

7-Hydroxycitronellal – Determination of 7-hydroxycitronellylic acid in urine by UPLC-MS/MS

Biomonitoring Method – Translation of the German version from 2021

Keywords

7-hydroxycitronellal;
7-hydroxycitronellylic acid;
fragrance; 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 7-hydroxycitronellal metabolite 7-hydroxycitronellylic acid is determined in urine after enzymatic cleavage. After adding the deuterated internal standard (ISTD) (D₆-7-hydroxycitronellylic acid) to one millilitre of urine, the conjugates are enzymatically cleaved with β -glucuronidase/arylsulfatase. The hydrolysate is then subjected to liquid-liquid extraction with dichloromethane. The extract is evaporated to dryness and reconstituted in the eluent, a mixture of ultra-pure water and methanol (1 : 1, (v/v)). The analytes are separated by UPLC and then analysed by tandem mass-spectrometry using negative electro-spray ionisation (ESI⁻).

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1 Characteristics of the method

Matrix	Urine
Analytical principle	UPLC-MS/MS

Parameter and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
7-Hydroxycitronellal (7-hydroxy-3,7-dimethyloctanal)	107-75-5	7-Hydroxycitronellylic acid (7-hydroxy-3,7-dimethyloctanoic acid)	56046-15-2

Reliability data

7-Hydroxycitronellylic acid

Within-day precision:	Standard deviation (rel.)	$s_w = 4.5\%, 3.5\%, \text{ or } 2.8\%$
	Prognostic range	$u = 12.5\%, 9.72\%, \text{ or } 7.77\%$
	at a concentration of 6.3 µg, 73.8 µg, or 515 µg 7-hydroxycitronellylic acid per litre of urine and n = 5 determinations	
Day-to-day precision:	Standard deviation (rel.)	$s_w = 9.6\%, 3.8\%, \text{ or } 3.7\%$
	Prognostic range	$u = 24.7\%, 9.77\%, \text{ or } 9.51\%$
	at a concentration of 5.9 µg, 71.8 µg, or 504 µg 7-hydroxycitronellylic acid per litre of urine and n = 6 determinations	
Accuracy:	Recovery rate (rel.)	$r = 97.8\%, 106\%, \text{ or } 103\%$
	at a nominal concentration of 1.0 µg, 5.0 µg, or 500 µg 7-hydroxycitronellylic acid per litre of urine and n = 5 determinations	
Detection limit:	0.17 µg 7-hydroxycitronellylic acid per litre of urine	
Quantitation limit:	0.5 µg 7-hydroxycitronellylic acid per litre of urine	

2 General information on 7-hydroxycitronellal

7-Hydroxycitronellal (for chemical structure, see [Figure 1](#)) is a synthetic fragrance with a lilac odour which is widely used in cosmetics, polishes, waxes, biocides, and detergents as well as other cleaning products (ECHA 2020). Each year, between 100 and 1,000 tons of 7-hydroxycitronellal are either produced in Europe or imported into the European Economic Area (ECHA 2020). Due to its widespread use, both consumers and workers are exposed to 7-hydroxycitronellal.

Frequent or regular contact with 7-hydroxycitronellal causes skin sensitisation, leading the Commission to designate this substance with “Sh” (Hartwig 2013). Threshold values in workplace air or in biological materials have not been derived by the Commission. The Scientific Committee on Consumer Safety (SCCS) investigated numerous studies on the sensitising effect of fragrances, where 7-hydroxycitronellal was also found to be a dermal sensitiser (SCCS 2012). As a result, the concentration levels of 7-hydroxycitronellal in consumer products which come into contact with human skin were limited in compliance with the International Fragrance Association (IFRA) Guideline (IFRA 2020). In addition, the use of 7-hydroxycitronellal 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 (European Commission 2019).

In an *in vitro* test, approximately 50% of the substance was absorbed after dermal application of ¹⁴C-7-hydroxycitronellal on rat skin ($T_{max} = 30$ h) (Tonge 1995). In *in vivo* tests on rabbits, the metabolites 7-hydroxycitronellol and 7-hydroxycitronellylic acid were detected in urine after single-dose oral application (Ishida et al. 1989). Within the BMU/VCI

cooperation (German Ministry for the Environment, Nature Conservation and Nuclear Safety; *Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit* (BMU) / German Chemical Industry Association; *Verband der Chemischen Industrie e. V.* (VCI)) to develop the biomonitoring method described herein, human metabolism of 7-hydroxycitronellal was also studied. The most important metabolites as well as their toxicokinetics were identified in a metabolism study in five volunteers. The metabolism study included a single oral intake as well as a single dermal application of 7-hydroxycitronellal. 7-Hydroxycitronellol and 7-hydroxycitronellylic acid could be detected as metabolites (Stoekelhuber et al. 2017). [Figure 1](#) shows the postulated metabolism of 7-hydroxycitronellal.

While only traces of 7-hydroxycitronellol were detected after the required derivatisation, 7-hydroxycitronellylic acid seems to be the major metabolite and could be found at high concentrations. Unmetabolised 7-hydroxycitronellal was not detected. The method described herein is therefore based on the quantitation of 7-hydroxycitronellylic acid as a biomarker of exposure to 7-hydroxycitronellal.

The developers of this method analysed 40 spontaneous urine samples collected from subjects not occupationally exposed to 7-hydroxycitronellal (23 men and 17 women; age: 18–83 years) for 7-hydroxycitronellylic acid. The mean 7-hydroxycitronellylic acid concentration was 33.6 µg/l with a median of 14.2 µg/l (range: 3.1–627 µg/l).

Pluym et al. (2020) analysed 329 urine samples (24-hour urine collection) from young adults (20–29 years) for 7-hydroxycitronellylic acid. The samples were taken from the *Umweltprobenbank* (Environmental Specimen Bank), and had been collected between 2000 and 2018. The mean 7-hydroxycitronellylic acid concentration in these samples was 14.9 µg/l with a median of 8.1 µg/l. Data on workplace exposure to 7-hydroxycitronellal were not available.

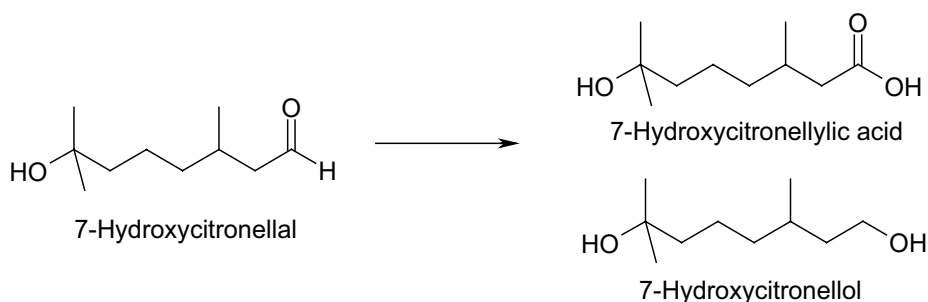


Fig. 1 Postulated metabolism of 7-hydroxycitronellal

3 General principles

The 7-hydroxycitronellal metabolite 7-hydroxycitronellylic acid is determined in urine following enzymatic cleavage. After adding the deuterated internal standard (ISTD) (D_6 -7-hydroxycitronellylic acid) (see [Figure 2](#)) to one millilitre of urine, the conjugates are enzymatically cleaved with β -glucuronidase/arylsulfatase. The hydrolysate is then subjected to liquid-liquid extraction with dichloromethane. The extract is evaporated to dryness and reconstituted in the eluent, a mixture of ultra-pure water and methanol (1 : 1, (v/v)). The analytes are separated by ultra-performance liquid chromatography (UPLC) and then analysed by tandem mass-spectrometry using negative electrospray ionisation (ESI⁻).

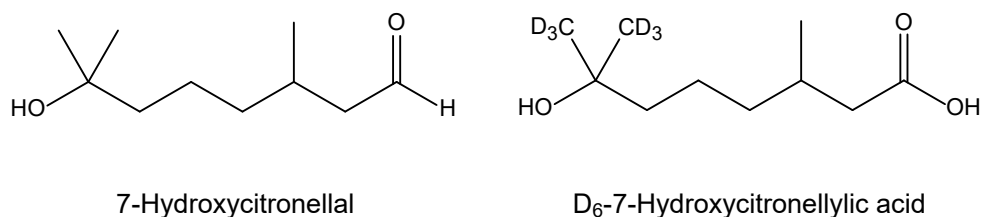


Fig. 2 Structures of 7-hydroxycitronellal and of D_6 -7-hydroxycitronellylic acid

4 Equipment, chemicals, and solutions

4.1 Equipment

- UPLC system (e.g. Acquity UPLC I-Class System, Waters GmbH, Eschborn, Germany) consisting of a sample manager (SM-FTN), I-Class binary solvent manager, column manager, and sample organiser connected to a Xevo TQ-S tandem-quadrupole mass-spectrometer (e.g. Waters GmbH, Eschborn, Germany)
- HPLC column (e.g. Acquity UPLC BEH C8 1.7 µm; 2.1 × 150 mm, Waters GmbH, Eschborn, Germany)
- Incubator with shaker (e.g. Incucell 111 with GFL 3005 shaker, MMM Medcenter Einrichtungen GmbH, Planegg, Germany)
- Vacuum centrifuge (e.g. Jouan GmbH, Unterhaching, Germany)
- Centrifuge (e.g. Rotixa KS, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Nitrogen generator (e.g. cmc Instruments GmbH, Eschborn, Germany)
- Precision balance (e.g. Sartorius AG, Göttingen, Germany)
- Tube roller (e.g. Stuart SRT 6, Cole Parmer Ltd, Stone, United Kingdom)
- Multi-tube vortex mixer (e.g. VWR International GmbH, Darmstadt, Germany)
- pH meter with pH electrode (e.g. CG 842, SCHOTT AG, Mainz, Germany)
- Multipipette® (e.g. Eppendorf AG, Hamburg, Germany)
- Pipettes (1–20 µl, 10–200 µl, 100–1000 µl, and 500–5000 µl) (e.g. Eppendorf AG, Hamburg, Germany)
- Volumetric pipette made of glass (e.g. BRAND GMBH & CO KG, Wertheim, Germany)
- Various volumetric flasks (e.g. SCHOTT AG, Mainz, Germany)
- Various measuring cylinders (e.g. SCHOTT AG, Mainz, Germany)
- 4-ml glass vials (e.g. No. 130400, BGB Analytik Vertrieb GmbH, Rheinfelden, Germany) with screw-caps (e.g. No. 2.301158, Klaus Ziemer GmbH, Langerwehe, Germany)
- 1.8-ml sample vials with micro-inserts (e.g. Klaus Ziemer GmbH, Langerwehe, Germany)
- Urine-collection containers (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)

4.2 Chemicals

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

Reference materials

- 7-Hydroxycitronellylic acid (custom synthesis, Dr. Belov, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)
- D₆-7-Hydroxycitronellylic acid (custom synthesis, Dr. Belov, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany)

Other chemicals

- Acetic acid, $\geq 99.8\%$ (e.g. No. 15633920, Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany)
- Acetonitrile, dry, 99.8% (e.g. No. 271004, Merck KGaA, Darmstadt, Germany)
- Dichloromethane (e.g. No. 15633730, Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany)
- β -Glucuronidase/arylsulfatase (from *Helix pomatia*) (30 U/ml; 60 U/ml) (e.g. No. 1041140002, Merck KGaA, Darmstadt, Germany)
- Methanol (e.g. No. 136878, Biosolve BV, Valkenswaard, Netherlands)
- *ortho*-Phosphoric acid, 85% (e.g. No. 5.43828, Merck KGaA, Darmstadt, Germany)
- Sodium hydroxide, pellets (e.g. No. 106469, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. No. 15634670, Honeywell Specialty Chemicals Seelze GmbH, Seelze, Germany)

Eluents

- Eluent A: Acetonitrile + 0.1% formic acid (e.g. No. 019378, Biosolve BV, Valkenswaard, Netherlands)
- Eluent B: Ultra-pure water + 0.1% formic acid (e.g. No. 232478, Biosolve BV, Valkenswaard, Netherlands)

4.3 Solutions

- Sodium hydroxide solution (5 mol/l)
Place 70 ml of ultra-pure water into a 250-ml beaker and dissolve 20 g of sodium hydroxide pellets while cooling on an ice bath. Transfer the sodium hydroxide solution into a 100-ml volumetric flask and make up to the mark with ultra-pure water. Afterwards, transfer the sodium hydroxide solution into a 100-ml amber glass bottle for storage.
- Sodium acetate buffer (pH = 5.1)
Place approximately 800 ml of ultra-pure water into a 1000-ml beaker and add 57 ml of acetic acid using a pipette. Afterwards, adjust the solution to pH 5.1 with 5 mol/l sodium hydroxide solution and transfer into a 1000-ml volumetric flask. Make up to the mark with ultra-pure water.
- *ortho*-Phosphoric acid (20%)
Place 70 ml of ultra-pure water into a 100-ml volumetric flask and add 23.5 ml of 85% *ortho*-phosphoric acid. Make up to the mark with ultra-pure water.

The solutions are stored in laboratory glass bottles at room temperature. Under these conditions, they are stable for at least six months.

- Enzyme suspension
Pipette 100 μ l of β -glucuronidase/arylsulfatase (30 U/ml; 60 U/ml) and 600 μ l of ultra-pure water into a 2-ml screw-neck vial and mix well. The enzyme suspension is stored in the refrigerator at 4 °C and is stable for at least six months.

4.4 Internal standard (ISTD)

- ISTD stock solution (10 000 mg/l)
Approximately 10 mg of D₆-7-hydroxycitronellylic acid are weighed exactly into a 1-ml volumetric flask and dissolved in acetonitrile. The solution is then made up to the mark with acetonitrile, and thoroughly mixed.
- ISTD working solution (100 mg/l)
100 µl of the ISTD stock solution are pipetted into a 10-ml volumetric flask, made up to the mark with acetonitrile, and thoroughly mixed.
- ISTD spiking solution (1 mg/l)
100 µl of the ISTD working solution are pipetted into a 10-ml volumetric flask, made up to the mark with acetonitrile, and thoroughly mixed.

The ISTD solutions are stored in amber screw-top glass vials in a dark place at –20 °C.

4.5 Calibration standards

- Stock solution (1000 mg/l)
Approximately 10 mg of 7-hydroxycitronellylic acid are weighted exactly into a 10-ml volumetric flask and dissolved in acetonitrile. The solution is then made up to the mark with acetonitrile and thoroughly mixed.
- Spiking solution I (100 mg/l)
500 µl of the stock solution are pipetted into a 5-ml volumetric flask. The solution is then made up to the mark with acetonitrile and thoroughly mixed.
- Spiking solution II (10 mg/l)
50 µl of the stock solution are pipetted into a 5-ml volumetric flask. The solution is then made up to the mark with acetonitrile and thoroughly mixed.
- Spiking solution III (1 mg/l)
500 µl of spiking solution II are pipetted into a 5-ml volumetric flask. The solution is then made up to the mark with acetonitrile and thoroughly mixed.
- Spiking solution IV (0.1 mg/l)
50 µl of spiking solution II are pipetted into a 5-ml volumetric flask. The solution is then made up to the mark with acetonitrile and thoroughly mixed.

The standard solutions are stored in amber screw-top vials at –20 °C in the dark.

The calibration standards are prepared in ultra-pure water, as no urine without background levels is available. The slope of the calibration curve prepared in water does not differ significantly from the slope of a calibration curve prepared in urine.

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 1](#). It is also possible to prepare larger batches of the calibration solutions and aliquot them to 1 ml each. In this case, the calibration solutions are stored in 4-ml screw-neck vials at –20 °C. Under these conditions, they are stable for at least six months. The calibration standards are processed in the same way as the samples to be analysed, as described in [Section 5](#).

Tab. 1 Pipetting scheme for the preparation of calibration standards for the determination of 7-hydroxycitronellylic acid in urine

Calibration standard	Spiking solution	Volume of spiking solution [μ l]	Volume of ultra-pure water [μ l]	Analyte concentration [μ g/l]
0	–	–	1000	0
1	IV	2	998	0.2
2	IV	5	995	0.5
3	IV	10	990	1
4	IV	20	980	2
5	IV	50	950	5
6	III	10	990	10
7	III	40	960	40
8	III	100	900	100
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. The samples can be stored at room temperature for 24 hours without loss of analyte. For longer term storage of the urine samples, freezing at $-20\text{ }^{\circ}\text{C}$ is recommended, even though the long-term stability of the analyte has not been investigated. However, the freeze-thaw stability of the quality-control samples over six freeze-thaw cycles indicates a certain stability of the analyte in samples stored at $-20\text{ }^{\circ}\text{C}$.

5.2 Sample preparation

Prior to analysis, the urine samples are thawed to room temperature and mixed thoroughly.

Pipette 1 ml of each urine into 4-ml glass vials and add 0.5 ml of sodium acetate buffer (pH = 5.1). After adding 10 μ l of the enzyme suspension and 10 μ l of the ISTD spiking solution (1 mg/l), mix the samples thoroughly and thermostate in an incubator with shaker for three hours at $37\text{ }^{\circ}\text{C}$ (approx. 100 rpm).

Add 50 μ l of phosphoric acid (20%) and 1.5 ml of dichloromethane to each sample and mix for 30 minutes on the tube roller. Afterwards, centrifuge the samples for 15 minutes at 3500 rpm, transfer the organic phase into 1.8-ml sample vials, and evaporate to dryness in the vacuum centrifuge. Dissolve the residues in 100 μ l of methanol/ultra-pure water (1 : 1, v/v) each. Transfer the sample solutions into 1.8-ml vials with micro-inserts and apply them for analysis by UPLC-MS/MS.

6 Operational parameters

Analysis is performed using a UPLC system coupled with a tandem mass-spectrometer.

6.1 Ultra-performance liquid chromatography

Analytical column:	C8, 1.7 μm ; 2.1 \times 150 mm
Eluent:	A: acetonitrile + 0.1% formic acid B: water + 0.1% formic acid
Column temperature:	50 $^{\circ}\text{C}$, isothermal
Autosampler:	10 $^{\circ}\text{C}$, cooled
Injection volume:	5 μl
Flow rate (binary pump):	0.5 ml/min
Gradient program:	see Table 2

Tab. 2 Gradient program for the determination of 7-hydroxycitronellylic acid in urine

Time [min]	Eluent A [%]	Eluent B [%]
0	80	20
1	80	20
5	20	80
5.5	20	80
5.51	80	20
7	80	20

All other parameters must be optimised in accordance with the manufacturer's specifications.

6.2 Tandem mass spectrometry

Ionisation mode:	Negative electrospray ionisation (ESI ⁻)
Source temperature:	150 $^{\circ}\text{C}$
Desolvation temperature:	500 $^{\circ}\text{C}$
Cone gas flow:	148 l/h
Desolvation gas flow:	798 l/h
Collision gas flow:	0.14 ml/min
Detection mode:	MRM
Parameter-specific settings:	see Table 3

Tab. 3 Parameter-specific settings for the determination of 7-hydroxycitronellylic acid in urine

Analyte	Retention time [min]	Parent ion [m/z]	Product ion [m/z]	Cone [V]	Collision energy [V]
7-Hydroxycitronellylic acid	2.73	187.03	127.09	24	16
			56.97 ^{a)}		20
D ₆ -7-Hydroxycitronellylic acid (ISTD)	2.73	193.16	62.93	2	18

^{a)} Qualifier

All settings are instrument-specific and must be adjusted individually by the user. The parameters specified above are therefore intended only as cursory guidance.

7 Analytical determination

5 μl each of the samples, prepared as described in Section 5, are injected into the UPLC-MS/MS system. Identification of the analytes is based on the specific ion transitions and retention times (see Table 3). The retention times given in Table 3 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.

As an example, Figure 3 shows a chromatogram of a urine sample spiked with the 7-hydroxycitronellal metabolite 7-hydroxycitronellylic acid.

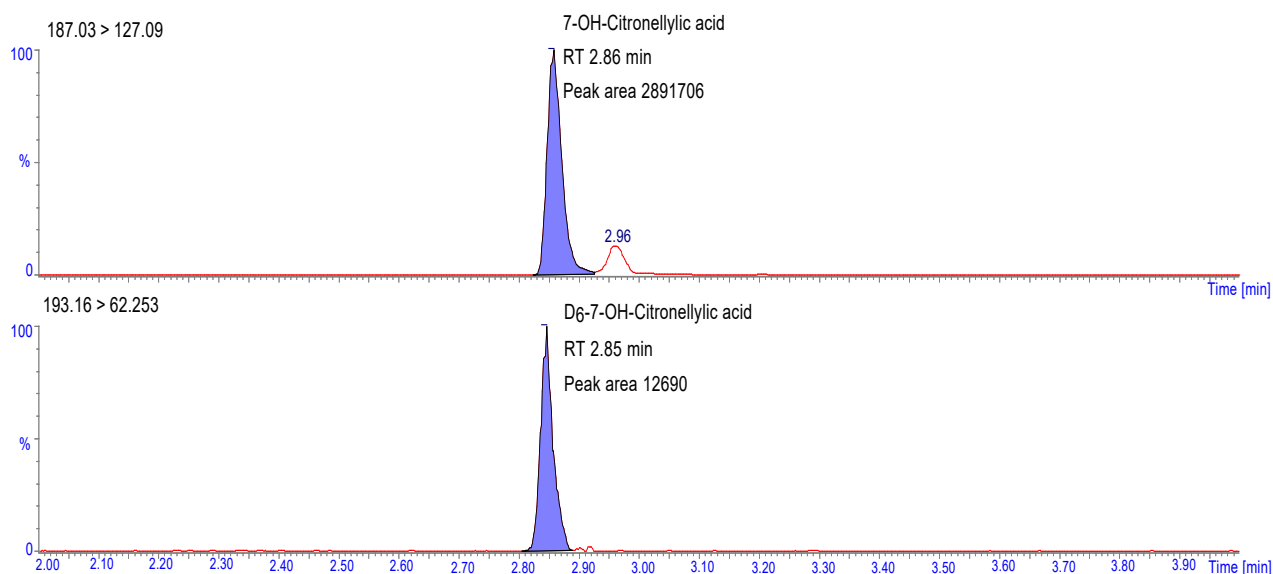


Fig. 3 Chromatogram of a urine sample spiked with 500 μg of 7-hydroxycitronellylic acid per litre

8 Calibration

For method calibration, the calibration standards described in Section 4.5 are prepared and processed in the same way as the samples (cf. Section 5) and analysed by UPLC-MS/MS (cf. Sections 6 and 7). The calibration curve is obtained by plotting the quotient of the peak area of the analyte and the peak area of the isotope-labelled ISTD against the spiked concentration of the respective calibration standard. Under the described analytical conditions, the calibration function is linear in the relevant concentration range from 0.2 to 2000 $\mu\text{g/l}$ with a correlation coefficient of $r \geq 0.999$. Figure 4 shows a representative calibration curve for the calibration standards prepared in water, used to quantify 7-hydroxycitronellylic acid in urine.

Signals are evaluated using the MassLynx V4.1 software (Waters GmbH, Eschborn, Germany).

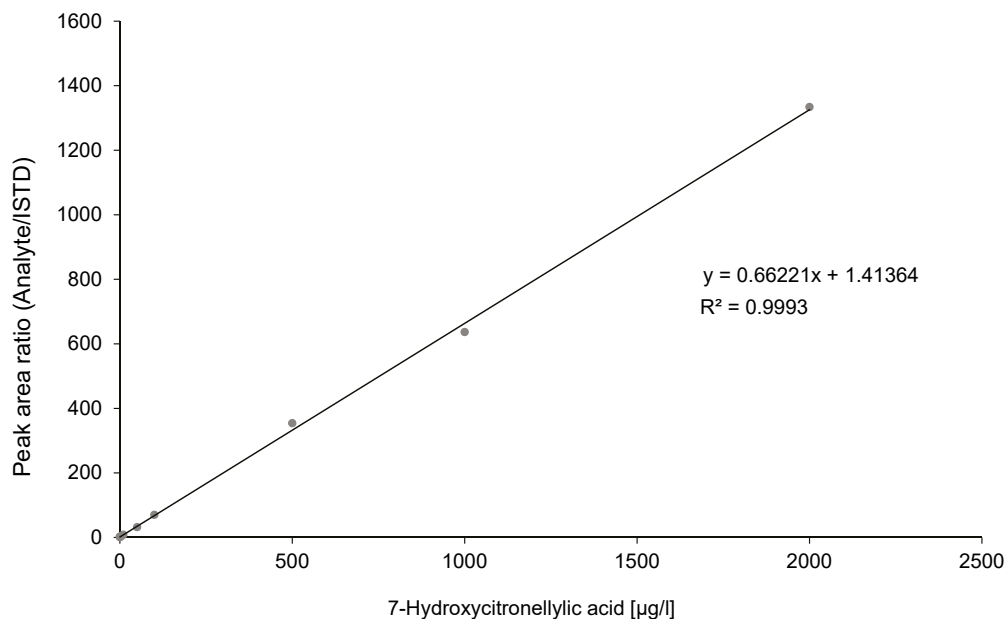


Fig. 4 Calibration curve for the determination of 7-hydroxycitronellylic acid in urine

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 ISTD. Using the calibration function corresponding to the relevant analytical run (cf. Section 8), the 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.

A reagent blank (ultra-pure water) is included in each analytical run. Any reagent blank values are subtracted from the analytical results.

10 Standardisation and quality control

Quality control of the analytical results is carried out as stipulated in the guidelines of the *Bundesärztekammer* (German Medical Association) and in a general chapter of the MAK Collection for Occupational Health and Safety (Bader et al. 2010; Bundesärztekammer 2014).

To check precision, at least three quality-control samples with consistent low, medium, and high analyte concentrations are included in each analytical run. As control material is not commercially available, it must be prepared in the laboratory by spiking pooled non-smoker urine with standard solutions of the analytes. The analyte concentration in the quality-control materials should be within the relevant concentration range (e.g. 1 µg/l, 5 µg/l, and 500 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 the 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 was determined at three different concentration levels. To this end, urine samples spiked with three different concentrations of 7-hydroxycitronellylic acid were processed and analysed five times in parallel. The precision data obtained are presented in [Table 4](#).

Tab. 4 Within-day precision for the determination of 7-hydroxycitronellylic acid in urine (n = 5)

Analyte	Determined concentration [$\mu\text{g/l}$]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
7-Hydroxycitronellylic acid	6.3	4.5	12.5
	73.8	3.5	9.72
	515	2.8	7.77

Day-to-day precision was determined by processing and analysing the same urine samples on six different days within a two-week timeframe. The results obtained are presented in [Table 5](#).

Tab. 5 Day-to-day precision for the determination of 7-hydroxycitronellylic acid in urine (n = 6)

Analyte	Determined concentration [$\mu\text{g/l}$]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
7-Hydroxycitronellylic acid	5.9	9.3	24.7
	71.8	3.8	9.77
	504	3.7	9.51

11.2 Accuracy

The accuracy of the method was determined by analysing urine spiked with 1 μg , 5 μg , or 500 μg 7-hydroxycitronellylic acid per litre. The lowest concentration was below three times the limit of quantitation, and the highest lied in the upper range of the calibration graph. Both the spiked and unspiked urine samples were processed and analysed. The relative recovery rates were calculated based on the determined concentrations in the spiked urine samples, subtracting any background levels in the unspiked samples. Five determinations were performed for each concentration level. The results of these determinations are presented in [Table 6](#).

Tab. 6 Mean relative recovery rates for the determination of 7-hydroxycitronellylic acid in urine (n = 5)

Analyte	Spiked concentration [$\mu\text{g/l}$]	Mean relative recovery r [%]	Range [%]
7-Hydroxycitronellylic acid	1.0	97.8	87.7–124
	5.0	106	102–109
	500	103	98.2–107

11.3 Absolute recovery

Absolute recovery was determined to estimate process-related losses, using urine spiked with 1 μg , 5 μg , and 500 μg 7-hydroxycitronellylic acid per litre, respectively. Recovery was calculated by comparison with a reference sample. The same urine matrix was used as a reference sample, but was only spiked with the analyte after processing. At all three concentration levels, the reference samples were analysed three times and the spiked samples six times. The absolute recovery lied between 76.7 and 95.5% (see [Table 7](#)).

Tab. 7 Absolute recovery rates for the determination of 7-hydroxycitronellylic acid in urine (n = 6)

Analyte	Spiked concentration [$\mu\text{g/l}$]	Mean absolute recovery r [%]	Range [%]
7-Hydroxycitronellylic acid	1.0	95.5	93.3–96.8
	5.0	88.2	86.4–89.3
	500	76.7	73.2–79.9

11.4 Matrix effects

Matrix effects observed during the UPLC-MS/MS analysis were assessed using three individual native urine samples. The urine samples were processed without spiking, and were only spiked with the ISTD and the analyte at a low or a high concentration level before the actual UPLC-MS/MS analysis. The matrix effects were calculated by comparing the peak areas of the analyte with the peak areas of the same quantities of analyte in pure solvent. The results obtained are presented in Table 8. The analyte signal intensities were significantly reduced due to a high matrix effect.

Tab. 8 Matrix effects for the determination of 7-hydroxycitronellylic acid in urine (n = 6)

Analyte	Spiked concentration [$\mu\text{g/l}$]	Mean relative recovery r [%]	Range [%]
7-Hydroxycitronellylic acid	2.0	21.3	20.0–23.7
	40	23.8	21.7–26.9
D ₆ -7-Hydroxycitronellylic acid (ISTD)	10	23.0	20.3–28.5

11.5 Limits of detection and quantitation

The limits of detection and quantitation of the analyte were calculated by determining the signal-to-noise ratio in different matrix samples using the corresponding feature in the MassLynx V4.1 evaluation software. The limit of quantitation was first calculated at a signal-to-noise ratio of 1 : 9 and then verified by determining the precision and accuracy in three different matrix samples spiked at the limit of quantitation. The criteria were a maximum precision of 20% and an accuracy of 80–120%.

The limit of detection was calculated based on the limit of quantitation and corresponds to the concentration with a signal-to-noise ratio of at least 1 : 3.

Tab. 9 Limits of detection and quantitation for the determination of 7-hydroxycitronellylic acid in urine

Analyte	Detection limit [$\mu\text{g/l}$]	Quantitation limit [$\mu\text{g/l}$]
7-Hydroxycitronellylic acid	0.17	0.5

11.6 Sources of error

Stability of the analyte was tested under different storage conditions: short-term stability by storing the samples for 24 hours at room temperature (n = 3); freeze-thaw stability by thawing the samples up to six times for at least six hours each and refreezing at $-20\text{ }^{\circ}\text{C}$ (n = 3); and the stability of already processed and analysed samples by storing the samples in the autosampler at $10\text{ }^{\circ}\text{C}$ for a period of two weeks before renewed analysis (n = 3). The storage tests revealed that the 7-hydroxycitronellal is stable under the examined conditions; the processed and analysed samples did not show any analyte loss.

As a further potential source of disturbing influence, carryover effects in the chromatographic system, such as injection carryover, were investigated. After five injections each of a high-concentration sample, a blank matrix sample was injected and analysed twice. No interfering signals with the retention times of the analyte or the ISTD were observed.

Regarding possible blank values, the sodium acetate buffer was found to be contaminated with 7-hydroxycitronellylic acid. To keep the resulting blank value constant from sample to sample, care must be taken to add the precise volume of buffer. It is also advisable to additionally process a reagent blank consistent with the descriptions in Section 5.

In the course of external method verification, it was observed that the linear working range can be smaller than indicated, depending on the analytical instruments used. In this case, samples with high analyte levels must be diluted, reprocessed, and newly analysed.

12 Discussion of the method

The biomonitoring method presented herein for determining the exposure of the general population to 7-hydroxycitronellal was developed as part of the BMU/VCI cooperation and has already been published internationally (Stoekelhuber et al. 2017).

This comprehensively validated UPLC-MS/MS method allows the sensitive quantitation of 7-hydroxycitronellylic acid as the main metabolite of 7-hydroxycitronellal. The method is selective with high levels of both accuracy and linearity. The relative and absolute recovery rates determined during method validation are satisfactory. The quantitation limit of 0.5 µg/l is sufficient to quantify the background levels of the analyte for the general population.

Instruments used UPLC system (Acquity UPLC I-Class System, Waters GmbH, Eschborn, Germany) consisting of a sample manager (SM-FTN), I-Class binary solvent manager, column manager, and sample organiser connected to a Xevo TQ-S tandem-quadrupole mass-spectrometer (Waters GmbH, Eschborn, Germany).

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