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Geraniol – Determination of 8-carboxygeraniol, Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid in urine by UPLC-MS/MS

Biomonitoring Method – Translation of the German version from 2021

Keywords

geraniol; citral; geraniol metabolites; 8-carboxygeraniol; Hildebrandt acid; geranic acid; 3-hydroxycitronellic acid; biomonitoring; urine; UPLC-MS/MS

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Abstract

The working group "Analyses in Biological Materials" of the Permanent Senate Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method.

The method described herein enables the selective and quantitative determination of geraniol metabolites in human urine into the concentration range relevant for environmental exposure. The following four metabolites can be determined using this method: 8-carboxygeraniol, Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid. Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid can also arise from the metabolism of citral. The buffered urine samples are enzymatically hydrolysed and, following addition of the internal standards (8-carboxygeraniol-d_5, Hildebrandt acid-d_5, and 2,4,6-octatrienoic acid), processed via liquid-liquid extraction with diethyl ether. The analytes are separated from any matrix components by liquid chromatography, and are then detected via tandem mass spectrometry using electrospray ionisation. The quantitative evaluation is carried out using external calibration in water.

1 Characteristics of the method

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

Parameters and corresponding hazardous substances

Hazardous substance	CAS No.	Parameter	CAS No.
Geraniol ((2*E*)-3,7-dimethyl-octa-2,6-dien-1-ol)	106-24-1	8-Carboxygeraniol ((2*E*,6*E*)-8-hydroxy-2,6-dimethylocta-2,6-dienoic acid)	26187-80-4
		Hildebrandt acid ((2*E*,6*E*)-2,6-dimethylocta-2,6-dienedioic acid)	123175-88-2
		Geranic acid ((2*E*)-3,7-dimethylocta-2,6-dienoic acid)	4698-08-2
		3-Hydroxycitronellic acid (3-hydroxy-3,7-dimethyl-6-octenoic acid)	87877-75-6
Citral ((2*E*)-3,7-dimethyl-octa-2,6-dienal)	5392-40-5	Hildebrandt acid ((2*E*,6*E*)-2,6-dimethylocta-2,6-dienedioic acid)	123175-88-2
		Geranic acid ((2*E*)-3,7-dimethylocta-2,6-dienoic acid)	4698-08-2
		3-Hydroxycitronellic acid (3-hydroxy-3,7-dimethyl-6-octenoic acid)	87877-75-6

Reliability data

8-Carboxygeraniol

Within-day precision:	Standard deviation (rel.)	s_w = 2.1–11.5%
	Prognostic range	u = 4.8–26.5%
	at a concentration of 50 µg or 500 µg 8-carboxygeraniol per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 5.5–22.3%
	Prognostic range	u = 13.5–54.7%
	at a concentration of 50 µg or 500 µg 8-carboxygeraniol per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 97%
	at a nominal concentration of 500 µg 8-carboxygeraniol per litre of urine and n = 10 determinations	
Detection limit:	0.41 µg 8-carboxygeraniol per litre of urine	
Quantitation limit:	1.50 µg 8-carboxygeraniol per litre of urine	

Hildebrandt acid

Within-day precision:	Standard deviation (rel.)	s_w = 2.9–5.5%
	Prognostic range	u = 6.7–12.8%
	at a concentration of 500 µg or 1500 µg Hildebrandt acid per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 6.7–9.8%
	Prognostic range	u = 16.4–24.0%
	at a concentration of 500 µg or 1500 µg Hildebrandt acid per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 106%
	at a nominal concentration of 500 µg Hildebrandt acid per litre of urine and n = 10 determinations	
Detection limit:	0.76 µg Hildebrandt acid per litre of urine	
Quantitation limit:	2.65 µg Hildebrandt acid per litre of urine	

Geranic acid

Within-day precision:	Standard deviation (rel.)	s_w = 3.3–6.2%
	Prognostic range	u = 7.5–14.3%
	at a concentration of 500 µg or 1500 µg geranic acid per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 7.2–15.7%
	Prognostic range	u = 17.8–38.4%
	at a concentration of 500 µg or 1500 µg geranic acid per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 134%
	at a nominal concentration of 500 µg geranic acid per litre of urine and n = 10 determinations	
Detection limit:	0.53 µg geranic acid per litre of urine	
Quantitation limit:	1.80 µg geranic acid per litre of urine	

3-Hydroxycitronellic acid

Within-day precision:	Standard deviation (rel.)	s_w = 2.2–5.8%
	Prognostic range	u = 5.2–13.3%
	at a concentration of 50 µg or 500 µg 3-hydroxycitronellic acid per litre of urine and n = 8 determinations	
Day-to-day precision:	Standard deviation (rel.)	s_w = 6.1–14.6%
	Prognostic range	u = 14.9–35.7%
	at a concentration of 50 µg or 500 µg 3-hydroxycitronellic acid per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	r = 117%
	at a nominal concentration of 500 µg 3-hydroxycitronellic acid per litre of urine and n = 10 determinations	
Detection limit:	0.78 µg 3-hydroxycitronellic acid per litre of urine	
Quantitation limit:	2.66 µg 3-hydroxycitronellic acid per litre of urine	

2 General information on geraniol

Geraniol ((2*E*)-3,7-dimethylocta-2,6-dien-1-ol) is an acyclic monoterpene allyl alcohol (see Figure 1) which is often used as a fragrance in cosmetics, perfumes, hygiene products, and in household cleaning agents. Geraniol is a natural component of essential oils and can be extracted therefrom via distillation. Industrially, geraniol can be produced by hydrogenating citral, among other processes (Lapczynski et al. 2008).

The toxicity of geraniol has been investigated in numerous in vivo and in vitro studies (Lapczynski et al. 2008). Geraniol has a relatively low acute toxicity. After long-term exposure, the dominant effect is the irritation of the skin and the eyes. Due to its skin-sensitising potential in humans, the MAK Commission has designated geraniol with "Sh" (Hartwig 2014).

In an animal experiment using male rats, Chadha and Madyastha (1984) identified the following metabolites after administration of 800 mg geraniol per kg of body weight per day, by gavage: 8-hydroxygeraniol, 8-carboxygeraniol, Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid (Figure 1). However, Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid are only partially suitable for the measurement of geraniol exposure, as these metabolites can also arise from other sources, such as citral, and are therefore not specific (Diliberto et al. 1990) (Figure 2).

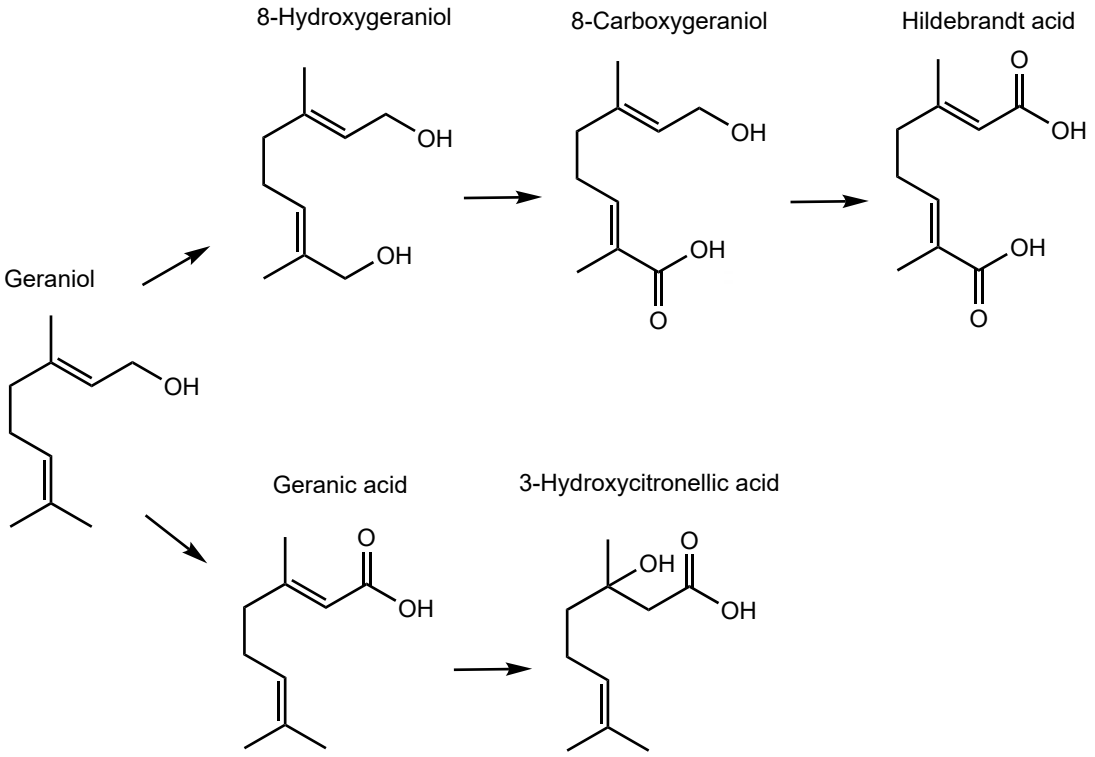

Fig. 1 Metabolism scheme of geraniol according to Chadha and Madyastha (1984)

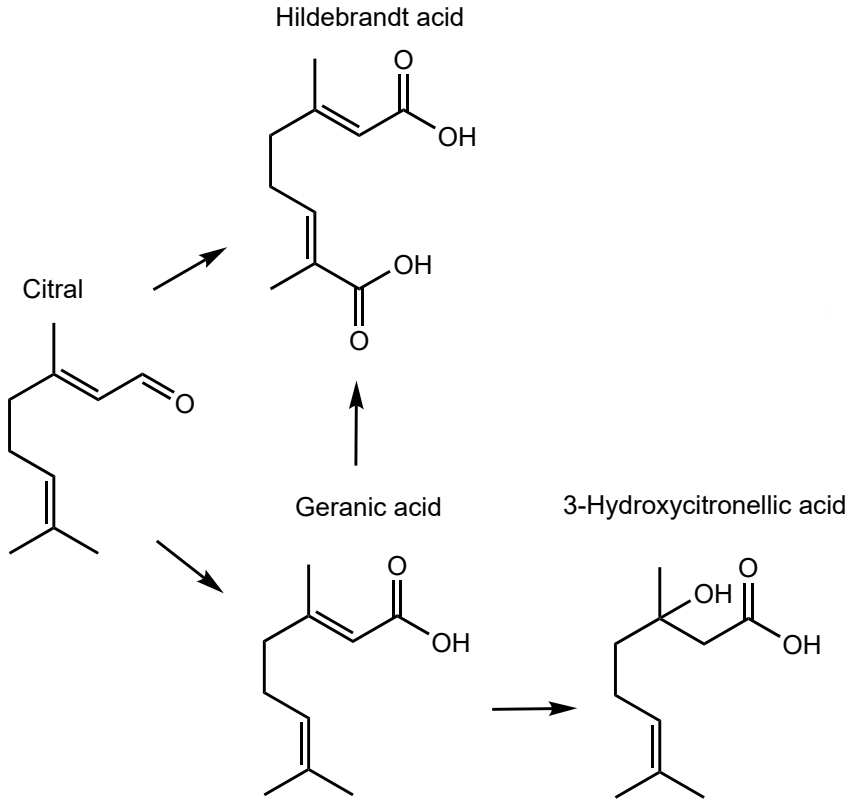

Fig. 2 Simplified metabolism scheme of citral according to Diliberto et al. (1990)

To clarify geraniol metabolism as well as toxicokinetics and metabolic conversion factors in humans, a metabolism study was performed within the framework of the cooperative project for the promotion of human biomonitoring between the *Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit* (Federal Ministry for the Environment, Nature Conservation and Nuclear Safety; BMU) and the *Verband der chemischen Industrie* (German Chemical Industry Association; VCI). For this purpose, after a single oral dosage of 25 mg or 250 mg of geraniol, the urine of three male subjects was continually and completely collected for 72 hours and subsequently analysed using the method described herein. The metabolism study showed that geraniol is predominantly excreted in the form of metabolites which are not strictly substance-specific, namely Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid. The concentrations of these metabolites reached their elimination maxima at 1.5–4 hours after exposure. The metabolites were subsequently eliminated with half-lives of 2–3 hours. The metabolite 8-hydroxygeraniol, which was described in rats, could not be detected in the urine of the human subjects after oral administration of geraniol (Jäger et al. 2016).

In the urine samples of 41 individuals with no known occupational exposure to geraniol, the following metabolites were detected: 8-carboxygeraniol, Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid. The concentrations thus ascertained are presented in Table 1. Although 8-carboxygeraniol accounts for only about 2% compared with the other metabolites determined, this metabolite is the most suitable biomarker for geraniol exposure, as it is the only specific metabolite of geraniol according to current knowledge (Jäger et al. 2020).

Tab. 1 Data on geraniol metabolites in the urine of individuals with no known occupational exposure to geraniol (n = 41) according to Jäger et al. (2020)

Analyte	n > LOQ	Mean ± SD [µg/l]	Range [µg/l]
8-Carboxygeraniol	34 (83%)	10 ± 9	< LOQ–46
Hildebrandt acid	41 (100%)	431 ± 385	37–1966
Geranic acid	41 (100%)	126 ± 118	9–477
3-Hydroxycitronellic acid	33 (81%)	21 ± 18	< LOQ–70

LOQ: limit of quantitation

3 General principles

The method described herein enables the detection of background exposure to geraniol by the selective determination of four geraniol metabolites in urine. The following metabolites can be determined using this method: 8-carboxygeraniol, Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid. Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid can also arise from the metabolism of citral. The buffered urine samples are enzymatically hydrolysed and, after addition of the internal standards (8-carboxygeraniol-d_5, Hildebrandt acid-d_5, and 2,4,6-octatrienoic acid), are processed via liquid-liquid extraction with diethyl ether. The analytes are separated by liquid chromatography and detected by tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out using external calibration in water.

4 Equipment, chemicals, and solutions

4.1 Equipment

- UPLC system (e.g. ACQUITY UPLC H-Class System PLUS, Waters GmbH, Eschborn, Germany)
- Tandem mass spectrometer (e.g. Xevo TQ, Waters GmbH, Eschborn, Germany)
- UPLC column: ACQUITY UPLC HSS T3, 1.8 µm, 2.1 mm × 150 mm (e.g. Waters GmbH, Eschborn, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Water-purification system (e.g. Milli-Q® Direct Water Purification System, Merck KGaA, Darmstadt, Germany)
- Rotary mixer (e.g. Multi Reax, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany)
- 250-ml and 1000-ml glass beakers (e.g. DURAN Group GmbH, Mainz, Germany)
- Separatory funnel (e.g. in-house production)
- 1000-ml laboratory glass bottle with dispenser (e.g. BRAND GmbH + CO KG, Wertheim, Germany)
- Glass centrifuge tubes with screw caps (e.g. DURAN Group GmbH, Mainz, Germany)
- 1.8-ml sample vials with crimp caps or screw caps (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Micro-inserts for sample vials (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- 100-ml amber glass bottle (e.g. BRAND GmbH + CO KG, Wertheim, Germany)
- Piston-stroke pipettes with variable volume setting (1–10 µl, 10–100 µl, and 100–1000 µl) with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)

- Rotation apparatus (e.g. in-house production)
- Rotary evaporator (e.g. BÜCHI Labortechnik GmbH, Essen, Germany)
- Heating block (e.g. Eppendorf AG, Hamburg, Germany)
- Transfer pipettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- Various volumetric flasks (e.g. witeg Labortechnik GmbH, Wertheim, Germany)
- 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.

- Acetic acid 100% (e.g. No. 24101.3000, Bernd Kraft GmbH, Duisburg, Germany)
- Acetonitrile (e.g. No. 11317080, Fisher Scientific GmbH, Schwerte, Germany)
- Diethyl ether (e.g. No. 07106.3700, Bernd Kraft GmbH, Duisburg, Germany)
- Formic acid 98–100% (e.g. No. 05314.2010, Bernd Kraft GmbH, Duisburg, Germany)
- β-Glucuronidase/arylsulfatase (e.g. No. 10127698001, Merck KGaA, Darmstadt, Germany)
- Methanol (e.g. No. 900688, Merck KGaA, Darmstadt, Germany)
- Sodium hydroxide (e.g. No. S5881, Merck KGaA, Darmstadt, Germany)
- Sulphuric acid 96% (e.g. No. 03062.3000, Bernd Kraft GmbH, Duisburg, Germany)
- Ultra-pure water (e.g. Milli-Q® Direct Water Purification System, Merck KGaA, Darmstadt, Germany)

4.3 Reference materials

- 8-Carboxygeraniol ((2*E*,6*E*)-8-hydroxy-2,6-dimethylocta-2,6-dienoic acid) (e.g. ChiroBlock GmbH, Wolfen, Germany)
- 8-Carboxygeraniol-d_5 ((2*E*,6*E*)-8-hydroxy-2-methyl-6-trideuteromethyl-5,5-dideuteroocta-2,6-dienoic acid) (e.g. ChiroBlock GmbH, Wolfen, Germany)
- Geranic acid ((2*E*)-3,7-dimethylocta-2,6-dienoic acid) (e.g. ChiroBlock GmbH, Wolfen, Germany)
- Hildebrandt acid ((2*E*,6*E*)-dimethylocta-2,6-dienedioic acid) (e.g. ChiroBlock GmbH, Wolfen, Germany)
- Hildebrandt acid-d_5 ((2*E*,6*E*)-6-trideuteromethyl-2-methylocta-5,5-dideutero-2,6-dienedioic acid) (e.g. ChiroBlock GmbH, Wolfen, Germany)
- 3-Hydroxycitronellic acid (3-hydroxy-3,7-dimethyl-oct-6-enoic acid) (e.g. ChiroBlock GmbH, Wolfen, Germany)
- 2,4,6-Octatrienoic acid (e.g. No. S439819, Merck KGaA, Darmstadt, Germany)

4.4 Solutions

- UPLC-Eluent A (Formic acid, 0.033%)

  2 ml of concentrated formic acid are filled up to 6 l with ultra-pure water (pH = 2.9).

- Sulphuric acid (1 mol/l)

  700 ml of ultra-pure water are filled into a 1000-ml volumetric flask, 98 g of sulphuric acid are added, and finally, the flask is filled to the mark with ultra-pure water. The solution is transferred into a 1000-ml laboratory glass bottle with dispenser.

- Sodium hydroxide solution (5 mol/l)

  20 g of sodium hydroxide are weighed and dissolved in 100 ml of ultra-pure water under ice cooling in a 250-ml glass beaker. The sodium hydroxide solution is transferred into a 100-ml amber glass bottle.

- Sodium acetate buffer (pH = 5.0)

  800 ml of ultra-pure water are given into a 1000-ml glass beaker and 1 ml of glacial acetic acid is added by pipetting; the solution is subsequently adjusted to a pH value of 5.0 using the sodium hydroxide solution (5 mol/l). The buffer is transferred into a 1000-ml laboratory glass bottle.

When stored at room temperature, the solutions are stable for at least twelve months.

4.5 Internal standards (ISTDs)

- ISTD stock solutions (1000 mg/l)

  Approximately 10 mg each of 8-carboxygeraniol-d_5, Hildebrandt acid-d_5, and 2,4,6-octatrienoic acid are weighed exactly into 10-ml volumetric flasks and dissolved in 5 ml of methanol. The volumetric flasks are then filled up to the mark with methanol.

- ISTD working solutions (100 mg/l)

  1 ml of each of the ISTD stock solutions are pipetted into a separate 10-ml volumetric flask, which is then filled up to the mark with methanol.

- ISTD spiking solution (1 or 2 mg/l)

  100 µl each of the ISTD working solutions for 8-carboxygeraniol-d_5 and Hildebrandt acid-d_5 and 200 µl of the ISTD working solution for 2,4,6-octatrienoic acid are pipetted into a 10-ml volumetric flask, which is then filled to the mark with ultra-pure water.

When stored in the refrigerator at 4 °C, the solutions of the internal standards are stable for at least twelve months.

4.6 Calibration standards

- Stock solutions (1000 mg/l)

  Approximately 10 mg each of 8-carboxygeraniol, Hildebrandt acid, geranic acid, or 3-hydroxycitronellic acid are weighed exactly into 10-ml volumetric flasks and dissolved in 5 ml of methanol. The volumetric flasks are then filled up to the mark with methanol.

- Spiking solution I (100 mg/l)

  1 ml of each stock solution is pipetted into a 10-ml volumetric flask, which is then filled up to the mark with ultra-pure water.

- Spiking solution II (10 mg/l)

  1 ml of spiking solution I is pipetted into a 10-ml volumetric flask, which is then filled up to the mark with ultra-pure water.

- Spiking solution III (1 mg/l)

  1 ml of spiking solution II is pipetted into a 10-ml volumetric flask, which is then filled up to the mark with ultra-pure water.

- Spiking solution IV (0,1 mg/l)

  1 ml of spiking solution III is pipetted into a 10-ml volumetric flask, which is then filled up to the mark with ultra-pure water.

When stored in the refrigerator at 4 °C, the stock and spiking solutions of the analytes are stable for at least twelve months.

To prepare the calibration standards, spiking solutions I to IV are mixed with ultra-pure water to a final volume of 1 ml according to the pipetting scheme given in Table 2. The calibration standards are processed analogously to the samples to be analysed as described in Section 5.2.

Tab. 2 Pipetting scheme for the preparation of calibration standards for the determination of geraniol metabolites in urine

Calibration standard	Spiking solution	Volume of spiking solution [µl]	Volume of ultra-pure water [µl]	Analyte concentration [µg/l]
0	–	0	1000	0
1	IV	10	990	1
2	IV	20	980	2
3	IV	50	950	5
4	III	10	990	10
5	III	20	980	20
6	III	50	950	50
7	II	10	990	100
8	II	20	980	200
9	II	50	950	500
10	I	10	990	1000
11	I	20	980	2000

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in sealable polypropylene containers and stored at 4 °C until sample preparation. For longer term storage (> three days), the urine samples should be frozen at –20 °C.

5.2 Sample preparation

The urine sample is brought to room temperature and thoroughly mixed. 1 ml of the sample is pipetted into a screw-top glass vial and diluted with 1 ml of the sodium acetate buffer (pH = 5.0). After adding 10 µl of the β-glucuronidase/arylsulfatase, the solution is incubated for three hours at 40 °C in a heating block. The sample is mixed with 100 µl of the ISTD spiking solution and, following the addition of 8 ml of ultra-pure water, is pipetted into a separatory funnel. After adding 1 ml of sulphuric acid (1 mol/l) and 25 ml of diethyl ether, the sample is extracted for ten minutes using a rotary mixer. The bottom layer (aqueous phase) is drained from the funnel and discarded. The organic phase is transferred into a 100-ml round-bottomed flask and mixed with 200 µl of 0.033% formic acid (Eluent A). The diethyl ether is

removed by evaporation at about 50 °C using a rotary evaporator. The sample is then transferred into a 1.8-ml sample vial with micro-insert, which is then sealed.

6 Operational parameters

Analytical determination is carried out using a UPLC system coupled with a tandem mass spectrometer (UPLC-MS/MS).

6.1 Ultra high-performance liquid chromatography

Separatory column:	ACQUITY UPLC HSS T3, 1.8 µm, 2.1 × 150 mm
Separation principle:	Reversed Phase
Injection volume:	20 µl
Column temperature:	40 °C
Flow rate:	0.3 ml/min
Eluent:	A: 0.033% formic acid B: acetonitrile
Runtime:	50 min

The gradient program is given in Table 3. All other parameters must be optimised in accordance with the specifications of the individual manufacturer.

Tab. 3 Gradient program for the determination of geraniol metabolites in urine

Time [min]	Eluent A [%]	Eluent B [%]
0.0	85	15
25.0	82	18
38.0	25	75
38.1	10	90
45.0	10	90
45.1	85	15
50.0	85	15

6.2 Tandem mass spectrometry

Ionisation mode:	Positive electrospray ionisation (ESI)
Detection mode:	Multiple Reaction Monitoring (MRM)
Capillary:	3.5 kV
Cone:	30 V
Ion-source temperature:	150 °C
Desolvation temperature:	500 °C
Cone gas flow:	50 l/h
Desolvation gas flow:	1000 l/h
Collision gas:	Argon
Collision gas flow:	0.15 ml/h
Parameter-specific settings:	see Table 4

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.

For each analyte, two mass transitions were selected. One transition serves the purpose of quantitation (quantifier) and the other is used for confirmation (qualifier). The selected transitions, along with retention times, are summarised in Table 4.

Tab. 4 Retention times and parameter-specific settings for the determination of geraniol metabolites in urine

Analyte / ISTD	Retention time [min]	Mass transition [*m/z*]	Cone [V]	Collision energy [V]	Dwell time [s]
8-Carboxygeraniol	18.98	167.0 ⟶ 93.0[a)]	16	14	0.028
	18.98	167.0 ⟶ 121.1[b)]	16	12	0.028
8-Carboxygeraniol-d_5	18.59	172.1 ⟶ 97.3[c)]	16	14	0.028
Hildebrandt acid	17.92	181.1 ⟶ 107.0[a)]	16	14	0.028
	17.92	181.1 ⟶ 163.1[b)]	16	6	0.028
Hildebrandt acid-d_5	17.22	186.1 ⟶ 167.8[c)]	22	10	0.028
Geranic acid	35.71	122.9 ⟶ 66.9[a)]	22	14	0.050
	35.71	122.9 ⟶ 80.9[b)]	22	12	0.050
3-Hydroxycitronellic acid	30.92	169.0 ⟶ 69.0[b)]	20	10	0.050
	30.92	169.0 ⟶ 109.1[a)]	20	10	0.050
2,4,6-Octatrienoic acid	31.64	139.0 ⟶ 93.0[c)]	12	18	0.050

[a)] Qualifier
[b)] Quantifier
[c)] ISTD

7 Analytical determination

20 µl of the processed urine sample (see Section 5.2) are injected into the UPLC-MS/MS system. Analytical separation is carried out by reversed-phase chromatography. Identification of the analytes is carried out using their specific mass transitions and retention times. The retention times given in Table 4 are intended only as a general guide. Users must ensure proper separation performance of the column used and the resulting retention behaviour of the analytes.

Figures 3 and 4 show representative chromatograms of a urine sample spiked with the geraniol metabolites. The spiked concentration of 8-carboxygeraniol and 3-hydroxycitronellic acid is 50 µg/l; that of Hildebrandt acid and geranic acid is 500 µg/l.

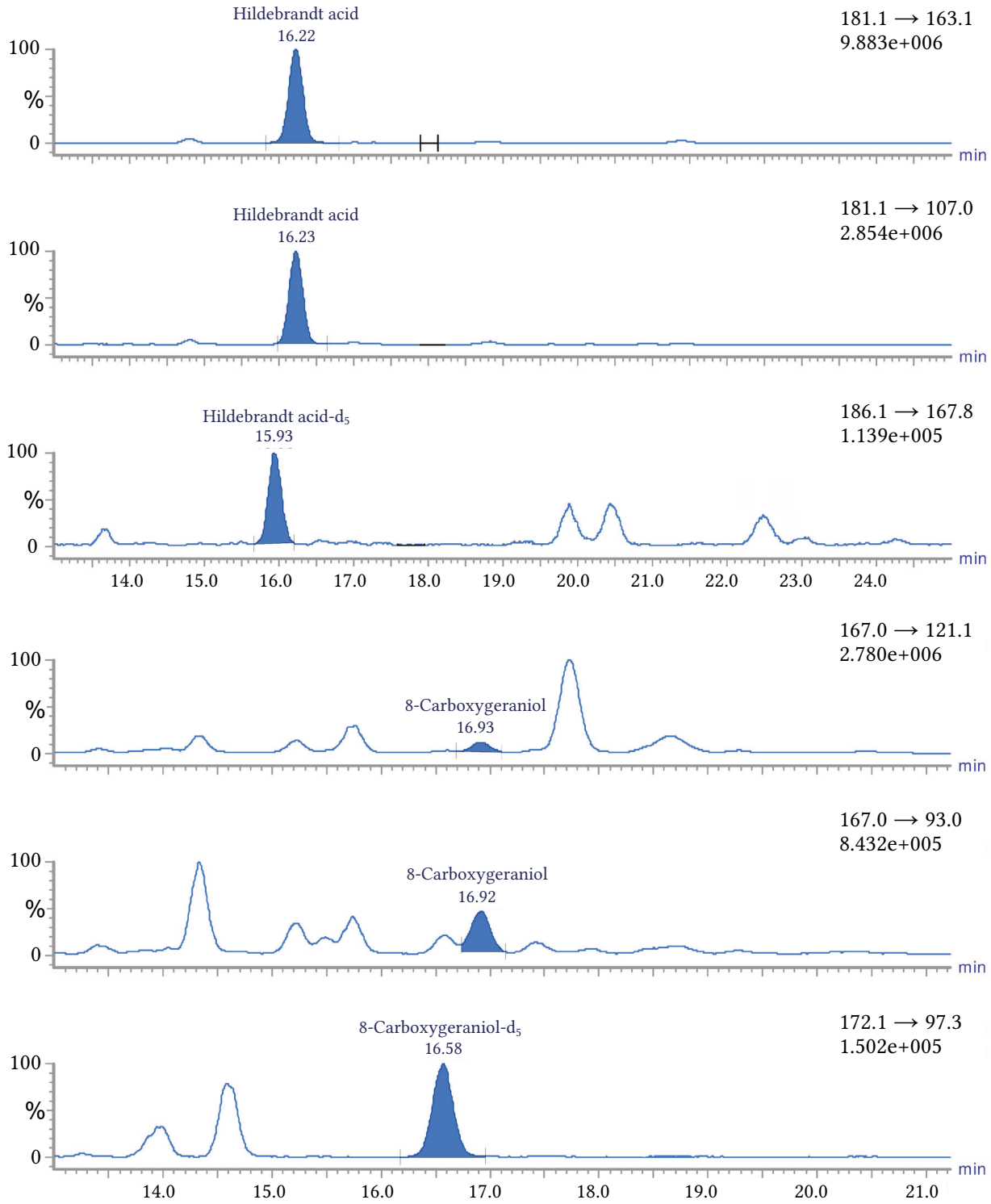

Fig. 3 Chromatograms of a urine spiked with geraniol metabolites (Hildebrandt acid: 500 µg/l, 8-carboxygeraniol: 50 µg/l)

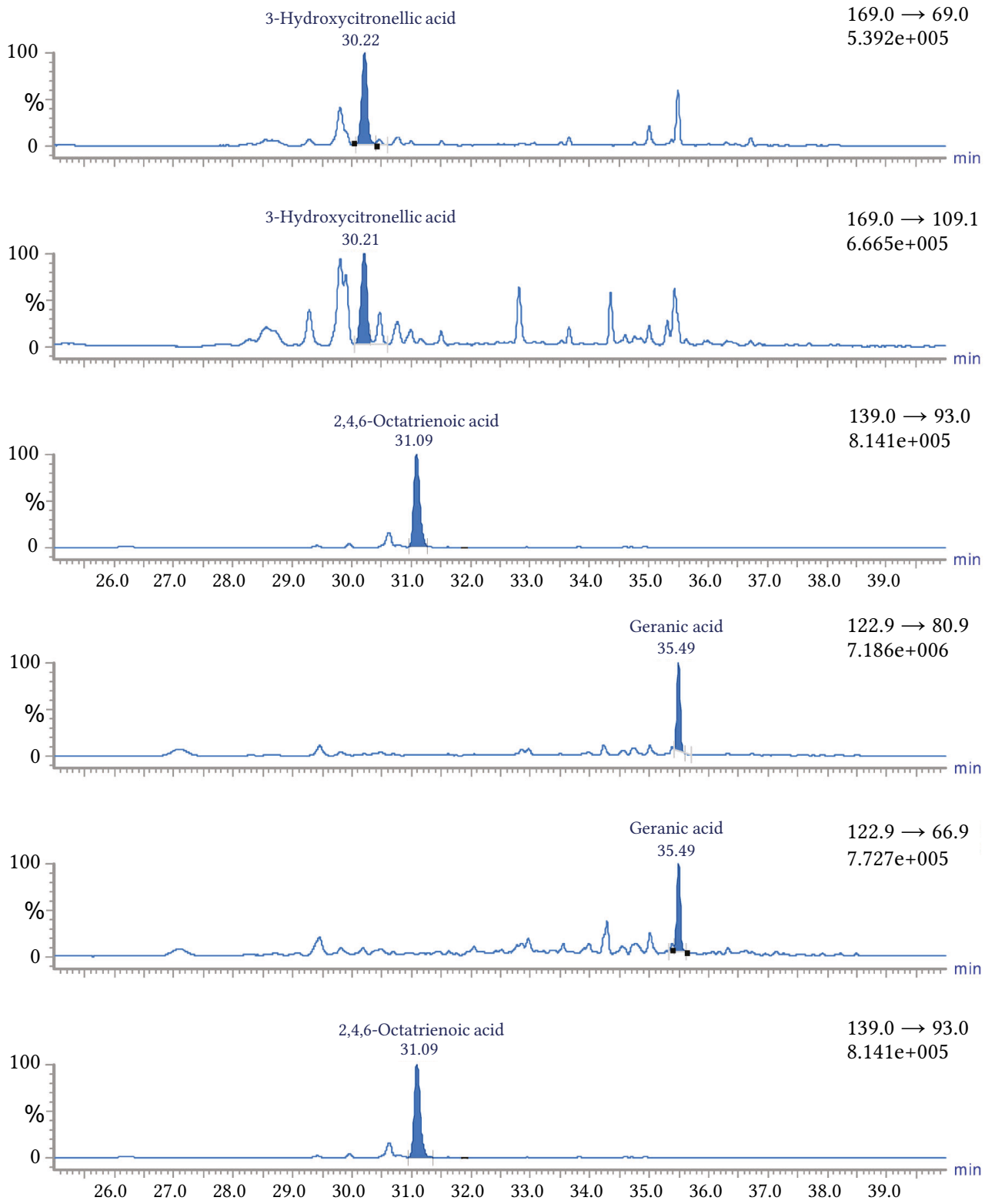

Fig. 4 Chromatograms of a urine spiked with geraniol metabolites (3-hydroxycitronellic acid: 50 µg/l, geranic acid: 500 µg/l)

8 Calibration

The calibration standards (see Section 4.6) are processed analogously to the urine samples (see Section 5.2), and analysed. The calibration curves are constructed by plotting the quotients of the peak area of the individual analyte and of the relevant ISTD against the spiked concentrations of the analytes. With regard to the analytical instrument used in method development, a quadratic relationship was found in the concentration range between the limit of quantitation and 2000 µg/l. Representative calibration curves of each analyte are shown in Figures 5 and 6.

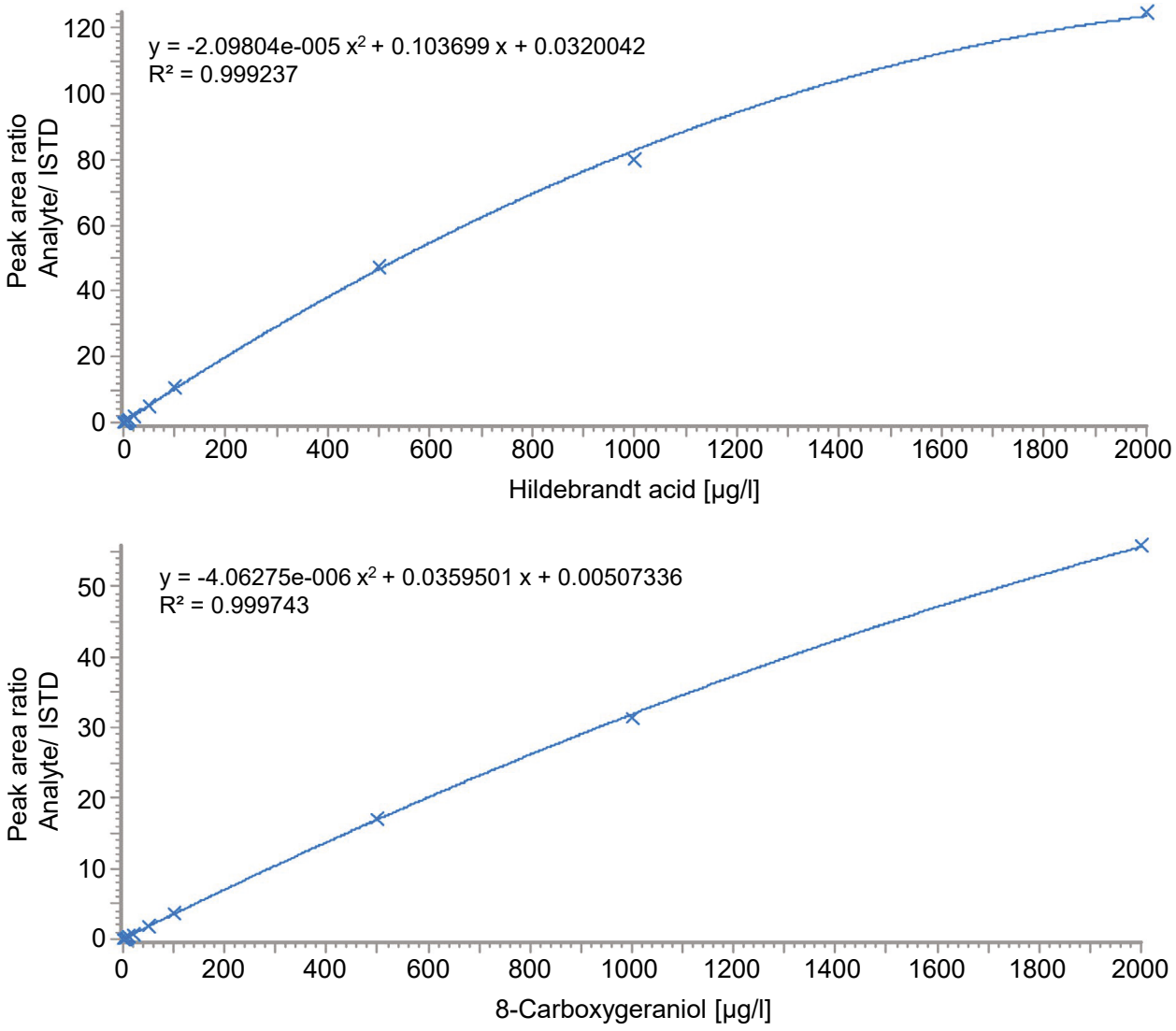

Fig. 5 Calibration curves for the determination of Hildebrandt acid and 8-carboxygeraniol in urine

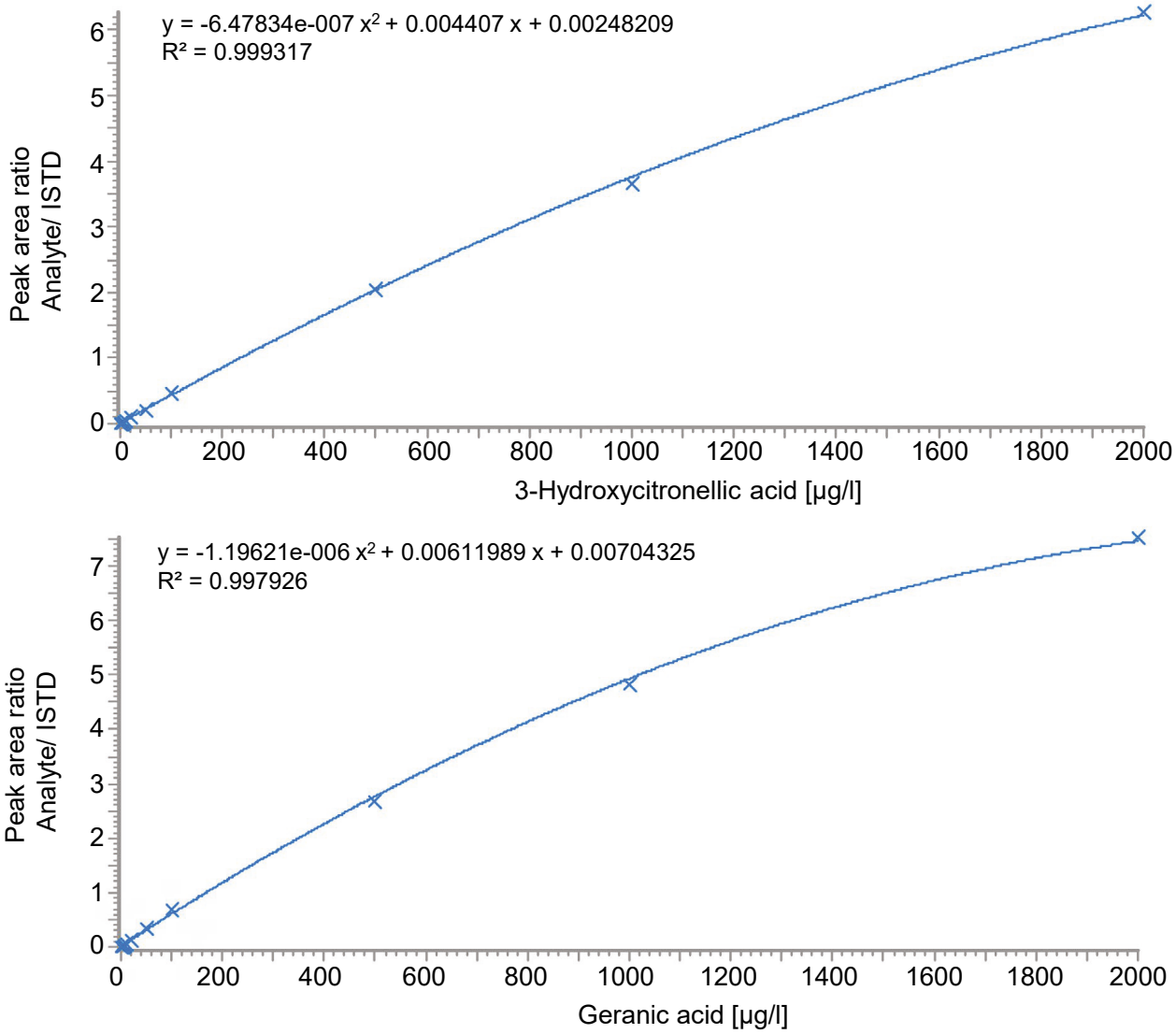

Fig. 6 Calibration curves for the determination of 3-hydroxycitronellic acid and geranic acid in urine

9 Calculation of the analytical results

The analyte concentration in a urine sample is calculated by dividing the peak area of the analyte by the peak area of the relevant ISTD. Using the calibration function corresponding to the analyte and the specific analytical run (see Section 8), analyte concentration in µg/l of urine can be determined from the quotient thus obtained. If the analytical result lies above the calibration range, the relevant sample is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

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

For quality assurance of the analytical results, at least three quality-control samples with different analyte levels are processed and analysed parallel to the samples. As there are currently no control materials commercially available, the material must be individually produced in-house. For this purpose, a pooled urine is used which is spiked with standard solutions of the analytes. Because of the varying analyte concentration levels detected in the urine samples of the general population, the pooled urine should be either unspiked or spiked with middle and high analyte concentration levels (see Table 5). The control materials are aliquoted and frozen until use at –20 °C.

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).

At the same time, at least one reagent blank (ultra-pure water) is included in each analytical run, in order to enable recognition of potential interferences by the reagents. Any reagent blank values which arise must be subtracted from the analytical results. No relevant reagent blank values arose during method development.

Tab. 5 Possible analyte concentration levels of the quality-control material for the determination of geraniol metabolites in urine

Quality-control material	8-Carboxygeraniol [µg/l]	Hildebrandt acid [µg/l]	Geranic acid [µg/l]	3-Hydroxycitronellic acid [µg/l]
Q_{low}	Urine blank	Urine blank	Urine blank	Urine blank
Q_{mid}	50	500	500	50
Q_{high}	500	1500	1500	500

11 Evaluation of the method

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

11.1 Precision

Within-day precision

In order to ascertain within-day precision, the quality-control materials (Q_{low}, Q_{mid}, Q_{high}) were processed and analysed eight times on the same day. The obtained within-day precision data is summarised in Table 6.

Tab. 6 Within-day precision for the determination of geraniol metabolites in urine (n = 8)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
8-Carboxygeraniol	0 (urine blank)	11.5	26.5
	50	7.8	18.0
	500	2.1	4.8
Hildebrandt acid	0 (urine blank)	4.9	11.4
	500	2.9	6.7
	1500	5.5	12.8

Tab. 6 *(continued)*

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
	0 (urine blank)	6.2	14.3
Geranic acid	500	4.3	10.0
	1500	3.3	7.5
	0 (urine blank)	5.8	13.3
3-Hydroxycitronellic acid	50	4.7	10.9
	500	2.2	5.2

Day-to-day precision

Day-to-day precision was investigated by processing and analysing the quality-control materials on six different days. The data thus obtained is presented in Table 7.

Tab. 7 Day-to-day precision for the determination of geraniol metabolites in urine (n = 6)

Analyte	Spiked concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
	0 (urine blank)	22.3	54.7
8-Carboxygeraniol	50	10.9	26.8
	500	5.5	13.5
	0 (urine blank)	9.4	23.1
Hildebrandt acid	500	9.8	24.0
	1500	6.7	16.4
	0 (urine blank)	15.7	38.4
Geranic acid	500	12.9	31.5
	1500	7.2	17.8
	0 (urine blank)	14.6	35.7
3-Hydroxycitronellic acid	50	9.0	22.1
	500	6.1	14.9

11.2 Accuracy

In order to evaluate the matrix effects of the method, urine samples (creatinine content in the range of 0.3–3.1 g/l) from ten different persons were each spiked with 500 µg/l of each analyte, subsequently processed, and measured. In addition, the unspiked urine samples were also processed and analysed. The relative recovery rates thus obtained are presented in Table 8.

Tab. 8 Mean relative recovery for the determination of geraniol metabolites in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Mean rel. recovery r [%]	Range [%]
8-Carboxygeraniol	500	97	79–111
Hildebrandt acid	500	106	77–124
Geranic acid	500	134	126–162
3-Hydroxycitronellic acid	500	117	99–137

11.3 Limits of detection and quantitation

The limits of detection and quantitation were calculated according to DIN 32645 (DIN 2008). To this end, an equidistant 10-point calibration (concentration range from 1–10 µg/l in water) was prepared, processed, and analysed in parallel, including an additional blank value (n = 3). The limits of detection and quantitation were calculated from the standard deviation of the derived calibration function. Table 9 shows the obtained detection and quantitation limits for all analytes.

Tab. 9 Limits of detection and quantitation for the determination of geraniol metabolites in urine (n = 3)

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
8-Carboxygeraniol	0.41	1.50
Hildebrandt acid	0.76	2.65
Geranic acid	0.53	1.80
3-Hydroxycitronellic acid	0.78	2.66

11.4 Sources of error

For certain mass transitions used as quantifiers and qualifiers, interfering peaks arose in the chromatograms. For this reason, it is important during method establishment to ensure a clean separation of the analytes from any matrix peaks which may be present. Furthermore, the investigation of matrix effects, especially for geranic acid and partially for 3-hydroxycitronellic acid, yielded an increased relative recovery rate (> 100%), which can presumably be attributed to the lack of structurally identical deuterated ISTDs. The use of 2,4,6-octatrienoic acid as a structurally similar ISTD can only partially compensate for the observed matrix effects. At this time, deuterated ISTDs for geranic acid and 3-hydroxycitronellic acid are not commercially available.

The quantitative determination of the geraniol metabolites is not susceptible to contamination. It is, of course, advisable to include reagent blanks in every analytical run in order to recognise potential contaminants.

Within the course of external method verification, a C18-phase with the same column dimensions was used as a UPLC column (Acquity UPLC BEH C18 1.7 µm; 2.1 × 150 mm (Waters GmbH, Eschborn, Germany)). With the column used in method verification, interfering peaks arose in some MRM transitions, especially for 2,4,6-octatrienoic acid. For this reason, ISTD concentrations were doubled as compared to the original method in order to clearly and reproducibly differentiate ISTD peaks from interference peaks.

Especially for the later eluting analytes, namely geranic acid and 3-hydroxycitronellic acid, the interfering peaks negatively affected method performance and made a consistent integration of the analyte peaks difficult especially at lower concentrations. For this reason, it is recommended that this method is carried out exclusively with the stationary phase indicated in the original method.

12 Discussion of the method

The biomonitoring procedure presented herein allows for the measurement of geraniol exposure of occupationally exposed persons as well as the general population. The method was developed within the framework of the BMU/VCI cooperative project and has already been published internationally (Jäger et al. 2020).

The comprehensively validated UPLC-MS/MS method allows for the sensitive determination of the four main metabolites of geraniol in urine, namely 8-carboxygeraniol, Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid. Hildebrandt acid, geranic acid, and 3-hydroxycitronellic acid can also arise from the metabolism of citral. The method is selective and displays good precision. The relative and absolute recovery rates ascertained within the course of

method development are excellent. The quantitation limits of 1.50 to 2.66 µg/l are sufficient to quantify background exposure in the general population.

The limits of detection and quantitation ascertained by the external verifier of the method lied above the values described in the original method by a factor of about 2–3. This was probably caused by the fact that a different procedure as well as a different matrix were used for the determination of the limits of detection and quantitation (external verification: pooled urine; method development: water).

Sample preparation can also be adjusted to account for lower sample volumes (down-scaling). For this process, 1 ml of urine is pipetted into a 4-ml glass vial and diluted with 0.5 ml of sodium acetate buffer (pH = 5.0). After adding 10 µl of the β-glucuronidase/arylsulfatase, the solution is incubated at 37 °C for three hours. The sample is then spiked with 20 µl of the ISTD spiking solution. After adding 50 µl of phosphoric acid (4 mol/l), the sample is then mixed with 2 ml of diethyl ether, shaken briefly, and centrifuged for ten minutes at 3500 rpm. The upper organic phase is transferred into a 4-ml glass vial, and the solvent is evaporated. The residue is then reconstituted in 100 µl of 0.033% formic acid (Eluent A), subsequently transferred into a sample vial with micro-insert, and sealed.

Instruments used ACQUITY UPLC H-Class System PLUS and tandem mass spectrometer XEVO TQ (both from 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.

References

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 (eds) The MAK-Collection for Occupational Health and Safety. Part IV: Biomonitoring Methods, vol 12. Wiley-VCH, Weinheim, 55–101. Also available from DOI: https://doi.org/10.1002/3527600418.bireliabe0012

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

Chadha A, Madyastha KM (1984) Metabolism of geraniol and linalool in the rat and effects on liver and lung microsomal enzymes. Xenobiotica 14(5): 365–374. DOI: https://doi.org/10.3109/00498258409151425

Diliberto JJ, Srinivas P, Overstreet D, Usha G, Burka LT, Birnbaum LS (1990) Metabolism of citral, an alpha,beta-unsaturated aldehyde, in male F344 rats. Drug Metab Dispos 18(6): 866–875

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

Hartwig A (ed) (2014) Geraniol. MAK Value Documentation, 2004. In: The MAK-Collection for Occupational Health and Safety. Part I: MAK Value Documentations. Wiley-VCH, Weinheim. Also available from DOI: https://doi.org/10.1002/3527600418.mb10624e3913

Jäger T, Bäcker S, Leibold E, Koch HM, Bader M (2016) Human biomonitoring of the fragrant compound geraniol – the challenge of exposure assessment of natural products and nature-identical chemicals. In: International Society of Exposure Science (ed) Abstract book. Congress of the International Society of Exposure Science (ISES). ISES, Utrecht, 711. https://intlexposurescience.org/wp-content/uploads/2019/02/ISES-2016-abstractbook.pdf, accessed 15 Jul 2021

Jäger T, Bäcker S, Brodbeck T, Leibold E, Bader M (2020) Quantitative determination of urinary metabolites of geraniol by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Anal Methods 12(47): 5718–5728. DOI: https://doi.org/10.1039/D0AY01582B

Lapczynski A, Bhatia SP, Foxenberg RJ, Letizia CS, Api AM (2008) Fragrance material review on geraniol. Food Chem Toxicol 46(Suppl 11): 160–170. DOI: https://doi.org/10.1016/j.fct.2008.06.048