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Bisphenol A, bisphenol F, and bisphenol S – Determination of bisphenol A, bisphenol F, and bisphenol S in urine by UPLC-ESI-MS/MS

Biomonitoring Method – Translation of the German version from 2023

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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. Bisphenol A (BPA), bisphenol F (BPF), and bisphenol S (BPS) are co-monomers for producing engineering plastics used in the automotive, food, and household sectors and are also used in paper chemicals and leather-tanning agents. Due to the wide range of applications, exposure to these bisphenols can occur both at the workplace and via the environment. The aim of this work was to develop a selective method for the determination of BPA, BPF, and BPS in human urine. This method has been comprehensively verified, and the reliability data have been confirmed by replication and validation of the procedure in a second, independent laboratory. The internal standards are added to the buffered urine samples, which are then subjected to enzymatic hydrolysis and processed via dispersive liquid-liquid microextraction (DLLME). The analytes are separated from matrix components by liquid chromatography and detected by tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in water. The good precision and accuracy data show that the method provides reliable and accurate measurement values. The method is selective and sensitive, and the quantitation limits of 0.25 µg/l, 0.10 µg/l, and 0.05 µg/l urine for BPA, BPF, and BPS, respectively, are sufficient to determine occupational as well as background exposure.

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 substances

Hazardous substance	CAS No.	Parameter	CAS No.
Bisphenol A	80-05-7	Bisphenol A	80-05-7
Bisphenol F	620-92-8	Bisphenol F	620-92-8
Bisphenol S	80-09-1	Bisphenol S	80-09-1

Reliability data

Bisphenol A (BPA)

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.4 µg, 2.0 µg, 10.0 µg, or 40.0 µg BPA per litre of urine and n = 10 determinations	s_w = 9.8%, 4.5%, 10.8%, or 7.9% u = 22.2%, 10.2%, 24.4%, or 17.9%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.4 µg, 2.0 µg, 10.0 µg, or 40.0 µg BPA per litre of urine and n = 6 determinations	s_w = 15.5%, 8.4%, 9.2%, or 12.5% u = 39.9%, 21.6%, 23.7%, or 32.1%
Accuracy:	Recovery rate (rel.) at a spiked concentration of 2.0 µg, 10.0 µg, or 40.0 µg BPA per litre of urine and n = 10 determinations	r = 85.2%, 88.2%, or 94.1%
Detection limit:	0.08 µg BPA per litre of urine	
Quantitation limit:	0.25 µg BPA per litre of urine	

Bisphenol F (BPF)

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.4 µg, 2.0 µg, 10.0 µg, or 40.0 µg BPF per litre of urine and n = 10 determinations	s_w = 13.4%, 7.7%, 5.5%, or 4.8% u = 30.3%, 17.4%, 12.4%, or 10.9%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.4 µg, 2.0 µg, 10.0 µg, or 40.0 µg BPF per litre of urine and n = 6 determinations	s_w = 12.9%, 7.3%, 6.8%, or 10.7% u = 33.2%, 18.8%, 17.5%, or 27.5%
Accuracy:	Recovery rate (rel.) at a spiked concentration of 2.0 µg, 10.0 µg, or 40.0 µg BPF per litre of urine and n = 10 determinations	r = 101%, 95.9%, or 97.1%
Detection limit:	0.03 µg BPF per litre of urine	
Quantitation limit:	0.10 µg BPF per litre of urine	

Bisphenol S (BPS)

Within-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.4 µg, 2.0 µg, 10.0 µg, or 40.0 µg BPS per litre of urine and n = 10 determinations	s_w = 2.9%, 3.3%, 3.2%, or 6.2% u = 6.6%, 7.5%, 7.2%, or 14.0%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a spiked concentration of 0.4 µg, 2.0 µg, 10.0 µg, or 40.0 µg BPS per litre of urine and n = 6 determinations	s_w = 7.4%, 2.8%, 4.9%, or 8.2% u = 19.0%, 7.2%, 12.6%, or 21.1%
Accuracy:	Recovery rate (rel.) at a spiked concentration of 2.0 µg, 10.0 µg, or 40.0 µg BPS per litre of urine and n = 10 determinations	r = 91.7%, 92.7%, or 97.2%
Detection limit:	0.02 µg BPS per litre of urine	
Quantitation limit:	0.05 µg BPS per litre of urine	

2 General information on bisphenol A, bisphenol F, and bisphenol S

Bisphenols are a group of chemical substances which are derived from the basic structure of two *p*-hydroxyphenyl groups bound by carbon, sulfur, or a benzene ring. The bisphenols described in this analytical method – BPA (4-[2-(4-hydroxyphenyl)propan-2-yl]phenol), BPF (4-[(4-hydroxyphenyl)methyl]phenol), and BPS (4-(4-hydroxyphenyl) sulfonylphenol) – are used as co-monomers for the manufacture of engineering-grade plastics (e.g. polycarbonates, polyethers, polysulfones, polyethersulfones, and epoxy resins) (Bousoumah et al. 2021). Engineering-grade plastics are resistant to high temperatures and have a wide spectrum of applications in the automotive industry as well as in the food and household sectors. BPA and BPS are furthermore used in paper chemicals, e.g. as colour developers in thermal papers (Björnsdotter et al. 2017). BPS is additionally used in leather-tanning agents (Ho et al. 2017). As a result of these many and varied areas of application, bisphenol exposure can take place in workplaces which manufacture or process bisphenols as well as by environmental exposure via contact with plastic products and thermal paper. The structural formulas of BPA, BPF, and BPS are presented in Figure 1.

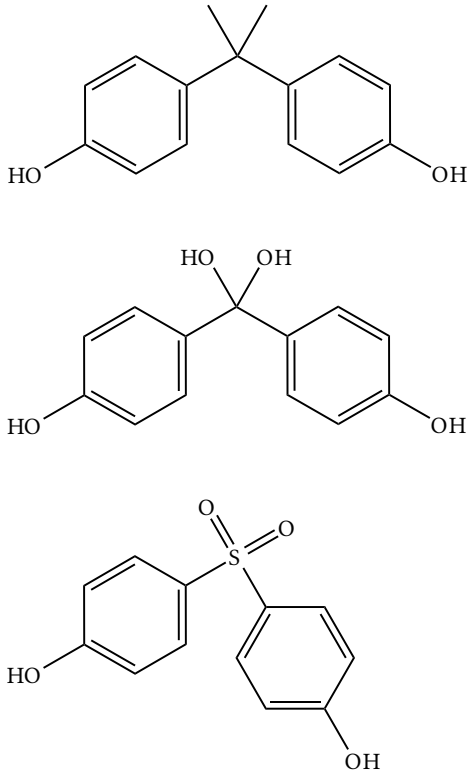

Fig. 1 Structural formulas of BPA, BPF, and BPS

BPA Numerous review articles on BPA have been published (EFSA 2007; Hartwig 2015). After oral ingestion in humans, BPA undergoes first-pass metabolism to form glucuronide conjugates, which are rapidly excreted with the urine. With oral administration of 5 mg (approx. 0.07 mg/kg body weight), the elimination half-life was less than six hours (Völkel et al. 2002). Only a small proportion of BPA is sulfated or excreted after hydroxylation of the phenyl ring (Völkel et al. 2002). Figure 2 shows a simplified metabolism scheme for bisphenols.

For BPA, the Commission has derived a biological guidance value (*Biologischer Leitwert*, BLW) of 80 mg/l urine (after hydrolysis) as well as a maximum workplace concentration (*maximale Arbeitsplatzkonzentration*, MAK) of 5 mg/m^3 I (inhalable fraction) (Peak Limitation Category I with an excursion factor of 1). The local effects on the respiratory tract were relevant for the establishment of the MAK value. Due to the substance's (photo)contact-sensitising effect in humans, it was additionally designated with "SP". Damage to the embryo or foetus is unlikely when the MAK value is observed (Pregnancy Risk Group C). Details on the toxicological evaluation can be found in the corresponding documentations by the Commission (DFG 2022; Greim 1999, 2000; Hartwig 2015; Nasterlack and Csanády 2016). The potential reprotoxic effect of bisphenol A is under discussion based on the substance's weak oestrogenic effects.

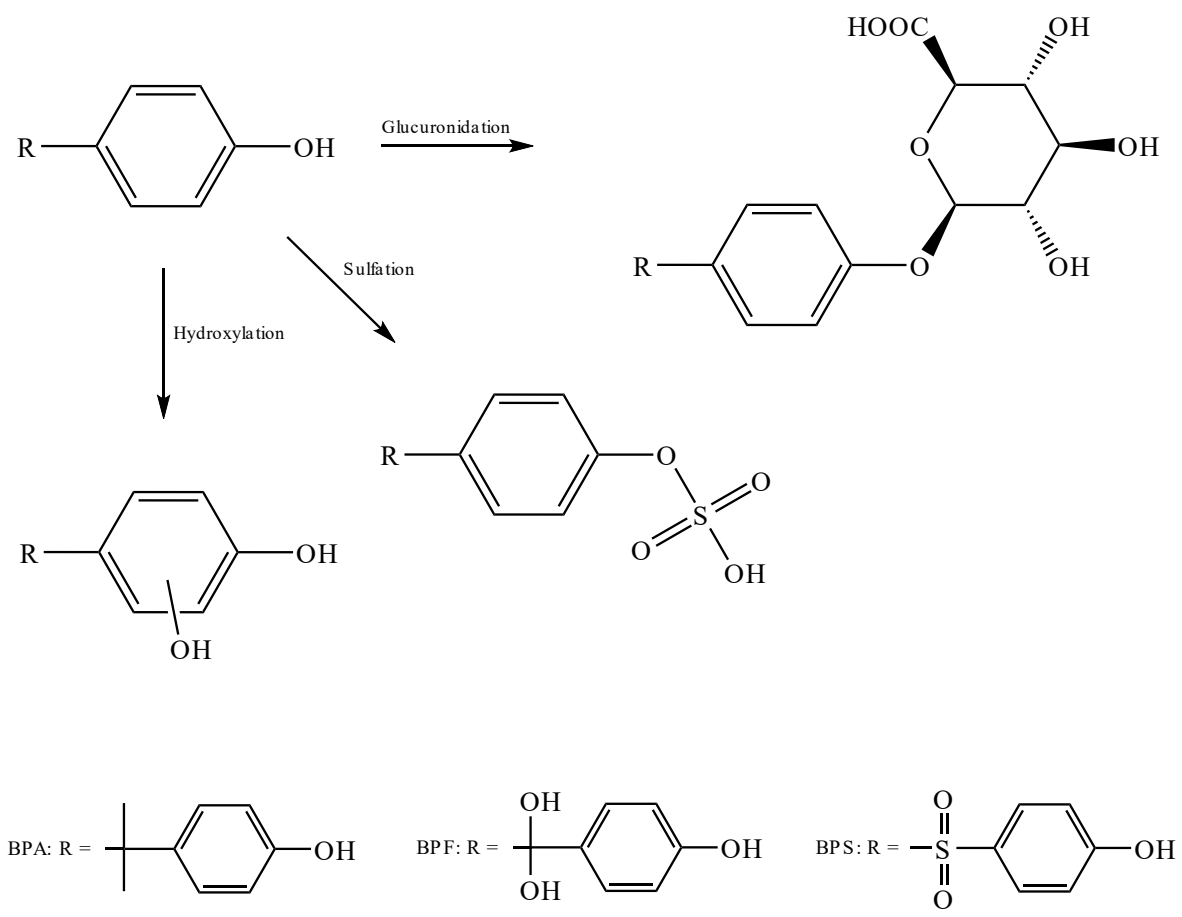

Fig. 2 Simplified metabolism scheme for bisphenols

BPF Animal experiments in rats have shown that 43–54% of an orally administered dose of BPF (7 or 100 mg/kg body weight) are excreted with the urine and 15–20% are excreted with the faeces. The main metabolite of BPF is its sulfate conjugate (Cabaton et al. 2006). The glucuronide as well as hydroxylated compounds such as *meta*- and *ortho*-dihydroxy-BPF have likewise been detected (Cabaton et al. 2008). The Commission has not published any documentations on BPF. The fact that BPF in (predominantly sweet and medium-spicy) mustard can be formed endogenously from sinalbin (Reger et al. 2017; Zoller et al. 2016) must be taken into account when assessing BPF concentrations in biological material.

BPS Numerous *in vitro* studies (Gramec Skledar et al. 2015; Gramec Skledar and Peterlin Mašič 2016; Grignard et al. 2012; Le Fol et al. 2015) as well as a human-metabolism study following oral administration (Oh et al. 2018) are available for BPS. Like the structurally similar BPA, BPS is rapidly and nearly completely glucuronidated or sulfated at the hydroxyl groups (Gramec Skledar and Peterlin Mašič 2016). Moreover, a study based on a microsomal assay described a 1,2-diol compound (Gramec Skledar et al. 2016). A human study by Oh et al. (2018) investigated serum concentrations and excretion of glucuronidated/sulfated and unchanged d_4-bisphenol S in urine after oral administration in four men and three women over a period of 48 hours. The half-lives of glucuronidated/sulfated and unchanged d_4-BPS in urine were observed to be 6.81 hours and 4.06 hours, respectively. In total, 82% (range: 59–104%) of the administered dose was excreted within 48 hours, 2.5% (range: 0.9–4.1%) of this amount as unchanged compound.

The Commission has derived a biological reference value (*Biologischer Arbeitsstoff-Referenzwert*, BAR) of 1 µg BPS (after hydrolysis)/l urine (Bader et al. 2020).

The method presented herein determines the concentrations of BPA, BPF, and BPS in urine for the evaluation of overall burden following occupational and/or environmental exposure.

Table 1 shows representative concentrations of BPA, BPF, and BPS in the urine of the non-occupationally exposed general population. Data on renal bisphenol excretion following occupational exposure are summarised in Table 2.

Tab. 1 BPA, BPF, and BPS background levels in urine samples from the general population

Collective (number of persons); country; study	Analyte	LOQ [µg/l]	Concentration [µg/l] ([µg/g creatinine])		Reference
			Geometric mean	Range	
Adults (116); China	BPA	0.1	1.1 (1.03)	<LOQ–29.4 (<LOQ–58.1)	Zhang et al. 2011
Adults (21); India	BPA	0.1	1.59 (2.51)	0.25–5.60 (0.31–39.2)	
Adults (36); Japan	BPA	0.1	0.84 (0.67)	0.10–23.2 (0.05–16.0)	
Adults (89); China	BPS	0.02	0.226 (0.223)	<LOQ–3.16 (<LOQ–6.64)	Liao et al. 2012
Adults (38); India	BPS	0.02	0.072 (0.098)	<LOQ–0.088 (<LOQ–4.72)	
Adults (36); Japan	BPS	0.02	1.18 (0.933)	0.147–0.57 (0.148–14.0)	
Adults (31); USA	BPS	0.02	0.299 (0.304)	<LOQ–21.0 (<LOQ–7.57)	
Children and adolescents, 3–17 a (515); Germany; GerES V	BPA	0.5	1.905 (1.669)	–	Tschersich et al. 2021
Adults, female (889); Italy	BPA	0.523	5.79 (5.36)	3.30–10.1 (3.14–9.73)[a]	Carli et al. 2022
Children, 6 a (488); Korea	BPA	0.212[b]	1.629	0.150–153	Lee et al. 2022
Children, 6 a (115); Korea	BPF	0.074[b]	0.157	<LOD–2.31	
Children, 6 a (205); Korea	BPS	0.020[b]	0.075	<LOD–21.5	
Children and adolescents, 6–17 a (745); USA; NHANES 2013–2014	BPA	0.2[b]	1.23[c]	0.63–2.36[a]	Liu et al. 2019
	BPF	0.2[b]	0.30[c]	0.14–0.93[a]	
	BPS	0.1[b]	0.28[c]	0.12–0.66[a]	
Adults (130); Saudi Arabia	BPA	–	4.92[c]	–	Asimakopoulos et al. 2016
	BPF	–	0.19[c]	–	
	BPS	–	13.3[c]	–	
Adults (1690); USA; NHANES 2015–2016	BPA	0.2[b]	1.08	–	CDC 2022 a
Adults (1812); USA; NHANES 2013–2014	BPF	0.2[b]	0.541	–	CDC 2022 b
Adults (1690); USA; NHANES 2015–2016	BPS	0.1[b]	0.496	–	CDC 2022 c

LOQ: limit of quantitation, LOD: limit of detection
[a] 25^{th}–75^{th} percentile
[b] LOD
[c] Median

Tab. 2 BPA and BPS concentrations in urine samples after occupational exposure

Collective (number of persons); sampling time; country	Analyte	LOD [µg/l]	Geometric mean (range) [µg/g creatinine]	Reference
Cashiers (33); after end of shift; USA	BPA	0.07–0.25	2.76 (0.44–187.96)	Thayer et al. 2016
	BPS	0.01–0.02	1.35 (0.29–20.38)	
Employees of a paint factory (9); after end of shift; Finland	BPA	0.1[a]	9.2 (0.9–187)	Heinälä et al. 2017
Employees of a thermal-paper factory (21); after end of shift; Finland	BPA	0.1[a]	35.5 (1.3–1001)	

Tab. 2 *(continued)*

Collective (number of persons); sampling time; country	Analyte	LOD [µg/l]	Geometric mean (range) [µg/g creatinine]	Reference
Cashiers (90); France	BPA	–	7.10 (0.68–704)	Ndaw et al. 2016
Cashiers (female) (17); USA	BPA	0.4	2.8	Braun et al. 2011
Employees of epoxide-resin manufacture (28); China	BPA	–	31.96 (4.61–1253.69)	Wang et al. 2012
Firefighters (101); USA	BPA	–	1.40 (up to 21.1)	Waldman et al. 2016

LOD: limit of detection
[a] limit of quantitation

3 General principles

After adding the internal standards (d_8-BPA, d_{10}-BPF, and d_8-BPS) to the urine samples, the samples are enzymatically hydrolysed. The analytes are then enriched by DLLME and simultaneously separated from matrix components. The analytes are separated by liquid chromatography and detected by tandem mass spectrometry using electrospray ionisation. Quantitative evaluation is carried out via external calibration in water.

4 Equipment, chemicals, and solutions

4.1 Equipment

- Ultra-high-performance liquid-chromatographic system (e.g. Waters UPLC I-Class comprised of a binary solvent manager, a sample manager, and a column oven, Waters GmbH, Eschborn, Germany)
- Mass-spectrometric detector (e.g. Waters Xevo-TQS, Waters GmbH, Eschborn, Germany) with MassLynx™ mass-spectrometric software
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Laboratory shaker (e.g. Multi Reax, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany)
- Vacuum concentrator with a cooling trap (e.g. Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany)
- Centrifuge (e.g. ROTINA 380R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Heating block (e.g. Barkey GmbH & Co. KG, Leopoldshöhe, Germany)
- 1000-ml glass bottles with dispensers (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 100-ml plastic bottle (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 100-ml amber glass bottles (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- Various volumetric flasks (e.g. witeg Labortechnik GmbH, Wertheim, Germany)
- Glass centrifuge tubes with screw caps (e.g. DURAN Group GmbH, Mainz, Germany)
- Various pipettes with matching pipette tips (e.g. Eppendorf AG, Hamburg, Germany)
- Transfer pipettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- 1.8-ml sample vials with 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)
- Water-purification system (e.g. Milli-Q® Direct Water Purification System, Merck KGaA, Darmstadt, 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.

- Acetone for HPLC (e.g. No. 10417440, Fisher Scientific GmbH, Schwerte, Germany)
- Dichloromethane for liquid chromatography, LiChrosolv® (e.g. Supelco®, No. 1.06044, Merck KGaA, Darmstadt, Germany)
- Acetic acid, puriss. (e.g. No. 33209-M, Merck KGaA, Darmstadt, Germany)
- β-Glucuronidase/arylsulfatase, 4.5 U/ml (e.g. SKU 10127698001, Roche Diagnostics GmbH, Mannheim, Germany)
- Methanol for liquid chromatography, LiChrosolv® (e.g. Supelco®, No. 1.06007, Merck KGaA, Darmstadt, Germany)
- Sodium hydroxide, pellets, EMSURE® (e.g. Supelco®, No. 1.06469, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Milli-Q® Direct Water Purification System, Merck KGaA, Darmstadt, Germany)
- BPA, ≥ 99% (e.g. Supelco®, No. 239658, Merck KGaA, Darmstadt, Germany)
- d_8-BPA (ring-d_8), 98% (e.g. No. D75807, Medical Isotopes, Inc., Pelham NH, USA)
- BPF, ≥ 98.0% (e.g. Supelco®, No. 51453, Merck KGaA, Darmstadt, Germany)
- d_{10}-BPF, 98.0% (e.g. No. B519557, Toronto Research Chemicals, Toronto, Canada)
- BPS, ≥ 98.0% (e.g. Supelco®, No. 43034, Merck KGaA, Darmstadt, Germany)
- d_8-BPS, 98.0% (e.g. No. B447392, Toronto Research Chemicals, Toronto, Canada)

4.3 Solutions

- Sodium hydroxide solution (about 5 mol/l)
  100 ml of ultra-pure water are placed in a beaker and 20 g of sodium hydroxide are dissolved under cooling with ice. The sodium hydroxide solution is transferred into a 100-ml amber glass bottle.
- Sodium acetate buffer (60 mmol/l, pH = 5.0)
  About 800 ml of ultra-pure water are placed in a 1000-ml beaker and 3.42 ml of glacial acetic acid are added by pipetting. The solution is then adjusted to a pH of 5.0 by adding sodium hydroxide solution, transferred into a 1000-ml volumetric flask, and made up to one litre.
- 15% Methanol (v : v)
  1.5 ml of methanol are pipetted into a 10-ml volumetric flask. The flask is made up to the mark with ultra-pure water.

4.4 Internal standards (ISTDs)

- d_8-BPA stock solution (1000 mg/l)
  10 mg of d_8-BPA are weighed into a 10-ml volumetric flask and dissolved in methanol. The flask is then made up to the mark with methanol.
- d_{10}-BPF stock solution (100 mg/l)
  For the d_{10}-BPF stock solution, 1 mg of the standard is dissolved in methanol and transferred into a 10-ml volumetric flask. The flask is then made up to the mark with methanol.
- d_8-BPS stock solution (100 mg/l)
  For the d_8-BPS stock solution, 1 mg of the standard is dissolved in methanol and transferred into a 10-ml volumetric flask. The flask is then made up to the mark with methanol.

- ISTD working solution (d_8-BPA: 5 mg/l, d_{10}-BPF: 5 mg/l, d_8-BPS: 1 mg/l)
  In a 10-ml volumetric flask, 50 µl of the d_8-BPA stock solution, 500 µl of the d_{10}-BPF stock solution, and 100 µl of the d_8-BPS stock solution are added by pipetting. The flask is then made up to the mark with methanol.
- ISTD spiking solution (d_8-BPA: 0.5 mg/l, d_{10}-BPF: 0.5 mg/l, d_8-BPS: 0.1 mg/l)
  1000 µl of the ISTD working solution are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with ultra-pure water.

The ISTD stock solutions can be stored at –18 °C for one year. The ISTD working solution can be stored at 4 °C for three months. The ISTD spiking solution should be freshly prepared.

4.5 Calibration standards

- Stock solutions (Single-substance standards of BPA, BPF, and BPS; 1000 mg/l)
  The stock solutions for BPA, BPF, and BPS are prepared as single-substance standards. To this end, 10 mg each of BPA, BPF, and BPS are weighed into individual 10-ml volumetric flasks and dissolved in 5 ml of methanol. The flasks are then made up to the mark with methanol.
- Working solution (Multi-analyte standard of BPA, BPF, and BPS; 10 mg/l)
  Of each stock solution, 100 µl are pipetted into a 10-ml volumetric flask. The flask is then made up to the mark with methanol.
- Spiking solution I (1.0 mg/l)
  1 ml of the working solution is pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.
- Spiking solution II (0.1 mg/l)
  100 µl of the working solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.
- Spiking solution III (0.01 mg/l)
  10 µl of the working solution are pipetted into a 10-ml volumetric flask, which is then made up to the mark with ultra-pure water.

The stock solutions can be stored at –18 °C for one year. The working solution can be stored at 4 °C for three months. The spiking solutions should be freshly prepared.

Since the slopes of the calibration curves in urine do not differ from the slopes in water (see Section 8), the calibration standards are prepared in ultra-pure water. The calibration standards are prepared by spiking water with the spiking solutions as indicated in the pipetting scheme given in Table 3. The calibration standards are processed analogously to the urine samples per Section 5.2. The concentration range of the calibration standards can be extended up to 100 µg/l for BPA and BPF or up to 50 µg/l for BPS if required.

Tab. 3 Pipetting scheme for the preparation of calibration standards for the determination of BPA, BPF, and BPS in urine

Calibration standard	Spiking solution I [µl]	Spiking solution II [µl]	Spiking solution III [µl]	Water [µl]	Concentration [µg/l]
0	–	–	–	500	0.0
1	–	–	5	495	0.1
2	–	–	10	490	0.2
3	–	–	25	475	0.5
4	–	–	50	450	1.0
5	–	10	–	490	2.0

Tab. 3 *(continued)*

Calibration standard	Spiking solution I [µl]	Spiking solution II [µl]	Spiking solution III [µl]	Water [µl]	Concentration [µg/l]
6	–	25	–	475	5.0
7	–	50	–	450	10.0
8	10	–	–	490	20.0

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in urine cups and stored at 4 °C until sample preparation. For longer storage (> 3 days), the urine samples should be frozen at –18 °C.

5.2 Sample preparation

The urine samples are brought to room temperature and thoroughly mixed. 500 µl of the sample are pipetted into a glass centrifuge tube and mixed with 100 µl of the ISTD spiking solution and 1 ml of the sodium acetate buffer. After adding 10 µl of glucuronidase/arylsulfatase, the preparation is then incubated in a heating block for three hours at 37 °C. The samples are then diluted with 2.5 ml of ultra-pure water. For liquid-liquid extraction, 750 µl of acetone and 750 µl of dichloromethane are added using a syringe or pipette. Subsequently, the preparation is shaken intensely for 10 seconds (emulsification). After 10 minutes, the preparation is centrifuged for 20 minutes at 4000 rpm (2600 × *g*). Of the organic phase, 500 µl are transferred into a 1.8-ml sample vial and concentrated to dryness in a vacuum concentrator (1000 rpm, 40 °C, 20 min). The residue is dissolved in 100 µl of 15% methanol and transferred into a 1.8-ml sample vial with a micro insert; the sample vial is then sealed.

6 Operational parameters

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

6.1 Ultra-high-performance liquid chromatography

Separation column:	Agilent Zorbax SB-C8 RRHD (1.8 µm; 2.1 × 150 mm)
Separation principle:	Reversed phase
Injection volume:	10 µl
Column temperature:	40 °C
Flow rate:	0.2 ml/min
Eluent:	A: Water B: Methanol
Runtime:	20 min
Gradient program:	see Table 4

Tab. 4 Gradient program for the determination of BPA, BPF, and BPS in urine

Time [min]	Eluent A [%]	Eluent B [%]
0.0	85	15
15.0	10	90
18.0	10	90
18.1	85	15
20.0	85	15

6.2 Tandem mass spectrometry

Ionisation: Electrospray, negative (ESI–)

Detection mode: Multiple Reaction Monitoring (MRM)

Capillary: 2.90 kV

Cone: –76 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

Parameter-specific settings: see Table 5

Instrument-specific parameters must be individually ascertained and adjusted by the user for the UPLC-MS/MS system used. The instrument-specific parameters given in this section were determined and optimised for the system used here (Waters Acquity UPLC, Waters Xevo-TQS tandem mass spectrometer, both from Waters GmbH, Eschborn, Germany).

Two fragment-ion transitions were selected for each of the analytes; one transition is used for quantification (quantifier) and the other for confirmation (qualifier). For the ISTDs, only one mass transition was used in each case. The selected fragment-ion transitions are summarised with the retention times in Table 5.

Tab. 5 Retention times, mass transitions and MRM parameters for the determination of BPA, BPF, and BPS in urine

Analyte/ISTD	Retention time [min]	Mass transition [m/z]	Status	Cone [V]	Collision energy [V]	Dwell time [s]
BPA	12.25	226.92 ⟶ 133.03	Quantifier	2	26	0.025
	12.25	226.92 ⟶ 211.99	Qualifier	2	18	0.025
BPA-d_8	12.19	234.99 ⟶ 220.05	ISTD	50	22	0.025
BPF	10.73	198.89 ⟶ 104.97	Quantifier	62	22	0.025
	10.73	198.89 ⟶ 76.95	Qualifier	62	20	0.025
BPF-d_{10}	10.62	208.83 ⟶ 110.03	ISTD	28	20	0.025
BPS	8.67	248.92 ⟶ 91.96	Quantifier	50	34	0.025
	8.67	248.92 ⟶ 107.95	Qualifier	50	26	0.025
BPS-d_8	8.61	256.86 ⟶ 111.98	ISTD	2	28	0.025

7 Analytical determination

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

Representative chromatograms for the individual analytes are presented in Figure 3.

8 Calibration

The calibration standards are prepared as described in Section 4.5, then processed and analysed analogously to the urine samples. The calibration curve is generated by plotting the quotients of the peak area of the analyte and the corresponding ISTD against the spiked analyte concentrations. With the analytical device used, a linear measurement range was achieved from the quantitation limit up to 50 µg/l (BPS) or 100 µg/l (BPA and BPF).

During method development, it was observed that the slopes of the calibration curves in urine do not differ from the slopes in water (Figure 4). For this reason, the calibration standards can be prepared in water; this approach has the added advantage that any background levels present in urine do not have to be taken into account. If reagent blank values do arise, these must be subtracted from the analytical results.

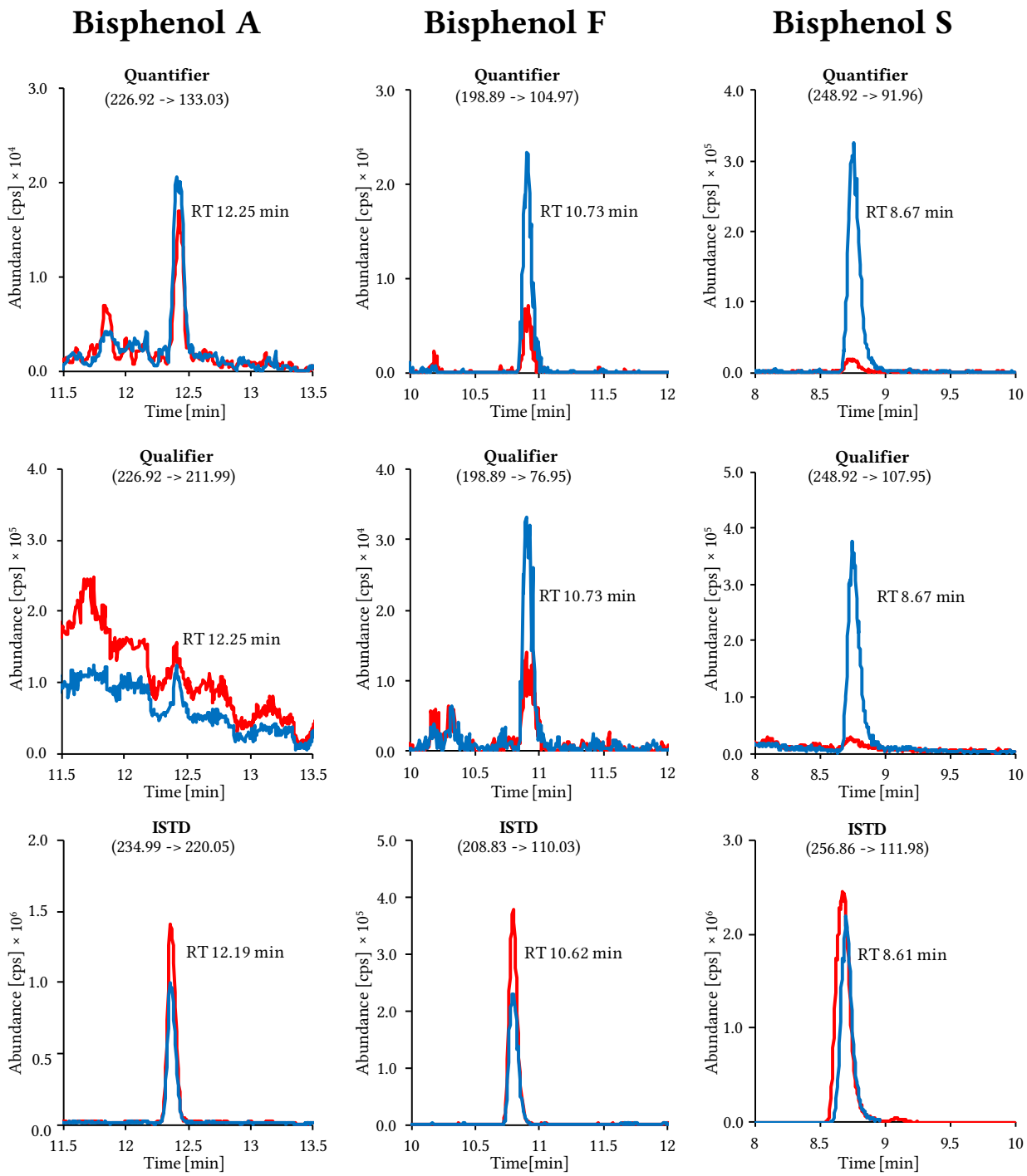

Fig. 3 Chromatograms of an unspiked human urine sample (red line; BPA: 0.87 µg/l, BPF: 0.31 µg/l, BPS: 0.07 µg/l) as well as a spiked human urine sample (blue line; BPA: 2 µg/l, BPF: 2 µg/l, BPS: 2 µg/l)

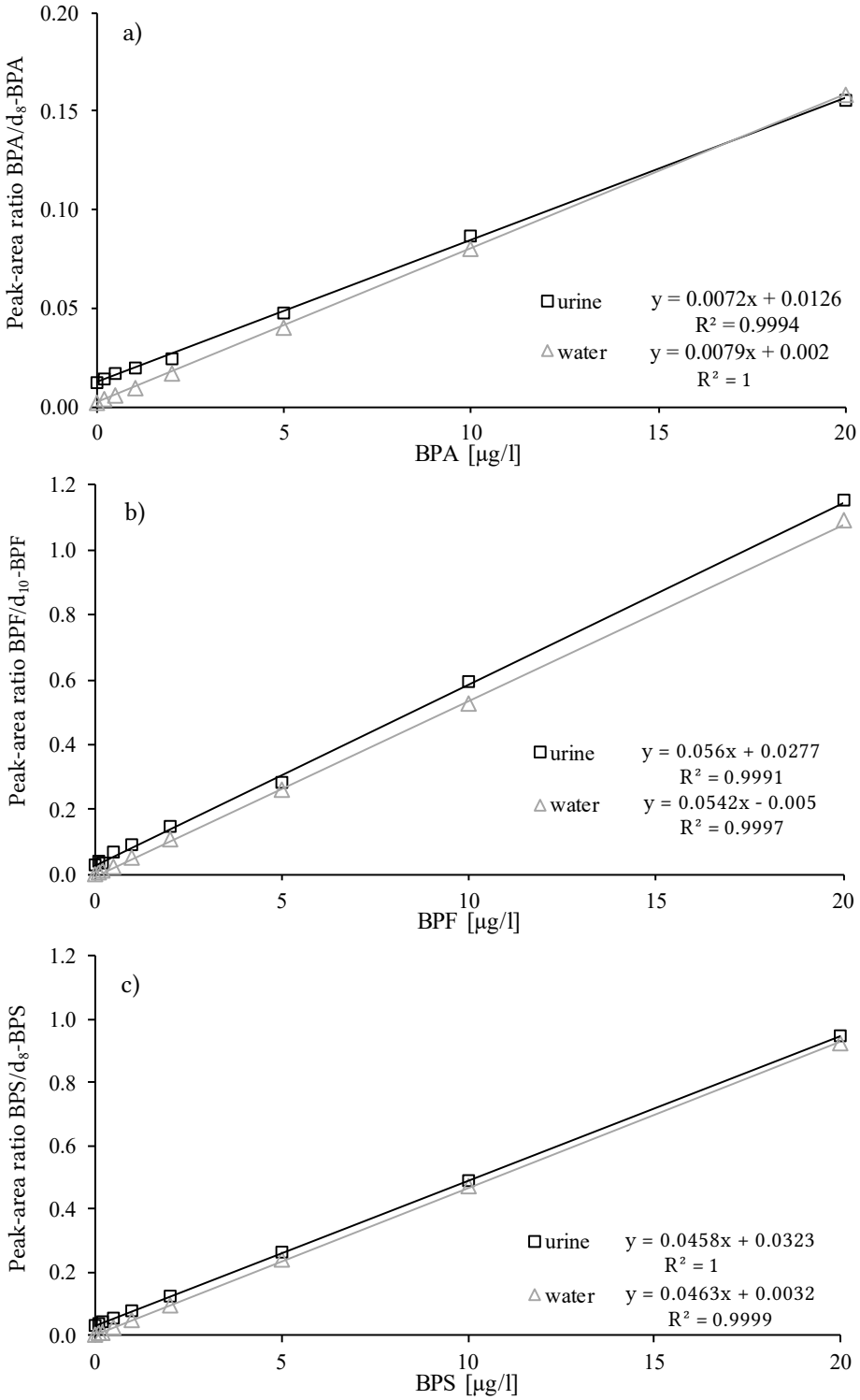

Fig. 4 Calibration curves in water and in urine for the determination of a) BPA, b) BPF, and c) BPS

9 Calculation of the analytical results

The peak-area ratio of the analyte and the corresponding ISTD is inserted into the calibration function of the analytical run in question in order to calculate the analyte concentration of a sample in µg/l. If the measured result exceeds the calibration range, the 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 concentrations are processed and analysed as part of each analytical run.

At present, no control material is commercially available for BPF and BPS; for this reason, the control material must be prepared by the in-house laboratory. To this end, pooled urine samples are spiked with three different analyte concentrations, aliquoted, and frozen at –18 °C until use. At the same time, at least one reagent blank is included in each analytical run (processing ultra-pure water instead of urine) to enable detection of any potential interferences from the reagents.

For external quality assurance, it is possible to participate in the interlaboratory-comparison program for occupational- and environmental-medical toxicological analyses G-EQUAS (German External Quality Assessment Scheme, https://app.g-equas.de/web/) offered by the German Society of Occupational and Environmental Medicine (*Deutsche Gesellschaft für Arbeitsmedizin und Umweltmedizin*, DGAUM) (see Section 11.2).

11 Evaluation of the method

The reliability of the 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, control urines were spiked with the analytes at four different concentrations. In one day, ten aliquots of each of these urines were processed and analysed in parallel. The within-day precision data thus obtained are summarised in Table 6.

Tab. 6 Within-day precision for the determination of BPA, BPF, and BPS in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Measured concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
BPA	0.4	0.78	9.8	22.2
	2.0	2.22	4.5	10.2
	10.0	9.34	10.8	24.4
	40.0	38.2	7.9	17.9

Tab. 6 *(continued)*

Analyte	Spiked concentration [µg/l]	Measured concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
BPF	0.4	0.29	13.4	30.3
	2.0	2.01	7.7	17.4
	10.0	9.59	5.5	12.4
	40.0	38.8	4.8	10.9
BPS	0.4	0.40	2.9	6.6
	2.0	1.90	3.3	7.5
	10.0	9.35	3.2	7.2
	40.0	39.0	6.2	14.0

Day-to-day precision

To determine day-to-day precision, the same control urines were used as for the determination of within-day precision. Aliquots of these urines were processed and analysed on six different days. The precision data thus calculated are given in Table 7.

Tab. 7 Day-to-day precision for the determination of BPA, BPF, and BPS in urine (n = 6)

Analyte	Spiked concentration [µg/l]	Measured concentration [µg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
BPA	0.4	0.70	15.5	39.9
	2.0	2.04	8.4	21.6
	10.0	8.29	9.2	23.7
	40.0	36.8	12.5	32.1
BPF	0.4	0.31	12.9	33.2
	2.0	1.97	7.3	18.8
	10.0	9.15	6.8	17.5
	40.0	40.4	10.7	27.5
BPS	0.4	0.40	7.4	19.0
	2.0	1.93	2.8	7.2
	10.0	8.74	4.9	12.6
	40.0	36.8	8.2	21.1

11.2 Accuracy

The accuracy of the method was investigated by analysing spiked pooled urine (2.0 µg/l, 10.0 µg/l, and 40.0 µg/l) while accounting for background levels. The relative recoveries thus obtained are presented in Table 8.

Tab. 8 Accuracy for the determination of BPA, BPF, and BPS in urine (n = 10)

Analyte	Spiked concentration [µg/l]	Rel. recovery r [%]
BPA	2.0	85.2
	10.0	88.2
	40.0	94.1

Tab. 8 *(continued)*

Analyte	Spiked concentration [µg/l]	Rel. recovery *r* [%]
BPF	2.0	101
	10.0	95.9
	40.0	97.1
BPS	2.0	91.7
	10.0	92.7
	40.0	97.2

The accuracy of the method was further proven by the developer of the method through successful participation in external quality-assurance programs (interlaboratory comparisons as part of the European Human Biomonitoring project "HBM4EU" and the German External Quality Assessment Scheme by the German Society of Occupational and Environmental Medicine (G-EQUAS), Jäger 2020). Table 9 shows exemplarily results of the interlaboratory comparisons from 2019 that were obtained using the method presented herein.

Tab. 9 Interlaboratory-comparison results for the determination of BPA, BPF, and BPS in urine

Trial	Parameter	Result [µg/l]	Nominal value [µg/l]	Deviation from nominal value [%]
HBM4EU – Round 2	BPA	1.93	1.89	2
		6.85	7.0	–2
	BPF	0.46	0.38	21
		3.66	3.40	8
	BPS	1.13	0.95	12
		6.07	5.47	11
HBM4EU – Round 3	BPA	0.94	1.10	–15
		6.98	8.40	–17
	BPF	0.18	0.23	–22
		3.76	3.35	12
	BPS	3.12	3.56	–13
		8.51	9.00	–5
63rd G-EQUAS	BPA	2.79	3.44	–19
		12.9	14.1	–8
64th G-EQUAS	BPA	1.18	0.89	32
		22.2	22.3	–1

To verify accuracy in individual urines, urine samples from ten individuals (creatinine concentrations in the range of 0.4–2.1 g/l) were each spiked at concentrations of 2.0 µg/l or 20 µg/l with BPA, BPF, and BPS and then processed and analysed as described above. The calculated mean relative recovery for the analytes is presented in Table 10.

Tab. 10 Relative recovery for the determination of BPA, BPF, and BPS in individual urines (n = 10)

Analyte	Concentration [µg/l]	Rel. recovery *r* [%]	
		Mean ± SD	Range
BPA	2.0	101 ± 7	87–115
	20.0	98 ± 5	91–104

Tab. 10 *(continued)*

Analyte	Concentration [µg/l]	Rel. recovery *r* [%]	
		Mean ± SD	Range
BPF	2.0	102 ± 15	79–126
	20.0	104 ± 12	82–118
BPS	2.0	98 ± 5	91–110
	20.0	102 ± 5	97–115

11.3 Matrix effects

The matrix effects which arose during measurement were investigated using six individual urines. The urine samples were processed and only spiked with the analytes at a low or high concentration (2 µg/l or 10 µg/l) directly before the actual LC-MS/MS analysis. Matrix effects were evaluated by comparing the corresponding peak areas with the signals of the same analyte amounts in pure solvent. The matrix factor (MF) and the ISTD-adjusted matrix factor ($MF_{adj.}$) were calculated from the results using Formulas 1 and 2, respectively. The coefficient of variation for the ISTD-adjusted matrix factor is less than 15% for all three analytes at both concentration levels (see Table 11).

Formula 1:

$$MF = \frac{Peak\ area_{Urine}}{Peak\ area_{Solvent}}$$

Formula 2:

$$MF_{adj.} = \frac{MF_{Analyte}}{MF_{ISTD}}$$

Tab. 11 ISTD-adjusted matrix factors for the determination of BPA, BPF, and BPS in urine (n = 6)

Analyte	Spiked concentration [µg/l]	ISTD-adjusted matrix factors		
		Mean ± SD	Range	Coefficient of variation [%]
BPA	2.0	1.12 ± 0.08	0.99–1.22	6.9
	10.0	0.98 ± 0.05	0.91–1.04	5.2
BPF	2.0	0.92 ± 0.12	0.80–1.10	12.6
	10.0	0.93 ± 0.07	0.80–0.98	7.8
BPS	2.0	1.01 ± 0.09	0.97–1.19	8.5
	10.0	1.05 ± 0.07	0.96–1.18	7.0

11.4 Limits of detection and quantitation

The limits of detection and quantitation were determined according to DIN 32645 (DIN 2008). To this end, equidistant 10-point calibrations (concentration range of 0.05–0.5 µg/l in aqueous solution) were established, processed in conjunction with a reagent blank, and analysed (see Figure 5).

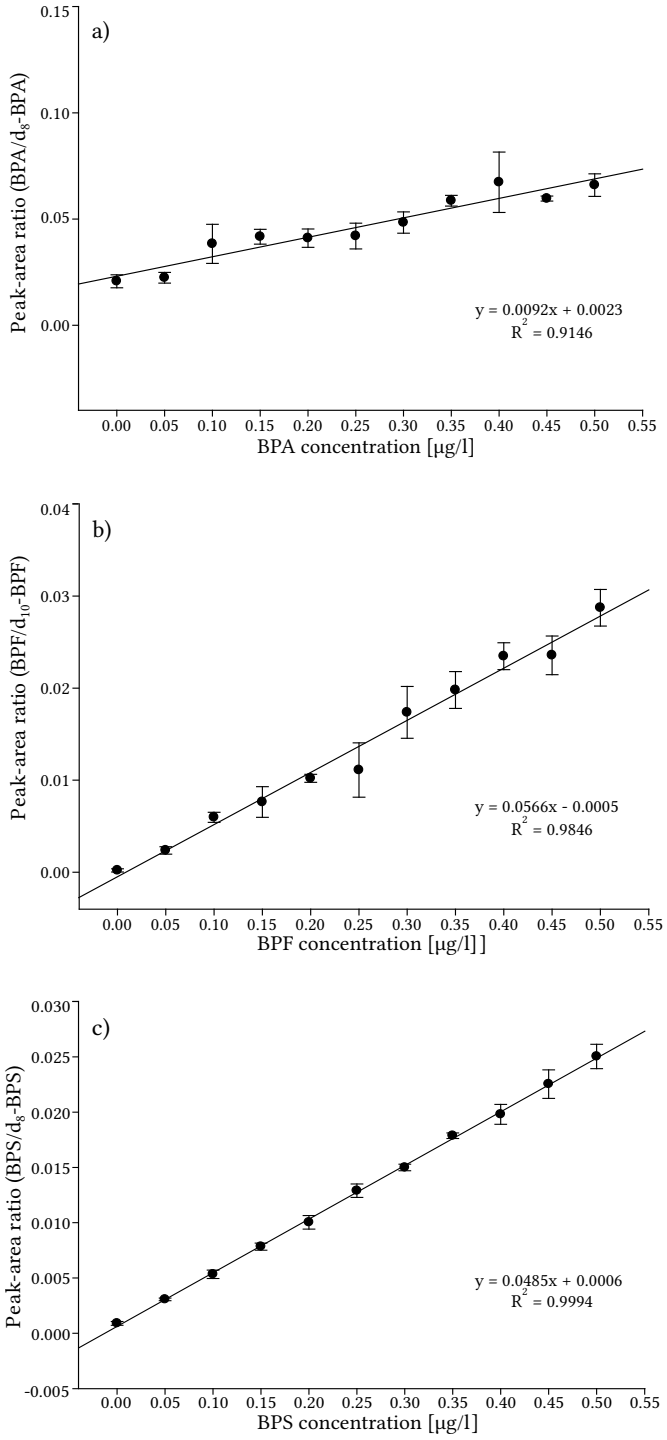

Fig. 5 10-point calibrations in the concentration range of 0.05–0.5 µg/l for the calculation of the detection and quantitation limits of a) BPA, b) BPF, and c) BPS

Per DIN 32645, the detection and quantitation limits were calculated from the standard deviation of the calibration function obtained at the blank value. Table 12 provides the limits of detection and quantitation obtained for the determination of BPA, BPF, and BPS in urine.

Tab. 12 Limits of detection and quantitation for the determination of BPA, BPF, and BPS in urine (n = 3)

Analyte	Detection limit [µg/l]	Quantitation limit [µg/l]
BPA	0.08	0.25
BPF	0.03	0.10
BPS	0.02	0.05

11.5 Carryover effects

Carryover effects in the chromatographic system were investigated by multiple injections of highly concentrated sample extracts (urine sample spiked with 100 µg analyte/l), followed by the injection of blank-value samples. After five injections of the highly concentrated sample each time, a reagent blank was injected and analysed twice. No interfering peaks were observed at the retention times of the analytes; likewise, no analyte peaks or increased peaks of ISTDs were observed in the reagent blank.

11.6 Sources of error

During method development, no significant risk of contamination was observed for the determination of BPA, BPF, and BPS in urine when using this method. While a blank value might be detected even in unspiked, aqueous samples, the concentrations are low and within the range of the detection limit. In order to recognise contamination caused by impurities in the chemicals or equipment used, a reagent blank should be included as part of each analytical run.

In general, the avoidance or minimisation of blank values is of great importance. This is shown by the fact that the method verifiers were not able to replicate the given quantitation limit for BPA. Moreover, excessively high values were sporadically obtained during the measurements for precision data; these results were deemed outliers. These problems, which arose during external method verification, show that the sensitive and precise determination of low bisphenol concentrations in urine, such as those found in the non-occupationally exposed general population, presents a considerable analytical challenge.

During method development and external verification, some serious matrix effects were observed, particularly for BPF, including both ion suppression and enhancement effects. The use of a deuterated internal standard (d_{10}-BPF) as well as the optimisation of elution conditions (reduction of the proportion of organic solvent in the injection and the extension of chromatographic runtime) have yielded satisfactory results. Nevertheless, strong enhancement effects have been observed in individual urine samples. For this reason, when implausibly high BPF concentrations are obtained, it is recommended to repeat analysis and perform quantitation by standard addition.

12 Discussion of the method

The method presented herein enables the sensitive and specific quantitation of BPA, BPF, and BPS in human urine. The use of isotope-labelled ISTDs increases precision by compensating for workup-related differences between individual samples. Moreover, due to the low quantitation limits of 0.25 µg/l (BPA), 0.10 µg/l (BPF), and 0.05 µg/l (BPS), this analytical procedure is suitable to measure background exposure in the non-occupationally exposed general population.

Instruments used Ultra-high-performance liquid-chromatographic system (Waters UPLC I-Class, Waters GmbH, Eschborn, Germany); Mass-spectrometric detector (Waters Xevo-TQS, 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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