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Benzo[*a*]pyrene – Determination of 3-hydroxybenzo[*a*]pyrene in urine by LC-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 German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area developed and verified the presented biomonitoring method. Benzo[*a*]pyrene (B[*a*]P) is a polycyclic aromatic hydrocarbon and is classified as carcinogenic to humans by the Commission. B[*a*]P originates, for example, from coal tar or from the incomplete combustion of organic material. The general population is primarily exposed through cigarette smoke, food, and ambient air. The aim of this work was to develop a selective method for the determination of 3-hydroxybenzo[*a*]pyrene (3-OH-B[*a*]P) in urine. The method is characterised by the use of glucuronidated standards, ascorbic acid as an antioxidant, and by the conversion of the analyte into a derivative which allows for sensitive measurement. The procedure has been comprehensively validated, and the reliability data have been confirmed by replication and verification of the procedure in a second, independent laboratory. For the determination of 3-OH-B[*a*]P, an internal standard is added to the urine samples, which undergo solid-phase extraction prior to analysis by liquid chromatography-tandem mass spectrometry. Quantitative evaluation is carried out via external calibration using pooled urine from persons with no known exposure to B[*a*]P. The good precision and accuracy data show that the method provides reliable and accurate analytical results. The method is both selective and sensitive, and the limit of detection of 17 pg/l urine is sufficient to determine both occupational exposure and exposure to B[*a*]P in the general population.

1 Characteristics of the method

Matrix	Urine
Analytical principle	Liquid chromatography with tandem mass spectrometry (LC-MS/MS)

Parameter and corresponding hazardous substance

Hazardous substance	CAS No.	Parameter	CAS No.
Benzo[*a*]pyrene	50-32-8	3-Hydroxybenzo[*a*]pyrene	13345-21-6

Reliability criteria

3-Hydroxybenzo[*a*]pyrene (3-OH-B[*a*]P)

Within-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 200 pg or 640 pg 3-OH-B[*a*]P per litre of urine and n = 5 determinations	s_w = 10.4% or 4.3% u = 28.9% or 11.9%
Day-to-day precision:	Standard deviation (rel.) Prognostic range at a concentration of 200 pg or 640 pg 3-OH-B[*a*]P per litre of urine and n = 6 determinations	s_w = 10.1% or 13.7% u = 26.0% or 35.2%
Accuracy:	Recovery (rel.) at a concentration of 200 pg or 640 pg 3-OH-B[*a*]P per litre of urine and n = 5 determinations	r = 102% or 92.6%
Detection limit:	16.7 pg 3-OH-B[*a*]P per litre of urine	
Quantitation limit:	50 pg 3-OH-B[*a*]P per litre of urine	

2 General information on benzo[*a*]pyrene

Polycyclic aromatic hydrocarbons (PAHs) are formed by the incomplete incineration of organic material (IARC 2010, 2012) and occur ubiquitously in the environment. Both in the workplace and in the environment, PAHs consistently arise as mixtures of up to 100 individual components. The relative distribution of individual PAHs in different substance mixtures varies depending on the source of exposure (IARC 2010).

The Commission has classified 17 PAHs, including benzo[*a*]pyrene (B[*a*]P), in Carcinogen Category 2 (considered to be carcinogenic to humans) because of their carcinogenicity in animal studies (DFG 2023; Hartwig 2012 c). B[*a*]P was also classified as a Category 2 germ cell mutagen and, due to the risk posed by percutaneous B[*a*]P resorption, which may contribute considerably to internal exposure, the substance was additionally designated with an "H" (DFG 2023).

Pyrolytic products from organic material which exhibit a high proportion of PAHs (such as lignite and coal tar, coal tar pitch, carbolineum, and raw coking-plant gas) have been designated as "carcinogenic to humans" (Category 1) by the Commission (DFG 2023). PAH-containing diesel-motor emissions have been classified in Carcinogen Category 2, and further pyrolytic products which contain individual PAHs evaluated by the Commission should be treated as Category 2 carcinogenic substances.

B[*a*]P has a unique position among occupationally and environmentally relevant PAHs (Hartwig 2012 b) and serves not only as an indicator for the presence of carcinogenic PAHs in emission measurements and air sampling, but is also viewed as a marker for carcinogenic exposure to the entire PAH group.

In its pure form, B[*a*]P presents as yellowish needles and platelets and is used as such only in the laboratory. B[*a*]P occurs almost ubiquitously in PAH mixtures (IFA 2023). In workplaces, B[*a*]P is not only a component of the pyrolytic products described above (Hartwig 2012 a), but is also formed in non-water miscible cooling lubricants if their mineral base oils are not sufficiently refined or hydrogenated (DFG 2023, Section X c).

Outside of the workplace, exposure to B[*a*]P-containing PAH mixtures may take place via the diet, by smoking, or by way of traffic exhaust gases and air pollution (Klotz 2021).

In the workplace, B[*a*]P is primarily absorbed, bound to particles, via the respiratory tract. Moreover, due to its lipophilia, the substance possesses good skin permeability, such that the dermal absorption of persons occupationally exposed to B[*a*]P presents another important route of uptake (Fustinoni et al. 2010). After oral uptake, about 10% of B[*a*]P is absorbed via the gastrointestinal tract in humans (Klotz 2021).

The lipophilic nature of PAHs prevents direct excretion of the absorbed compounds. The metabolism of the PAHs contributes to the conversion of the lipophilic compounds into more water-soluble, excretable metabolites (ATSDR 1995). Figure 1 shows selected biotransformation pathways of B[*a*]P (ATSDR 1995; Hartwig 2012 c; Klotz 2021; Marquardt and Schäfer 2004; Simpson et al. 2000). B[*a*]P which is absorbed via the pulmonary, transdermal, and gastrointestinal routes is converted, in Phase I metabolism, to intermediary epoxides by cytochrome P450 (CYP)-dependent monooxygenases, a reaction which represents bioactivation. With microsomal epoxide hydrolases, arene oxides can be metabolised to the vicinal dihydrodiol (Klotz 2021). A second oxidation of the dihydrodiol via CYP enzymes initiates metabolisation to the highly reactive dihydrodiol epoxide, which may react with endogenous macromolecules and is considered the ultimate carcinogen in B[*a*]P metabolism (Hartwig 2012 c; Marquardt and Schäfer 2004). In addition, the conversion of arene oxides into less reactive dihydrodiols and phenols as well as the metabolisation of the downstream products of dihydrodiols, dihydrodiol epoxides, to tetrols is possible (Klotz 2021). Finally, a hydroxylation of position 3 of the aromatic hydrocarbon skeleton catalysed by CYP1A1 and CYP1B1 can also take place, in which 3-hydroxybenzo[*a*]-pyrene (3-OH-B[*a*]P) is formed (Klotz 2021). The analytical determination of 3-OH-B[*a*]P presents one possibility to perform a B[*a*]P-related biomonitoring.

In Phase II of the biotransformation, the formed hydroxylated compounds are converted to sulfates and glucuronides by sulfotransferases and UDP-glucuronosyltransferases in order to increase water solubility (Klotz 2021).

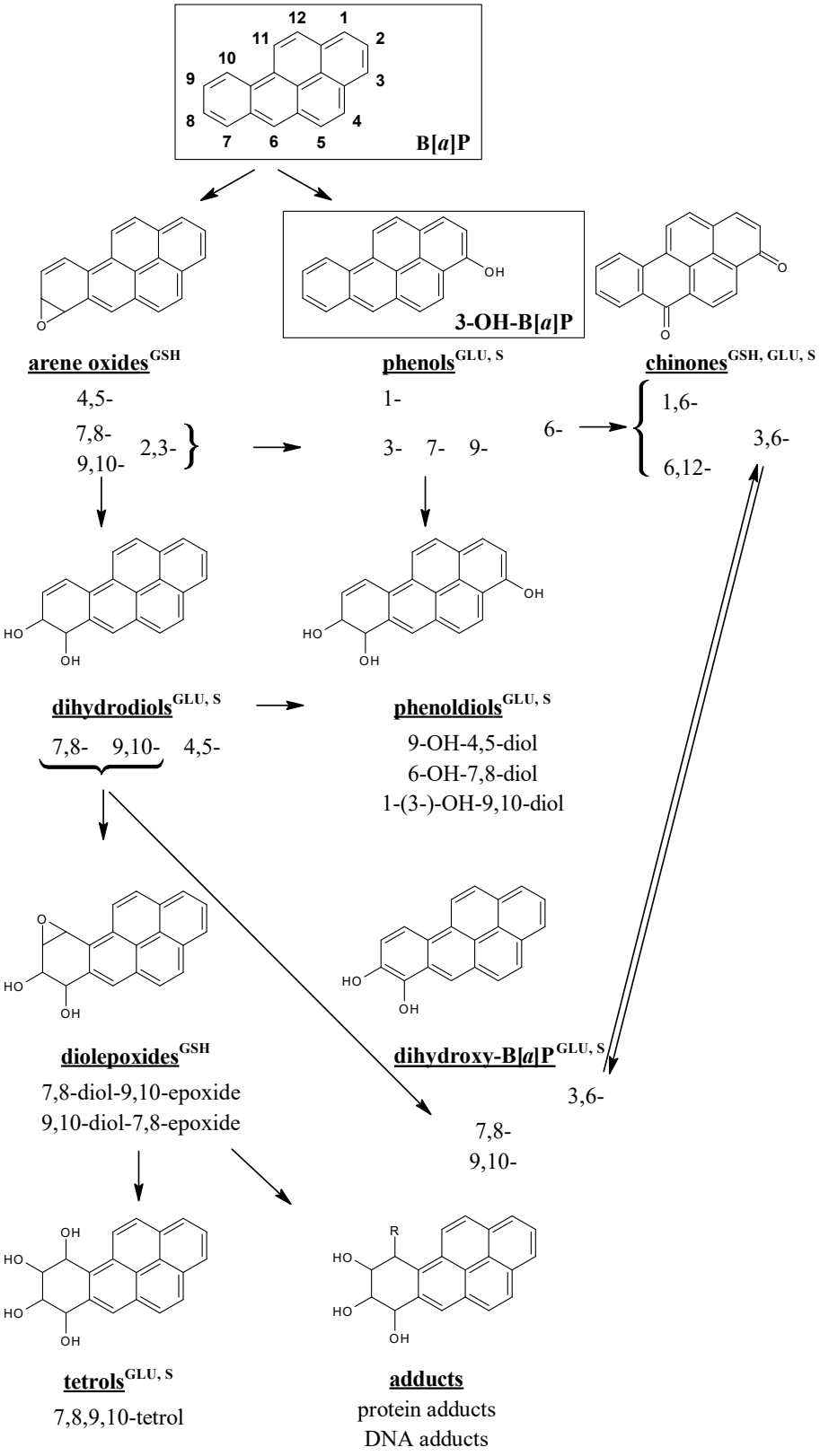

Fig. 1 Simplified metabolism scheme of B[a]P based on Klotz (2021) and Simpson (2000), modified. Conjugation is possible with GSH (glutathione), GLU (glucuronic acid), and S (sulfate).

Investigations on rats and mice have shown that only 0.1–0.35% of an applied B[*a*]P dose is excreted in the urine as 3-OH-B[*a*]P (Strickland et al. 1996). It can be assumed that the renally excreted portion of 3-OH-B[*a*]P is similarly low in humans. As a result, analytical procedures on the measurement of hydroxylated metabolites in urine must be specific and highly sensitive in order to detect the small amounts present in the urine with a sufficient level of diagnostic validity (Hartwig 2012 c). In urine samples from the general population, the largest proportion of OH-PAHs is present as conjugates and less than 10% in free form (Gaudreau et al. 2016). Consequently, it can be assumed that 3-OH-B[*a*]P is largely excreted in humans as conjugate with the urine. The unconjugated portion is difficult to identify, as free 3-OH-B[*a*]P is much less stable, because it is subject to oxidative degradation and strong adsorption effects (Nikolaou et al. 1984).

On average, the half-life for the renal excretion of 3-OH-B[*a*]P is 8.8 hours (range: 3.1–16.2 h) in humans (Lafontaine et al. 2004), such that a sampling at the beginning of the next shift is recommended (DFG 2023).

The Commission established exposure equivalents for carcinogenic substances (*Expositionsäquivalente für krebserzeugende Arbeitsstoffe*, EKA) in order to assess exposure in cases of occupational handling of B[*a*]P. The EKA for PAHs can be used to determine which urinary 3-OH-B[*a*]P concentrations are to be expected when B[*a*]P is exclusively inhaled (DFG 2023; Klotz 2021). Details on the toxicological evaluation can be found in the corresponding documentations published by the Commission (DFG 2023; Hartwig 2012 a, b, c; Klotz 2021).

The history of the analysis of 3-OH-B[*a*]P in human urine goes back several decades, with inconsistent results due to the difficulties mentioned above. Table 1 shows publications from 1994 to 2023 which describe analytical methods for the determination of 3-OH-B[*a*]P in urine and their application in occupationally exposed persons. An overview of further methods and data on the background exposure of occupationally unexposed persons is shown in Table 10 at the end of Section 12. For both the methods in Table 1 and Table 10, sample preparation included –unless otherwise indicated– enzymatic hydrolysis with glucuronidase/arylsulfatase from *Helix pomatia* as well as solid-phase extraction with C18 cartridges. To facilitate comparison of study results, the creatinine-related values given by the authors were in both tables converted to ng/l urine according to Bader et al. (2020). To this end, a creatinine concentration of 1.4 g/l urine was assumed for predominantly male subjects (workers) and 1.2 g/l urine was assumed for study collectives with balanced gender ratios.

Tab. 1 Published analytical methods for the determination of 3-OH-B[*a*]P in urine and their application on occupationally exposed individuals

Study collective (n, smoker status)	Method	LOD	LOQ	3-OH-B[*a*]P		References
				Mean ± SD	Range	
Coke-oven workers (14, incl. 9 S)	Derivatisation (methylation), HPLC-FD (LIF) and laser-excited Shpol'skii spectrometry (ShS)	8 ng/l (HPLC-FD) or 0.5 ng/l (ShS)	n. a.	4.83 ng/l[a] (HPLC-FD; n = 6, incl. 5 < LOQ), 17.41 ng/l (ShS; n = 8)	< LOQ–9 ng/l (HPLC-FD), 1.7–80 ng/l (ShS)	Ariese et al. 1994
Controls/office (6, incl. 3 S)				8.3 ng/l (ShS)	2.3–19.2 ng/l (ShS)	
Workers/manufacturing refractory materials (19)	Column switching with a phthalocyanine precolumn, HPLC-FD	6 ng/l	n. a.	37 ± 56 ng/g crea[b] (51.8 ± 78.4 ng/l)	3–198 ng/g crea (4.2–277 ng/l)	Gündel et al. 2000; Gündel and Angerer 2000
Workers (3)	Automated column-switching for purification/enrichment, HPLC-FD	0.1 ng/l	0.4 ng/l	5.03 ng/l	1.7–9.5 ng/l	Simon et al. 2000
Controls (3 S)				0.5 ng/l[c]	0.1–0.8 ng/l	
Controls (3 N, 2 < LOD)				0.1 ng/l[a]	< LOQ–0.2 ng/l	
Workers in six factories doing 45 different jobs (39)	Automated column-switching for purification/enrichment, HPLC-FD	0.1 ng/l	0.4 ng/l	–	0.14–35 nmol/mol crea (0.46–116 ng/l)	Lafontaine et al. 2004
Workers/asphalt pavers (75, incl. 32 S)	Liquid-liquid extraction with diethyl ether, HPLC-FD	60 ng/l	n. a.	< LOD	< LOD	Buratti et al. 2007
Workers/road construction (37, incl. 25 S)				< LOD	< LOD	
Workers (225, 3 < LOD)	Automated column-switching for purification/enrichment, HPLC-FD	0.05 ng/l	n. a.	1.74 ng/g crea (2.44 ng/l)	< LOD–19.53 ng/g crea (< LOD–27.3 ng/l)	Förster et al. 2008
Workers/converter-infeed (26)				3.69 ng/g crea (5.17 ng/l)	0.08–19.53 ng/g crea (0.12–27.3 ng/l)	
Workers/production of refractory materials (86)				2.00 ng/g crea (2.80 ng/l)	0.17–17.08 ng/g crea (0.24–23.9 ng/l)	
Workers/coking-plant (87, 3 < LOD)				0.71 ng/g crea (0.99 ng/l)	< LOD–8.05 ng/g crea (< LOD–11.3 ng/l)	
Workers/graphite-electrode production (26)				2.43 ng/g crea (3.40 ng/l)	0.21–14.58 ng/g crea (0.29–20.4 ng/l)	
Workers/anode-production (35, incl. 3 S)	HPLC-FD	n. a.	0.05 ng/l	0.74 nmol/mol crea[b] (2.46 ng/l)[b]	0.10–5.05 nmol/mol crea (0.33–16.7 ng/l)	Barbeau et al. 2014
Workers/graphite-cathode production (50, incl. 18 S)				0.28 nmol/mol crea[b] (0.93 ng/l)[b]	0.02–5.27 nmol/mol crea (0.07–17.5 ng/l)	
Workers/silicone-production (44, incl. 22 S)				0.20 nmol/mol crea[b] (0.66 ng/l)[b]	0.03–1.14 nmol/mol crea (0.10–3.79 ng/l)	
Chimney sweeps (151, incl. 53 S, 19 < LOD)	LC-MS/MS	2 ng/l	n. a.	4.75 ng/l[b]	< LOD–42.86 ng/l	Alhamdow et al. 2017
Controls (152, incl. 29 S, 22 < LOD)				1.38 ng/l[b]	< LOD–16.51 ng/l	

Crea: creatinine; HPLC-FD: high-performance liquid chromatography with fluorescence detection; LC-MS/MS: liquid chromatography with tandem mass spectrometry; LIF: laser-induced fluorescence; LOD: limit of detection; LOQ: limit of quantitation; n. a.: not available; N: non-smokers; S: smokers; SD: standard deviation

[a] Values < LOD were included as LOD/2.

[b] median

[c] Values < LOQ were included as LOQ/2.

3 General principles

For the determination of 3-OH-B[*a*]P in urine, the samples are mixed with acetate buffer and the pH value of the solution is adjusted to a range of 5.0–5.5. After adding the internal standard (ISTD), 3-OH-B[*a*]P-$^{13}C_6$-glucuronide, and glucuronidase/arylsulfatase to the samples, they are incubated overnight (37 °C, 16–18 h) in order to cleave the conjugates. The samples are subsequently subject to solid-phase extraction for analyte concentration and for the separation of matrix components. After derivatisation with 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FMPT), 3-OH-B[*a*]P is analysed and quantified by LC-MS/MS in MRM mode. Calibration is performed with similarly processed spiked samples of pooled urine.

4 Equipment, chemicals, and solutions

4.1 Equipment

- HPLC-MS/MS system with an autosampler (e.g. Agilent 1200, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany) and a triple-quadrupole mass spectrometer (e.g. API 6500, AB SCIEX Germany GmbH, Darmstadt, Germany) with a data-processing system (e.g. Analyst 1.5.2, AB SCIEX Germany GmbH, Darmstadt, Germany)
- HPLC separation column (e.g. Acquity UPLC BEH C18 column, 1.7 µm × 2.1 mm × 50 mm, No. 186002350, Waters GmbH, Eschborn, Germany)
- Tabletop centrifuge (e.g. ROTINA 380R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany)
- Vacuum concentrator (e.g. SpeedVac™ SPD120 V, Thermo Fisher Scientific GmbH, Dreieich, Germany)
- Incubator (e.g. Incucell 111, MMM Medcenter GmbH, Planegg, Germany)
- Water-purification system (e.g. Arium®, Sartorius AG, Göttingen, Germany)
- Test-tube shaker (e.g. Standard Multi-Tube Vortexer, VWR International GmbH, Darmstadt, Germany)
- Analytical balance (e.g. Sartorius AG, Göttingen, Germany)
- Sample-processing manifold (e.g. Biotage® VacMaster™, Biotage Sweden AB, Uppsala, Sweden) with a membrane pump (e.g. KNF DAC GmbH, Hamburg, Germany)
- 20-ml, 100-ml, and 1000-ml volumetric flasks (e.g. BRAND GMBH + CO KG, Wertheim, Germany)
- 10-ml threaded test tubes with screw caps (e.g. No. 212-7549, VWR International GmbH, Darmstadt, Germany)
- 4-ml glass vials with screw caps (e.g. No. 1.300820-01, Klaus Ziemer GmbH, Langerwehe, Germany)
- Autosampler vials (e.g. No. 18525267, Klaus Ziemer GmbH, Langerwehe, Germany)
- 325-µl crimp-top vials (e.g. No. 1.301038, Klaus Ziemer GmbH, Langerwehe, Germany)
- Variably adjustable microlitre pipettes with matching tips (e.g. Eppendorf AG, Hamburg, Germany)
- Pasteur pipettes (e.g. Sarstedt AG & Co. KG, Nümbrecht, Germany)
- SPE (solid phase extraction) cartridges, 200 mg, 3 ml (e.g. No. 12105025, Bond Elut-LMS, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany)
- Screw-top 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.

- Acetonitril, ULC/MS - CC/SFC (e.g. No. 012041, Biosolve BV, Valkenswaard, Netherlands)

- Formic acid, ≥ 99% (e.g. No. 069141, Biosolve BV, Valkenswaard, Netherlands)
- Ascorbic acid (e.g. No. 33034, Merck KGaA, Darmstadt, Germany)
- Dichloromethane, Promochem Picograde® (e.g. No. SO-1185-C011, neoLab Migge GmbH, Heidelberg, Germany)
- *N,N*-Dimethylethylamine, ≥ 99% (e.g. No. B23992, Fisher Scientific GmbH, Schwerte, Germany)
- Dimethyl sulfoxide (DMSO), ≥ 99.5% (e.g. No. D4540, Merck KGaA, Darmstadt, Germany)
- Acetic acid, ReagentPlus®, ≥ 99% (e.g. No. A6283, Merck KGaA, Darmstadt, Germany)
- 2-Fluoro-1-methylpyridinium *p*-toluenesulfonate (FMPT), ≥ 98% (e.g. No. F0225, TCI Deutschland GmbH, Eschborn, Germany)
- Glucuronidase/arylsulfatase from *Helix pomatia* (e.g. No. 10127698001, Roche Diagnostics GmbH, Mannheim, Germany)
- Methanol for HPLC, Promochem Optigrade® (e.g. No. SO-3041-C012, neoLab Migge GmbH, Heidelberg, Germany)
- Sodium hydroxide pellets, ≥ 98.0% (e.g. No. 71690, Merck KGaA, Darmstadt, Germany)
- Hydrochloric acid, fuming, 37% (e.g. No. 100317, Merck KGaA, Darmstadt, Germany)
- Ultra-pure water (e.g. Arium®, Sartorius AG, Göttingen, Germany)

4.3 Standards

- 3-OH-Benzo[*a*]pyrene-O-*β*-glucuronide (3-OH-B[*a*]P-Gluc), purity 98.7% (e.g. custom synthesis, Synthèse AptoChem Inc., Montreal, Canada or No. B-5218, TLC Pharmaceutical Standards Ltd., Ontario, Canada)
- 3-OH-Benzo[*a*]pyrene-O-*β*-glucuronide-$^{13}C_6$ (3-OH-B[*a*]P-Gluc-$^{13}C_6$), purity 97.9% (e.g. custom synthesis, Synthèse AptoChem Inc., Montreal, Canada)

4.4 Solutions

- Acetate buffer (1 mol/l, pH 5.1)
  57.0 ml of acetic acid are placed in a 1000-ml volumetric flask. The volumetric flask is subsequently made up to the mark with ultra-pure water. The pH value of the solution is adjusted to 5.1 by adding approximately 30 g of sodium hydroxide pellets.
- Ascorbic acid solution (150 g/l)
  3 g of ascorbic acid are weighed into a 20-ml volumetric flask and dissolved in ultra-pure water. The volumetric flask is subsequently made up to the mark with ultra-pure water.
- Formic acid (1%)
  In a 100-ml volumetric flask, 50 ml of methanol are mixed with 49 ml of ultra-pure water and one millilitre of formic acid.
- *N,N*-Dimethylethylamine (0.2%)
  20 µl of *N,N*-dimethylethylamine are placed in a 10-ml volumetric flask. The volumetric flask is subsequently made up to the mark with acetonitrile.
- FMPT stock solution (2.5 g/l)
  50 mg of FMPT are weighed into a 20-ml volumetric flask and dissolved in a few millilitres of acetonitrile. The volumetric flask is subsequently made up to the mark with acetonitrile.
- FMPT working solution (0.5 g/l)
  4 ml of the FMPT stock solution are placed in a 20-ml volumetric flask. The volumetric flask is subsequently made up to the mark with acetonitrile.

- Methanol/water (50 : 50, v/v)
  In a 1000-ml volumetric flask, 500 ml of methanol are mixed with 500 ml of ultra-pure water.
- Methanol/acetonitrile (50 : 50, v/v)
  In a 1000-ml volumetric flask, 500 ml of methanol are mixed with 500 ml of acetonitrile.
- Hydrochloric acid (1 mol/l)
  50 ml of ultra-pure water are placed in a 100-ml volumetric flask, then 8.3 ml of fuming hydrochloric acid are carefully added. The volumetric flask is subsequently made up to the mark with ultra-pure water.

4.5 HPLC eluents

- Eluent A (0.5% formic acid in water)
  5 ml of formic acid are placed in a 1000-ml volumetric flask. The volumetric flask is subsequently made up to the mark with ultra-pure water and the eluent is degassed.
- Eluent B (0.5% formic acid in acetonitrile)
  5 ml of formic acid are placed in a 1000-ml volumetric flask. The volumetric flask is subsequently made up to the mark with acetonitrile and the eluent is degassed.

4.6 Internal standards (ISTDs)

- Stock solution of 3-OH-B[*a*]P-Gluc-$^{13}C_6$ (250 mg/l)
  5 mg of 3-OH-B[*a*]P-Gluc-$^{13}C_6$ are weighed into a 20-ml volumetric flask and dissolved in a little ultra-pure water. The volumetric flask is subsequently made up to the mark with ultra-pure water.
- Working solution 1 of 3-OH-B[*a*]P-Gluc-$^{13}C_6$ (2.5 mg/l)
  3960 µl of ultra-pure water are placed in a 4-ml screw-top glass vial and 40 µl of the ISTD stock solution are added.
- Working solution 2 of 3-OH-B[*a*]P-Gluc-$^{13}C_6$ (25 µg/l)
  3960 µl of ultra-pure water are placed in a 4-ml screw-top glass vial and 40 µl of ISTD working solution 1 are added.
- Spiking solution of 3-OH-B[*a*]P-Gluc-$^{13}C_6$ (100 ng/l)
  About 5 ml of ultra-pure water are placed in a 20-ml volumetric flask and 80 µl of ISTD working solution 2 are added. The volumetric flask is subsequently made up to the mark with ultra-pure water.

The stock solution as well as the ISTD working solutions are stored at –20 °C and are stable for at least three years. The ISTD spiking solution can be stored at –20 °C for one month.

4.7 Calibration standards

- Stock solution (50 mg 3-OH-B[*a*]P-Gluc/l)
  1.0 mg of 3-OH-B[*a*]P-Gluc (corresponding to 0.929 mg of the pure substance) is dissolved in a little ultra-pure water in a 20-ml volumetric flask. The volumetric flask is subsequently made up to the mark with ultra-pure water.
- Working solution 1 (500 µg 3-OH-B[*a*]P-Gluc/l)
  3960 µl of ultra-pure water are placed in a 4-ml screw-top glass vial and 40 µl of the 3-OH-B[*a*]P-Gluc stock solution are added.
- Working solution 2 (5 µg 3-OH-B[*a*]P-Gluc/l)
  3960 µl of ultra-pure water are placed in a 4-ml screw-top glass vial and 40 µl of the 3-OH-B[*a*]P-Gluc working solution 1 are added.

- Spiking solution 1 (SP 1) (500 ng 3-OH-B[*a*]P-Gluc/l)
  3600 µl of ultra-pure water are placed in a 4-ml screw-top glass vial and 400 µl of the 3-OH-B[*a*]P-Gluc working solution 2 are added.
- Spiking solution 2 (SP 2) (50 ng 3-OH-B[*a*]P-Gluc/l)
  3600 µl of ultra-pure water are placed in a 4-ml screw-top glass vial and 400 µl of the 3-OH-B[*a*]P-Gluc spiking solution 1 are added.
- Spiking solution 3 (SP 3) (5 ng 3-OH-B[*a*]P-Gluc/l)
  3600 µl of ultra-pure water are placed in a 4-ml screw-top glass vial and 400 µl of the 3-OH-B[*a*]P-Gluc spiking solution 2 are added.

The stock solution and the working solutions of 3-OH-B[*a*]P-glucuronide are stored at –20 °C and are stable for at least three years. Spiking solutions 1–3 can be stored at –20 °C for one month.

The calibration standards are prepared in pooled urine according to the pipetting scheme given in Table 2. To prepare the pooled urine, urine is collected from persons who are not exposed to B[*a*]P and mixed. The pooled urine is stored at –20 °C; prior to use, it is brought to room temperature and centrifuged for 10 min at 5000 × *g*. During workup, 50 µl of the ISTD (3-OH-B[*a*]P-Gluc-$^{13}C_6$, c = 100 ng/l water) are added to each of the calibration standards S0–S8. The standard S00 is processed without the addition of ISTD.

Tab. 2 Pipetting scheme for the preparation of calibration standards for the determination of 3-OH-B[a]P in urine

Calibration standard	SP 1 [µl]	SP 2 [µl]	SP 3 [µl]	Pooled urine [µl]	Concentration with respect to 3-OH-B[*a*]P-Gluc [pg/l]	Concentration with respect to free 3-OH-B[*a*]P [pg/l]
S00	–	–	–	6000	0	0
S0	–	–	–	6000	0	0
S1	–	–	100	6000	83.3	50.3
S2	–	20	–	6000	167	101
S3	–	40	–	6000	333	201
S4	–	80	–	6000	667	403
S5	–	128	–	6000	1067	644
S6	–	160	–	6000	1333	805
S7	32	–	–	6000	2667	1611
S8	64	–	–	6000	5333	3221

5 Specimen collection and sample preparation

5.1 Specimen collection

Urine samples are collected in sealable urine cups (e.g. made of polypropylene). If the samples are not immediately processed, they can be stored at –20 °C and are stable for several months under these conditions.

5.2 Sample preparation

Frozen urine samples are thawed slowly at room temperature and thoroughly mixed. 6 ml of each sample are pipetted into 10-ml threaded test tubes and mixed with 400 µl of the acetate buffer. The pH value of the buffered samples is adjusted to a range of 5.0–5.5 using hydrochloric acid (1 mol/l). After adding 50 µl of the ISTD spiking solution, 100 µl of the ascorbic acid solution, and 20 µl of glucuronidase/arylsulfatase, the threaded test tubes are sealed with screw

caps. The preparations are mixed by careful swivelling, taking caution to avoid the formation of foam, and are incubated at 37 °C overnight (16–18 hours). If the sediment has not dissolved during incubation, the sample is centrifuged (5000 × *g*, 10 min) and the supernatant transferred into a new screw-top glass vial.

During the subsequent process of solid-phase extraction, it is important to ensure that the cartridges do not run dry at any point in time. For solid-phase extraction, the SPE cartridges are first conditioned with 3 ml of dichloromethane, then with 6 ml of methanol, and finally with 3 ml of ultra-pure water. The urine samples are applied in 2–3 aliquots using Pasteur pipettes. The washing steps are then carried out: first with 3 ml of ultra-pure water, then with 3 ml of methanol/water (50 : 50, v/v), followed by 1 ml of methanol, and finally with 2 ml of methanol/acetonitrile (50 : 50, v/v). The analytes are thereafter eluated from the SPE cartridges twice with 2 ml of dichloromethane each time, whereby the remaining approximately 300 µl of dichloromethane are pushed through using the appropriate syringe plunger. The eluates are collected in 4-ml vials, mixed with 20 µl DMSO, and, within about 30 min, are concentrated down to 20 µl (DMSO fraction) using a SpeedVac concentrator. The residues are mixed with 250 µl of the FMPT working solution and 50 µl of *N*,*N*-dimethylethylamine (0.2% in acetonitrile). These solutions are incubated for 20 min at 45 °C, subsequently transferred into microvials, and, within about 15 min, are concentrated down to 20 µl (DMSO fraction) using a SpeedVac concentrator. The residues are reconstituted in 250 µl of 1% formic acid and vigorously mixed on the test-tube shaker. Of each of the samples thus prepared, 15 µl is applied for LC-MS/MS analysis.

The processed samples can be stored at –20 °C and are stable under these conditions for at least 6 months.

6 Operational parameters

Analytical determination was performed using a device configuration comprised of an HPLC system with a column oven and a degasser, coupled to a triple-quadrupole mass spectrometer and an autosampler.

6.1 Liquid chromatography

Separation column:	Waters Acquity UPLC BEH C18 (1.7 µm, 2.1 mm × 50 mm)
Separation principle:	Reversed phase
Injection volume:	15 µl
Column-oven temperature:	50 °C
Flow rate:	0.6 ml/min
Eluents:	A: 0.5% formic acid in water B: 0.5% formic acid in acetonitrile
Runtime:	15 min
Gradient program:	see Table 3

Tab. 3 Gradient program for the determination of 3-OH-B[*a*]P in urine

Time [min]	Eluent A [%]	Eluent B [%]
Initial	80	20
1.0	80	20
7.0	69	31
8.5	69	31
10.0	10	90
13.0	10	90

Tab. 3 *(continued)*

Time [min]	Eluent A [%]	Eluent B [%]
13.1	80	20
15.0	80	20

6.2 Tandem mass spectrometry

Ionisation mode: Electrospray ionisation, positive (ESI)

Detection mode: Multiple Reaction Monitoring (MRM)

Carrier gas: Nitrogen

Ion-spray voltage: 5500 V

Source temperature: 550 °C

Entrance potential: 10.0 V

Curtain gas: 10.0 psi (0.69 bar)

Nebuliser gas: 130 psi (8.96 bar)

Heating gas: 220 psi (15.2 bar)

Collision gas: 7.0 psi (0.48 bar)

The instrument-specific parameters must be ascertained and adjusted by the user for the MS/MS system used. The device-specific parameters given in this section have been determined and optimised for the system used during method development (API 6500, AB SCIEX Germany GmbH, Darmstadt, Germany).

Two mass transitions were selected for 3-OH-B[*a*]P. One transition serves the purpose of quantification (quantifier) and the other of confirmation (qualifier). Only one mass transition was used for the ISTD. The selected transitions are summarised in Table 4 alongside the retention times and further MRM parameters. The retention times given below are only intended as a point of reference. The user must ensure the separation performance of the LC column used as well as the resulting retention behaviour of the analysed substances.

Tab. 4 Retention times, mass transitions, dwell times, declustering potential (DP), collision energy (CE), and cell-exit potential (CXP) for the determination of 3-OH-B[*a*]P in urine

Analyte / ISTD	Retention time [min]	Mass transition [*m/z*]	Status	Dwell time [ms]	DP [V]	CE [V]	CXP [V]
3-OH-B[*a*]P	6.74	360.2 ⟶ 251.1	Quantifier	150	161	45	18
	6.74	360.2 ⟶ 267.1	Qualifier	150	161	45	18
3-OH-B[*a*]P-$^{13}C_6$ (ISTD)	6.74	366.2 ⟶ 257.1	ISTD	150	161	45	18

7 Analytical determination

For analytical determination, 15 µl of each of the urine samples processed according to Section 5.2 are injected into the HPLC-MS/MS system and analysed under the conditions given in Section 6. The analyte is identified by its retention time (RT) and characteristic ion transition.

Figure 2 shows a representative chromatogram of a urine spiked with a concentration of 50 pg 3-OH-B[*a*]P/l.

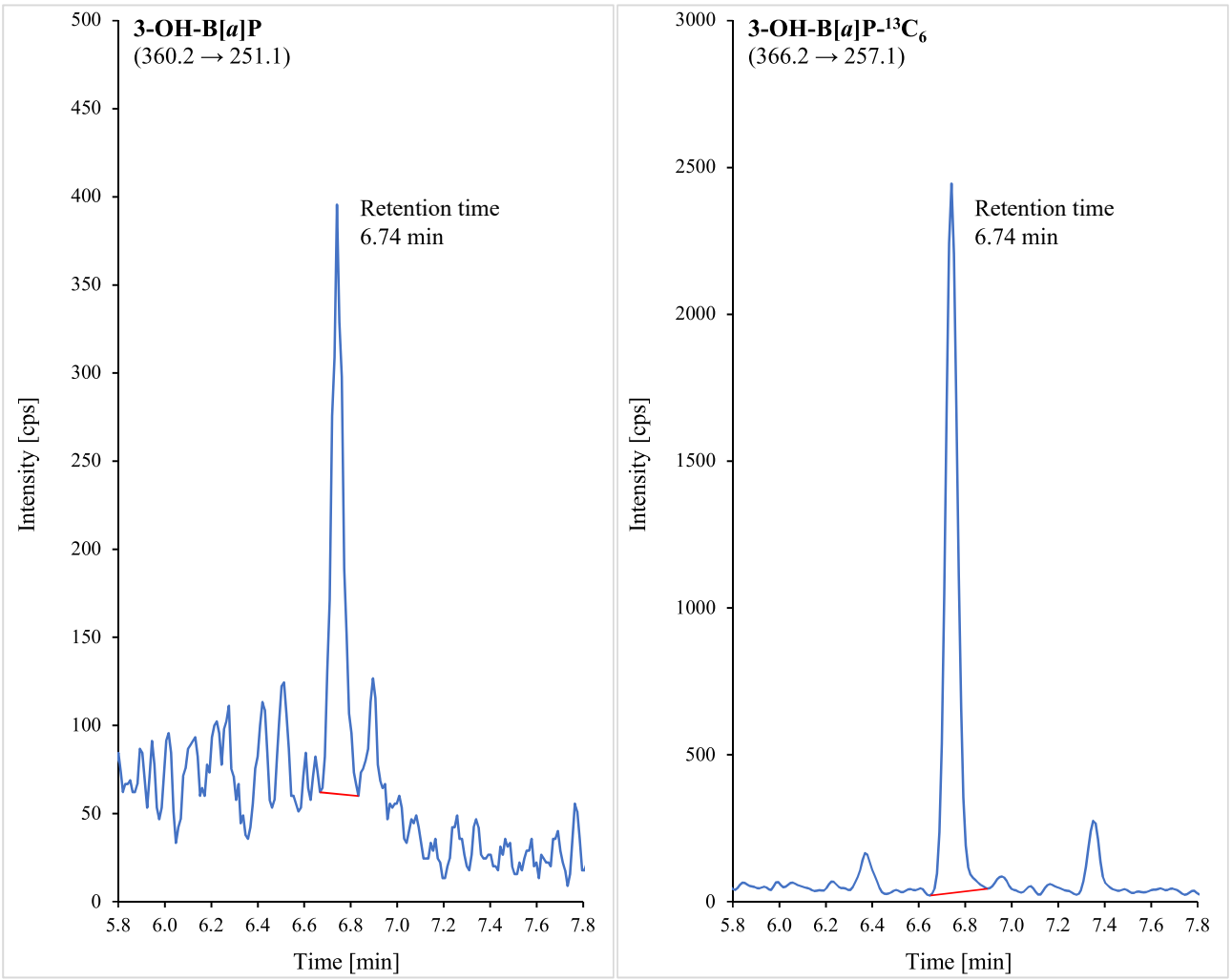

Fig. 2 Representative chromatogram of a urine spiked with 50 pg 3-OH-B[a]P/l

8 Calibration

The calibration solutions prepared according to Section 4.7 are processed analogously to the urine samples (see Section 5.2) and analysed according to the operational parameters described in Section 6. The peak area of the analyte 3-OH-B[*a*]P is normalised to the peak area of the ISTD 3-OH-B[*a*]P-$^{13}C_6$. The quotients thus obtained are plotted against the spiked concentrations of the corresponding calibration standards. The calibration curve for 3-OH-B[*a*]P is linear in the concentration range of 50–3221 pg/l (see Figure 3). When measuring real-world samples, the calibration range may need to be adjusted to the expected concentration range.

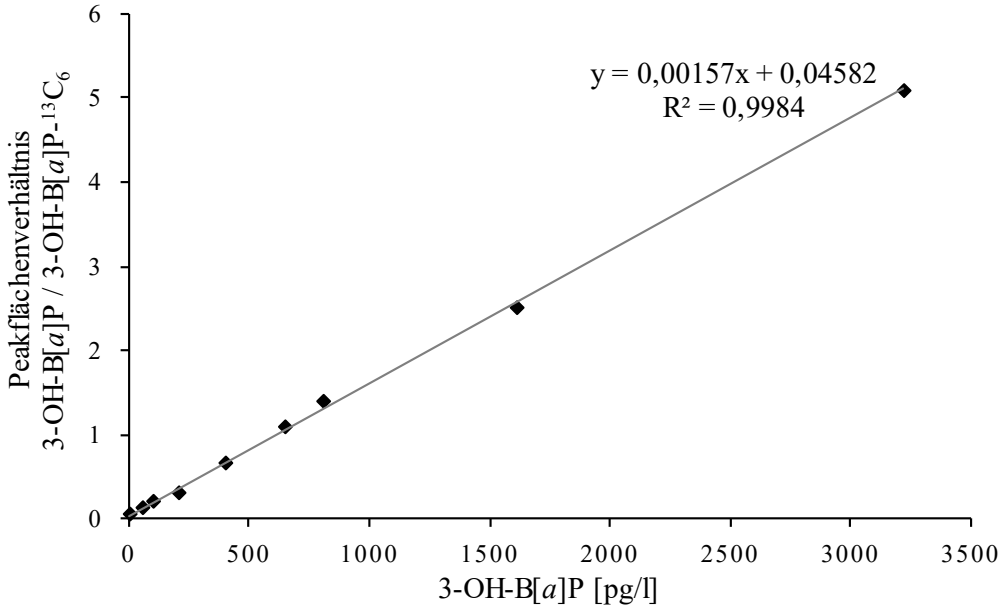

Fig. 3 Calibration curve for the determination of 3-OH-B[*a*]P in urine using 3-OH-B[*a*]P-$^{13}C_6$ as ISTD

9 Calculation of the analytical results

The slopes of the calibration curves are calculated by linear regression and applied for quantification. As such, the axis intercept of the calibration curve corresponds to the background level in the pooled urine and is not considered in the calculation.

If the measurement result lies above the calibration range, the urine sample is diluted with ultra-pure water, reprocessed, and newly analysed.

10 Standardisation and quality control

Quality assurance of the analytical results is carried out as stipulated 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, quality-control samples are processed and analysed parallel to the samples as part of each analytical run. The control material is prepared by spiking pooled urine with three concentrations of 3-OH-B[*a*]P-Gluc, aliquoted, and stored at –20 °C. The spiked concentrations should lie within the expected concentration range. The method developers included two samples of each of the three quality-control materials in every analytical run.

The nominal values and tolerance ranges of the quality-control materials were determined in a pre-analytical period (one analysis of each control material on ten different days) (Bader et al. 2010).

11 Evaluation of the method

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

11.1 Precision

Within-day precision

To determine within-day precision, pooled urine is spiked at concentrations of 200 pg or 640 pg 3-OH-B[*a*]P/l urine, processed five times in parallel, and analysed. The precision data obtained from the measurement results are given in Table 5.

Tab. 5 Within-day precision for the determination of 3-OH-B[*a*]P in urine (n = 5)

Analyte	Spiked concentration [pg/l]	Measured concentration [pg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
3-OH-B[*a*]P	200	203.6	10.4	28.9
	640	592.8	4.3	11.9

Day-to-day precision

Day-to-day precision was likewise determined using samples of pooled urine spiked at concentrations of 200 pg or 640 pg 3-OH-B[*a*]P/l. Aliquots of these spiked pooled urines were stored at –20 °C and processed and analysed on six different days (within a timeframe of 11 days). The precision data thus obtained are presented in Table 6.

Tab. 6 Day-to-day precision for the determination of 3-OH-B[*a*]P in urine (n = 6)

Analyte	Spiked concentration [pg/l]	Measured concentration [pg/l]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
3-OH-B[*a*]P	200	202.5	10.1	26.0
	640	530.9	13.7	35.2

11.2 Accuracy

The mean relative recovery of the method was calculated from the within-day precision data. The recovery rates thus obtained are summarised in Table 7.

Tab. 7 Relative recovery for the determination of 3-OH-B[*a*]P in urine (n = 5)

Analyte	Spiked concentration [pg/l]	Recovery (rel.) r [%]	Prognostic range u [%]
3-OH-B[*a*]P	200	102	94.6–121
	640	92.6	88.0–97.3

11.3 Absolute recovery

Process-related analyte losses were quantified by determining absolute recovery at two concentrations. To this end, pooled urine was spiked at concentrations of 200 pg or 640 pg of the free analyte (3-OH-B[*a*]P) per litre, processed, and analysed. Furthermore, pooled urine was spiked with 200 pg or 640 pg of the free analyte (3-OH-B[*a*]P) per litre after complete workup and then analysed. Absolute recovery rates were calculated by comparison of the corresponding peak areas (see Table 8).

Tab. 8 Absolute recovery for the determination of 3-OH-B[*a*]P in urine (n = 6)

Analyte	Spiked concentration [pg/l]	Reference peak area	Mean measured peak area	Absolute recovery [%]	Standard deviation (rel.) s_w [%]	Prognostic range u [%]
3-OH-B[*a*]P	200	2777	3368	121	11.7	30.1
	640	7487	8152	109	1.6	4.2

11.4 Limits of detection and quantitation

The limit of quantitation for 3-OH-B[*a*]P in urine is 50 pg/l at a signal-to-noise ratio of 12 : 1. The accuracy at the quantitation limit was verified using analyte-free urine samples from six individuals. The urine samples were each spiked with 100 pg 3-OH-B[*a*]P-Gluc/l, processed and analysed. The accuracies of the individual values lied in the range of 96.2–116.2% and the mean of the determined concentrations (106 pg/l) varied by 6.6%.

The limit of detection was defined as 1/3 of the limit of quantitation and was therefore determined to be 16.7 pg/l urine. Table 9 provides the limits of detection and quantitation calculated for the determination of 3-OH-B[*a*]P in urine.

Tab. 9 Limits of detection and quantitation for the determination of 3-OH-B[*a*]P in urine

Analyte	Detection limit [pg/l]	Quantitation limit [pg/l]
3-OH-B[*a*]P	16.7	50

11.5 Sources of error

Interferences may arise due to the varying matrix from sample to sample and, especially in the lower concentration range, may make the quantification of 3-OH-B[*a*]P more difficult. Correct signal identification is, however, ensured by the 3-OH-B[*a*]P qualifier.

Many challenges arose during method verification. Process-related losses–with otherwise excellent validation data–were observed and lied in the range of 1–70%. As the method verifiers used a non-glucuronidated, deuterated ISTD and as no ascorbic acid was used during sample workup, these work-up related losses may have been caused by oxidation as well as by adsorption to the solid-phase column. Moreover, in one analytical run, the signal intensities were quite low as a result of ion suppression; in this case, a poor batch of acetonitrile was found to be the cause.

Users of this method are therefore recommended to use 3-OH-B[*a*]P-Gluc-$^{13}C_6$ as ISTD, to follow the sample workup instructions exactly (addition of ascorbic acid, individual SPE steps), and to ensure the purity of the reagents.

In addition to the Acquity UPLC C18 column (1.7 µm × 2.1 mm × 100 mm with a 0.2-µm prefilter, Waters GmbH, Eschborn, Germany), the method verifiers also tested a Kinetex Biphenyl column (2.6 µm Biphenyl 100 Å, 2.1 mm × 100 mm, No. 00D-4622-AN, Phenomenex Ltd. Deutschland, Aschaffenburg, Germany). The analyte and the ISTD were easily detectable with both separation columns, and the calibration curves were linear. With the biphenyl phase, however, a baseline was observed which was more stable and exhibited less noise at simultaneously higher signal intensities.

12 Discussion of the method

The method presented herein is based on the analysis of the renally excreted 3-OH-B[*a*]P, which is enzymatically released from its glucuronide. After purification and enrichment by SPE, the 3-OH-B[*a*]P is derivatised and analysed by LC-MS/MS. The addition of ascorbic acid as an antioxidant counteracts the oxidative degradation of 3-OH-B[*a*]P, which is released during hydrolysis, and thereby improves the sensitivity and replicability of the method. The ISTD 3-OH-B[*a*]P-$^{13}C_6$ is likewise applied as a glucuronide in order to ensure a stable calibration.

To date, only a few analytical procedures have been published which achieve the necessary quantitation limit for low, environmentally relevant B[*a*]P exposures. Using HPLC-fluorescence detection and automated column-switching, Simon et al. (2000) achieved a quantitation limit of 100 pg/l (see Table 1), whereby it was possible to differentiate between smokers and non-smokers. Table 10 shows an overview of further methods as well as data on the background exposure of occupationally unexposed persons. Barbeau et al. (2011, 2014) also published a very laborious analytical method combining chromatographic separation procedures and subsequent fluorescence detection. In this way, a quantitation limit of 50 pg/l human urine could be reached. Yao et al. (2014) and Raponi et al. (2017) worked with LC-MS/MS (with as well as without derivatisation of 3-OH-B[*a*]P) and ascertained quantitation limits of 250 pg/l and 390 pg/l, respectively, which are sufficient for the field of occupational medicine.

From the results summarised in Table 10, it can be concluded that, for the quantitation of non-occupational exposure to B[*a*]P (primarily from smoking, diet, and ambient air), a quantitation limit of at least 0.1 ng 3-OH-B[*a*]P/l urine is necessary; a quantitation limit of < 0.05 ng/l would be even better. For the measurement of occupational exposure, according to current knowledge, a quantitation limit of 0.2–0.5 ng/l would be sufficient. Based on the data available today, the reported 3-OH-B[*a*]P concentrations of > 1 ng/l urine for non-occupationally exposed persons (Ariese et al. 1994; Fan et al. 2006; Luo et al. 2015; Raponi et al. 2017; Zhang et al. 2015) must be considered methodological artefacts.

With a quantitation limit of 50 pg 3-OH-B[*a*]P per litre of urine, the method presented herein is extremely sensitive, such that 3-OH-B[*a*]P can be quantified in 50–60% of the urine samples from the occupationally non-exposed general population. In order to achieve this low limit of quantitation, the analytical instrument must be in very good condition. The ionisation chamber and the column must be clean; moreover, it may be necessary to maintain the Q1 system of the MS/MS. Regarding detection sensitivity, the selection of the suitable derivatisation reagent was particularly relevant, since the sole purpose of the derivatisation step is to increase the response of the detector and thereby the sensitivity of the method.

To measure extremely low background levels in the general population, it would be necessary to reduce the quantitation limit to 5 pg/l urine. To this end, the method developers have tested certain procedural changes, such as the use of higher volumes of urine; this particular change proved inexpedient as it also led to increased background levels.

The method presented herein enables the sensitive and valid measurement of 3-OH-B[*a*]P in urine. In cases of occupational exposure to B[*a*]P, it is possible to estimate and assess exposure–for example, as part of a preliminary occupational-health examination–with the help of the EKA established by the Commission (DFG 2023). This analytical method and the corresponding EKA together create the scientifically based prerequisite for the detection and assessment of B[*a*]P exposure for the protection of individuals occupationally exposed to carcinogenic PAH mixtures.

Instruments used HPLC-MS/MS system with an autosampler (Agilent 1200, Agilent Technologies Germany GmbH & Co. KG, Waldbronn, Germany) and a triple-quadrupole mass spectrometer (API 6500, AB SCIEX Germany GmbH, Darmstadt, Germany) with a data-processing system (Analyst 1.5.2, AB SCIEX Germany GmbH, Darmstadt, Germany), HPLC separation column (Acquity UPLC BEH C18 column, 1.7 µm × 2.1 mm × 50 mm, No. 186002350, Waters GmbH, Eschborn, Germany)

Tab. 10 Published analytical methods for the determination of 3-OH-B[*a*]P in urine and their application on occupationally non-exposed individuals

Study collective: country, gender (number of persons, smoker status)	Method	LOD	LOQ	3-OH-B[*a*]P		References
				Mean ± SD	Range	
France, ♂ (27 S, 9 < LOD)	Automated column-switching for purification/enrichment, HPLC-FD	0.1 ng/l	0.4 ng/l	0.030 nmol/mol crea[a)] (0.100 ng/l)	< 0.01–0.084 nmol/mol crea (< 0.033–0.279 ng/l)	Lafontaine et al. 2006
France, ♂ (27 N, 22 < LOD)				0.014 nmol/mol crea[a)] (0.046 ng/l)	< 0.01–0.045 nmol/mol crea (< 0.033–0.149 ng/l)	
USA, ♂/♀ (30 S with max. 30 cigarettes/d during the study)	Derivatisation (FMPT), LC-MS/MS	n. a.	0.05 ng/l	0.155 ± 0.091 ng/24 h	–	Sarkar et al. 2010
USA, ♂/♀ (59 S with ≤ 50% cigarettes/day and *ad libitum* snuff use during the study)				0.078 ± 0.045 ng/24 h	–	
USA, ♂/♀ (15 S with *ad libitum* snuff use during the study)				0.079 ± 0.051 ng/24 h	–	
USA, ♂/♀ (11 R without tobacco consumption during the study)				0.056 ± 0.022 ng/24 h	–	
France, ♂/♀ (25 N, 224 of the 284 measurements < LOD)	HPLC-FD	0.08 ng/l	0.25 ng/l	< LOD[b)]	< LOD–0.095 nmol/mol crea (< LOD–0.270 ng/l)	Leroyer et al. 2010
France, ♂ (13 S, 1 < LOD)	Column-switching with a "heart-cut", HPLC-FD	0.02 ng/l	0.05 ng/l	0.029 ± 0.024 nmol/mol crea (0.096 ± 0.080 ng/l)	< LOD–0.075 nmol/mol crea (< LOD–0.249 ng/l)	Barbeau et al. 2011
France, ♂ (23 N, 18 < LOD)				0.010 ± 0.005 nmol/mol crea (0.033 ± 0.017 ng/l)	< LOD–0.023 nmol/mol crea (< LOD–0.076 ng/l)	
China, ♂ (4 S)	Derivatisation (dansyl chloride), LC-MS/MS	0.1 ng/l	0.25 ng/l	0.45 ng/l	0.32–0.67 ng/l	Yao et al. 2014
China, ♂/♀ (4 N, 1 < LOD)				0.25 ng/l[a)]	< LOD–0.35 ng/l	
China (7, 2 < LOD)	Derivatisation (dansyl chloride), UPLC-MS/MS	0.1 ng/l	0.3 ng/l	1.35 ng/l[a)]	< LOD–2.30 ng/l	Luo et al. 2015
China, ♂ (81 S, 195 of the 243 measurements < LOD)	LC-MS/MS	n. a.	2 ng/l	6 ± 3 ng/l	–	Zhang et al. 2015
China, ♂ (58 N, 56 < LOD)				2 ± 0.2 ng/l	–	
Italy (200)	HPLC-MS/MS	0.13 ng/l	0.39 ng/l	0.03 ± 0.05 nmol/l (8.05 ± 13.4 ng/l)	< LOQ–0.39 nmol/l (< LOQ–105 ng/l)	Raponi et al. 2017
Italy (39 S)				0.04 ± 0.07 nmol/l (10.7 ± 18.8 ng/l)	< LOQ–0.39 nmol/l (< LOQ–105 ng/l)	
Italy (97 N)				0.03 ± 0.04 nmol/l (8.05 ± 10.7 ng/l)	< LOQ–0.296 nmol/l (< LOQ–79.4 ng/l)	
Italy (64 ex-S)				0.03 ± 0.04 nmol/l (8.05 ± 10.7 ng/l)	< LOQ–0.19 nmol/l (< LOQ–51.0 ng/l)	
Italy, ♂/♀ (269 S)	HPLC-MS/MS	0.07 ng/g crea (0.2 ng/l)	n. a.	< LOD[b)]	< LOD–24 ng/l[c)]	Tombolini et al. 2018
Italy, ♂/♀ (747 N)				< LOD[b)]	< LOD–32 ng/l[c)]	

Tab. 10 *(continued)*

Study collective: country, gender (number of persons, smoker status)	Method	LOD	LOQ	3-OH-B[*a*]P		References
				Mean ± SD	Range	
Germany, ♂/♀ (242 S/N/ex-S, 155 < LOD	Glucuronides as standards, derivatisation with FMPT, HPLC-MS/MS	0.017 ng/l	0.05 ng/l	0.081 ng/l	–	Rögner et al. 2021
Germany, ♂/♀ (45 S, 27 < LOD)				0.059 ± 0.069 ng/l	–	
Germany, ♂/♀ (42 ex-S, 42 < LOD)				< LOD	–	
Germany, ♂/♀ (37 N, 37 < LOD)				< LOD	–	

Crea: creatinine; ex-S: former smokers; FMPT: 2-fluoro-1-methylpyridinium *p*-toluenesulfonate; HPLC-FD: high-performance liquid chromatography with fluorescence detection; HPLC-MS/MS: high-performance liquid chromatography with tandem mass spectrometry; LC-MS/MS: liquid chromatography with tandem mass spectrometry; LOD: limit of detection; LOQ: limit of quantitation; n. a.: not available; N: non-smokers; S: smokers; SD: standard deviation; UPLC-MS/MS: ultra-high-performance liquid chromatography with tandem mass spectrometry

[a] Values < LOD were included as LOD/2.

[b] median

[c] 5^{th}–95^{th} percentile

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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