and measured in parallel.

The calculation of matrix effects was performed by comparing the peak areas with the signals of the same analyte amounts in pure solvent (Table 8).

Tab. 8 Matrix effects for the determination of lysmeral metabolites in urine (n = 3)

Analyte	Spiked concentration [µg/l]	Matrix effects [%]
Hydroxylysmerylic acid	0.5	75.9
	10.0	72.2
	10.0 (ISTD)	109
TBBA	0.5	111
	10.0	108
	10.0 (ISTD)	105
TBHA	0.5	89.6
	10.0	90.0
	10.0 (ISTD)	105
Lysmerylic acid	0.5	99.9
	10.0	99.7
	10.0 (ISTD)	115
Lysmerol	0.5	265
	10.0	504
	10.0 (ISTD)	271

11.5 Limits of detection and quantitation

The limits of detection and quantitation of the analytes were ascertained by determining the signal-to-noise ratio using the corresponding function of the evaluation software MassLynx V4.1. For this, three individual analyte-free urine samples were spiked in the lower calibration range and the signal-to-noise ratio was calculated. The concentrations at a signal-to-noise-ratio of three were established as the limits of detection. The limits of quantitation were taken from the signal-to-noise ratio of nine and subsequently verified by the determination of precision and accuracy in five individual urine samples spiked at the limit of quantitation. The criteria for this process included a precision of a maximum 20% and an accuracy of 80–120%. Table 9 shows the determined limits of detection and quantitation for all analytes.

Tab. 9 Limits of detection and quantitation of the analytes for the determination of lysmeral metabolites in urine

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
Hydroxylysmerylic acid	0.15	0.45
TBBA	0.14	0.42
TBHA	0.13	0.39
Lysmerylic acid	0.12	0.36
Lysmerol	0.035	0.10

11.6 Stability

Three different tests were performed in order to investigate the stability of the analytes: short-term stability, long-term stability, and freeze/thaw stability. Two urine samples, spiked at either low or high concentrations, were used to ascertain the aforementioned parameters.

Short-term stability of the analytes was determined at room temperature and at a storage period of 24 h, which corresponds with the maximum storage period of the sample (during sample collection and processing) at room temperature. The accuracy for the five lysmeral metabolites thus ascertained lied between 85% and 115%.

Long-term stability was determined at a storage temperature of ≤ –20 °C. Aliquoted samples were processed and measured at regular intervals, three times in parallel, whereby accuracy lied between 85% and 115% of the corresponding nominal values. TBBA, lysmerylic acid, and hydroxylysmerylic acid are stable for ten months at –20 °C.

Freeze-thaw stability was determined using a sample aliquot which was processed and measured in triplicate directly following preparation and after six freeze-thaw cycles. The accuracy of these determinations lied within 85–115% of the corresponding nominal values. TBBA, lysmerylic acid, lysmerol, and hydroxylysmerylic acid were stable over the course of six freeze-thaw cycles.

Stability data for the remaining analytes was not yet available at the time of this publication.

11.7 Carry-over effects

Carry-over effects in the chromatographic system were investigated by multiple injections of highly concentrated samples and blank-value samples. A blank urine was analysed twice after every five injections of a highly concentrated sample. For lysmerol, small interfering signals were observed at the retention time of the analyte and the internal standard, but the concentration of the carry-over was lower than the limit of quantitation.

11.8 Sources of error

During method development, an interfering peak was observed at the retention time of lysmerylic acid. This disturbance could, however, be eliminated by optimising the pH values of the eluents. It was ascertained that a pH value of pH 9.2 yields robust results; for this reason, an exact adherence to this pH value during chromatography is absolutely necessary.

During the external method verification, whereby an HPLC system was used in place of a UPLC system, interferences were observed for all analytes as well as partially significant blank values. The enzyme exchange from *E. coli* to *Helix pomatia* glucuronidase (Sigma-Aldrich No. G7017) could be excluded as the cause of these disturbances. A second verification, using a freshly cleaned HPLC-MS/MS device and samples processed exclusively by the developers of the method showed lower interferences and significantly lower blank values.

12 Discussion of the method

The UPLC-MS/MS method described herein allows for the determination of five lysmeral metabolites (hydroxylysmerylic acid, TBBA, TBHA, lysmerylic acid, and lysmerol). The method is fast, selective, and displays sufficient accuracy and linearity. The limits of quantitation are sufficient to quantify analyte concentrations in the urine of the general population. With regard to lysmerylic acid and hydroxylysmerylic acid, the background levels in urine of the occupationally non-exposed general population are below the limit of quantitation in 80% of the samples.

During method development, the metabolite TBHA displayed high variances in the lower concentration range. For this analyte, the user of the method must either ensure measurement accuracy, especially in the lower concentration range, or exclude TBHA as a biomarker for the determination of lysmeral exposure.

External method verification was performed in a second independent laboratory using an HPLC-MS/MS device. Due to peak broadening, the verifiers of the method found significantly higher quantitation limits for lysmerylic acid and hydroxylysmerylic acid when compared to those of the developers of the method. Moreover, high background levels were found for TBBA. Validation data for lysmerol were confirmed during method verification.

Due to the concentrations of lysmeral metabolites observed in the urine of the general population (Pluym et al. 2016; Scherer et al. 2017), the limits of quantitation for lysmerol and TBBA, as achieved using HPLC separation, are sufficient for biomonitoring.

Instruments used UPLC system: Acquity UPLC I-Class System comprised of a Sample Manger (SM-FTN), an I-Class Binary Solvent Manager, a Column Manager and a Sample Organiser as well as an Xevo TQ-S Tandem Quadrupole Mass Spectrometer (Waters GmbH, Eschborn, Germany).

Notes

Competing interests

The established rules and measures of the Commission to avoid conflicts of interest (https://www.dfg.de/en/dfg_profile/statutory_bodies/senate/health_hazards/conflicts_interest/index.html) ensure that the content and conclusions of the publication are strictly science-based.

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