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2-Phenoxyethanol – Determination of phenoxyacetic acid and 4-hydroxyphenoxyacetic acid in urine by UPLC-ESI-MS/MS

Biomonitoring Method – Translation of the German version from 2023

Keywords

2-phenoxyethanol; phenoxyacetic acid; 4-hydroxyphenoxyacetic acid; biomonitoring; urine; UPLC-ESI-MS/MS

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Abstract

The working group "Analyses in Biological Materials" of the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented method for the biomonitoring of exposure to 2-phenoxyethanol, an important biocide. The method described herein allows for the determination of the two main metabolites of 2-phenoxyethanol, specifically phenoxyacetic acid (PhAA) and 4-hydroxyphenoxyacetic acid (4-OH-PhAA), in urine. The urine samples are mixed with diluted formic acid and the internal standards. The analytes are separated from matrix components by liquid chromatography and subsequently analysed by tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in ultra-pure water. Good precision data with standard deviations below 7.19% for PhAA and below 8.07% for 4-OH-PhAA as well as good accuracy data with mean relative recoveries in the range of 98.7–109% for PhAA and of 92.6–107% for 4-OH-PhAA show that the method provides reliable and accurate analytical results. The method is both selective and sensitive, and the limits of quantitation of 8.2 µg PhAA/l urine and 7.6 µg 4-OH-PhAA/l urine are sufficient to determine occupational exposure as well as background exposure of the general population to 2-phenoxyethanol.

1 Characteristics of the method

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

Parameters and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
2-Phenoxyethanol	122-99-6	Phenoxyacetic acid	122-59-8
		4-Hydroxyphenoxyacetic acid	1878-84-8

Reliability data

Phenoxyacetic acid (PhAA)

Within-day precision:	Standard deviation (rel.) Prognostic range at a mean concentration of 0.16 mg, 0.83 mg, or 15.3 mg PhAA per litre of urine and n = 10 determinations	s_w = 2.79%, 1.08%, or 2.04% u = 6.23%, 2.40%, or 4.54%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a mean concentration of 0.14 mg, 0.80 mg, or 15.0 mg PhAA per litre of urine and n = 11 determinations	s_w = 7.19%, 5.08%, or 4.48% u = 16.0%, 11.3%, or 10.0%
Accuracy:	Recovery (rel.) using ten native urine samples and at spiked concentrations of 0.07 mg, 0.65 mg, or 6.50 mg PhAA per litre of urine	r = 98.7%, 108%, or 109%
Detection limit:	2.5 µg PhAA per litre of urine	
Quantitation limit:	8.2 µg PhAA per litre of urine	

4-Hydroxyphenoxyacetic acid (4-OH-PhAA) with 4-hydroxymethyl-PhAA as ISTD

Within-day precision:	Standard deviation (rel.) Prognostic range at a mean concentration of 0.08 mg, 0.21 mg, or 5.38 mg 4-OH-PhAA per litre of urine and n = 10 determinations	s_w = 4.90%, 3.16%, or 3.24% u = 10.9%, 7.06%, or 7.22%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a mean concentration of 0.08 mg, 0.21 mg, or 5.65 mg 4-OH-PhAA per litre of urine and n = 11 determinations	s_w = 8.07%, 4.01%, or 4.61% u = 18.0%, 8.94%, or 10.3%
Accuracy:	Recovery (rel.) using ten native urine samples and at spiked concentrations of 0.07 mg, 0.65 mg, or 6.50 mg 4-OH-PhAA per litre of urine	r = 95.0%, 107%, or 104%
Detection limit:	3.1 µg 4-OH-PhAA per litre of urine	
Quantitation limit:	10.2 µg 4-OH-PhAA per litre of urine	

4-Hydroxyphenoxyacetic acid (4-OH-PhAA) with d_4-4-OH-PhAA as ISTD[a)]

Within-day precision:	Standard deviation (rel.) Prognostic range at a mean concentration of 0.04 mg, 0.11 mg, or 1.78 mg 4-OH-PhAA per litre of urine and n = 10 determinations	s_w = 4.02%, 1.77%, or 2.14% u = 9.01%, 4.01%, or 4.84%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a mean concentration of 0.04 mg, 0.11 mg, or 1.80 mg 4-OH-PhAA per litre of urine and n = 6 determinations	s_w = 6.31%, 3.85%, or 3.00% u = 16.2%, 9.89%, or 7.72%
Accuracy:	Recovery (rel.) using ten native urine samples and at spiked concentrations of 0.07 mg, 0.65 mg, or 6.50 mg 4-OH-PhAA per litre of urine	r = 92.6%, 96.3%, or 98.5%
Detection limit:	2.2 µg 4-OH-PhAA per litre of urine	
Quantitation limit:	7.6 µg 4-OH-PhAA per litre of urine	

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

2 General information on phenoxyethanol

2-Phenoxyethanol is a colourless, slightly viscous liquid with a weak rose-like odour and is obtained by the hydroxyethylation of phenol (Fiege et al. 2000). 2-Phenoxyethanol is produced in or imported into the European Economic Area in amounts ranging from ≥ 10 000 t/a to < 100 000 t/a (ECHA 2023).

2-Phenoxyethanol acts as a biocide and is employed as the active substance in biocidal products for the small-scale disinfection of surfaces by professional users (BPC 2018 b, c). Due to its biocidal effects, it is also permitted as an active substance in hand disinfectants (BPC 2018 a) and is used in cosmetics and pharmaceutical products as a preservative, whereby a maximum content of 1% has been established (EU 2009; Uhr et al. 2013). 2-Phenoxyethanol is further applied as a biocide in metalworking fluids (Uhr et al. 2013).

In addition, 2-phenoxyethanol is employed as an intermediate in the production of plasticisers, medicines, and fragrances as well as in the perfume industry as a fixing agent. 2-Phenoxyethanol is moreover marketed as a solvent for cellulose acetate (Fiege et al. 2000).

Per the GHS, 2-phenoxyethanol is classified as acutely toxic (Category 4; H 302: "harmful if swallowed") and irritating to the eyes (Category 2; H 319: "causes serious eye irritation") (EU 2008). For 2-phenoxyethanol, the Commission has established a MAK value of 1 ml/m³ (5.7 mg/m³) with peak limitation category I and an excursion factor of 1. The substance has further been assigned to Pregnancy Risk Group C (DFG 2023). A detailed description and discussion of the toxicological characteristics of 2-phenoxyethanol can be found in the corresponding documentations published by the Commission (Hartwig and MAK Commission 2019) and elsewhere (BPC 2018 a, b, c; SCCS 2016; Scognamiglio et al. 2012).

There are no data on the primary route of uptake of 2-phenoxyethanol in the workplace. Inhalation exposure may occur under normal conditions due to the substance's low vapour pressure, mainly by way of aerosols rather than via vapours (IFA 2023). No data have been published on absorption following inhalation (Hartwig and MAK Commission 2019).

After dermal or oral exposure, 2-phenoxyethanol is easily absorbed by humans and rats (Howes 1988). After absorption, it is rapidly metabolised and excreted renally. Animal studies on female Wistar rats showed that 2-phenoxyethanol is mainly metabolised to PhAA (about 60% of the applied dose). Both free and conjugated forms of PhAA were present in the urine. Unmetabolised 2-phenoxyethanol and its conjugates were only detected in the urine in trace (< 1% of the applied dose) or small (about 5% of the applied dose) amounts, respectively. Ring-hydroxylated metabolites and

their conjugates were also found in only small amounts in the urine (Hartwig and MAK Commission 2019). Figure 1 depicts the structural formulas of 2-phenoxyethanol and its metabolites. An overview of the postulated metabolism is given in Figure 2.

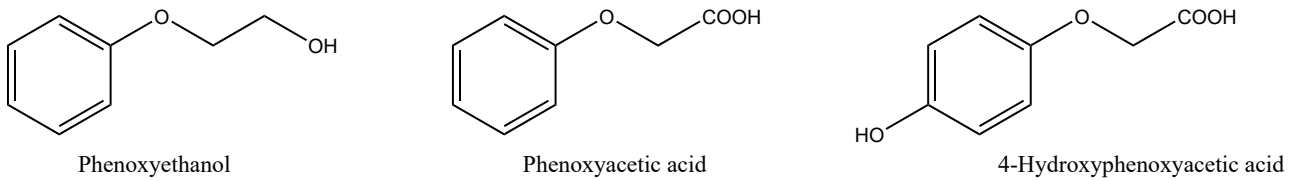


Fig. 1 Structural formulas of 2-phenoxyethanol, phenoxyacetic acid, and 4-hydroxyphenoxyacetic acid

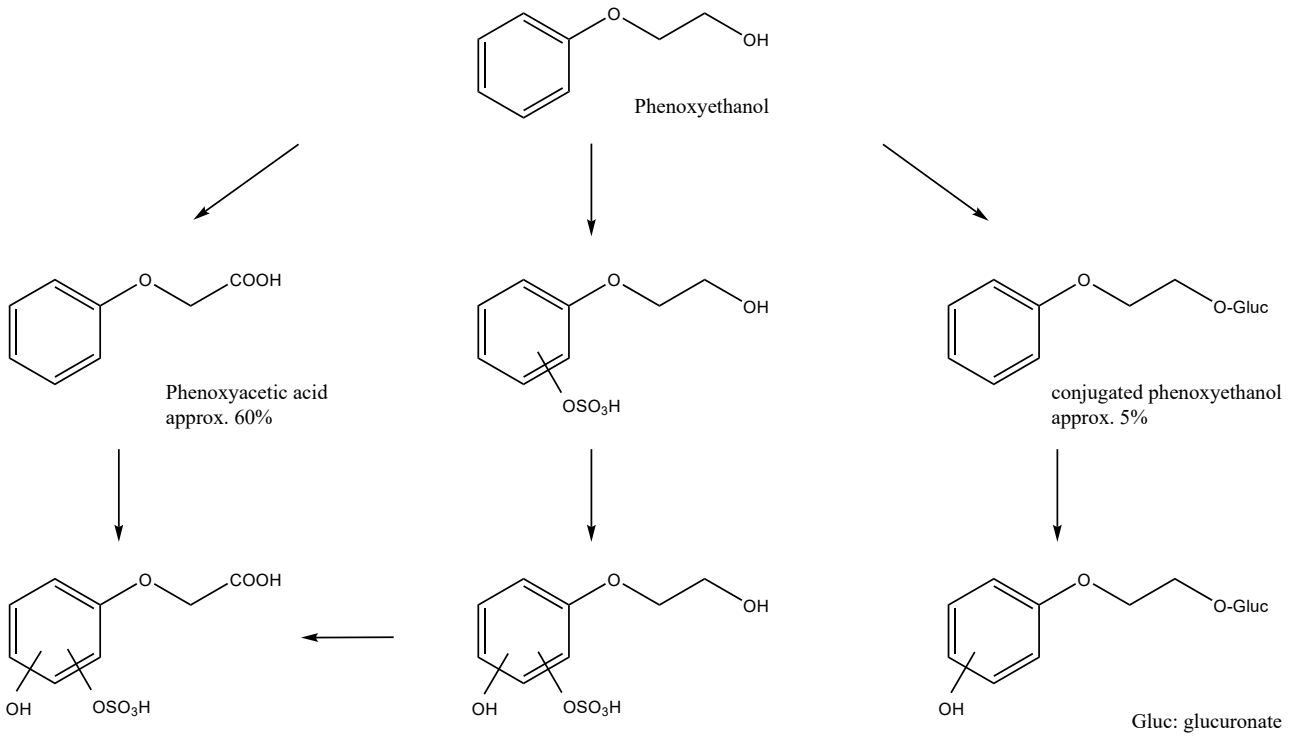


Fig. 2 Scheme of the postulated metabolism of 2-phenoxyethanol

In humans, PhAA is also the main metabolite, while 4-OH-PhAA is formed in significantly lower concentrations (Hartwig and MAK Commission 2019; Jäger et al. 2022). Unmetabolised 2-phenoxyethanol has not yet been detected after oral or dermal application (Hartwig and MAK Commission 2019).

Table 1 and Table 2 provide data on background exposure in the general population. While there are some studies on PhAA levels in the urine of the occupationally non-exposed general population, data on 4-OH-PhAA have so far only been published by Jäger et al. (2022).

Tab. 1 PhAA in urine samples from the occupationally non-exposed general population

Study collective	LOD	LOQ	Phenoxyacetic acid			References
			Median	GM	Range	
Men, France, 53, n = 22 > LOD	0.05 mg/l	–	< LOD	–	< LOD–2.03 mg/g crea	Multigner et al. 2007
Pregnant women, France, 200, n = 194 > LOD	0.001 mg/l	–	–	0.58 mg/g crea	< LOD–73.91 mg/g crea	Labat et al. 2008
General population, Germany, 31 ♀, 13 ♂, n = 44 > LOD	–	0.01 mg/l	1.33 mg/g crea	–	0.02–79.1 mg/g crea	Fromme et al. 2013
Pregnant women, France, 519, n = 484 > LOD	0.05 mg/l	–	0.48 mg/l	–	< LOD–36.0 mg/l	Garlantézec et al. 2013
General population, Germany, 94 ♂, 59 ♀, n = 151 > LOQ	–	0.01 mg/l	1.27 mg/g crea (0.99 mg/l)	–	< LOQ–84.11 mg/g crea (< LOQ–53.83 mg/l)	Jäger et al. 2022

Crea: creatinine; GM: geometric mean; LOD: limit of detection; LOQ: limit of quantitation

Tab. 2 4-OH-PhAA in urine samples from the occupationally non-exposed general population

Study collective	LOD	LOQ	4-Hydroxyphenoxyacetic acid			References
			Median	GM	Range	
General population, Germany, 94 ♂, 59 ♀, n = 144 > LOQ	–	0.02 mg/l	0.15 mg/g crea (0.11 mg/l)	–	< LOQ–4.98 mg/g crea (< LOQ–6.55 mg/l)	Jäger et al. 2022

Crea: creatinine; GM: geometric mean; LOD: limit of detection; LOQ: limit of quantitation

The Commission has already published a biomonitoring method on the determination of PhAA in urine. At 0.1 mg/l, the detection limit of this method is, however, insufficient to measure background exposure in the general population (Göen et al. 2006).

3 General principles

The method described below allows for the determination of the two most important metabolites of 2-phenoxyethanol, specifically PhAA and 4-OH-PhAA, in urine. The urine samples are mixed with diluted formic acid and internal standards (ISTDs). As ISTDs, d_5-PhAA and 4-hydroxymethylphenoxyacetic acid or d_4-4-OH-PhAA are used. The analytes are separated from matrix components by liquid chromatography and subsequently analysed by tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in aqueous medium.

4 Equipment, chemicals, and solutions

4.1 Equipment

- Ultra-high-performance liquid chromatograph (e.g. Waters UPLC H-Class, Waters GmbH, Eschborn, Germany) with a mass-spectrometric detector (e.g. Waters Xevo-TQS, Waters GmbH, Eschborn, Germany)
- UPLC column (e.g. ACQUITY UPLC HSS T3, 100 Å, 1.8 µm × 2.1 mm × 150 mm, No. 186003540, Waters GmbH, Eschborn, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Laboratory shaker (e.g. Multi Reax, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany)
- Water-purification system (e.g. Milli-Q®, Merck KGaA, Darmstadt, Germany)

- 1000-ml glass bottles with dispensers (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 1.8-ml screw-top injection bottles (e.g. Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- 100-ml plastic bottles (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 100-ml amber glass bottles (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- Various pipettes with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- 1.5-ml reaction vials (e.g. Eppendorf AG, Hamburg, 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.

Reference materials

- Phenoxyacetic acid, 98% (e.g. No. 158518, Merck KGaA, Darmstadt, Germany)
- d_5-Phenoxyacetic acid, > 98% (e.g. No. D-6655, C/D/N Isotopes Inc., Quebec, Canada)
- 4-Hydroxyphenoxyacetic acid, > 98% (e.g. No. 11429773, Fisher Scientific GmbH, Schwerte, Germany)
- d_4-4-Hydroxyphenoxyacetic acid, ≥ 95% (e.g. custom synthesis, ChiroBlock GmbH, Wolfen, Germany)
- 4-Hydroxymethylphenoxyacetic acid, ≥ 97% (e.g. No. 55730, Merck KGaA, Darmstadt, Germany)

Other chemicals

- Acetonitrile, ≥ 99.9%, CHROMASOLV™ (e.g. No. 34851, Fluka™, Honeywell Deutschland Holding GmbH, Offenbach, Germany)
- Formic acid, 98–100%, pure, for analysis (e.g. No. 05311, AnalytiChem GmbH, Duisburg, Germany)
- Sodium hydroxide solution, min. 10%, for analysis, EMSURE® (e.g. No. 105588, Merck KGaA, Darmstadt, Germany)
- Methanol, for liquid chromatography (e.g. No. M/4058/17, Fisher Chemicals, Geel, Belgium)
- Nitrogen 5.0 (e.g. Nippon Gases Deutschland GmbH, Biebesheim am Rhein, Germany)
- Argon 5.0 (e.g. Linde GmbH, Pullach, Germany)
- Ultra-pure water (e.g. Milli-Q® water-purification system, Merck KGaA, Darmstadt, Germany)

4.3 Solutions

- Formic acid (0.033%; pH = 2.9)
  2 ml of concentrated formic acid are made up to 6 l with ultra-pure water and, if necessary, adjusted to a pH value of 2.9 using the sodium hydroxide solution.

The diluted formic acid is stored at room temperature and is stable for one year.

4.4 Internal standards (ISTDs)

A different ISTD was used for the quantification of 4-OH-PhAA during method development than in external verification. The method developers used the structurally analogous 4-hydroxymethyl-PhAA, whereas the external verifiers used a structurally identical, deuterated ISTD (d_4-4-OH-PhAA) produced by custom synthesis. In the following sections, both options for ISTDs are mentioned; the reliability criteria for 4-OH-PhAA are also given for both ISTDs.

- ISTD stock solutions (1000 mg/l)
  10 mg of d_5-PhAA and 10 mg of d_4-4-OH-PhAA (or 4-hydroxymethyl-PhAA) are weighed exactly into separate 10-ml volumetric flasks and dissolved in 5 ml of methanol. The volumetric flasks are subsequently made up to the mark with methanol.
- ISTD spiking solution (10 mg/l)
  100 µl of the d_5-PhAA stock solution and 100 µl of the d_4-4-OH-PhAA stock solution (or 4-hydroxymethyl-PhAA stock solution) are pipetted into a 10-ml volumetric flask. The volumetric flask is subsequently made up to the mark with ultra-pure water.

The ISTD stock solutions and spiking solution are stored at –18 °C and are stable for one year at this temperature.

4.5 Calibration standards

- Stock solutions (1000 mg/l)
  10 mg of PhAA or 4-hydroxymethyl-PhAA are weighed into individual 10-ml volumetric flasks and dissolved in 5 ml of methanol each. The volumetric flasks are subsequently made up to the mark with methanol.
- Spiking solution I (100 mg/l)
  100 µl of each stock solution are pipetted into a 1.5-ml reaction vial and 800 µl of ultra-pure water are added.
- Spiking solution II (10 mg/l)
  100 µl of each stock solution are pipetted into a 10-ml volumetric flask. The volumetric flask is subsequently made 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. The volumetric flask is subsequently made up to the mark with ultra-pure water.

The calibration standards are prepared according to the pipetting scheme given in Table 3, stored in the refrigerator at 4 °C, and used for a maximum of four weeks. For workup, 20 µl of each calibration standard are used. Since 4-OH-PhAA concentrations are expected to be significantly lower than the concentrations of PhAA, it may be advisable to use a lower concentration range for the determination of 4-OH-PhAA.

Tab. 3 Pipetting scheme for the preparation of calibration standards for the determination of PhAA und 4-OH-PhAA in urine

Calibration standard	Spiking solution I [µl]	Spiking solution II [µl]	Spiking solution III [µl]	Ultra-pure water [µl]	Concentration [mg/l]
0	–	–	–	1000	0.0
1	–	–	10	990	0.01
2	–	–	20	980	0.02
3	–	–	50	950	0.05
4	–	10	–	990	0.1
5	–	20	–	980	0.2
6	–	50	–	950	0.5

Tab. 3 *(continued)*

Calibration standard	Spiking solution I [µl]	Spiking solution II [µl]	Spiking solution III [µl]	Ultra-pure water [µl]	Concentration [mg/l]
7	10	–	–	990	1.0
8	20	–	–	980	2.0
9	50	–	–	950	5.0
10	100	–	–	900	10.0
11	200	–	–	800	20.0
12	400	–	–	600	40.0

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in sealable polypropylene containers and stored in the refrigerator at 4 °C until sample workup. For long-term storage (> three days), the urine samples should be frozen at –18 °C.

5.2 Sample preparation

The urine samples are brought to room temperature and subsequently thoroughly mixed. 1000 µl of diluted formic acid (0.033%) are placed in a 1.8-ml injection bottle, then 50 µl of the ISTD spiking solution and 20 µl of the urine sample are added by pipetting. The bottle is closed and intensely shaken (10 s on the laboratory shaker). Of the urine sample thus diluted, 10 µl are injected into the UPLC-MS/MS.

6 Operational parameters

Analytical determination was performed using a device configuration comprised of a UPLC system and a tandem mass spectrometer using negative electrospray ionisation (UPLC-ESI-MS/MS).

6.1 Ultra-high-performance liquid chromatography

Separation column:	Waters Acquity UPLC HSS T3 (1.8 µm, 2.1 × 150 mm)
Separation principle:	Reversed phase
Injection volume:	10 µl
Column temperature:	30 °C
Flow rate:	0.2 ml/min
Eluent:	A: Formic acid (0.033%) B: Acetonitrile
Runtime:	15 min
Gradient program:	see Table 4

Tab. 4 Gradient program for the determination of PhAA and 4-OH-PhAA in urine

Time [min]	Eluent A [%]	Eluent B [%]
0.0	80	20
10.0	10	90
13.0	10	90
13.1	80	20
15.0	80	20

6.2 Tandem mass spectrometry

Ionisation: Electrospray, negative (ESI–)

Detection mode: Multiple Reaction Monitoring (MRM)

Capillary voltage: 3.0 kV

Cone voltage: 38 V

Source temperature: 150 °C

Desolvation temperature: 500 °C

Cone-gas flow: 150 l/h

Desolvation-gas flow: 1000 l/h

Collision gas: Argon

Collision-gas flow: 0.15 ml/h

The instrument-specific parameters must be ascertained and adjusted by the user for the individual MS/MS system in question. The device-specific parameters indicated above have been determined and optimised for the system used during method development. Two fragment-ion transitions were selected for PhAA and 4-OH-PhAA. One transition serves the purpose of quantification (quantifier) and the other of confirmation (qualifier). Only one mass transition was selected for the corresponding ISTDs. The selected fragment-ion transitions are presented alongside the retention times in Table 5. Figure 3 shows the structural formulas of the selected ions (parent, quantifier, qualifier).

Tab. 5 MRM parameters for the determination of PhAA and 4-OH-PhAA in urine

Analyte/ISTD	Retention time [min]	Mass transition [*m/z*]	Status	Cone [V]	Collision energy [V]	Dwell time [s]
PhAA	5.48	150.7 ⟶ 93.0	Quantifier	32	12	0.025
		150.7 ⟶ 107.0	Qualifier	32	10	0.025
d_5-PhAA (ISTD)	5.43	155.7 ⟶ 98.1	Quantifier	32	12	0.025
4-OH-PhAA	3.32	166.8 ⟶ 109.1	Quantifier	34	12	0.025
		166.8 ⟶ 81.0	Qualifier	34	22	0.025
4-Hydroxymethyl-PhAA (ISTD)	3.21	181.0 ⟶ 105.0	Quantifier	38	20	0.025
d_4-4-OH-PhAA (ISTD)	3.30	170.8 ⟶ 113.1	Quantifier	30	14	0.025

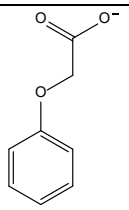
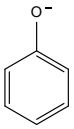
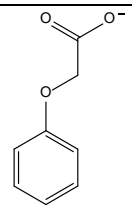
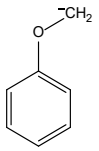
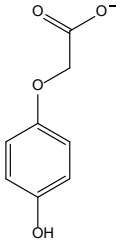
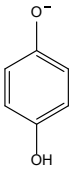
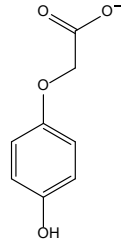
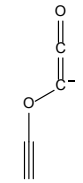
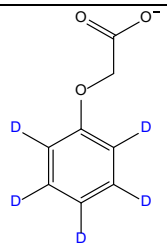
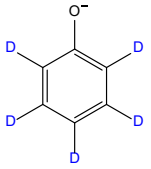
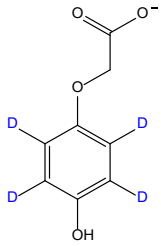
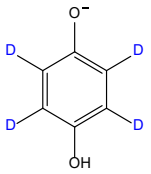
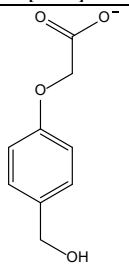
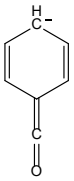
	Quantifier		Qualifier	
PhAA				
	m/z 150.7 [M–H]⁻	*m/z* 93.0 [M–CH₂-COO]⁻	*m/z* 150.7 [M–H]⁻	*m/z* 107.0 [M–COO]⁻
4-OH-PhAA				
	m/z 166.8 [M–H]⁻	*m/z* 109.0 [M–CH₂-COO]⁻	*m/z* 166.8 [M–H]⁻	*m/z* 81.0 [M–C₄H₄O₂]⁻
	ISTDs			
d_5-PhAA				
	m/z 155.7 [M–H]⁻	*m/z* 98.1 [M–CH₂-COO]⁻		
d_4-4-OH-PhAA				
	m/z 170.8 [M–H]⁻	*m/z* 113.1 [M–CH₂-COO]⁻		
4-Hydroxymethyl-PhAA				
	m/z 181.0 [M–H]⁻	*m/z* 105.1 [M–CH₂-COO]⁻		

Fig. 3 Structural formulas of the selected ions (parent, quantifier, qualifier) for the determination of PhAA and 4-OH-PhAA in urine

7 Analytical determination

Of the diluted urine sample (see Section 5.2), 10 µl are injected into the UPLC-MS/MS system. Analytical separation is conducted by reversed-phase chromatography. The analytes are identified by their individual retention times and specific mass transitions. The retention times for the analytes and ISTDs given in Table 5 are only intended as a point of reference. The user must ensure the separation performance of the column used as well as the resulting retention behaviour of the analytes.

Representative chromatograms for the individual analytes are depicted in Figures 4, 5, and 6. For the determination of 4-OH-PhAA, both chromatograms using the structurally identical isotope-labelled ISTD d_4-4-OH-PhAA (Figure 5) and using the structurally analogous ISTD 4-hydroxymethyl-PhAA (Figure 6) are shown.

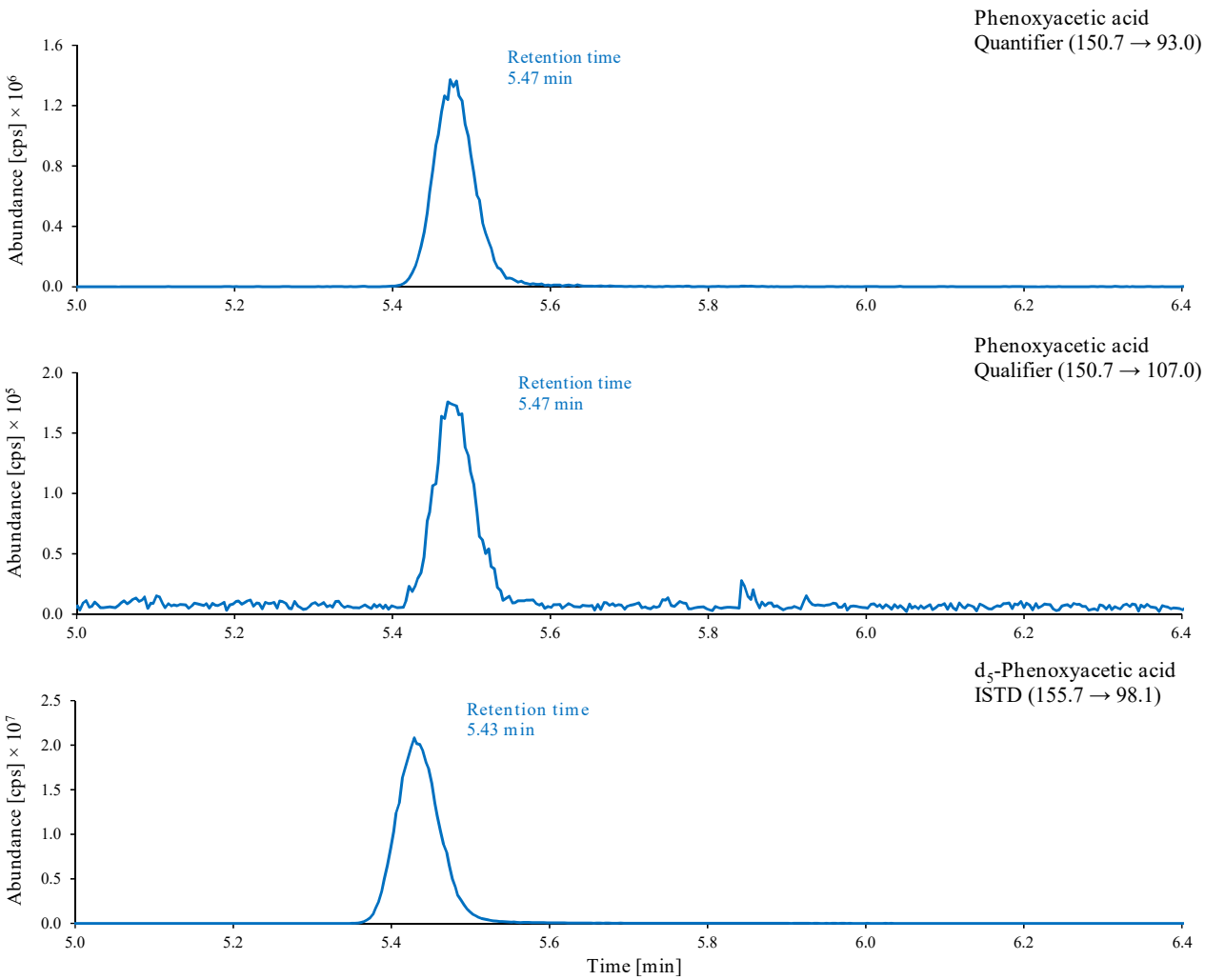


Fig. 4 Chromatograms of a urine sample spiked with 0.98 mg PhAA per litre (ISTD: d_5-PhAA)

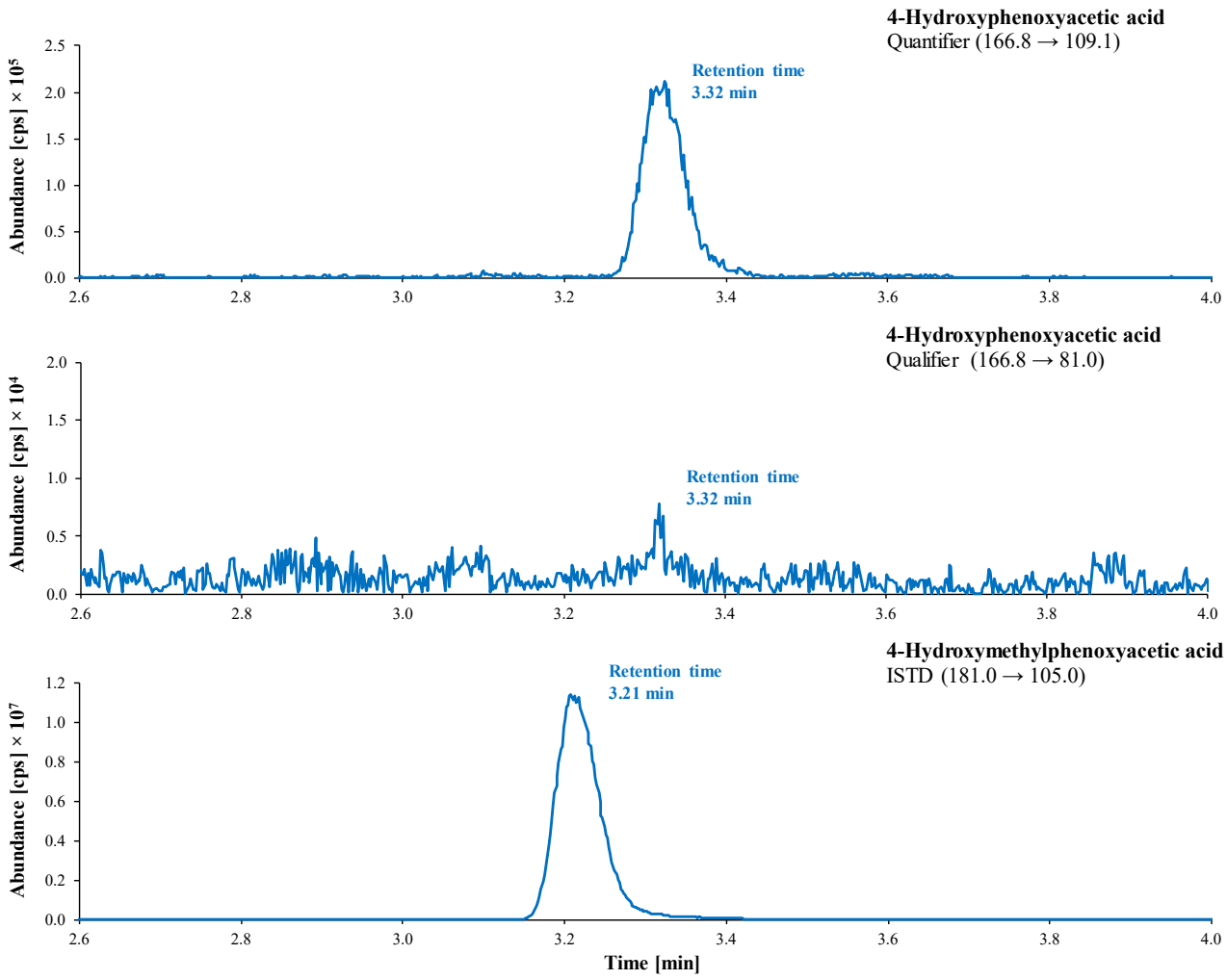


Fig. 5 Chromatograms of a urine sample spiked with 0.20 mg 4-OH-PhAA per litre (ISTD: 4-hydroxymethyl-PhAA)

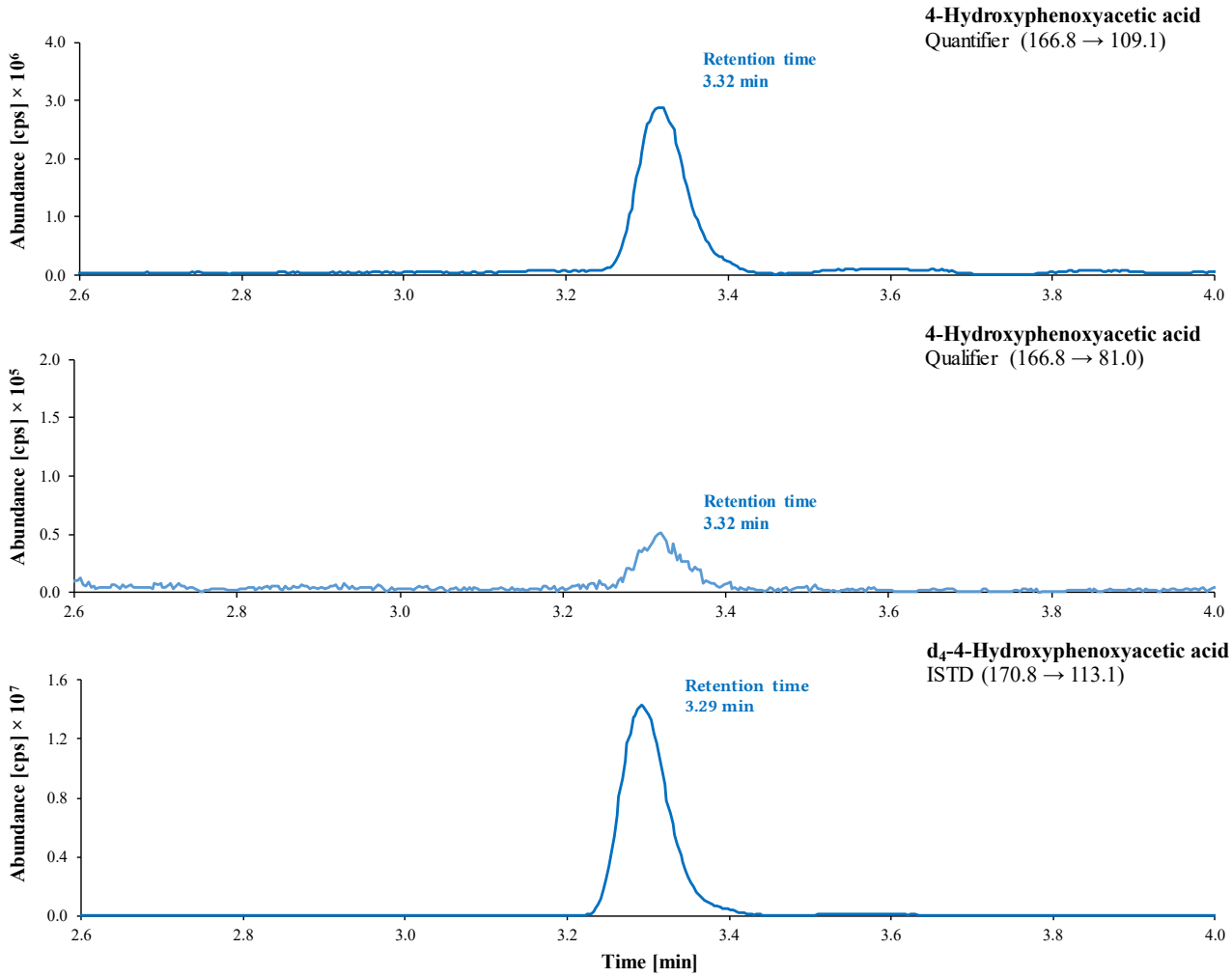


Fig. 6 Chromatograms of a urine sample spiked with 5.0 mg 4-OH-PhAA (ISTD: d_4-4-OH-PhAA)

8 Calibration

The calibration solutions are prepared as described in Section 4.5, processed analogously to the urine samples (see Section 5.2), and analysed. The calibration curve is generated by plotting the quotient of the peak area of the analyte and that of its ISTD against the corresponding spiked concentration of the analyte. With the analytical instrumentation used during method development, a linear measurement range was observed from the quantitation limit up to 40.0 mg/l (see Figure 7). If necessary, reagent blank values must be accounted for by subtraction.

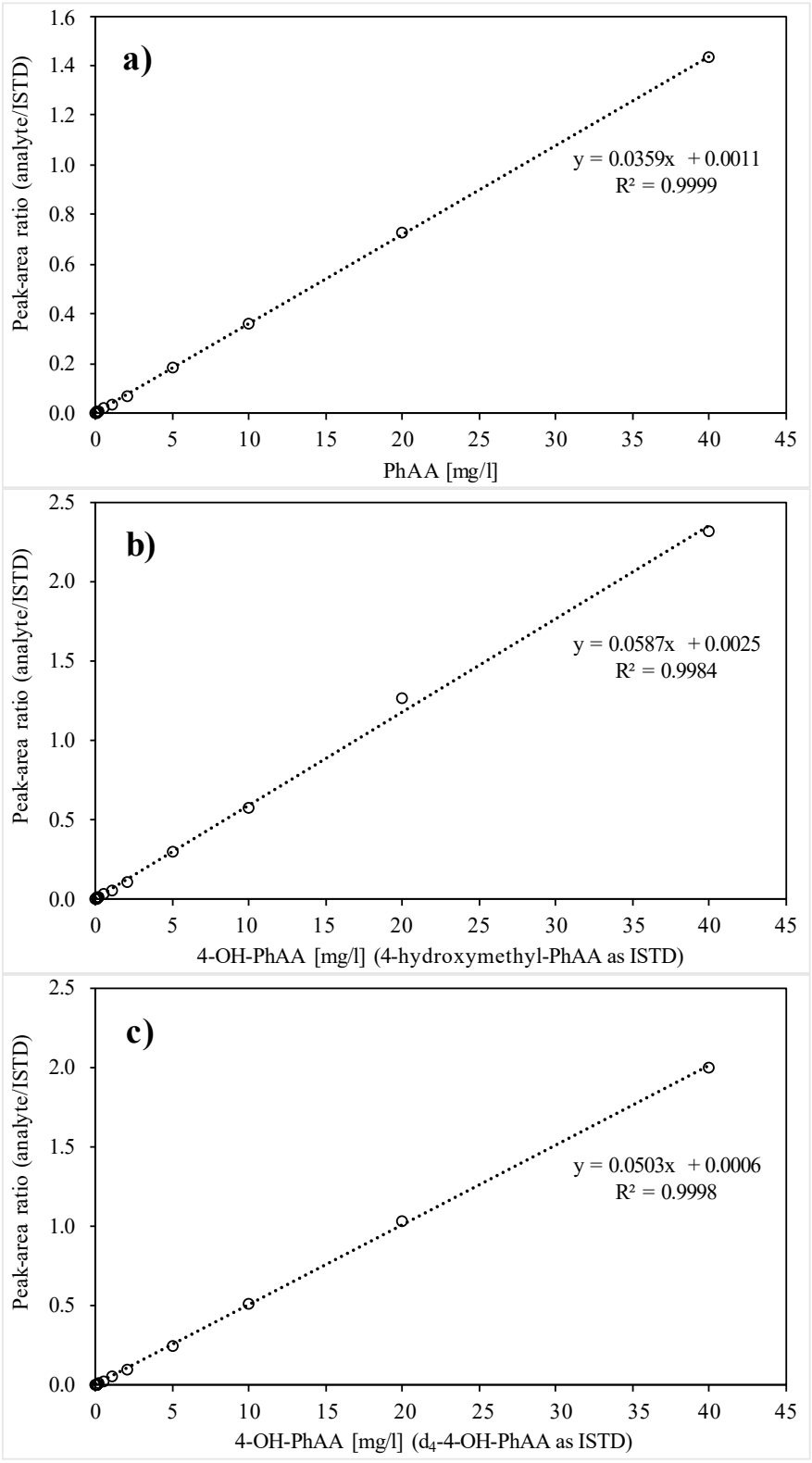


Fig. 7 Calibration curves for the determination of a) PhAA, b) 4-OH-PhAA using 4-hydroxymethyl-PhAA as ISTD, and c) 4-OH-PhAA using d_4-4-OH-PhAA as ISTD in urine (linear regression)

9 Calculation of the analytical results

The analyte content of a sample in mg/l urine is calculated by inserting the peak-area ratio of the analyte to its ISTD into the calibration function of the corresponding analytical run. The calibration range may need to be adjusted to the expected concentration ranges. If the analyte concentration lies above the calibration range, the sample must be newly analysed. To this end, urine aliquots are diluted with ultra-pure water in such a way that the analyte concentration lies within the calibration range and are then processed and measured.

10 Standardisation and quality control

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

For each analytical run, at least three control-urine samples with known analyte concentrations (low, medium, and high concentrations) are processed and analysed parallel to the samples to be analysed. Since control material is not commercially available for PhAA or 4-OH-PhAA, it must be prepared in the in-house laboratory. To this end, native urine samples with varying background levels of PhAA and 4-OH-PhAA are pooled or pooled urine samples are spiked with varying analyte concentrations. The quality-control material thus prepared is aliquoted and frozen at –18 °C until use. Simultaneously, a reagent blank is included as part of each analytical run.

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.

11.1 Precision

Within-day precision

To determine within-day precision, three native urine samples with varying PhAA and 4-OH-PhAA concentrations were processed and analysed ten times in parallel on one day. The within-day precision results thus obtained are summarised in Table 6. To ascertain the precision data, the method developers used 4-hydroxymethyl-PhAA as the ISTD for 4-OH-PhAA. During external verification, the structurally identical, isotope-labelled standard d_4-4-OH-PhAA was used. Of the data from external method verification, only the values for 4-OH-PhAA are given in Table 6.

Tab. 6 Within-day precision for the determination of PhAA and 4-OH-PhAA in urine (n = 10)

Analyte	Concentration [mg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
PhAA	0.16	2.79	6.23
	0.83	1.08	2.40
	15.3	2.04	4.54
4-OH-PhAA (4-Hydroxymethyl-PhAA as ISTD)	0.08	4.90	10.9
	0.21	3.16	7.06
	5.38	3.24	7.22

Tab. 6 *(continued)*

Analyte	Concentration [mg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
4-OH-PhAA[a] (d_4-4-OH-PhAA as ISTD)	0.04	4.02	9.08
	0.11	1.77	4.01
	1.78	2.14	4.84

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

Day-to-day precision

Day-to-day precision was determined by processing and analysing three native urine samples with varying PhAA and 4-OH-PhAA concentrations. The method developers used 4-hydroxymethyl-PhAA as ISTD for 4-OH-PhAA. As part of method development, the samples were processed and analysed on eleven different days. During method verification, the structurally identical, isotope-labelled standard d_4-4-OH-PhAA was used for 4-OH-PhAA and the samples were processed and analysed on six different days. The data thus calculated are given in Table 7. Of the data from external method verification, only the values for 4-OH-PhAA are given.

Tab. 7 Day-to-day precision for the determination of PhAA and 4-OH-PhAA in urine (n = 11 or n = 6)

Analyte	Concentration [mg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
PhAA	0.14	7.19	16.0
	0.80	5.08	11.3
	15.0	4.48	10.0
4-OH-PhAA (4-Hydroxymethyl-PhAA as ISTD)	0.08	8.07	18.0
	0.21	4.01	8.94
	5.65	4.61	10.3
4-OH-PhAA[a] (d_4-4-OH-PhAA as ISTD)	0.04	6.31	16.2
	0.11	3.85	9.89
	1.80	3.00	7.72

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

11.2 Accuracy

To check for matrix effects, the method developers spiked each of ten native urine samples (creatinine concentrations of 0.56–1.96 g/l) with 0.07 mg, 0.65 mg, or 6.50 mg PhAA as well as 4-OH-PhAA per litre of urine. Both the spiked and unspiked urine samples were then processed and analysed as described above.

To check for matrix effects using d_4-4-OH-PhAA as ISTD, the method verifiers spiked each of ten native urine samples (creatinine concentrations of 0.13–1.43 g/l) with 0.07 mg, 0.65 mg, or 6.50 mg PhAA as well as 4-OH-PhAA per litre of urine. Both the spiked and unspiked urine samples were then processed and analysed as described above.

The relative recovery data thus obtained is summarised in Table 8, whereby, of the data from external method verification, only the values for 4-OH-PhAA are given.

Tab. 8 Relative recovery of PhAA and 4-OH-PhAA in individual urines (n = 10)

Analyte	Spiked concentration [mg/l]	Recovery (rel.) *r* [%]	
		Mean ± SD	Range
PhAA	0.07	98.7 ± 13.0%	81.3–120
	0.65	108 ± 8.3%	88.9–116
	6.50	109 ± 5.4%	99.3–116
4-OH-PhAA (4-Hydroxymethyl-PhAA as ISTD)	0.07	95.0 ± 11.7%	76.6–110
	0.65	107 ± 5.4%	98.1–114
	6.50	104 ± 8.9%	89.3–122
4-OH-PhAA[a] (d_4-4-OH-PhAA as ISTD)	0.07	92.6	86.3–97.3
	0.65	96.3	89.0–103
	6.50	98.5	91.7–102

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

11.3 Limits of detection and quantitation

The limits of detection and quantitation were ascertained per DIN 32645. According to DIN 32645, the detection and quantitation limit is calculated from the standard deviation of the calibration function obtained at the blank value (DIN 2008).

An equidistant 10-point calibration was created by the method developers in a concentration range of 0.005–0.05 mg/l in either aqueous solution as well as in pooled urine, then processed and analysed in conjunction with a blank value (n = 3). To this end, 4-hydroxymethyl-PhAA was used as ISTD for 4-OH-PhAA.

During method verification, the equidistant 10-point calibrations were generated in both ultra-pure water and in an individual urine sample in a concentration range of 0.01–0.1 mg/l, then processed and analysed in conjunction with a blank value (n = 3). The method verifiers used d_4-4-OH-PhAA as the ISTD for 4-OH-PhAA.

The calibration curves of the 10-point calibrations for PhAA and 4-OH-PhAA, using both ISTDs, are depicted in Figure 8. The background levels of both PhAA and 4-OH-PhAA in urine are easily recognisable. *Table 9* shows the limits of detection and quantitation determined for the urine matrix. Of the data from external method verification, only the values for 4-OH-PhAA are given in Figure 8 and Table 9.

Tab. 9 Limits of detection and quantitation for the determination of PhAA und 4-OH-PhAA in urine

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
PhAA	2.5	8.2
4-OH-PhAA (4-Hydroxymethyl-PhAA as ISTD)	3.1	10.2
4-OH-PhAA[a] (d_4-4-OH-PhAA as ISTD)	2.2	7.6

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

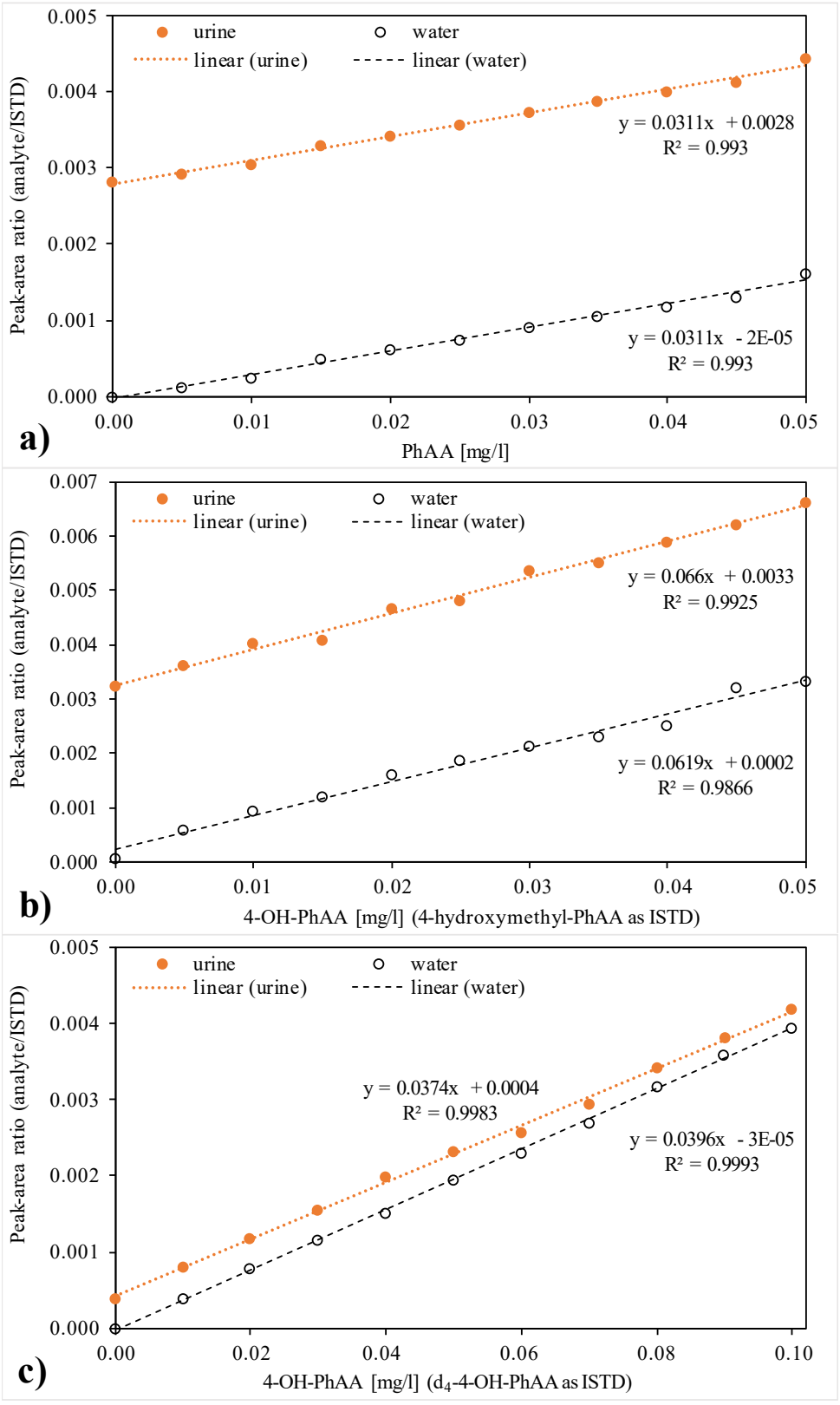


Fig. 8 Calibration curves in water or urine for the determination of the detection and quantitation limits according to DIN 32645: a) PhAA, b) 4-OH-PhAA using 4-hydroxymethyl-PhAA as ISTD, and c) 4-OH-PhAA using d_4-4-OH-PhAA as ISTD

11.4 Sources of error

Experiences with the method for the determination of PhAA and 4-OH-PhAA herein described have shown no significant risk of external contamination. It is, however, advisable to include a reagent blank as part of every analytical run in order to recognise potential impurities or contaminants.

12 Discussion of the method

The method hereby presented allows for the rapid, sensitive, and specific quantification of PhAA and 4-OH-PhAA in human urine. The low quantitation limits obtained enable the measurement of both phenoxyethanol metabolites in well over 90% of urine samples from the general population. The method hereby presented was used to investigate 153 spontaneous urine samples (creatinine concentrations of 0.09–4.87 g/l) from 153 workers from the chemical industry without occupational exposure to 2-phenoxyethanol. PhAA could be detected in 151 of 153 samples (99%) and 4-OH-PhAA in 146 of 153 samples (95%). The median was found to be 0.99 mg/l (range: < LOQ–53.8 mg/l, 95th percentile: 14.9 mg/l) for the metabolite PhAA and 0.11 mg/l (< LOQ–4.98 mg/l, 95th percentile: 2.08 mg/l) for 4-OH-PhAA. The method herein described is more sensitive and, in the low concentration range, more precise than the more laborious, previously published method for the determination of PhAA (Göen et al. 2006).

During method development, the structurally analogous 4-hydroxymethyl-PhAA was used for the quantification of 4-OH-PhAA. After the completion of method validation by the method developers, the synthesis of an isotope-labelled ISTD for 4-OH-PhAA (d_4-4-OH-PhAA) was ordered. During external method verification, this deuterated standard was then used for the quantification of 4-OH-PhAA concentrations.

If a non-isotope-labelled compound is used, it may not be possible to reliably compensate all interferences which may arise, especially when using LC-MS/MS analysis. In order to verify whether the ISTD used for the quantification of 4-OH-PhAA concentrations may have an influence on the analytical results, comparative measurements on 87 urine samples were performed. The results are given as both a linear correlation and a Bland-Altman plot in Figure 9. The investigation showed neither a clear trend nor individual matrix-related outliers, such that both ISTDs can generally be used.

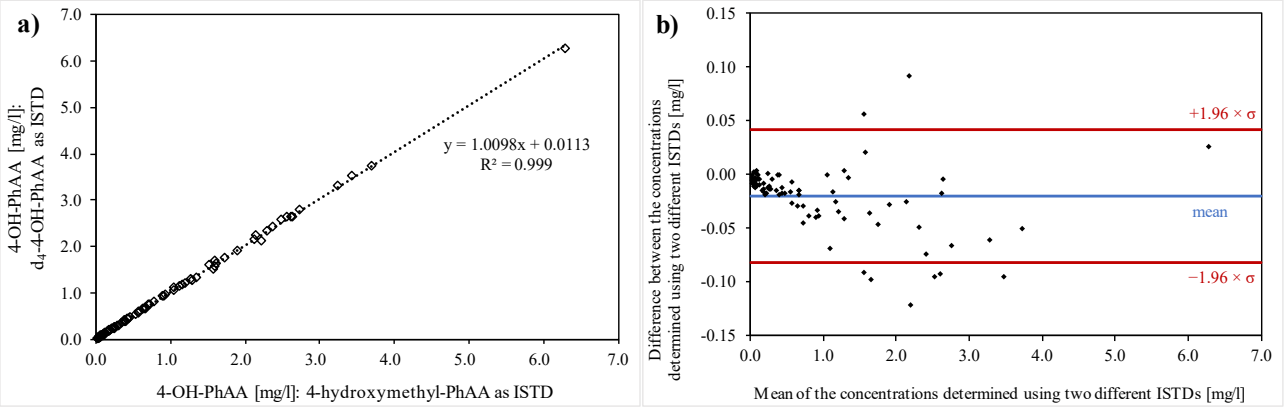


Fig. 9 Comparative determination of 4-OH-PhAA using 4-hydroxymethyl-PhAA or d_4-4-OH-PhAA as ISTD: a) linear correlation and b) Bland-Altman plot

The literature on PhE metabolism describes that PhAA is excreted by rats and rabbits in both conjugated and unconjugated forms (Hartwig and MAK Commission 2019). In order to verify the necessity of a hydrolysis step as part of sample preparation for human samples, a total of 29 urine samples were analysed both without and after acid hydrolysis. For acid hydrolysis, 200 µl of urine are heated for one hour at 95 °C in an aluminium heating block after the addition

of 80 µl hydrochloric acid (37%). After cooling to room temperature, the sample is mixed with 70 µl sodium hydroxide (10 ml/l) and 50 µl ultra-pure water. Exactly 40 µl of the sample solution are diluted with 980 ml formic acid (0.033%). After adding 50 µl of the ISTD spiking solution, the sample mixture is thoroughly mixed and 10 µl of the solution is injected into the UPLC-MS/MS.

Acid hydrolysis did not lead to increased analyte concentrations for either metabolite (see Figure 10). It must therefore be assumed that PhAA and 4-OH-PhAA are excreted in their unconjugated forms and that a hydrolysis step is not necessary.

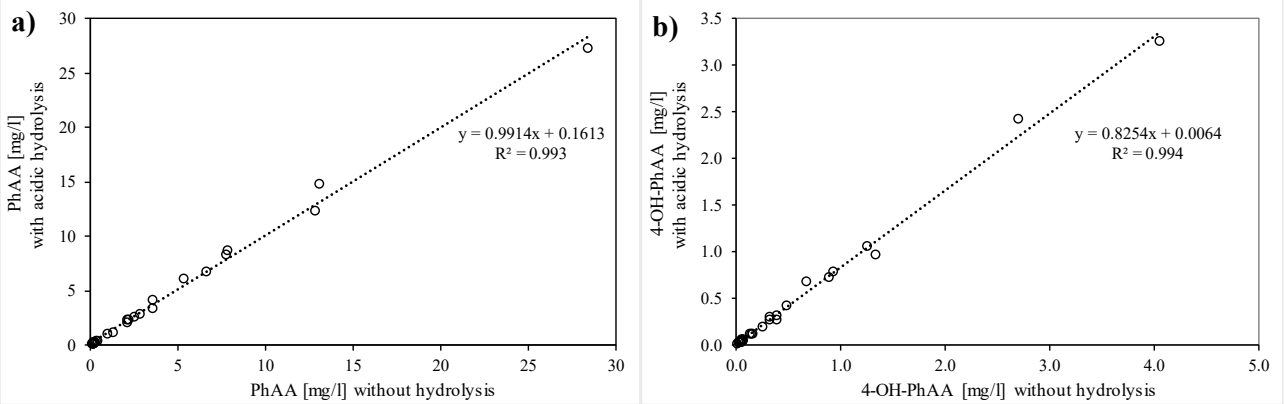


Fig. 10 Results of the comparative measurements with and without hydrolysis: a) PhAA and b) 4-OH-PhAA using 4-hydroxymethyl-PhAA as ISTD

Instruments used Ultra-high-performance liquid chromatograph (Waters UPLC H-Class, Waters GmbH, Eschborn, Germany) with a mass-spectrometric detector (Waters Xevo-TQS, Waters GmbH, Eschborn, Germany), UPLC column (ACQUITY UPLC HSS T3, 100 Å, 1.8 µm × 2.1 mm × 150 mm, No. 186003540, Waters GmbH, Eschborn, Germany)

Notes

Competing interests

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

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