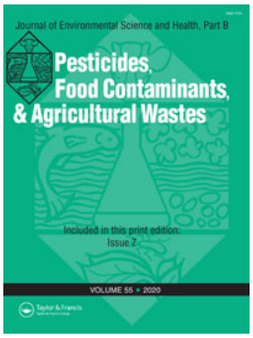


Taylor & Francis
Taylor & Francis Group

Journal of Environmental Science and Health, Part B
Pesticides, Food Contaminants, and Agricultural Wastes

ISSN: 0360-1234 (Print) 1532-4109 (Online) Journal homepage: https://www.tandfonline.com/loi/lesb20

Determination of free and total bisphenol A in the urine and feces of orally and subcutaneously dosed sheep by high-performance liquid chromatography with fluorescence detection

Sabina Šturm, Andrej Škibin, Milan Pogačnik & Vesna Cerkvenik-Flajs

To cite this article: Sabina Šturm, Andrej Škibin, Milan Pogačnik & Vesna Cerkvenik-Flajs (2020) Determination of free and total bisphenol A in the urine and feces of orally and subcutaneously dosed sheep by high-performance liquid chromatography with fluorescence detection, Journal of Environmental Science and Health, Part B, 55:7, 655-668, DOI: 10.1080/03601234.2020.1759329

To link to this article: https://doi.org/10.1080/03601234.2020.1759329

Published online: 07 May 2020.

JOURNAL OF ENVIRONMENTAL SCIENCE AND HEALTH, PART B
2020, VOL. 55, NO. 7, 655–668
https://doi.org/10.1080/03601234.2020.1759329

Determination of free and total bisphenol A in the urine and feces of orally and subcutaneously dosed sheep by high-performance liquid chromatography with fluorescence detection

Sabina Šturm[a], Andrej Škibin[b], Milan Pogačnik[a], and Vesna Cerkvenik-Flajs[a]

[a]Veterinary Faculty, Institute of Pathology, Wild Animals, Fish and Bees, University of Ljubljana, Ljubljana, Slovenia; [b]Veterinary Faculty, Clinic of Reproduction and Farm Animals, Infrastructure Centre for Sustainable Recultivation Vremščica, University of Ljubljana, Ljubljana, Slovenia

ABSTRACT

An analytical procedure has been introduced to enable a study of the excretion of free bisphenol A (BPA), total BPA and its main metabolite bisphenol A glucuronide (BPA–GLUC). In the experiment, in which 100 μg/kg b. w. BPA was administered daily to one Istrian Pramenka sheep for 5 days with consecutive urine and feces samples being taken, BPA and total BPA were determined in samples using high-performance liquid chromatography (HPLC) with fluorescence detection. Because of their good recovery, precision, and sensitivity, the methods have also proved applicable to further ecotoxicological studies of free BPA, BPA–GLUC and total BPA. The results were subsequently compared with reported field studies of BPA in livestock excreta.

ARTICLE HISTORY
Received 6 January 2020
Accepted 7 April 2020

KEYWORDS
Environmental; emerging contaminants; analytical chemistry; HPLC–analysis; phenolic compounds; livestock; farm animals; ruminants; excreta; manure

Introduction

Bisphenol A (BPA) (Table 1), one of the most widely produced and used chemicals in the world, is a well-known xenobiotic present in numerous daily products, mainly in plastics, particularly in polycarbonate resins and epoxy resins.[1]

Recognized as an endocrine disruptor, BPA is capable of altering the endocrine function by imitating or blocking the endogenous hormones.[2] Studies regarding its impact were mainly directed toward vertebrate species[1] or aquatic invertebrate species.[3] In recent years, some studies were also directed toward the impact of BPA on the edaphic environment and soil life. These studies were particularly conducted on isopods[4,5] and earthworms,[6–9] due to the detected presence of BPA in agricultural soils.[3]

BPA can reach soil life through sewage sludge or biosolids that are used to fertilize farmland.[10] However, little data is available about BPA in farm animal excreta (Table 2),[11–16] even though livestock excreta are still directly applied to the soil in some farming practices. In addition, the different policies of various countries allow the producers of animal feed to include waste food, still packaged, in animal feed. It is thus highly likely that BPA could enter animal feed and could be subsequently eliminated with animal excreta. As such, animal excreta would be, together with biosolids, responsible for the BPA contamination of the agricultural soil. Yet, information on the occurrence of BPA in animal feed for livestock is lacking.

The majority of studies in which the excretion of BPA is evaluated, are toxicokinetic studies. Most of these determine BPA excretion in the urine of different animal models (sheep, rats, monkeys, pigs), and some even report fecal excretion (rats, monkeys).[17–20]

There are a few published analytical methods for the determination of bisphenols in animal urine and feces, and those that do exist are principally derived[21] from the methods used in human occurrence and exposure studies.[22] A combination of scintillation counting and a radio-high performance liquid chromatography (HPLC) was used for determination of both free and total (free + conjugated) ^{14}C-BPA in experimentally dosed monkeys[19] and rats.[18] Two liquid chromatography—tandem mass spectrometric (LC-MS/MS) methods in Twaddle et al.[23] and Yang et al.[24] determined total deuterated BPA (d6-BPA) and bisphenol AF (BPAF) in both the urine and feces of experimentally dosed rats, respectively, while an LC-MS/MS method of Zhang et al.[11,12] determined free (aglycone) BPA in the feces obtained in field studies of various livestock animals. With regard to the differences in these earlier methods that all utilized enzymatic hydrolysis by ß-glucuronidase for the deconjugation step, Lacroix et al.[17] pioneered the simultaneous quantification of BPA and BPA–GLUC in sheep's urine by LC-MS/MS.

The objective of our work was to introduce a sensitive and selective analytical method for determination of BPA and total BPA in urine and feces after consequent dietary and subcutaneous administration of 100 μg/kg b. w. of BPA

CONTACT Sabina Šturm sabina.sturm@vf.uni-lj.si Veterinary Faculty, Institute of Pathology, Wild Animals, Fish and Bees, University of Ljubljana, Ljubljana, Slovenia.

Table 1. Chemical names, CAS numbers, synonyms, and chemical structures of the bisphenol A (BPA) and BPA–glucuronide (BPA–GLUC).

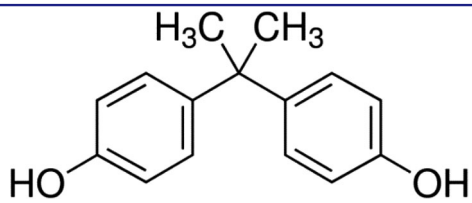
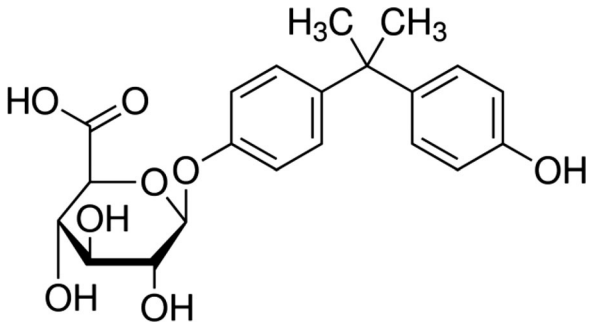
Chemical, abbreviation	CAS number	Synonym	Chemical structure
Bisphenol A, BPA	80-05-7	2,2-bis(4-hydroxyphenyl)propane, 4,4′-isopropylidenediphenol	
Bisphenol A mono-β-D-glucuronide, BPA–GLUC	267244-08-6	4-[1-(4-hydroxyphenyl)-1-methylethyl]phenyl β-D-glucopyranosiduronic acid	

Table 2. The determined (free) BPA concentrations in livestock excreta from the available field studies.

Sample	Animal species	Free BPA concentration	Reference
Feces (μg/kg)	Milking cow	4.1–10.9	[11]
	Replacement cow	7.4–10.8	
	Piglet	ND	
	Barrow	ND–6.0	
	Sow	ND	
	Broiler (female)	11.8–12.0	
	Broiler (male)	0.9–13.0	
	Laying hen	5.4–9.8	
	Brood hen	ND	
Urine (μg/L)	Replacement cow	0.23–0.31	
	Barrow	0.42–0.45	
	Sow	0.22–0.26	
Fresh feces (μg/kg)	Milking cow	2.3–2.7	[12]
	Beef cattle (bull)	3.3–4.1	
	Sow	ND	
	Broiler chicken	ND	
Fresh urine (μg/L)	*Sheep**	*1.0–1.6 ng/L*	
	Milking cow	0.35–0.41	
	Beef cattle	1.95–2.12	
	Sow	0.35–0.41	
Manure (μg/kg)	Poultry	up to 207	[13]
Manure (μg/kg)	Hen	ND–166.5	[15]
	Duck	ND–178.9	
	Swine	ND–361.8	
	Cow	ND–33.3	
Manure (μg/kg)	Swine	ND	[16]
Liquid manure (μg/kg d. w.)	Pig and cow-fattening facilities	61.1–1,112	[14]

ND = not detected
*There is dispute in the article regarding the BPA concentrations in sheep, whether the concentration was measured in urine or in feces.

to a sheep, and compare the obtained concentrations of BPA, total BPA and of the main metabolite bisphenol A glucuronide (BPA–GLUC) (Table 1) with those in the reported field studies in the literature.

Materials and methods

Experimental design

Chemicals

Bisphenol A of $\geq$ 99% purity (Merck, Sigma-Aldrich, Darmstadt, Germany) was dissolved in absolute ethanol for the dietary route and in corn oil for the subcutaneous route of administration. The dosage administered to the sheep was adjusted to the body weight recorded on the day of the administration. For dietary administration BPA solution in absolute ethanol was spilled onto the pellet ration (on average 1.18 mL of solution on 50 g of pellets), and applied on the morning dosage of pellets (400 g). For subcutaneous administration, injection of BPA solution was performed in the shoulder area (2.9 mL). BPA solutions were stored in sealed amber glass bottles at the ambient temperature for the entire experiment. Solution preparation, sample

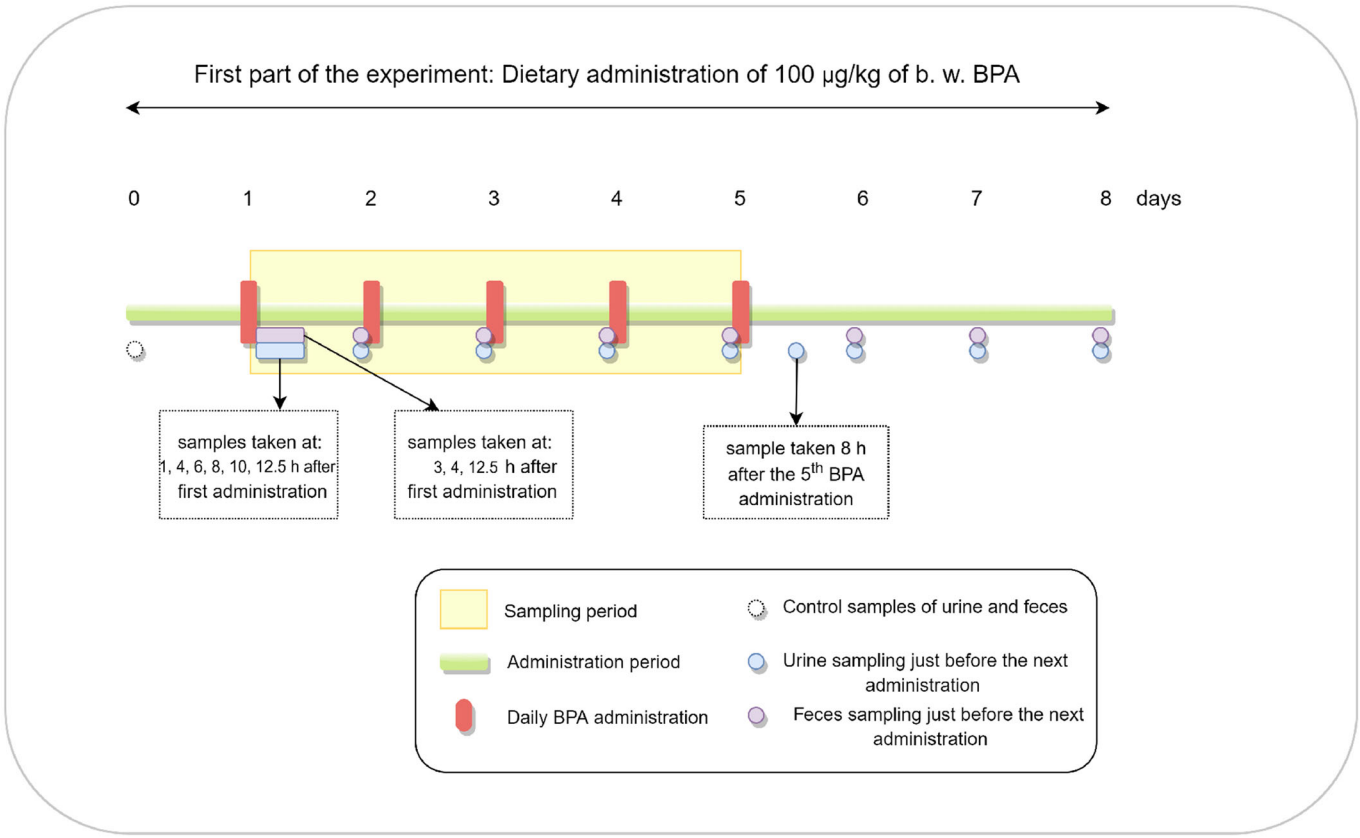


Figure 1. Study design with dietary experimental period, BPA administration (100 µg/kg b. w. per day), urine and feces sampling scheme for the ewe.

processing and assays were performed with the materials either made of glass or of BPA-free plastics.

Sampling regime

All animal procedures were carried out in accordance with ethical standards and approved by the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection with permission no. U34401-3/2015/8.

The study was performed on one stabled, healthy, lactating Istrian Pramenka sheep in a sheepfold at the Infrastructure Center for Sustainable Recultivation at Vremščica belonging to the Veterinary Faculty of the University of Ljubljana, Slovenia. The 6-year-old ewe weighed 59 kg. It was clinically healthy, as indicated by medical (temperature, breathing and rumination frequency, pulse rate), haematological and biochemical examination. It was penned individually and kept under natural temperature and photoperiodic conditions, with free access to water, hay and salt. The sheep was fed twice a day with 400 g plant-based pellets (SchafKorn Lac, Unser Lagerhaus Warenhandels Ges., Austria). Eventual contamination of the experimental environment was checked by testing of drinking water and pellets by HPLC analysis prior to the experiment. The analysis revealed the slight presence of BPA of 0.02 µg/L in drinking water and 5 µg/kg in pellets.

In the first period, the ewe received BPA in its diet (100 µg/kg b. w.) for five consecutive days. The ewe ingested all pellets within 2–9 minutes. During the second period, after a 13-day wash-out period, the same ewe was injected in the shoulder area with 100 µg/kg b. w. of BPA subcutaneously per day for five consecutive days. The sampling scheme for urine and feces after dietary administration of BPA is shown in Fig. 1.

The experimental design was the same for the part of the experiment with subcutaneous administration of BPA, with only small differences in some sampling times for the feces samples, as follows. On the first day of the experiment there were different time periods of feces collection, which were 4.5, 5, 6.15 and 8 hours after the first subcutaneous administration of BPA, and on the second day, sampling was done 12 hours after the administration of BPA.

Urine samples were collected with the stimulation of sheep to urinate. They were collected in a clean glass and stored in a laboratory screw cap bottle. Feces samples were collected fresh from the barn floor, right after defecation. The samples were stored in polypropylene (PP) tubes. Immediately after the sampling, urine and feces samples were frozen at −20 °C. The samples were kept frozen until analysis.

For study sample concentration measurements, the samples from the ewe, taken just before the start of the experiment were used as baseline samples. Additional urine and feces samples from multiple other stabled sheep from the same herd of the same breed, nutrition and physiological status, were also taken simultaneously to enable enough biological material for validation series. These sheep were not treated with BPA and were dislocated from the ewe with a lamb included in the experiment.

Analytical method

Reference standard materials

The certified reference standard of BPA was obtained as a powder of 99.0% analytical purity from Sigma-Aldrich (Merck, Darmstadt, Germany). The reference standard solutions were prepared in high-quality dark brown glassware. The stock standard solution was prepared at a concentration of 200 μg/mL in acetonitrile (MeCN) and kept frozen (at −20 °C). Working standard solutions for the calibration curve and fortification of the samples determining free BPA were prepared in 35% (v/v) MeCN in H_2O. At concentrations of ≥ 50 ng/mL they were kept refrigerated (at 4–8 °C), whereas at concentrations below 50 ng/mL they were prepared on a daily basis. The working standard solutions used for fortification of the samples determining total BPA, were prepared by dissolution of a reference powder of BPA in H_2O or by proper dilution of a stock standard solution (in MeCN) with H_2O. At concentrations from 10,000–100,000 ng/mL they were kept frozen (at −20 °C), whereas at concentrations ≤ 500 ng/mL they were prepared on a daily basis.

Reagents and consumables

The high-purity deionized water used with resistivity of 18.2 MΩ.cm was obtained using a PureLab Option and PureLab Classic water purification system (Elga, Woodridge, Illinois, USA). The MeCN and methanol (MeOH) used, which were HPLC gradient-grade purity, were supplied by J.T. Baker (Center Valley, PA, USA). The aqueous solution of enzyme ß-glucuronidase from *Helix pomatia* Type HP − 2, with ≥100,000 U/mL (and ≤7,500 U/mL of sulfatase activity), the sodium acetate anhydrous for analysis, acetic acid (glacial) 100% anhydrous for analysis and formic acid 98–100% for analysis, were supplied by Merck (Darmstadt. Germany). Sodium acetate buffer of 1.1 M and with pH values of 4.8 and 4.9 was prepared by mixing of aqueous 1.1 M sodium acetate and 1.1 M acetic acid (glacial) in a ratio of 59:41 (v/v) and 64.5:35.5 (v/v), respectively.

The solid-phase extraction (SPE) columns used were Chromabond HR-X, 6 mL, PP, with 85 μm particle size and 200 mg of sorbent, which were supplied by Macherey-Nagel (Düren, Germany), and molecularly imprinted polymer (MIP) AFFINIMIP® SPE Bisphenols, 6 mL, with 100 mg of sorbent, which were supplied by AFFINISEP (Petit-Couronne, France). The centrifuge tubes (15 mL, conical, screw cap, PP) were supplied by Isolab (Wertheim, Germany), and the centrifuge tubes (15 mL, conical, glass) were purchased from Brand (Wertheim, Germany). The dark glass, 1.5−mL vials for HPLC were purchased from La-Pha-Pack (Langerweche, Germany).

Equipment

The homogenization of the sheep's feces was performed using a Tube Mill control with a metal mixing chamber (Ika, Staufen, Nemčija). An electronic balance Vibra AJ − CE/AJH − CE (± 0.001 g), an incubator shaker Vibromix 403 RVI, a Vibromix 10 Vortex mixer and a centrifuge Centric 350 were obtained from Domel (Železniki, Slovenia). A Transsonic 460/H ultrasonic bath was acquired from Elma (Singen, Germany). An SPE vacuum manifold Visiprep with 24 flow control valves was supplied by Merck (Darmstadt, Germany), and an N-EVAP 111 evaporator was provided by Organomation Associates (Berlin, MA, USA). The HPLC system used was a Varian ProStar (Varian Analytical Instruments, Walnut Creek, CA, USA), which comprised a tertiary pump (240 model), an automatic injector (410 model), a fluorescence detector (363 model), a degasser and Galaxie 1.7.4.5 analytical software.

Sample extraction and clean-up

Samples of the sheep's urine and feces were tested for the presence of both free (aglycone) and total (a sum of free and conjugated) BPA. Conjugated BPA was determined by an enzymatic deconjugation of the glucuronide bond, followed by subtraction of the free BPA from the total BPA.

Regarding the sheep's urine, for the determination of the free BPA an aliquot of 1 ± 0.005 mL of the homogenized sample was transferred into a 15-mL plastic (PP) centrifuge tube and diluted by 4 mL of H_2O, while for the determination of the total BPA a sample aliquot of 0.5 ± 0.005 mL was diluted by 1.5 mL of 1.1 M sodium acetate buffer with pH 4.8, 20 μL of the ß-glucuronidase from *Helix pomatia* and incubated by shaking for 16 hours at 37°C. A further clean-up of both free and total BPA was performed by solid phase extraction (SPE) using two SPE sorbents, namely Chromabond HR − X and molecularly imprinted polymer (MIP) AFFINIMIP® SPE Bisphenols according to the procedure developed by Deceuninck et al.[25] with some modifications and omitting the derivatization step. In detail, urine samples were applied slowly under gravity (1 drop/5 sec) onto the Chromabond HR-X cartridge, pre-conditioned by 10 mL of H_2O and 10 mL of MeOH. The cartridge was then washed under gravity (1 drop/sec) with 6 mL of H_2O, 8 mL of MeOH/H_2O (10/90, v/v) and 4 mL of MeOH/H_2O (60/40, v/v), and was shortly sucked by vacuum at the end of washing step. The solid phase extract was eluted by 10 mL of MeOH under gravity (1 drop/2 sec) into a 15-mL glass tube and was evaporated under a N_2 stream at 40 °C just to dryness. The residue was re-dissolved in 0.2 mL of MeCN, ultrasonicated for 5 min, diluted by 5 mL of H_2O, vibromixed, and applied slowly under gravity (1 drop/5 sec) onto the AFFINIMIP® SPE Bisphenols cartridge, pre-conditioned by 10 mL of formic acid/MeOH (2/98, v/v, prepared on a daily basis), 4 mL of MeCN and 4 mL of H_2O. The cartridge was washed under gravity (1 drop/sec) with 5 mL of H_2O, 3 mL of MeCN/H_2O (40/60, v/v) and 3 mL of MeCN, and was sucked by vacuum for 2–3 min at the end of washing step. The solid phase extract was eluted by 4 mL of MeOH under gravity (1 drop/2 sec) into a 15 mL glass tube and was evaporated under a N_2 stream at 40 °C just to dryness.

Final free BPA extracts were re-dissolved in 0.8 mL of MeCN/H_2O (35/65, v/v), ultrasonicated for 2 min, and vibromixed, while total BPA extracts were re-dissolved in 1 mL of the same solution, ultrasonicated for 2 min,

vibromixed, and further diluted in a volume ratio of 1:24 or 1:4 (v/v), depending on the analyte concentration level. An aliquot of the final sample extract was transferred into a HPLC vial.

Regarding the sheep's feces, an aliquot of 0.5 ± 0.003 g of the homogenized moist sample was put into a 15−mL plastic (PP) centrifuge tube. For the determination of the free BPA, it was extracted by 8 mL of MeCN, by vigorous vibromixing for 2 min and ultrasonication for 13 min. After repeated vibromixing for 1 min, the sample was centrifuged at room temperature for 10 min at 2640×***g*** and re-extracted using 2 mL of MeCN. For the determination of the total BPA, a sample was diluted by 1.5 mL of 1.1 M sodium acetate buffer with pH 4.9, then 20 μL of the ß-glucuronidase from *Helix pomatia* was added, and the sample was incubated by shaking for 4 hours at 37°C. Total BPA was extracted from the buffered feces using 6 and 2 mL of MeCN in the same manner as free BPA. The combined MeCN supernatant for both free and total BPA residue was transferred into a 15−mL glass centrifuge tube and evaporated under a N_2 stream at 40°C to an aqueous residue. The free BPA sample extract was re-dissolved in 0.3 mL of MeOH, vibromixed, ultrasonicated and diluted by 4.7 mL of H_2O, while the total BPA sample extract was re-dissolved in 3 mL of MeOH/H_2O (10/90, v/v) solution. From this step onward the procedure basically followed the analytical method of Deceuninck et al.,[25] in the same manner as described for urine samples. Final sample extracts were re-dissolved in 0.5 mL of MeCN/H_2O (35/65, v/v), ultrasonicated for 5 min, vibromixed, centrifuged at room temperature for 10 min at 2640×***g***, and transferred into a HPLC vial.

HPLC analysis

A 50 μL aliquot of the final urine and feces sample extract was taken for the high-performance liquid chromatography (HPLC) analysis. A Hypersil GOLD C18 (150 × 4.6 mm, 3 μm particle size) analytical column was used which was protected by a Hypersil GOLD 3 μ drop in guard cartridges (Thermo Scientific, Waltham, MA, USA). The chromatographic process was performed at room temperature in a gradient manner using the two HPLC methods. Method no. 1, which was used for the analysis of the free BPA in the sheep's urine, pumped the mobile phase of H_2O (constituent A) and MeCN (constituent B) at a flow rate of 1.0 mL/min in the following volume ratios: time 0–2 min (35% B), time 2–12 min (gradient 35–50% B), time 12–20 min (50% B), time 20–20.5 min (gradient 50–35% B), time 20.5–21 min (35% B).[26] HPLC method no. 2, which was used for the analysis of the total BPA in the sheep's urine and of the both free and total BPA in sheep's feces, pumped the mobile phase at a flow rate of 0.9 mL/min and used the two constituents of the mobile phase, i.e., H_2O (constituent A) and MeCN:MeOH = 1:1 (v/v) (constituent B) in the following volume ratios: time 0–2 min (35% B), time 2–22 min (gradient 35–60% B), time 22–35 min (60% B), time 35–40 min (gradient 60–75% B), time 40–42 min (gradient 75–35% B) and time 42–43 min (35% B).[27] For both HPLC methods the excitation and emission wavelengths of the fluorescence spectrophotometry analysis were set at 230 and 315 nm, respectively.[26] The results were evaluated according to an external standard method using a solvent standard calibration curve, which was constructed by plotting the peak area as a function of the analyte concentration. The measured BPA concentration in the baseline sample, taken just before the first administration of BPA to the sheep, was subtracted from all measured samples in the series (study and spiked samples—these were used to calculate recovery of the series). The baseline corrected study sample concentrations (C_{corr}) were then additionally corrected for the mean recovery rate of the series (rec_{mean}, %) as follows: $C = C_{corr}/rec_{mean}*100$. As they were measured in parallels, the mean value was used as a final result.

Quality assurance procedures

Each study sample series consisted of a baseline sample, the study samples (in duplicate) and two recovery samples. These were obtained by fortification of the baseline sample with BPA at a reasonable level. Daily standard calibration curves were constructed from 6 or 7 calibration points. The baseline concentrations for the first, dietary part of the experiment were, in the urine sample, 0.5 μg/L and <10 μg/L, and in feces sample, <1 μg/kg and <1 μg/kg of free and total BPA, respectively. The baseline concentrations for the second, subcutaneous part of the experiment were, in urine sample, 0.15 μg/L and <10 μg/L, and in feces sample, <1 μg/kg and <1 μg/kg of free and total BPA, respectively.

Efficiency of the SPE step was included within the recovery control over the whole procedure, which included extraction from the matrix, SPE and concentration step. Recovery samples were spiked at the beginning of the procedure with the free BPA, also for determination of the total BPA. The recovery was calculated as follows:

$$\text{Rec}\ (\%) = \frac{(\text{BPA}_{\text{found}} - \text{BPA}_{\text{baseline}})}{\text{BPA}_{\text{added}}} \times 100\%$$

Rec … recovery (%)

BPA_{found} … BPA found in the spiked sample (μg/L or μg/kg)

$BPA_{baseline}$ … BPA found in the baseline sample (μg/L or μg/kg)

BPA_{added} … BPA added in the spiked sample (μg/L or μg/kg)

Validation of BPA analysis

The analytical methodology was validated to demonstrate its fitness for determination of the time profile of BPA excretion in sheep's urine and moist feces. Validation was done separately for free and total BPA in both matrices investigated. Linearity was determined on both a standard and a matrix level by the least squares method, giving the regression and correlation parameters of the calibration lines. Solvent standard concentrations ranged for HPLC method no. 1 from 1.0–50 ng/mL with 6 concentration points per calibration line, while for HPLC method no. 2 they ranged

within 1.5–200 ng/mL with 4–8 concentration points per calibration line. Linearity in matrix, recovery, and precision of BPA determination in sheep's urine and feces were evaluated by fortification of a baseline sample. Linearity on a matrix level was evaluated as an intra-day correlation between the mean measured and added BPA concentrations (n = 2/fortification level), for free and total BPA in urine over a concentration range of 0.5–20 μg/L and 100–15,000 μg/L, respectively, and for both free and total BPA in moist feces over a concentration range of 2–50 μg/kg. Recovery, repeatability and intra-laboratory reproducibility were tested on two BPA concentration levels. Urine samples for determination of the free and total BPA were fortified by 5 and 10 μg BPA/L, and 500 and 2000 μg BPA/L, respectively. Feces samples for determination of both free and total BPA were fortified by 5 and 10 μg BPA/kg. Repeatability was evaluated by 2–5 fortified replicate samples per concentration, tested on the same time occasion, while intra-laboratory reproducibility was evaluated by in total 6–11 fortified samples tested on the 2–4 separate time occasions. The precision of the methods was evaluated using the standard deviation (SD) and coefficient of variation (CV) of the determined values, and assessed in accordance with the Horwitz coefficients (CV_H) according to the European Commission Decision 2002/657/EC.[28] The limit of detection (LOD) value was estimated as the minimum detectable amount of BPA from matrix samples with signal-to-noise ratio of 3:1 and was corrected for the baseline matrix response.

Results

Performance characteristics of BPA analysis

The analytical methodology presented in this work comprises four separate assays for particular BPA category/matrix combinations, and is used for the determination of free BPA in urine/feces and total BPA in urine/feces, which varied in the sample preparation and clean-up, followed by the HPLC analysis. Representative HPLC chromatograms of determining free BPA in sheep's urine and total BPA in sheep's feces are presented in Figs. 2 and 3, demonstrating an appropriate chromatographic resolution and a BPA retention time of around 8.0 and 15.5 min, respectively.

The analytical HPLC methodology demonstrated good linearity, as shown by the obtained correlation coefficients. The "R-squared" values of the solvent standard calibration lines for HPLC method no. 1 over a concentration range of 1–50 ng/mL were $\geq$ 0.9993, whereas for HPLC method no. 2 over and within a concentration range of 1.5–200 ng/mL they were $\geq$ 0.9983 (Table 3). The linearity of determining the level of BPA in both matrices is presented in Fig. 4, based on the correlation curves between the found and added values of BPA in sheep's urine and moist feces. The "R-squared" values were 0.9987 and 0.9982 for the free and total BPA in urine, respectively, and 0.9941 and 0.9918 for the free and total BPA in feces, respectively.

The recovery and precision of the method are presented in Table 4 and were determined on two levels of content for particular BPA category/matrix combinations. The recovery values for determination of BPA in urine and feces ranged from 52 to 67% and from 41 to 81%, respectively. The repeatability and within-laboratory reproducibility of the measurements, represented by the CV values, ranged from 1.3 to 27.4% and from 8.8 to 32%, respectively. Regarding urine, the estimated LOD values for determination of free and total BPA were 0.1 and 10 μg/L, respectively. With regard to the feces analysis, the difference in the chromatographic background was smaller between the free and total BPA analysis, resulting in equal LOD value of 1 μg/kg for both free and total BPA, respectively.

BPA, total BPA and BPA–GLUC excretion in urine and feces of sheep after dietary and subcutaneous administration

The free BPA, total BPA and BPA–GLUC concentrations in urine and free BPA, total BPA and BPA–GLUC concentrations in the feces of the sheep after dietary and subcutaneous administration are presented in the Tables 5 and 6, respectively.

After the first day of both experiments, only the trough concentrations were measured in urine each following day, with the exception of the sampling on the fifth day of the dietary and subcutaneous part of experiment, when urine was also taken 8 hours (Table 5) and 7 hours (Table 6) after the last BPA administration, respectively. Another exception was the additional feces sampling which occurred 12 hours after the second BPA administration in the second, subcutaneous part of the experiment (Table 6).

The results in Table 5 demonstrate that there was a very small amount of free BPA excreted in the urine, and that the administered BPA was mostly excreted as BPA–GLUC. On the first day, Cmax (16.6 μg/L) for free BPA was reached after 8 hours of BPA administration and Cmax for BPA–GLUC (15,141 μg/L) was reached after 4 hours. From the end of BPA administration, on the 5th day, the concentrations of free BPA and BPA–GLUC began to decline sharply and got under the analytical LOD for both total BPA and BPA–GLUC on the 8th day, which was three days after the last BPA administration.

Regarding the concentrations of free BPA and total BPA in sheep's feces, Tables 5 and 6 demonstrate that the concentrations of the parent compound (free BPA) were approximately the same as of the total BPA or slightly higher after subcutaneous determination, presumably due to the variability of the analytical method used. Nevertheless, the results demonstrated a general absence of BPA–GLUC in sheep's feces samples irrespective of the form of BPA administration used. Thus, BPA was preferably excreted in feces in its free (aglycone) form.

The results in Table 6 also demonstrate that, as with dietary administration, the BPA in urine was mostly excreted as the BPA–GLUC. On the first day, Cmax for both free BPA

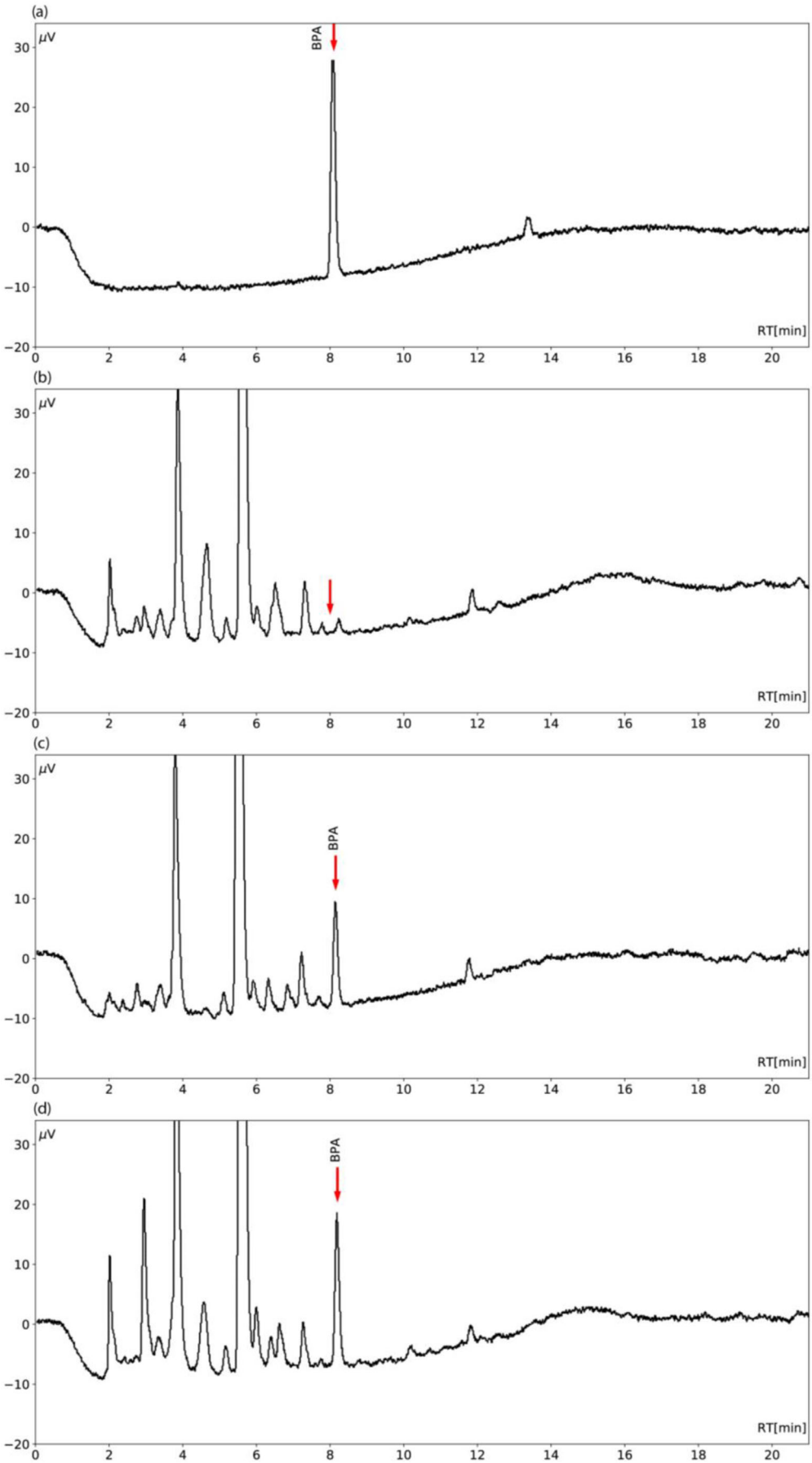


Figure 2. Representative HPLC chromatograms for the determination of free (aglycone) bisphenol A (BPA) in the urine of one sheep: (a) standard solution of 25 ng BPA/mL (b) baseline sample (before BPA administration) (c) baseline sample spiked with 10 μg BPA/L (d) sample containing 15.8 μg BPA/L, obtained 4 hours following p. o. administration to the ewe at a dose of 100 μg BPA/kg b. w.

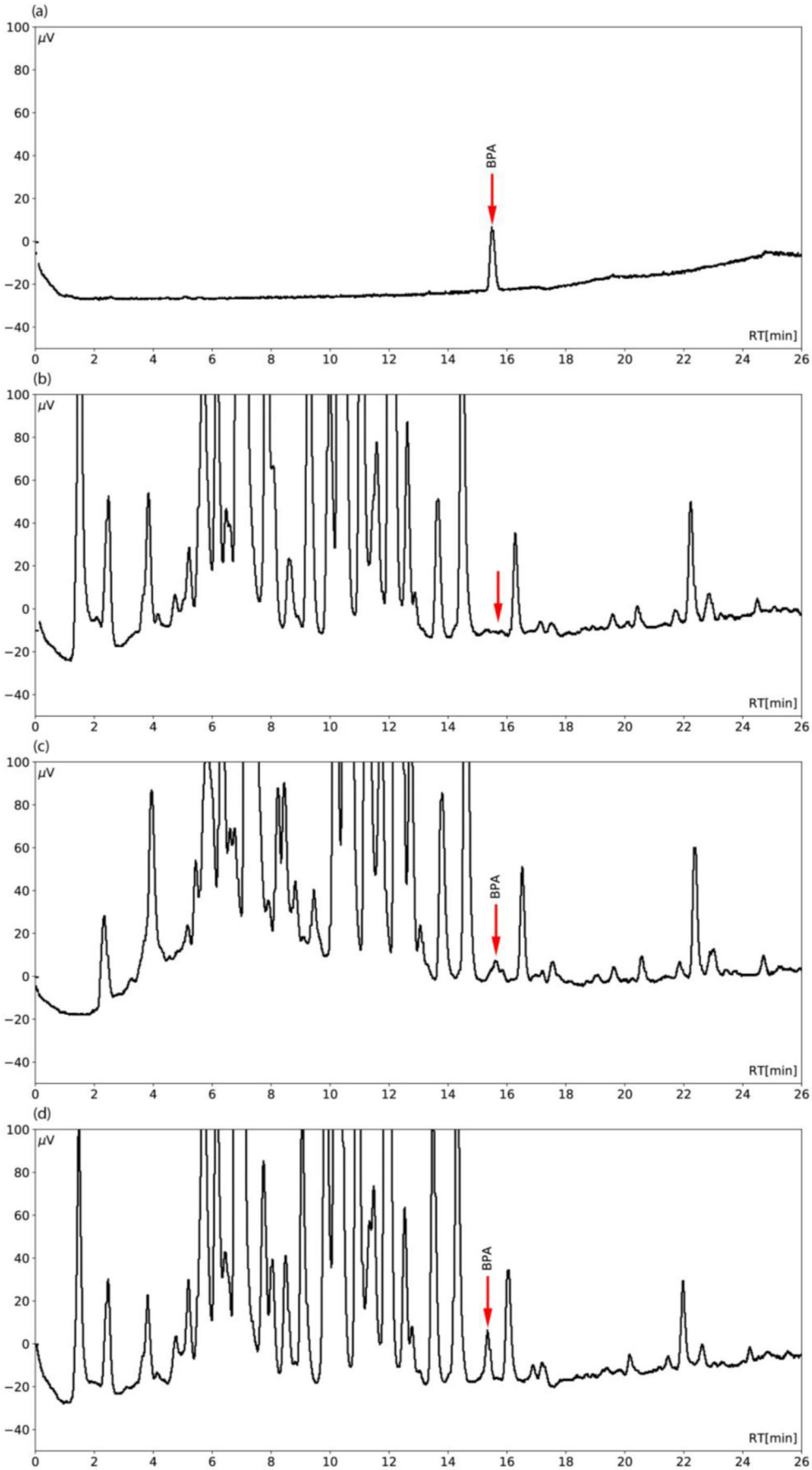


Figure 3. Representative HPLC chromatograms for the determination of the total (free + conjugated) bisphenol A (BPA) in the fresh feces of one sheep: (a) standard solution of 25 ng BPA/mL (b) baseline sample (before BPA administration to the ewe) (c) baseline sample spiked with 10 μg BPA/kg (d) sample containing 34.9 μg BPA/kg, obtained 12.5 hours following p. o. administration to the ewe at a dose of 100 μg BPA/kg b. w.

Table 3. Linearity of bisphenol A (BPA) standard calibration curves obtained by correlation between area of chromatographic peaks and concentration (ng/mL).

HPLC method* (run time)	Concentration range (ng/mL)	Points/curve (no. of curves)	Linear correlation	
			[a]r	r^2
No. 1 (21 min)	1 to 50	6 (9)	0.9996–0.9999	0.9993–0.9999
No. 2 (43 min)	1.5 to 25	4 (1)	0.9973	0.9945
	1.5 to 50	6 (11)	0.9992–1.0000	0.9984–1.0000
	1.5 to 200	8 (1)	0.9991	0.9983
	2.5 to 100	5 (1)	0.9997	0.9994
	2.5 to 200	6 (3)	0.9992–0.9999	0.9983–0.9998
	5 to 200	6 (1)	0.9994	0.9988

[a]correlation coefficient
*HPLC methods are described under Analytical method.

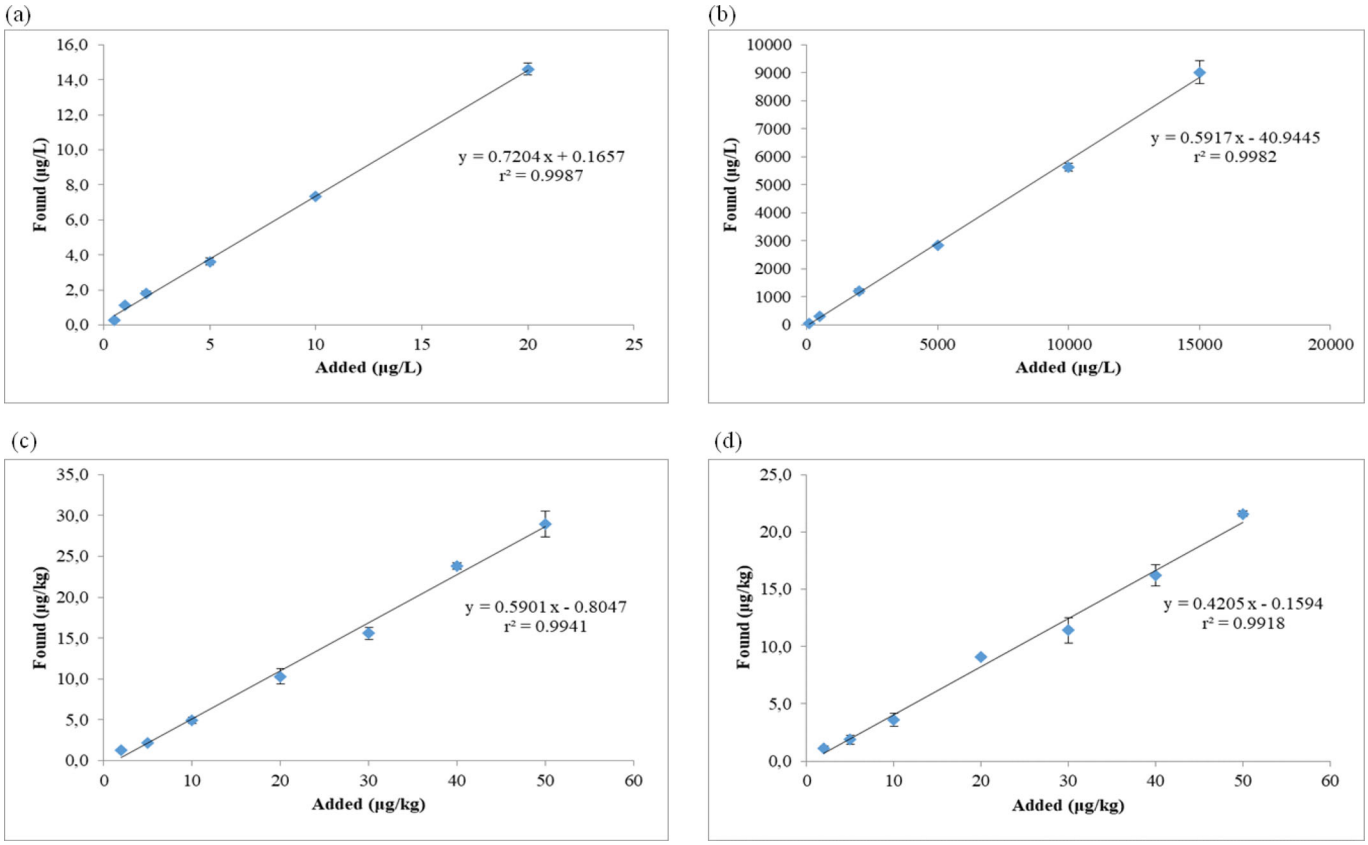


Figure 4. Linearity of analytical HPLC determining the level of bisphenol A (BPA) in sheep's urine and fresh feces, evaluated as an intra-day correlation between the mean measured and added BPA concentrations (n = 2/fortification level); difference bars of both parallels are also presented: (a) free (aglycone) BPA in urine, fortification range from 0.5–20 µg/L (b) total (free + conjugated) BPA in urine, fortification range from 100–15,000 µg/L (c) free (aglycone) BPA in feces, fortification range from 2–50 µg/kg (d) total (free + conjugated) BPA in feces, fortification range from 2–50 µg/kg.

(11.5 µg/L) and BPA–GLUC (13,834 µg/L) was reached after 4 hours of BPA administration. From the last administration of BPA on the 5th day, the concentrations of free BPA and BPA–GLUC were declining sharply and they fell below the analytical LOD on the 7th day, being the 2nd day after the last BPA administration.

Discussion

Development and performance of BPA analysis

An analytical procedure was developed in this work for determination of the time profile of BPA excretion in sheep's urine and feces. Optimizations of the BPA analysis were made mainly regarding the enzymatic deconjugation, extraction from the matrix, concentration of the extract and chromatographic separation.

The aliquot mass of the feces samples was optimized according to the capacity of the SPE Chromabond HR-X, 200 mg cartridges. Therefore, preliminary testing was done taking from 0.25 to 1.5 g of the sample mass. The results clearly demonstrated that sample mass of 1.5 g exceeded the sorbent capacity, as only 26% recovery was obtained. The optimal mass of weighted feces, which enabled as low LOD as possible, was 0.5 g, which gave an average recovery of 51%.

Regarding the deconjugation step, high quality certified reference enzyme with defined activity was used and was for any case added in excess volume of about 2,000 U of the ß-glucuronidase per sample of 0.5 mL of urine compared to

Table 4. Limit of detection (LOD), recovery, repeatability and intra-laboratory reproducibility of bisphenol A (BPA) determination in sheep's urine and moist feces.

Matrix	BPA_{tested}	LOD	Precision category	Fortification level	No. of samples	Mean found ± SD	[a]$Recovery_{mean}$ (%)	CV (%)	[b]CV_H (%)
Urine	Free (aglycone)	0.1 μg/L	Repeatability	10 μg/L	5	5.99 ± 0.49 μg/L	59.9	8.2	32
				5 μg/L	5	3.04 ± 0.32 μg/L	60.7	10.6	36
			Intra-laboratory reproducibility	10 μg/L	11	6.72 ± 0.87 μg/L	67.2	13.0	32
				5 μg/L	10	3.01 ± 0.27 μg/L	60.3	9.0	36
	Total (sum of free + conjugated)	10 μg/L	Repeatability	2,000 μg/L	5	1,215.3 ± 22.3 μg/L	60.8	1.8	14
				500 μg/L	2	313.6 ± 4.0 μg/L	62.7	1.3	18
			Intra-laboratory reproducibility	2,000 μg/L	8	1,037.7 ± 132.5 μg/L	51.9	12.8	14
				500 μg/L	6	289.4 ± 25.3 μg/L	57.9	8.8	18
Feces	Free (aglycone)	1 μg/kg	Repeatability	10 μg/kg	5	5.09 ± 0.89 μg/kg	50.9	15.9	32
				5 μg/kg	5	2.03 ± 0.56 μg/kg	40.7	27.4	36
			Intra-laboratory reproducibility	10 μg/kg	8	6.30 ± 1.82 μg/kg	63.0	28.9	32
				5 μg/kg	7	2.08 ± 0.48 μg/kg	41.6	23.1	36
	Total (sum of free + conjugated)	1 μg/kg	Repeatability	10 μg/kg	5	8.06 ± 1.09 μg/kg	80.6	13.5	32
				5 μg/kg	4	2.96 ± 0.56 μg/kg	59.2	19.1	36
			Intra-laboratory reproducibility	10 μg/kg	7	6.76 ± 2.16 μg/kg	67.6	32.0	32
				5 μg/kg	8	2.82 ± 0.47 μg/kg	56.3	16.7	36

[a]recovery was determined on the basis with free BPA spiking.
[b]Horwitz coefficient of variation.[28]

Table 5. Detected free, total and BPA–GLUC concentrations in urine and feces samples after dietary BPA administration of 100 μg/kg b. w./day to an ewe.

			BPA concentration				
			Urine (μg/L)			Feces (μg/kg)	
Day	Daily BPA adm.*	Hours after BPA adm.	Free BPA	Total BPA	BPA–GLUC**	Free BPA	Total BPA
1	YES	1 h	4.51	8,385	8,380	/	/
		3 h	/	/	/	<1.00	<1.00
		4 h	15.81	15,157	15,141	<1.00	<1.00
		6 h	12.86	7,368	7,356	/	/
		8 h	16.55	6,434	6,417	/	/
		10 h	5.15	4,811	4,805	/	/
		12 h 30 min	3.25	3,967	3,964	24.41	34.93
2	YES	22 h 20 min	1.60	2,225	2,224	17.41	32.56
3	YES	22 h 15 min	2.11	1,643	1,641	/	/
4	YES	22 h 46 min	1.43	2,196	2,195	45.25	53.40
5	YES	23 h	1.09	1,058	1,057	18.89	19.19
		8 h	1.94	2,577	2,576	/	/
6	NO	23 h 45 min	1.38	1,022	1,021	25.23	49.01
7	NO	2 days	0.79	17.88	17.09	3.96	4.93
8	NO	3 days	0.41	<10.0	<10.0	<1.00	<1.00

*adm. is referring to administration.
**BPA–GLUC is calculated as the difference between total BPA and free BPA, as the BPA–GLUC was determined indirectly by enzymatic conversion of the BPA–GLUC to free BPA.
/not tested.

the reported values by literature.[29,30] As recovery samples for determination of the total BPA were also spiked at the beginning of the procedure with the free BPA due to aggravated availability of the certified reference BPA–GLUC, we did our own testing to control and optimize the time duration of the deconjugation process. We took a real study urine sample taken at 4 hours post p. o. administration of BPA at a dose of 0.1 mg/kg b. w. to the ewe and performed the enzymatic deconjugation within different incubation time periods, which justified the time of 16–17 hours, taken for the analysis of urine, being also in line with references in the literature.[24,29,30,31] Regarding feces, the incubation time was shortened to 4 hours due to significantly lower expected concentrations in the study samples. At the end of incubation, the feces samples were totally decomposed as a result of an extremely effective enzymatic activity of the β-glucuronidase used. Additionally, the BPA standard for recovery testing of the total BPA was diluted preferably in H_2O, with an MeCN share of 0–0.25% (v/v), which prevented the denaturation of the enzyme used. This is in accordance with Markham et al.,[30] who demonstrated that the enzyme viability is affected when the organic level exceeds 0.5% of the sample volume, particularly at lower concentrations (<2 ng/mL).

While urine samples were applied directly onto the SPE cartridge, after dilution with water or sodium acetate buffer, BPA from feces had to be extracted using an organic solvent. MeCN was used because of its excellent solubility for the BPA and its strong ability to denature enzymes and precipitate proteins to produce an acceptable matrix background. Re-extraction was also included to increase effectiveness, and no hydrolysis was observed. The following two SPE steps were carried out using modern sorbent materials, prepared with advanced technology, and this is the spherical, hydrophobic polystyrene–divinylbenzene (PS/DVB) copolymer Chromabond HR-X and molecularly

Table 6. Detected free, total and BPA–GLUC concentrations in urine and feces samples after subcutaneous BPA administration of 100 μg/kg b. w./day to an ewe.

			BPA concentration				
			Urine (μg/L)			Feces (μg/kg)	
Day	Daily BPA adm.*	Hours after BPA adm.	Free BPA	Total BPA	BPA–GLUC**	Free BPA	Total BPA
1	YES	1 h	3.42	5,000	4,996	/	/
		2 h	4.24	8,683	8,679	/	/
		4 h	11.53	13,846	13,834	/	/
		4 h 30 min	/	/	/	<1.00	<1.00
		5 h	/	/	/	<1.00	<1.00
		6 h	0.43	3,262	3,261		
		6 h 15 min	/	/	/	4.14	4.41
		8 h	0.63	2,578	2,577	<1.00	<1.00
		10 h	0.88	1,602	1,601		
2	YES	23 h 55 min	0.60	<10.0	<10.0		
		12 h	/	/	/	3.36	2.72
						3.94	3.39
3	YES	23 h 55 min	0.10	15.63	15.52	/	/
4	YES	23 h 25 min	0.41	/	/	26.65	26.51
5	YES	22 h	1.21	456.0	454.8	22.45	20.98
		7 h	2.91	1,723	1,720	/	/
6	NO	23 h 25 min	0.22	18.13	17.91	8.27	5.32
7	NO	2 days	<0.1	<10.0	<10.0	<1.00	<1.00
8	NO	3 days	<0.1	<10.0	<10.0	<1.00	<1.00

*adm. is referring to administration.
**BPA–GLUC is calculated as the difference between total BPA and free BPA, as the BPA–GLUC was determined indirectly by enzymatic conversion of the BPA–GLUC to free BPA.
/not tested.

imprinted polymer (MIP) AFFINIMIP® SPE Bisphenols. The two SPE steps basically followed the analytical method of Deceuninck et al.,[25] developed for the analysis of the free BPA in a large set of food items. However, the final derivatisation step, intended for gas chromatography tandem mass spectrometry (GC-MS/MS) determination, as used in their method, was omitted due to the HPLC-fluorescence analysis used by the method presented in this work. The final sample extracts were dissolved in 0.5–1.0 mL of the starting HPLC mobile phase, containing 35% (v/v) MeCN in H_2O, which had to be additionally diluted (5–25 fold) in the case of analysis of the total BPA in urine to achieve the chromatographic results within the standard calibration curve used in this study.

We did not observe matrix effect due to physicochemical specificity of the fluorescence detection (for the difference of the mass spectrometric detection where such an effect is frequently reported). The greatest complexity of the chromatographic background was observed by urine analysis, preferably of the total BPA, resulting in a 100-fold higher LOD concentration level than for the free BPA. Nevertheless, the composition of the organic component in HPLC method no. 2, comprising MeOH and MeCN in a volume ratio of 1:1 according to Petersen et al.,[32] significantly improved the chromatographic selectivity of BPA from the comprehensive matrix background, as observed for the total BPA in urine and both free and total BPA in the feces.

The importance of appropriate quality control of sample testing was considered, as baseline controls, fortified controls, and study sample replicates were included with each analysis set to avoid and minimize possible artifacts or contamination and ensure appropriate performance characteristics of the BPA analysis. In addition, storage devices with declared absence of BPA were used, high quality glassware was used where possible, and the solvents used in the study were mainly of HPLC grade and screened via reagent blanks.

The analytical procedure for the determination of the total BPA was more demanding than for the determination of only free BPA due to the deconjugation step within the enzymatic decomposition of the sample, which consequently gave a more comprehensive matrix background, and influenced the validation parameters. The moderate recovery levels in sheep's urine and feces, ranging from 52 to 67%, and from 41 to 81% (Table 4), were a consequence of a comprehensive clean-up, including two SPE purification steps needed for isolation of the analyte from the complex biological matrices. Moreover, these moderate recovery levels were absolute levels, as an internal standard was not used, and this is a difference with the LC-MS/MS methods reported in the literature,[12,17,21,23] with these methods also used in human biomonitoring studies.[29,30,33] The repeatability and within-laboratory reproducibility yielded with the CV values, ranging from 1.3 to 27.4% and from 8.8 to 32%, respectively, was generally higher for feces than for urine analysis due extraction of BPA from feces, but did not exceed the CV_H values from the Horwitz equation.[28] Moreover, in 75% of cases the CV values were below two-thirds of the corresponding CV_H values, and thus fully acceptable (Table 4). The analytical LOD value of 0.1 μg/L for free BPA in urine was the same as reported by Zhang et al.,[11,12] while the reported LOD values for total BPA by LC-MS/MS[21,23] were two concentration orders of magnitude lower than the reported value of 10 μg/L obtained by our method, which was a consequence of the better sensitivity and selectivity of mass spectrometric detection in comparison with fluorescence detection at emission and excitation wavelengths below/around 300 nm for the analysis of very complex biofluids. Regarding feces, our LOD value

of 1 μg/kg obtained for both free and total BPA was in the same concentration order of magnitude as the values for BPA of 5 μg/kg and BPAF of 3 μg/kg, being reported by Twaddle et al.[23] and Yang et al.,[24] respectively.

Excretion of BPA, BPA–GLUC and total BPA with urine and feces from an experimentally dosed sheep

Based on the appropriate performance characteristics of the analytical method, we tested BPA excretion in one experimentally dosed sheep. Our study has been one of the few to measure the BPA in the samples of urine in a sheep model,[17,21,34] and to the best of our knowledge no experimental studies were conducted on a sheep model to analyze the excretion of BPA in feces. In our work both dietary and subcutaneous administrations were performed. As biological samples and experimental settings are highly valuable nowadays due to the 3 R (replacement, reduction, refinement) principle, as laid down by the Directive 2010/63/EU of the European Parliament and Council on the protection of animals used for scientific purposes,[35] the detected concentrations of BPA in the urine and feces after subcutaneous administration are reported in this paper as well, even though farm animals would rarely be exposed to subcutaneous administration of BPA. Unfortunately, as neither a metabolic cage nor catheter was used in the experiment, the samples of urine and feces were not total, and thus the percentage of the administered dose was not calculated to omit poor estimation.

The results in our study are comparable with those of studies performed on sheep,[17,34] monkeys[19] and pigs,[20,36] as the BPA in our study was mostly excreted in urine as BPA–GLUC and only a small fraction was excreted in urine as free BPA. Nevertheless, it is important to state that in our work the concentrations of BPA–GLUC in urine were probably slightly overestimated, as the enzyme ß-glucuronidase from *Helix pomatia* Type HP-2 was used for total BPA determination, containing both glucuronidase (≥100,000 U/mL) and sulfatase (≤7,500 U/mL) activity. The overestimation of BPA–GLUC obtained with enzymatic deconjugation was reported by Lacroix et al.,[17] who measured BPA–GLUC directly and compared its concentration with the results of enzymatic deconjugation of BPA–GLUC. As stated above, BPA has been mostly excreted through the kidneys as BPA–GLUC not only in sheep, but in monkeys and pigs as well. In rats, however, the fraction of total BPA excreted in urine was much lower,[18] due to the suggested enterohepatic recirculation of BPA, which was consequently excreted with feces. Regarding other farm animal species, however, one would speculate that the excretion route as reported in sheep is similar for all ruminants (cows, goats, etc.) due to the similarities in their gastrointestinal tracts. To the best of our knowledge, there is no research done on horses and other equids or poultry, and only two studies were conducted on pigs,[20,36] where the researchers reported that after oral dosing BPA was predominately excreted as BPA–GLUC, and approximately half of the dose was excreted 3 hours after administration.[36]

Experimental studies in which BPA was determined in the feces of animal models are even rarer. To the best of our knowledge, such studies were only performed on monkeys[19] and rats.[18] In the feces samples of the monkeys, a much smaller fraction of the dose was excreted via the feces than in rats. Nevertheless, in both species, monkeys and rats, only free BPA was detected in the samples. In our study, the detected free BPA concentrations in feces were similarly low as in monkeys. Interestingly, in our work the total BPA concentrations were approximately of the same levels as found for free BPA, meaning there is a general absence of BPA–GLUC in the sheep's feces samples irrespective of the form of BPA administration, and that BPA is preferably excreted in feces in its free form. However, the concentrations of free BPA in feces at some sampling points were slightly higher then of total BPA after subcutaneous determination, presumably due to variability of the analytical method used, yet hydrolysis in these samples can not be ruled out.

Beside experimental studies, a couple of field studies were conducted, in which the samples of fresh urine, fresh feces, manure or liquid manure were taken directly from the farms.[11–16] In Table 2, the determined concentrations in urine and feces from the field studies are reported. It can be seen that the reported concentrations of free BPA in our study (<1–45.25 μg/kg for feces and <0.1–16.55 μg/L for urine) are in the same concentration range as the reported concentrations in urine and feces[11,12] but generally lower than the concentrations determined in manure samples.[13–16]

In Zhang et al. BPA was found in urine samples in the range from 218 to 446 ng/L[11] and from 1 to 2,120 ng/L[12] and in feces samples in the range from not detected (nd) to 13 μg/kg[11] and from nd to 4 μg/kg.[12] Zhang et al. believed that the BPA found in the samples of urine and feces most likely originated from materials used to coat the inner surfaces of animal food containers.[11] In Kinney et al. BPA was not detected in swine manure,[16] while in Aznar et al. BPA was detected in poultry manure at levels up to 207 μg/kg[13] and in Xu et al. it was detected in hen, duck and swine manure at levels up to 167 μg/kg, 179 μg/kg and 362 μg/kg, respectively, while the concentrations of BPA were lower only in cow manure and were at levels up to 33 μg/kg.[15] Regarding liquid manure, in Fromme et al. the BPA levels ranged between 61 and 1,112 μg/kg of dry weight (d. w.).[14] The authors believed that BPA presence in the liquid manure was most likely the consequence of migration from the inner surface coating of the manure tanks, yet they speculated that contribution entering via animal feed could not be ruled out. It is important, however, to consider that in Fromme et al.[14] the researchers measured BPA based on dry weight, and thus the BPA in dried samples was very concentrated. Hence, it is not relevant to compare their results with other research.

Interestingly, in all the field studies only the free BPA was measured in the fresh urine, fresh feces and manure samples. That seems relevant for fresh feces, as presumably there is only free BPA excreted in it. However, it is assumed

that, like natural estrogens, BPA is deconjugated prior degeneration,[37] and it was found that in humans, monkeys, sheep and pigs BPA is predominantly excreted in urine as its main metabolite, with only a small fraction excreted as free BPA.[17,19,20,38] It is important to be aware of this when using detected concentrations in risk assessments, as concentrations of free BPA could be higher or lower depending on the time of sampling. That is especially true for urine, meanwhile in manure it also depends on the type of manure (liquid or not) and the content of urine in it. In addition, it depends on many other physiological, microbiological and chemical factors, which influence the sample. Thus, it would be of great help, if researchers specified the collection protocol and approximate composition of the manure in the future studies.

Conclusion

The analytical strategy presented in this work enabled the analysis of both free and total BPA in urine and feces samples from a biological experiment by using HPLC-fluorescence technology, which evaluated the BPA concentration profiles by both dietary and subcutaneous administration to one ewe. The results obtained in this work show that the method could also be applied to other ecotoxicological studies of BPA, BPA–GLUC and total BPA in urine and feces. There is currently not much research devoted to the testing of BPA in animal excreta, nor animal feed, although the ingestion of BPA contaminated feed might contribute to the burden of BPA in our environment.

Acknowledgements

We would like to thank our colleagues from the Infrastructure Center of Sustainable Recultivation Vremščica and Institute of Pathology, Wild Animals, Fish and Bees for their technical assistance.

Disclosure statement

The authors declare no conflict of interest.

Funding

This research was supported by the Slovenian Research Agency (ARRS) Program P4-0092 and the Slovenian Research Agency postgraduate (PhD) research funding.

References

[1] Michalowicz, J. Bisphenol A—Sources, Toxicity and Biotransformation. *Environ. Toxicol. Pharmacol.* **2014**, *37*, 738–758.

[2] Chapin, R. E.; Adams, J.; Boekelheide, K.; Gray, L. E.; Hayward, S. W.; Lees, P. S.; McIntyre, B. S.; Portier, K. M.; Schnorr, T. M.; Selevan, S. G.; et al. NTP-CERHR Expert Panel Report on the Reproductive and Developmental Toxicity of Bisphenol A. *Birth. Defects. Res. B. Dev. Reprod. Toxicol.* **2008**, *83*, 157–395.

[3] Flint, S.; Markle, T.; Thompson, S.; Wallace, E. Bisphenol A Exposure, Effects, and Policy: A Wildlife Perspective. *J. Environ. Manage.* **2012**, *104*, 19–34. DOI: 10.1016/j.jenvman.2012.03.021.

[4] Lemos, M. F. L.; Van Gestel, C. A. M.; Soares, A. M. V. M. Reproductive Toxicity of the Endocrine Disrupters Vinclozolin and Bisphenol A in the Terrestrial Isopod *Porcellio Scaber* (Latreille, 1804). *Chemosphere* **2010**, *78*, 907–913. DOI: 10.1016/j.chemosphere.2009.10.063.

[5] Lemos, M.; Gestel, C.; Soares, A. Endocrine Disruption in a Terrestrial Isopod under Exposure to Bisphenol A and Vinclozolin. *J. Soils* Sediments **2009**, *9*, 492–500. DOI: 10.1007/s11368-009-0104-y.

[6] Babić, S.; Barišić, J.; Bielen, A.; Bošnjak, I.; Sauerborn Klobučar, R.; Ujević, I.; Strunjak-Perović, I.; Topić Popović, N.; Čož-Rakovac, R. Multilevel Ecotoxicity Assessment of Environmentally Relevant Bisphenol A Concentrations Using the Soil Invertebrate *Eisenia Fetida*. *J. Hazard. Mater.* **2016**, *318*, 477–486. DOI: 10.1016/j.jhazmat.2016.07.017.

[7] Babić, S.; Barišić, J.; Malev, O.; Klobučar, G.; Popović, N. T.; Strunjak-Perović, I.; Krasnići, N.; Čož-Rakovac, R.; Klobučar, R. S. Sewage Sludge Toxicity Assessment Using Earthworm *Eisenia Fetida*: can Biochemical and Histopathological Analysis Provide Fast and Accurate Insight? *Environ. Sci. Pollut. Res.* **2016**, *23*, 12150–12163. DOI: 10.1007/s11356-016-6097-3.

[8] Novo, M.; Verdu, I.; Trigo, D.; Martinez-Guitarte, J. L. Endocrine Disruptors in Soil: Effects of Bisphenol A on Gene Expression of the Earthworm *Eisenia Fetida*. *Ecotoxicol. Environ. Saf.* **2018**, *150*, 159–167. DOI: 10.1016/j.ecoenv.2017.12.030.

[9] Verdu, I.; Trigo, D.; Martinez-Guitarte, J. L.; Novo, M. Bisphenol A in Artificial Soil: Effects on Growth, Reproduction and Immunity in Earthworms. *Chemosphere* **2018**, *190*, 287–295. DOI: 10.1016/j.chemosphere.2017.09.122.

[10] Careghini, A.; Mastorgio, A. F.; Saponaro, S.; Sezenna, E. Bisphenol A, Nonylphenols, Benzophenones, and Benzotriazoles in Soils, Groundwater, Surface Water, Sediments, and Food: A Review. *Environ. Sci. Pollut. Res.* **2015**, *22*, 5711–5741. DOI: 10.1007/s11356-014-3974-5.

[11] Zhang, H.; Shi, J.; Liu, X.; Zhan, X.; Chen, Q. Occurrence and Removal of Free Estrogens, Conjugated Estrogens, and Bisphenol A in Manure Treatment Facilities in East China. *Water. Res.* **2014**, *58*, 248–257.

[12] Zhang, H.; Shi, J.; Liu, X.; Zhan, X.; Dang, J.; Bo, T. Occurrence of Free Estrogens, Conjugated Estrogens, and Bisphenol A in Fresh Livestock Excreta and Their Removal by Composting in North China. *Environ. Sci. Pollut. Res.* **2014**, *21*, 9939–9947. DOI: 10.1007/s11356-014-3002-9.

[13] Aznar, R.; Albero, B.; Perez, R. A.; Sanchez-Brunete, C.; Miguel, E.; Tadeo, J. L. Analysis of Emerging Organic Contaminants in Poultry Manure by Gas Chromatography-Tandem Mass Spectrometry. *J. Sep. Sci.* **2018**, *41*, 940–947. DOI: 10.1002/jssc.201700883.

[14] Fromme, H.; Kuchler, T.; Otto, T.; Pilz, K.; Muller, J.; Wenzel, A. Occurrence of Phthalates and Bisphenol A and F in the Environment. *Water. Res.* **2002**, *36*, 1429–1438. DOI: 10.1016/S0043-1354(01)00367-0.

[15] Xu, P.; Zhou, X.; Xu, D.; Xiang, Y.; Ling, W.; Chen, M. Contamination and Risk Assessment of Estrogens in Livestock Manure: A Case Study in Jiangsu Province, China. *IJERPH.* **2018**, *15*, 125. DOI: 10.3390/ijerph15010125.

[16] Kinney, C. A.; Furlong, E. T.; Kolpin, D. W.; Burkhardt, M. R.; Zaugg, S. D.; Werner, S. L.; Bossio, J. P.; Benotti, M. J. Bioaccumulation of Pharmaceuticals and Other Anthropogenic Waste Indicators in Earthworms from Agricultural Soil Amended with Biosolid or Swine Manure. *Environ. Sci. Technol.* **2008**, *42*, 1863–1870. DOI: 10.1021/es702304c.

[17] Lacroix, M. Z.; Puel, S.; Collet, S. H.; Corbel, T.; Picard-Hagen, N.; Toutain, P. L.; Viguié, C.; Gayrard, V. Simultaneous Quantification of Bisphenol A and Its Glucuronide Metabolite (BPA-G) in Plasma and Urine: Applicability to Toxicokinetic Investigations. *Talanta* **2011**, *85*, 2053–2059. DOI: 10.1016/j.talanta.2011.07.040.

[18] Kurebayashi, H.; Betsui, H.; Ohno, Y. Disposition of a Low Dose of 14C-Bisphenol A in Male Rats and Its Main Biliary Excretion as BPA Glucuronide. *Toxicol. Sci.* **2003**, *73*, 17–25. DOI: 10.1093/toxsci/kfg040.

[19] Kurebayashi, H.; Harada, R.; Stewart, R. K.; Numata, H.; Ohno, Y. Disposition of a Low Dose of Bisphenol A in Male and Female Cynomolgus Monkeys. *Toxicol. Sci.* **2002**, *68*, 32–42. DOI: 10.1093/toxsci/68.1.32.

[20] Tijdeman, J. N. M. Evaluation of the Interspecific Variations of BPA Disposition in Pigs and Ewes. P.H.D. Thesis, University of Utrecht, **2010**. https://dspace.library.uu.nl/handle/1874/41124 (accessed: June 1, 2019).

[21] Gingrich, J.; Pu, Y.; Ehrhardt, R.; Karthikraj, R.; Kannan, K.; Veiga-Lopez, A. Toxicokinetics of Bisphenol A, Bisphenol S, and Bisphenol F in a Pregnancy Sheep Model. *Chemosphere* **2019**, *220*, 185–194. DOI: 10.1016/j.chemosphere.2018.12.109.

[22] Liao, C.; Liu, F.; Alomirah, H.; Loi, V. D.; Mohd, M. A.; Moon, H. B.; Nakata, H.; Kannan, K. Bisphenol S in Urine from the United States and Seven Asian Countries: Occurrence and Human Exposures. *Environ. Sci. Technol.* **2012**, *46*, 6860–6866. DOI: 10.1021/es301334j.

[23] Twaddle, N. C.; Churchwell, M. I.; Vanlandingham, M.; Doerge, D. R. Quantification of Deuterated Bisphenol A in Serum, Tissues, and Excreta from Adult Sprague-Dawley Rats Using Liquid Chromatography with Tandem Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 3011–3020. DOI: 10.1002/rcm.4733.

[24] Yang, Y.; Yin, J.; Yang, Y.; Zhou, N.; Zhang, J.; Shao, B.; Wu, Y. Determination of Bisphenol AF (BPAF) in Tissues, Serum, Urine and Feces of Orally Dosed Rats by Ultra-High-Pressure Liquid Chromatography-Electrospray Tandem Mass Spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci.* **2012**, *901*, 93–97. DOI: 10.1016/j.jchromb.2012.06.005.

[25] Deceuninck, Y.; Bichon, E.; Durand, S.; Bemrah, N.; Zendong, Z.; Morvan, M. L.; Marchand, P.; Dervilly-Pinel, G.; Antignac, J. P.; Leblanc, J. C.; Le Bizec, B. Development and Validation of a Specific and Sensitive Gas Chromatography Tandem Mass Spectrometry Method for the Determination of Bisphenol A Residues in a Large Set of Food Items. *J. Chromatogr. A* **2014**, *1362*, 241–249. DOI: 10.1016/j.chroma.2014.07.105.

[26] AFFINISEP. AFFINIMIP® SPE Bisphenol A, Q1. **2014**. http://www.polyintell.com (accessed July 9, 2014).

[27] Cerkvenik-Flajs, V.; Volmajer Valh, J.; Gombač, M.; Švara, T. Analysis and Testing of Bisphenol A, Bisphenol A Diglycidyl Ether and Their Derivatives in Canned Dog Foods. *Eur. Food Res. Technol.* **2018**, *244*, 43–56. DOI: 10.1007/s00217-017-2930-2.

[28] European Commission. Commission Decision 2002/657/EC of 12 August 2002 Implementing Council Directive 96/23/EC concerning the Performance of Analytical Methods and the Interpretation of Results. *Off. J. Eur. Commun. L* **2002**, *L221*, 8–36.

[29] Buscher, B.; van de Lagemaat, D.; Gries, W.; Beyer, D.; Markham, D. A.; Budinsky, R. A.; Dimond, S. S.; Nath, R. V.; Snyder, S. A.; Hentges, S. G. Quantitative Analysis of Unconjugated and Total Bisphenol A in Human Urine Using Solid-Phase Extraction and UPLC-MS/MS: method Implementation, Method Qualification and Troubleshooting. *J. Chromatogr. B. Analyt. Technol. Biomed. Life. Sci* **2015**, *1005*, 30–38. DOI: 10.1016/j.jchromb.2015.09.020.

[30] Markham, D.; Waechter, J.; Budinsky, R.; Gries, W.; Beyer, D.; Snyder, S.; Dimond, S.; Rajesh, V. N.; Rao, N.; Connolly, P.; et al. Development of a Method for the Determination of Total Bisphenol A at Trace Levels in Human Blood and Urine and Elucidation of Factors Influencing Method Accuracy and Sensitivity. *J. Anal. Toxicol.* **2014**, *38*, 194–203. DOI: 10.1093/jat/bku012.

[31] Pizarro, N.; Ortuno, J.; Farre, M.; Hernandez-Lopez, C.; Pujadas, M.; Llebaria, A.; Joglar, J.; Roset, P. N.; Mas, M.; Segura, J.; et al. Determination of MDMA and Its Metabolites in Blood and Urine by Gas Chromatography-Mass Spectrometry and Analysis of Enantiomers by Capillary Electrophoresis. *J. Anal. Toxicol.* **2002**, *26*, 157–165. DOI: 10.1093/jat/26.3.157.

[32] Petersen, H.; Schaefer, A.; Buckow, C. A.; Simat, T. J.; Steinhart, H. Determination of Bisphenol A Diglycidyl Ether (BADGE) and Its Derivatives in Food: Identification and Quantification by Internal Standard. *Eur. Food Res. Technol.* **2003**, *216*, 355–364. DOI: 10.1007/s00217-002-0648-1.

[33] Nicolucci, C.; Rossi, S.; Menale, C.; del Giudice, E. M.; Perrone, L.; Gallo, P.; Mita, D. G.; Diano, N. A High Selective and Sensitive Liquid Chromatography-Tandem Mass Spectrometry Method for Quantization of BPA Urinary Levels in Children. *Anal. Bioanal. Chem.* **2013**, *405*, 9139–9148. DOI: 10.1007/s00216-013-7342-y.

[34] Gauderat, G.; Picard-Hagen, N.; Toutain, P. L.; Corbel, T.; Viguie, C.; Puel, S.; Lacroix, M. Z.; Mindeguia, P.; Bousquet-Melou, A.; Gayrard, V. Bisphenol A Glucuronide Deconjugation is a Determining Factor of Fetal Exposure to Bisphenol A. *Environ. Int.* **2016**, *86*, 52–59. DOI: 10.1016/j.envint.2015.10.006.

[35] European Parliament, Council of the European Union. Directive 2010/63/EU of the European Parliament and of the Council of September 2010 on the Protection of Animals Used for Scientific Purposes. *Off. J. Eur. Union* **2010**, *L276*, 33–77.

[36] Gayrard, V.; Lacroix, M. Z.; Grandin, F. C.; Collet, S. H.; Mila, H.; Viguie, C.; Gély, C. A.; Rabozzi, B.; Bouchard, M.; Léandri, R.; et al. Oral Systemic Bioavailability of Bisphenol A and Bisphenol S in Pigs. *Environ. Health Perspect.* **2019**, *127*, 77005. DOI: 10.1289/EHP4599.

[37] Wang, H.; Liu, Z. H.; Zhang, J.; Huang, R. P.; Yin, H.; Dang, Z.; Ping-Xiao, W.; Yu, L. Insights into Removal Mechanisms of Bisphenol A and Its Analogues in Municipal Wastewater Treatment Plants. *Sci. Total. Environ.* **2019**, *692*, 107–116. DOI: 10.1016/j.scitotenv.2019.07.134.

[38] Thayer, K. A.; Doerge, D. R.; Hunt, D.; Schurman, S. H.; Twaddle, N. C.; Churchwell, M. I.; Garantziotis, S.; Kissling, G. E.; Easterling, M. R.; Bucher, J. R.; Birnbaum, L. S. Pharmacokinetics of Bisphenol A in Humans following a Single Oral Administration. *Environ. Int.* **2015**, *83*, 107–115. DOI: 10.1016/j.envint.2015.06.008.