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Multiclass method to determine emerging pollutants in bats using a non-invasive approach based on guano matrix

Julia Martín [a,*], Slawomir Gonkowski [b], Annemarie Kortas [c], Przemysław Sobiech [c], Liliana Rytel [c], Juan Luis Santos [a], Irene Aparicio [a], Esteban Alonso [a]

[a] Departamento de Química Analítica, Universidad de Sevilla, C/ Virgen de África, 7, E-41011 Sevilla, Spain
[b] Department of Clinical Physiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Street Oczapowskiego 14, 10-719 Olsztyn, Poland
[c] Department of Internal Diseases with Clinic, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, ul. Oczapowskiego 14, 10-719 Olsztyn, Poland

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ABSTRACT

Emerging pollutants have been ubiquitously found in environmental compartments, while there is scarce information about these substances and their effects on health status in wild terrestrial mammals. Bat species are very sensitive animals to any changes in the environment and are considered one of the best bioindicators of the quality of the environment to terrestrial wildlife. To acquire a better knowledge of the environmental exposure to these animals, a multiclass method is proposed to determine 20 emerging pollutants (six perfluoroalkyl substances (PFAS), four parabens (PB), four benzophenones (BP), a plasticizer (BPA), and five surfactants (four linear alkylbenzene sulfonates (LAS) and nonylphenol (NP)) in bats using a non-invasive approach based on guano matrix. Sample treatment involved ultrasonic solvent extraction and dispersive solid phase extraction prior to analysis in a single run with liquid chromatography-tandem mass spectrometry. The main variables affecting the extraction and clean-up steps were evaluated using single and multivariate strategies. Under the optimized conditions, satisfactory analytical characteristics in terms of linearity, recoveries (>80 %), precision (RSD < 24 %) and method quantification limits (from 0.01 to 64 ng/g dry weight) were obtained. Furthermore, and as a proof of concept, guano samples from a bat reference population were collected from a colony located in Brenna village, in south Poland. The results confirm the exposure of wild bats to emerging pollutants (LAS, PFAS, and PB compounds were frequently detected in the samples) and the suitability of the bat guano matrix for understanding the environmental exposure in terrestrial mammals.

1. Introduction

The ongoing and significant release of pollutants of emerging concern into the aquatic environment is a problem that has long been known [1]. The presence and occurrence of emerging compounds has been widely described in abiotic matrices [2,3], aquatic wildlife (especially fish and mussels) [4–7] and humans [8–11]. However, there is scarce information about these substances and their associations with health parameters in wild terrestrial mammals [4,12–15]. This is probably explained by sampling troubles together with matrix complexity and difficult analysis.

Substances such as plasticizers, pharmaceuticals and personal care products, surfactants or perfluoroalkyl compounds (PFAS) are particularly relevant causing disturbances in the functioning organs and systems of wildlife organisms [16]. They are exposed to these substances mainly through the gastrointestinal tract, but also through the respiratory system [14,16,17]. Toxicological studies have provided evidence that low concentration levels of some these pollutants are associated with animal damage ranging from genotoxicity and cytotoxicity to tissue malfunction, altered organism physiology, and disease development [4,12,16]. For example, recently, Wang et al. [17] quantified PFAS in the blood of captive Siberian tigers (Harbin, China) and observed a positive relationship between higher PFAS levels and a marker of liver damage.

Bat species are animals very sensitive to any changes in the environment, including the content of toxic substances in it [18,19]. Therefore, bats are considered one of the best bioindicators of the quality of the environment to terrestrial wildlife [18,20,21]. Some

* Corresponding author.
E-mail address: jbueno@us.es (J. Martín).

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authors [19,22] have proposed the use of bats as bioindicators in the detection of environmental contamination. Anthropogenic changes and progressive environmental pollution lead to a continuous drastic decline in their population [21]. Alfonso et al. [19] found a positive correlation between measured levels of Cd^{2+} and Zn^{2+} and the fecal prevalence of *E. hessei* from bat populations. Kannan et al. [23] quantified high concentrations of PCB, PBDE, DDT, and chlordanes in the tissues diseased bats in New York. The presence of pesticides in bats have also been considered as a significant factor contributing to its declines [22,24,25]. However, to our knowledge, it is not known what degree of exposure bats are to other pollutants of emerging concern, as well as their possible impact on bat health status and the number of their population.

Environmental pollutants are highly heterogeneous compounds. Exposome research advocates for more powerful analytical and computational tools for the characterization and quantification of exposures simultaneously to multiple environmental pollutants, anticipating possible health outcomes that an organism can develop throughout life. The additive impact of multiple compounds may result in toxic or other biological effects that otherwise will not be induced by individual compound. For this, advanced comprehensive multiclass analytical methods covering a single determination are needed [26–29]. The use of individual class-specific methods, although usually have excellent recoveries, they cannot provide an overview of the exposure to emerging pollutants in the matrix analyzed and are not practical in long-term studies involving many samples [28]. Till now analytical methodologies focused on terrestrial wildlife integrate no more than two different classes and are mainly proposed for the analysis of persistent organic compounds [13,30–34]. On the other hand, matrices other than blood and urine have not frequently used for biomonitoring studies [17,35,36]. Non-invasive matrices such as hair, nail or feces seem to be especially interesting and useful to assess the exposure of living organisms [9,37–40].

A critical first step to recognize the exposure of pollutants of emerging concern in terrestrial wildlife is to develop and validate analytical methods. Therefore, and considering recent developments in non-invasive approaches in wildlife studies, in this work a multiclass method is proposed to determine twenty emerging pollutants (six PFAS, four parabens (PB), four benzophenones (BP), a plasticizer (bisphenol A (BPA)), and five surfactants (four linear alkylbenzene sulfonates (LAS C10-C13) and nonylphenol (NP)) in bat guano matrix. The proposed method was then applied to guano samples collected from bats summer colonies located in Poland.

2. Experimental

2.1. Target analytes

Selected compounds were perfluorobutanoic acid (PFBuA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorooctane sulfonic acid (PFOS), methylparaben (MeP), ethylparaben (EtP), propylparaben (PrP), butylparaben (PuP), BPA, BP1, BP2, BP3, BP8, NP and LAS C10-13. Their physicochemical properties are shown in the supplementary material (Table S1). The purity of standards and reagents used are also described in the supplementary material. Individual standard stock solutions were prepared in methanol (MeOH) to obtain 1000 mg/L concentration and stored in dark glass vials at –18 °C. Working mixture concentrations of standards for the calibration curve and fortification of the samples were prepared by diluting stock solutions with a mixture methanol:water, 50:50 (v/v) or methanol, respectively. All the intermediate standard solutions were prepared weekly from stock solutions and kept refrigerated at 5 °C.

2.2. Dropping collection and storage

Bat guano samples were collected from the colony located in Brenna - a village in South Poland (number of citizens: 6134), colony location: school attic, coordinates: 49°43′37.4″N 18°53′46.3″E; approximate colony size: 250 bats. Sampling was performed in August and September 2021. For sampling, glass litter boxes were placed on different parts of the floor of the rooms where there were colonies of bats. After 48 h, the cuvettes were removed and the guano from the cuvettes was placed in glass containers, frozen and stored at −20° C until further analysis.

During sampling, particular emphasis was placed on avoiding disturbing and stressing the bats. The collection of guano samples was made according to the Act for the Protection of Animals for Scientific or Educational Purposes of 15 January 2015 (Official Gazette 2015, No. 266), applicable in the Republic of Poland. Because guano sample collection was a non-invasive procedure, which was not associated with stressing and scaring the bats and did not affect their welfare, consent was not required, approval from the Bioethical Committee for the present study was not required.

2.3. Analytical method

2.3.1. Preparation of spiked samples

Fortified commercial guano samples were prepared containing target analytes in different concentration ranges according to their instrument sensibility: from the method quantification limits (MQL) to 100 ng/g dry weight (dw), except for LAS mixture to 1500 ng/g dw. The spiked volume of the mixture of the standards was adjusted to 1 ml in MeOH for each concentration to soak the guano matrix and thus to ensure contact with the analytes. Once spiked, samples were left to stand for 24 h before treatment to evaporate the MeOH solvent at room temperature.

2.3.2. Sample treatment

Before treatment, guano samples were homogenized in a grinder, freeze dried and sieved (<100 μm). An aliquot of 1.0 g of the pretreated guano was weighed in a 12 ml glass tube. The sample was spiked with a mixture of the ISs perfluorooctanoic acid-$^{13}C_4$, propylparaben-$^{13}C_6$, benzophenone-d_{10} and bisphenol A-d_{16} (final concentration 25 ng/g each) and subjected to the extraction method as follow. The sample was sonicated with 7 ml of MeOH (0.5 % v/v, formic acid) as extraction solvent in a bath for 5 min and centrifuged for 5 min (4050 × g). The extraction procedure was repeated three times and the supernatants were combined.

To remove interferences from the matrix, a cleaning step was performed with 0.3 g of C18 sorbent, shaking for 2 min and centrifugation for 5 min (4050 × g). The solvent was evaporated under a N_2 stream at room temperature to a dry residue and then reconstituted in 0.25 ml of a mixture MeOH:H_2O (50:50 v/v). A 10 μL aliquot of the extract, filtered through a 0.22 μm, was injected into the liquid chromatography tandem mass spectrometry instrument (LC-MS/MS).

2.3.3. Liquid chromatography tandem mass spectrometry conditions

Chromatographic analyses were performed on an Agilent 1260 Infinity II (Agilent, Santa Clara, CA, USA). Compounds were analyzed using a method previously described by Martín et al. [9] slightly modified. An HALO C18 Rapid Resolution (50 × 4.6 mm id, 2.7 μm) analytical column was used which was protected by a HALO C18 guard column (4.6 × 5 mm, 2.7 um) (Teknokroma, Spain). The chromatographic process was performed at room temperature in a gradient manner using MeOH (solvent A) and a buffer solution of acetic acid/ammonium acetate (pH 4.4) (solvent B) at 0.6 ml/min flow. The elution program was as follows: 0–14 min, gradient from 28 to 70 % of solvent A, increased to 80 % of A in 5 min and to 100 % of A in 6 min and held for 2 min.

A 6495 triple quadrupole mass spectrometer with an electrospray ionization source operated in negative mode was coupled to the LC system. Two MRM transitions, for identification and quantification purposes, were used for each analyte. Ionization and fragmentation conditions are summarized in Table S2.

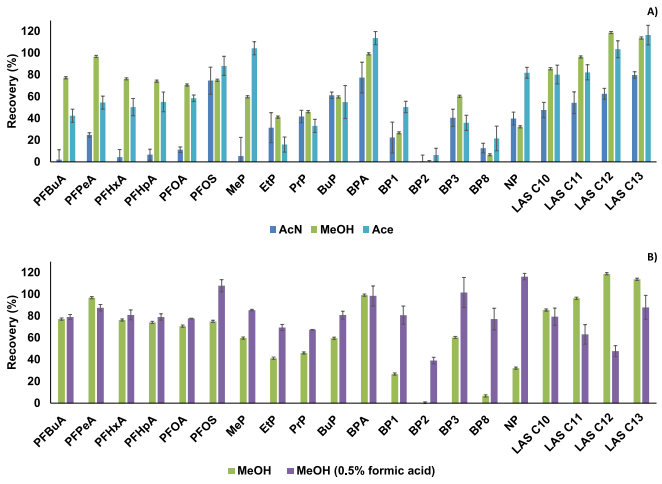

Fig. 1. Optimization of the extraction solvent (A) and acidification (B) (n = 3).

2.4. Validation requirements

The method was in-house validated using matrix effect, linearity, sensitivity, accuracy, and specificity:

First, to assess the impact of the matrix on the suppression/enhancement ionization of the analytes, we compared the slopes obtained in the calibration with matrix-matched standards to those obtained in pure solvent (methanol:water, 50:50 v/v), containing 100 ng/mL of the ISs. For calibration purposes, eight concentration levels including 0.01, 0.05, 0.10, 2.00, 5.00, 25.0, 50.0 and 100 ng/g dw were prepared for most of compounds while 25.0, 50.0, 100, 500, 750, 1000, 1250 and 1500 ng/g dw were used for LAS mixture. Calibration curve was built from the MQL to 100 ng/g dw, except for LAS mixture to 1500 ng/g dw. Linearity was determined by the least squares method, giving the regression and correlation parameters of the calibration curves. The method detection limits (MDL) and MQL were calculated as the concentrations of each compound corresponding to a signal-to-noise ratio of 3:1 and 10:1, respectively, using guano spiked samples at low concentration levels.

Accuracy (trueness and precision) of the method was assessed using commercial guano samples spiked at three concentration levels and in triplicate (5 ng/g dw (low), 25 ng/g dw (medium) and 50 ng/g dw (high), except LAS mixture 100 ng/g dw (low), 500 ng/g dw (medium) and 1000 ng/g dw (high). Accuracy was evaluated by a recovery control over the whole procedure, which included extraction from the matrix, d-SPE and concentration step. The precision is expressed through the relative standard deviation (%, RSD) of measurements on different days.

To guarantee the quality assurance of the results, a protocol involving the use of control samples including fortified commercial guano samples (at 25 ng/g dw, except ΣLAS mixture at 250 ng/g dw), a mixture of the standards of the target compounds in pure solvent (at 100 ng/ml, except LAS mixture at 1000 ng/mL), solvent (methanol:water 50:50 v/v) and procedural blanks (without guano) injections) were included in each analytical batch.

3. Results and discussion

3.1. Optimization of the extraction procedure

Ultrasonic solvent extraction (USE) was selected as the extraction method and dispersive solid phase extraction (d-SPE) for the purification of the extracts according to previous studies [5]. The main variables affecting the extraction step (solvent type, volume, time, and steps) and clean-up (disperser sorbent and amount of disperser solvent) were

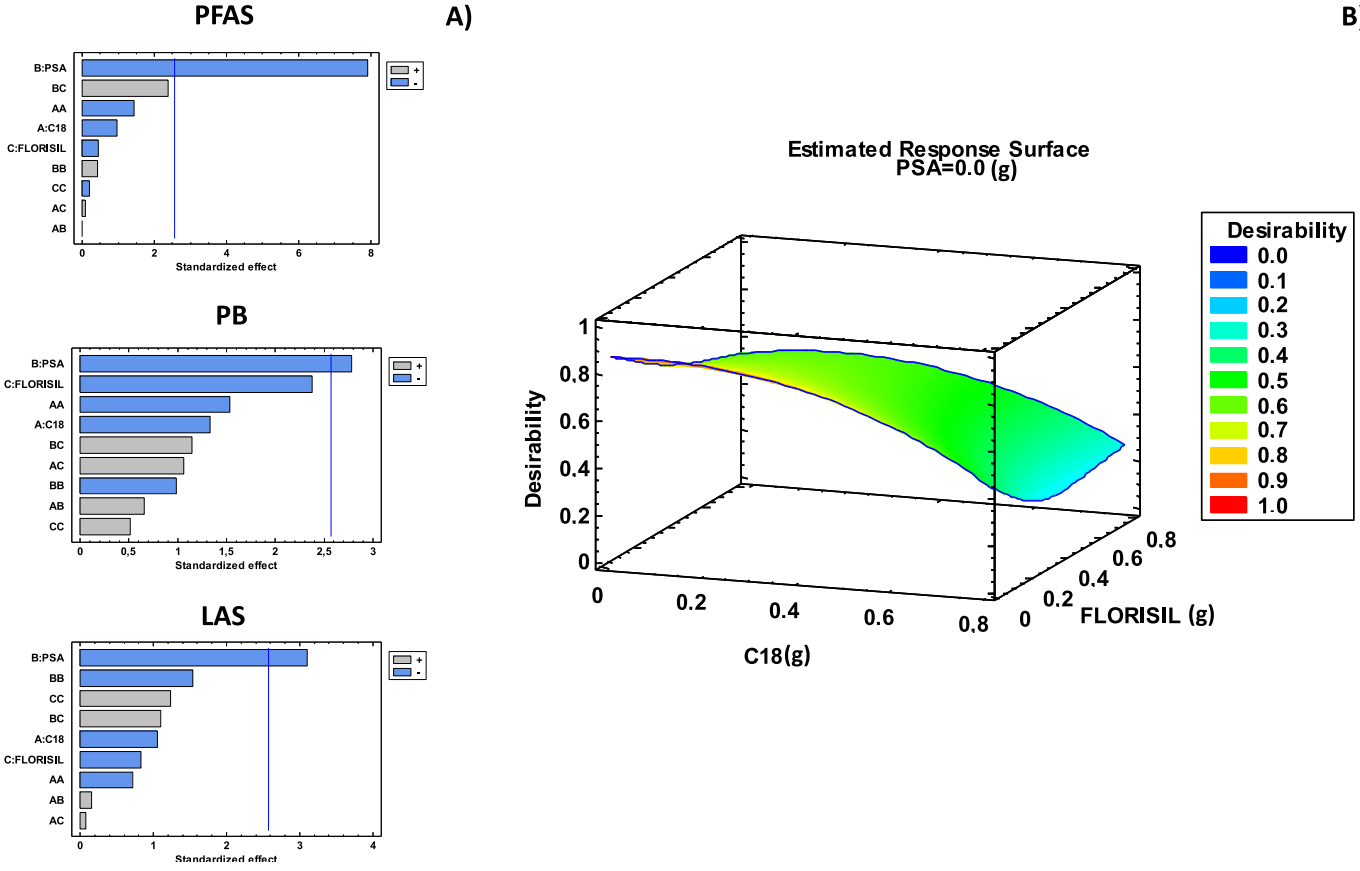

Fig. 2. Pareto charts (A) and global response surface plot (B), corresponding to the desirability function, when optimizing the d-SPE clean-up step.

evaluated individually and in multiple variables design experiments. Experiments were carried out using commercial guano samples fortified at 25 ng/g dw, except for ΣLAS mixture at 250 ng/g dw.

3.1.1. Selection of the extraction solvent

First, acetonitrile (AcN), MeOH, and acetone (Ace) were tested as organic solvents. To optimize it, the spiked sample was mixed with 5 ml of each solvent studied and extracted for 10 min in the ultrasonic bath. Fig. 1A shows the results obtained. The best extraction recoveries for most investigated compounds were clearly obtained when using MeOH, especially for PFAS (mean extraction efficiency: 78, 58 and 21 % with MeOH, Ace, and AcN, respectively) and LAS (mean extraction efficiency: 104, 96, 61 % with MeOH, Ace and AcN, respectively). However, in case of NP Ace seems to be the optimal solvent. The BP group was the worst recovered with all solvents tested (from 29 % with Ace to 19 % with AcN). The effect of acidification was tested in a second step by comparison of MeOH and MeOH (0.5 % in formic acid) (see. Fig. 1B). For most of the analytes, the extraction efficiencies were increased using the MeOH acidified mixture, especially those worse extracted without acidification: PB (76 %), BP (75 %) and NP (112 %). Mixtures of organic solvents with acidic modifiers have been reported to improve the extraction of compounds [5,41–43]. The increase of extraction recoveries of compounds could be explained by the prevalence of their nonionic form under acidic conditions facilitating their transference to the organic solvent. These results are consistent with those reported for PFAS by Abril et al. [41] and Li et al. [42] and for PB and BP by Abril et al. [41] and Luque-Muñoz et al. [43]. Therefore, MeOH (0.5 % in formic acid) was selected as the optimum solvent to extract analytes from guano samples.

3.1.2. Selection of the cleaning sorbent and amount: experimental design

A cleaning step was optimized to remove possible interference from the USE extract using dispersive adsorbents (d-SPE) by means of a Box–Behnken experimental design. Reversed phase sorbent (C18), primary-secondary amine bonded silica (PSA), and florisil, in amounts between 0, 0.4 and 0.8 g, were evaluated, resulting in a total of 15 analytical experiments. The spiked guano samples were treated following the optimization procedure previously described. All experiments were performed in randomized order and three center-point experiments were incorporated.

According with Pareto charts, the amount of PSA resulted the most significant variable, using a Student's *t*-test, being notable the negative effect on the extraction efficiency of most of the target compounds at 95 % of confidence. This can be explained by its higher adsorption and affinity by selected compounds. Florisil sorbent was also discarded. Pareto charts of some of the target compounds can be seen in Fig. 2A. On the other hand, different behaviors were found with respect to the amount of C18 according to the chemical class studied. The extraction efficiencies remain essentially unchanged for BPA and PB of shorter chain with the amount of C18, while for LAS a pronounced increase was observed when increasing the amount of C18. This can be explained considering that this sorbent is able to remove important apolar and moderately polar compounds such as lipophilic interferences, which could decrease the degree of signal suppression of the latter eluted analytes. PFAS, BP, NP, and PB of longer-chain groups presented better results when the amount of C18 increased up to 0.22–0.33 g. The optimized results are shown in three-dimensional response surface images to better comprehend the effects of the three variables and their interaction (Fig. 2B). The global desirability function (D) was obtained as a geometric mean of the individual desirability functions between 0 (least desirable) and 1 (most desirable). A compromise value of 0.3 g of C18 sorbent was selected to reduce matrix effect and maximize the simultaneous extraction of compounds (desirability function of 0.872).

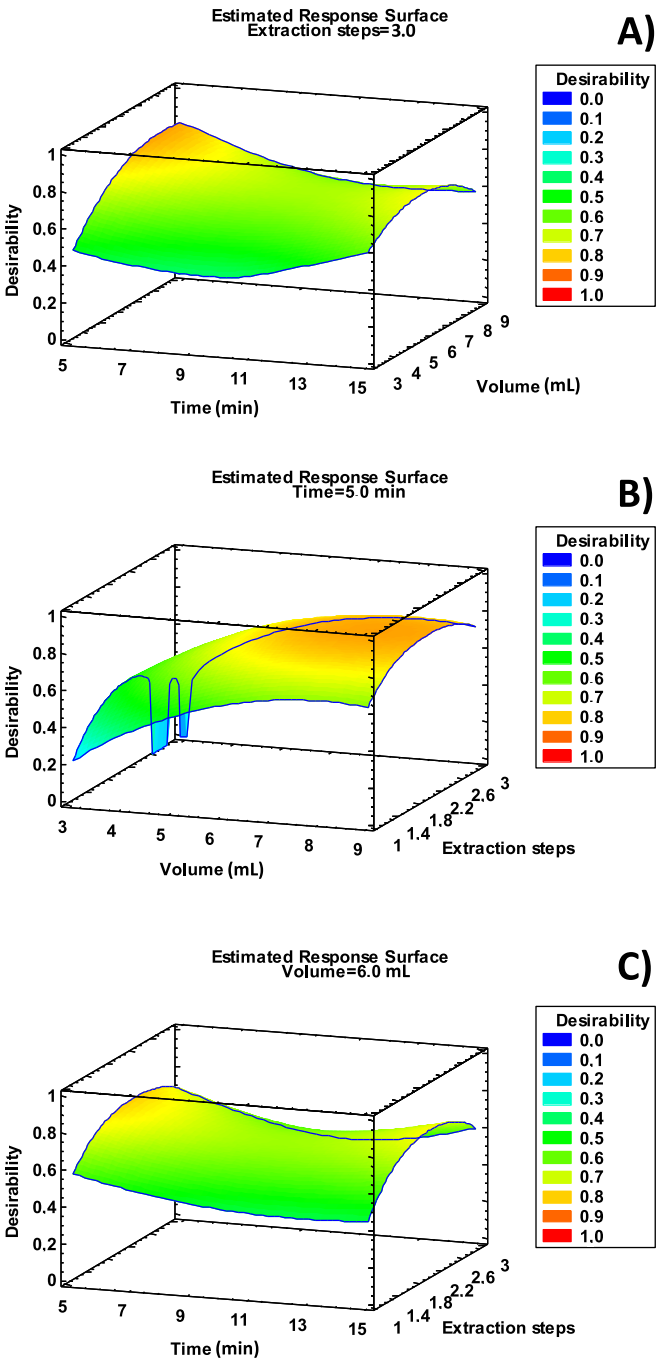

Fig. 3. Global response surface plots, corresponding to the desirability function, when optimizing the following pair of factors from the extraction procedure: time vs volume (A); volume vs extraction steps (B); time vs extraction steps (C).

3.1.3. Selection of the extraction steps, solvent volume, and extraction time: experimental design

In the already optimized conditions, MeOH (0.5 % in formic acid) and C18 (0.3 g) as solvent extraction and cleaning sorbent and amount, respectively, the effect of solvent volume, extraction time, and number of extraction steps were optimized using a second Box–Behnken experimental design. Three levels were selected for each factor (volume (3, 6 and 9 ml), extraction steps (1, 2 y 3) and extraction time (5, 10 and 15 min) resulting 15 set of experiments.

The solvent volume followed by the number of extraction steps were the most significant variables, highlighting the positive interaction.

Table 1
Linear ranges, calibration curves and MDL and MQL values of studied compounds in guano matrix.

Compound	LDR (ng/g dw)	Calibration curve	r^2	MDL (ng/g dw)	MQL (ng/g dw)
PFBuA	0.01–100	Y = 0.0008 X + 0.0007	0.999	0.004	0.01
PFPeA	0.01–100	Y = 0.0021 X + 0.0090	0.996	0.004	0.01
PFHxA	0.01–100	Y = 0.0045 X + 0.0358	0.997	0.004	0.01
PFHpA	0.01–100	Y = 0.0042 X + 0.0071	0.999	0.004	0.01
PFOA	0.01–100	Y = 0.0088 X + 0.0290	0.998	0.004	0.01
PFOS	0.01–100	Y = 0.0005 X + 0.0002	0.996	0.004	0.01
MeP	0.05–100	Y = 0.0029 X + 0.0071	0.998	0.02	0.05
EtP	0.05–100	Y = 0.0038 X + 0.0021	0.998	0.02	0.05
PrP	0.05–100	Y = 0.0061 X + 0.0017	0.999	0.02	0.05
BuP	0.05–100	Y = 0.0079 X + 0.0111	0.998	0.02	0.05
BPA	5.00–100	Y = 0.0117 X + 0.1392	0.997	2.00	5.00
BP1	0.10–100	Y = 0.0362 X + 0.7986	0.999	0.04	0.10
BP2	0.10–100	Y = 0.0101 X + 0.0262	0.990	0.04	0.10
BP3	0.10–100	Y = 0.0643 X + 1.0932	0.996	0.04	0.10
BP8	0.10–100	Y = 0.0318 X + 0.1160	0.999	0.04	0.10
NP	2.00–100	Y = 0.0001 X + 0.0173	0.996	0.60	2.00
LAS C10	24.6–738	Y = 0.0129 X + 2.3321	0.993	7.40	24.6
LAS C11	64.2–1926	Y = 0.0139 X + 8.2275	0.999	19.3	64.2
LAS C12	62.6–1848	Y = 0.0141 X + 8.0920	0.997	18.5	62.6
LAS C13	47.8–1404	Y = 0.0117 X + 2.6665	0.999	14.0	47.8

LDR: Linear dynamic range; r^2: determination coefficient; MDL: method detection limit; MQL: method quantification limit.

Diagrams of the desirability function: (A) time vs volume; (B) volume vs extraction steps; (C) time vs extraction steps are shown in Fig. 3.

Increasing the extraction volume from 3 ml to 9 ml improved extraction efficiencies, especially by the groups of PB (from 0.25 to 0.90), BP (from 0.30 to 0.80), PFAS (D from 0.15 to 0.55), BPA (from 0.65 to 0.95) and NP (from 0.60 to 0.98). A third extraction step also resulted to have a positive effect on the extraction efficiencies of PFAS (D from 0.45 to 0.95), NP (D from 0.40 to 0.80) and LAS (D from 0.20 to 0.60) whereas for the rest of the compounds the difference between the second and third extraction was not significant. As a result, a compromise of 7 ml, three extraction steps and 5 min was used to maximize simultaneous extraction of the compounds with a global desirability function of 0.838.

3.2. Method validation

Student's *t* test was used to assess statistical differences between calibration in pure solvent and calibration with a matrix match. Table S2 shows the statistical and analytical parameters obtained for each compound. Statistical differences among slope values for the calibration curves were observed for most of the analytes because of the presence of co-extracted matrix components, which typically suppress the analyte signal in mass spectrometry. Therefore, a matrix-matched calibration was needed. The calibration curves and sensibility of the method are reported in Table 1. Linearity of the curves, estimated by the determination coefficients, was > 0.990. The MQLs found ranged from 0.01 ng/g dw for PFAS to 64 ng/g dw for LAS C11.

The accuracy of the proposed analytical method is shown in Table 2. Trueness, in terms of relative recoveries, was adequate for all analytes with values ranging from 82 % to 124 %. The intra and inter-day precision was<19 % and 24 %, respectively. Data indicate that the proposed methodology is accurate.

Fig. S1 shows a LC-MS/MS chromatogram of a fortified guano sample. Method selectivity was assessed by comparing this chromatogram with the corresponding blank sample by studying endogenous substances at the same retention time that analytes. No interferences from blanks were observed at the retention time of the studied compounds. These findings suggest that the spectrometric multiple reaction mode used ensure the high selectivity of the method.

Table 2
Recovery assay, precision and trueness of target compounds in bat guano samples.

Compound	Spiked (ng/g dw)	Found (ng/g dw)	RSD (%)	Rec (%)	Spiked (ng/g dw)	Found (ng/g dw)	RSD (%)	Rec (%)	Spiked (ng/g dw)	Found (ng/g dw)	RSD (%)	Rec (%)
PFBuA	5	4.1	6.2/9.2	82.2	25	27.4	2.0/12.1	109.4	50	45	0.7/6.0	90.0
PFPeA	5	4.2	6.1/7.2	84.2	25	26.1	2.4/15.1	104.3	50	42.9	1.0/5.4	85.9
PFHxA	5	4	4.7/13.3	79.4	25	24.8	2.2/15.0	99.3	50	44	0.9/4.5	88.1
PFHpA	5	4	3.6/19.1	80.6	25	22.7	1.2/16.7	90.9	50	43.4	1.5/9.6	86.8
PFOA	5	4.8	1.8/10.7	95.8	25	24.6	3.1/17.7	98.3	50	47.7	1.6/6.1	95.4
PFOS	5	5.2	8.4/4.2	104.8	25	21.3	1.9/17.5	85.2	50	45.6	1.6/6.9	91.2
MeP	5	4.5	2.5/7.1	89.4	25	24.6	1.4/12.1	98.6	50	46.1	2.9/5.5	92.2
EtP	5	4.3	0.7/15.2	86.4	25	27	2.5/11.2	108.1	50	44.3	2.7/5.7	88.6
PrP	5	4.4	2.4/10.5	87.8	25	26.8	3.8/14.2	107.2	50	44.8	3.0/7.1	89.6
BuP	5	4.2	10.2/9.3	83.0	25	29.7	3.4/9.5	118.8	50	47	2.7/5.9	94.0
BPA	5	6.1	7.5/14.7	122.0	25	34.8	4.7/15.9	139.3	50	48.6	2.2/8.3	97.2
BP1	5	6.05	18.8/23.8	121.0	25	26.9	3.7/18.6	107.7	50	49.4	4.4/12.8	98.8
BP2	5	4.4	12.4/15.7	88.3	25	30.1	7.5/13.5	120.3	50	58	15.5/13.8	116.0
BP3	5	2.5	9.7/9.9	49.8	25	28.0	4.9/14.0	111.9	50	54.7	1.8/2.2	109.3
BP8	5	4	5.7/12.9	80.1	25	25.5	4.1/9.2	102.1	50	51.5	6.6/5.6	103.0
NP	5	5.92	12.1/15.8	118.4	25	25.8	10.1/19.5	103.2	50	60.0	17.4/18.1	120.0
LAS C10	49	61	4.8/12.9	123.7	246	208	3.5/14.7	84.6	492	521	1.8/7.0	105.9
LAS C11	128	160	3.7/9.6	124.5	642	584	2.9/17.4	91.0	1284	1284	3.4/3.7	100.0
LAS C12	123	107	6.4/8.4	87.0	616	501	6.2/20.9	81.4	1232	1010	9.2/8.1	82.0
LAS C13	94	94	3.4/0.7	100.8	468	512	10.4/12	109.5	936	825	10.1/12.1	88.1

RSD: Relative Standard Deviation intra-day/inter-day; Rec: Recovery.

Table 3
Concentrations of selected pollutants determined in bat guano samples (N = 5).

Compound	Concentration levels (ng/g dw)				
	1	2	3	4	5
PFBuA	11.0	11.0	7.83	7.15	17.7
PFPeA	91.3	45.6	51.9	42.4	170
PFHxA	49.9	42.8	61.8	37.0	55.2
PFHpA	41.0	35.0	43.1	30.9	50.1
PFOA	7.23	5.87	6.94	5.17	7.36
PFOS	11.1	11.8	11.1	11.0	13.1
MeP	31.1	25.5	27.8	20.7	52.7
EtP	0.68	0.65	0.75	0.23	35.3
PrP	0.41	0.30	0.48	0.08	18.6
BuP	<MDL	<MDL	<MDL	<MDL	<MDL
BPA	<MDL	<MDL	<MDL	<MDL	<MDL
BP1	10.5	13.2	13.7	20.8	34.8
BP2	<MDL	<MDL	<MDL	<MDL	<MDL
BP3	<MQL	<MQL	<MQL	<MQL	15.5
BP8	<MDL	<MDL	<MDL	<MDL	<MDL
NP	37.9	4.49	4.72	<MQL	<MQL
LAS C10	96.4	78.4	76.4	78.4	103
LAS C11	196	124	132	146	216
LAS C12	483	397	421	478	611
LAS C13	66.6	49.6	53.6	91.6	118

<MQL: Below method detection limit; <MQL: Below method quantification limit.

3.3. Method application

Guano samples from a reference population of wild greater mouse-eared bats (*Myotis myotis*) living in Poland were collected for the application and monitoring purposes of the method (Table 3). A total of 16 out of 20 compounds were quantified and a similar concentration profile was observed in the samples analyzed. In general, LAS, PFAS, and PB were frequently detected in guano samples at concentration levels of 0.08 (propylparaben) to 611 (LAS C12) ng/g dw. The group at the highest concentrations was LAS (49.6–611 ng dw) followed by PFAS (5.17–91.3 ng/g dw), NP (4.49–37.9 ng/g dw) and PB (0.08–52.7 ng/g dw). BuP, BPA, and the BP group (except BP1 and less frequently BP3) were rarely quantified in samples. The results obtained suggest that wild bats are exposed to emerging pollutants to a relatively large extent, suggesting that bats may be good bioindicators in the detection of environmental contamination to terrestrial wildlife. Fig. 4 shows a MRM chromatogram obtained for a guano sample (number 04). It can be assumed that sources of some selected pollutants such as PFAS acting on bats are connected not only with polluted drinking water and/or food, but also with the building materials of attics, where bat colonies live.

Information on exposure from emerging pollutants on terrestrial mammals' wildlife is lacking compared with legacy persistent organic making a comparison difficult with other studies. Most of reported studies have been focused on livestock animals especially on antibiotics [34]. In a recent study, using snow bunting eggs from the artic terrestrial environment, Warner et al. [13] found concentration levels of PFOS of 0.5 ng/g wet weight (ww) and perfluoroalkyl acids between 0.02 and 0.06 ng/g ww, significatively higher than those observed in the surrounding settlements. Authors attributed this concentration to the atmospheric transport and subsequent degradation of the fluorotelomer alcohols to perfluoroalkyl acids. High levels of PFAS have also been reported in artic seabird (up to 19.3 ng/ml for PFOS in plasma [12]) or in top marine mammals such as dolphin and porpoise (136–15,300 and 30.5–2,720 ng/g dw, respectively, in the animal liver [44]). Mean concentration values of 85.4 ng/g and 54.7 ng/g of PFAS have also been found in the feces of dogs and cats, respectively, from USA [40]. Wang et al. [17] (2020) found PFOA (0.667–3.963 ng/ml) and PFOS (0.163–1.399 ng/ml) in the blood of captive Siberian tigers in Harbin (China). Similar levels to those found in this study were also quantified of PB (0.93–18.7 ng/mL) in plasma of wild fish from the Yangtze River from China [35] or BP (up to 14 ng/g dw) in wild mussels collected from mariculture farms in Hong Kong [45]. Concentrations up to 19.8 mg/kg dw, significantly higher, were quantified of NP in marine fish, prawns, and mollusks of the East China Sea [46]. However, most studies were conducted in aquatic organisms. The knowledge of LAS exposure in wildlife organisms is scarce.

These findings indicate that the guano matrix can be a useful bioindicator offering the exposure load to emerging pollutants in terrestrial wildlife. And, considering the increasing need to propose animal models, guano samples represent a promising matrix since it is an invasive sample and relatively easy to collect without stressing the animals and without interfering with their welfare, which has importance in case of protected species.

4. Conclusions

A multiclass and efficient analytical method was proposed for the determination of exposure levels to a wide group of emerging pollutants in bats using a simplified sample treatment involving USE and d-SPE steps on guano matrix previous a single run by LC–MS/MS. The best analytical features were obtained using 1 g of freeze-dried sample, 7 ml of MeOH (0.5 % in formic acid), three extraction steps, and 0.3 g of C18. Good linearities, adequate precision (RSD < 24 %), recovery close to 100 % were achieved. The high specificity and sensitivity of the method allowed us to determine emerging pollutants in guano at very low concentrations (from 0.01 ng/g dw (for PFAS) to 64 ng/g dw (for LAS)). The method was successfully applied to know the concentration levels of selected contaminants in bats summer (nursery) colonies located in Poland. The results confirm the exposure of bats to emerging pollutants and the suitability of bat guano matrix in biomonitoring studies for understanding the environmental exposure in terrestrial wildlife. Guano is an invasive matrix and relatively easy to collect without stressing the animals. This investigation is the first step to further studies on factors that affect the exposure of wild bats to emerging pollutants and their toxic impact on this mammal species.

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CRediT authorship contribution statement

Julia Martín: Conceptualization, Methodology, Writing – review & editing. **Slawomir Gonkowski:** Investigation, Supervision, Funding acquisition, Project administration. **Annemarie Kortas:** Investigation. **Przemysław Sobiech:** Investigation. **Liliana Rytel:** Investigation. **Juan Luis Santos:** Conceptualization, Methodology, Validation, Formal analysis. **Irene Aparicio:** Resources, Validation, Writing – review & editing. **Esteban Alonso:** Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

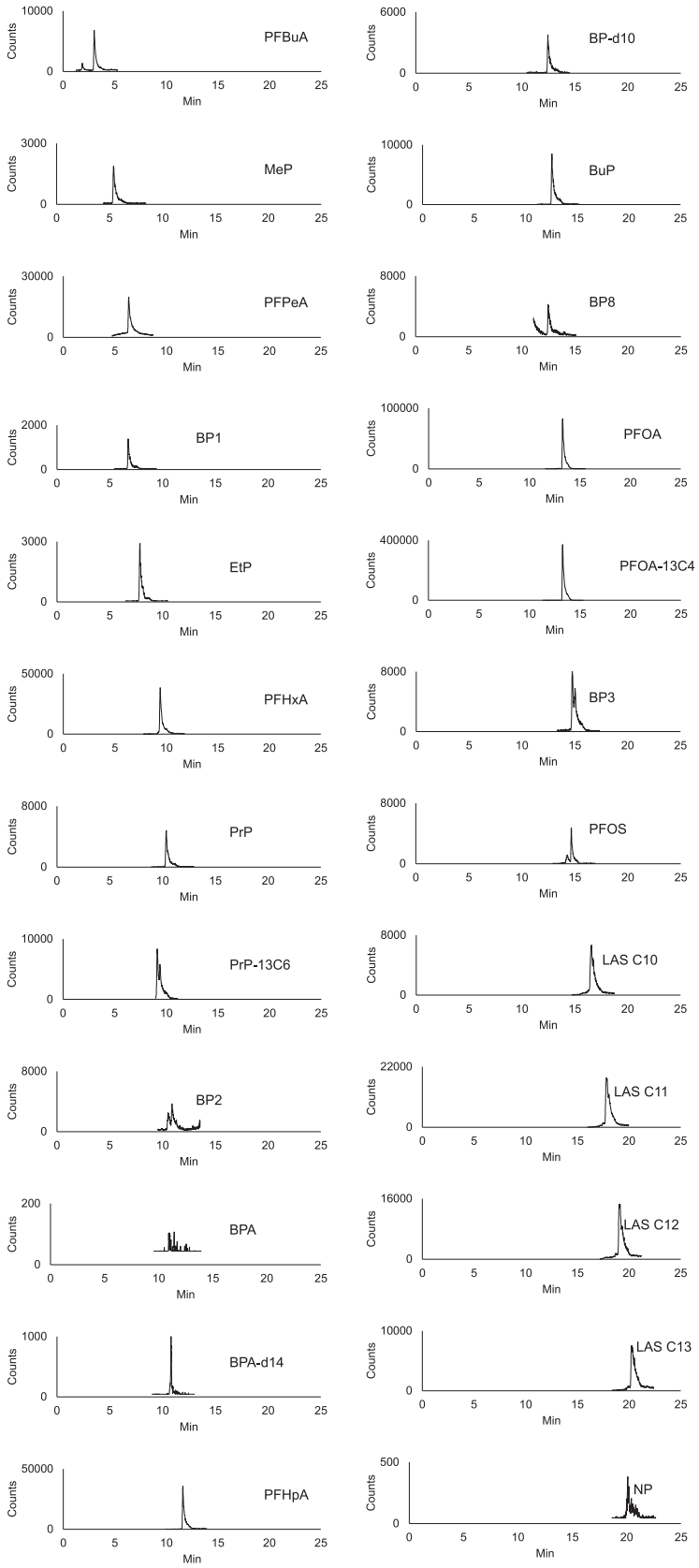

Fig. 4. MRM chromatogram of a guano sample.

org/10.1016/j.microc.2023.108486.

References

[1] R. Álvarez-Ruiz, Y. Picó, Analysis of emerging and related pollutants in aquatic biota, Trends Environ. Anal. Chem. 25 (2020) e00082.

[2] S.C. Cunha, R. Ferreira, I. Marmelo, L.R. Vieira, P. Anacleto, A. Maulvault, A. Marques, L. Guilhermino, J.O. Fernandes, Occurrence and seasonal variation of several endocrine disruptor compounds (pesticides, bisphenols, musks and UV-filters) in water and sediments from the estuaries of Tagus and Douro Rivers (NE Atlantic Ocean coast), Sci. Total Environ. 838 (2022), 155814, https://doi.org/10.1016/j.scitotenv.2022.155814.

[3] A. Yusuf, D. O'Flynn, B. White, L. Holland, A. Parle-Mcdermott, J. Lawler, T. McCloughlin, D. Harold, B. Huerta, F. Regan, Monitoring of emerging contaminants of concern in the aquatic environment: A review of studies showing the application of effect-based measures, Anal. Methods 13 (2021) 5120–5143, https://doi.org/10.1039/d1ay01184g.

[4] G. Delbes, M. Blázquez, J.I. Fernandino, P. Grigorova, B.F. Hales, C. Metcalfe, L. Navarro-Martín, L. Parent, B. Robaire, A. Rwigemera, G. van der Kraak, M. Wade, V. Marlatt, Effects of endocrine disrupting chemicals on gonad development: Mechanistic insights from fish and mammals, Environ. Res. 204 (2022), 112040, https://doi.org/10.1016/j.envres.2021.112040.

[5] J. Martín, A. Zafra-Gómez, F. Hidalgo, A.J. Ibáñez-Yuste, E. Alonso, J.L. Vilchez, Multi-residue analysis of 36 priority and emerging pollutants in marine echinoderms (Holothuria tubulosa) and marine sediments by solid-liquid extraction followed by dispersive solid phase extraction and liquid chromatography-tandem mass spectrometry analysis, Talanta 166 (2017) 336–348, https://doi.org/10.1016/j.talanta.2017.01.062.

[6] B. Huerta, A. Jakimska, M. Llorca, A. Ruhí, G. Margoutidis, V. Acuña, S. Sabater, S. Rodriguez-Mozaz, D. Barcelò, Development of an extraction and purification method for the determination of multi-class pharmaceuticals and endocrine disruptors in freshwater invertebrates, Talanta 132 (2015) 373–381, https://doi.org/10.1016/j.talanta.2014.09.017.

[7] H. Nakata, R.I. Shinohara, Y. Nakazawa, T. Isobe, A. Sudaryanto, A. Subramanian, S. Tanabe, M.P. Zakaria, G.J. Zheng, P.K.S. Lam, E.Y. Kim, B.Y. Min, S.U. We, P. H. Viet, T.S. Tana, M. Prudente, D. Frank, G. Lauenstein, K. Kannan, Asia-Pacific mussel watch for emerging pollutants: Distribution of synthetic musks and benzotriazole UV stabilizers in Asian and US coastal waters, Mar. Pollut. Bull. 64 (10) (2012) 2211–2218, https://doi.org/10.1016/j.marpolbul.2012.07.049.

[8] M. Li, S. Zhou, Y. Wu, Y. Li, W. Yan, Q. Guo, Y. Xi, Y. Chen, Y. Li, M. Wu, J. Zhang, J. Wei, S. Wang, Prenatal exposure to propylparaben at human-relevant doses accelerates ovarian aging in adult mice, Environ. Pollut. 285 (2021), 117254, https://doi.org/10.1016/j.envpol.2021.117254.

[9] J. Martín, J.L. Santos, I. Aparicio, E. Alonso, Exposure assessment to parabens, bisphenol A and perfluoroalkyl compounds in children, women and men by hair analysis, Sci. Total Environ. 695 (2019), 133864, https://doi.org/10.1016/j.scitotenv.2019.133864.

[10] E.M. Sunderland, X.C. Hu, C. Dassuncao, A.K. Tokranov, C.C. Wagner, J.G. Allen, A review of the pathways of human exposure to poly- and perfluoroalkyl substances (PFASs) and present understanding of health effects, J. Expo. Sci. Environ. Epidemiol. 29 (2) (2019) 131–147, https://doi.org/10.1038/s41370-018-0094-1.

[11] I. Jiménez-Díaz, F. Vela-Soria, R. Rodríguez-Gómez, A. Zafra-Gómez, O. Ballesteros, A. Navalón, Analytical methods for the assessment of endocrine disrupting chemical exposure during human fetal and lactation stages: A review, Anal. Chim. Acta 892 (2015) 27–48, https://doi.org/10.1016/j.aca.2015.08.008.

[12] A.V. Ask, B.M. Jenssen, S. Tartu, F. Angelier, O. Chastel, G.W. Gabrielsen, Per- and Polyfluoroalkyl Substances Are Positively Associated with Thyroid Hormones in an Arctic Seabird, Environ. Toxicol. Chem. 40 (3) (2021) 820–831, https://doi.org/10.1002/etc.4978.

[13] N.A. Warner, K. Sagerup, S. Kristoffersen, D. Herzke, G.W. Gabrielsen, B. M. Jenssen, Snow buntings (*Plectrophenax nivealis*) as bio-indicators for exposure differences to legacy and emerging persistent organic pollutants from the Arctic terrestrial environment on Svalbard, Sci. Total Environ. 667 (2019) 638–647, https://doi.org/10.1016/j.scitotenv.2019.02.351.

[14] J. Kowalczyk, J. Numata, B. Zimmermann, R. Klinger, F. Habedank, P. Just, H. Schafft, M. Lahrssen-Wiederholt, Suitability of wild boar (Sus scrofa) as a bioindicator for environmental pollution with perfluorooctanoic acid (PFOA) and perfluorooctanesulfonic acid (PFOS), Arch. Environ. Contam. Toxicol. 75 (4) (2018) 594–606, https://doi.org/10.1007/s00244-018-0552-8.

[15] S. Persson, U. Magnusson, Environmental pollutants and alterations in the reproductive system in wild male mink (*Neovison vison*) from Sweden, Chemosphere 120 (2015) 237–245, https://doi.org/10.1016/j.chemosphere.2014.07.009.

[16] R. Kanda, Reproductive Impact of Environmental Chemicals on Animals. In Advances in Experimental Medicine and Biology 1200 Reproductive Sciences in Animal Conservation Second Edition. Comizzoli, P. Brown, J.L. & Holt Editors, W. v. (Eds). 2019. http://www.springer.com/series/5584.

[17] Y. Wang, J. Yao, J. Dai, L. Ma, D. Liu, H. Xu, Q. Cui, J. Ma, H. Zhang, Per- and polyfluoroalkyl substances (PFASs) in blood of captive Siberian tigers in China: Occurrence and associations with biochemical parameters, Environ. Pollut. 265 (2020), 114805, https://doi.org/10.1016/j.envpol.2020.114805.

[18] D. Russo, V.B. Salinas-Ramos, L. Cistrone, S. Smeraldo, L. Bosso, L. Ancillotto, Do we need to use bats as bioindicators? Biology (Basel) 10 (8) (2021) 10080693, https://doi.org/10.3390/biology10080693.

[19] E. Afonso, P. Tournant, J.C. Foltête, P. Giraudoux, P.E. Baurand, S. Roué, V. Canella, D. Vey, R. Scheifler, Is the lesser horseshoe bat (*Rhinolophus hipposideros*) exposed to causes that may have contributed to its decline? A non-invasive approach, Global Ecol. Conservat. 8 (2016) 123–137, https://doi.org/10.1016/j.gecco.2016.09.002.

[20] D. Russo, L. Ancillotto, Sensitivity of bats to urbanization: a review, Mamm. Biol. 80 (3) (2015) 205–212, https://doi.org/10.1016/j.mambio.2014.10.003.

[21] D.F. Sotero, M. Benvindo-Souza, R. Pereira de Freitas, E. de Melo, D. Silva, Bats and pollution: Genetic approaches in ecotoxicology, Chemosphere 307 (2) (2022), 135934, https://doi.org/10.1016/j.chemosphere.2022.135934.

[22] O. Kuzukiran, I. Simsek, T. Yorulmaz, B. Yurdakok-Dikmen, O. Ozkan, A. Filazi, Multiresidues of environmental contaminants in bats from Turkey, Chemosphere 282 (2021), 131022, https://doi.org/10.1016/j.chemosphere.2021.131022.

[23] K. Kannan, S.H. Yun, R.J. Rudd, M. Behr, High concentrations of persistent organic pollutants including PCBs, DDT, PBDEs and PFOS in little brown bats with white-nose syndrome in New York, USA, Chemosphere 80 (6) (2010) 613–618, https://doi.org/10.1016/j.chemosphere.2010.04.060.

[24] D.R.C. Jr., DDT and the Decline of Free-Tailed Bats (*Tadarida brasiliensis*) at Carlsbad Cavern, New Mexico, Arch. Environ. Contam. Toxicol. 40 (4) (2001) 537–543.

[25] R.R. Eidels, D.W. Sparks, J.O. Whitaker, C.A. Sprague, Sub-lethal effects of chlorpyrifos on big brown bats (*Eptesicus fuscus*), Arch. Environ. Contam. Toxicol. 71 (3) (2016) 322–335.

[26] X. Li, Y.i. Zhong, W. He, S. Huang, Q. Li, C. Guo, S. Ma, G. Li, Y. Yu, Co-exposure and health risks of parabens, bisphenols, triclosan, phthalate metabolites and hydroxyl polycyclic aromatic hydrocarbons based on simultaneous detection in urine samples from guangzhou, south China, Environ. Pollut. 272 (2021) 115990.

[27] S. Schanzer, E. Kröner, G. Wibbelt, M. Koch, A. Kiefer, F. Bracher, C. Müller, Miniaturized multiresidue method for the analysis of pesticides and persistent organic pollutants in non-target wildlife animal liver tissues using GC-MS/MS, Chemosphere 279 (2021), 130434, https://doi.org/10.1016/j.chemosphere.2021.130434.

[28] K. Preindl, D. Braun, G. Aichinger, S. Sieri, M.L. Fang, D. Marko, B. Warth, A generic liquid chromatography-tandem mass spectrometry exposome method for the determination of xenoestrogens in biological matrices, Anal. Chem. 91 (2019) 11334–11342.

[29] B. Warth, S. Spangler, M. Fang, C.H. Johnson, E.M. Forsberg, A. Granados, R. L. Martin, X. Domingo-Almenara, T. Huan, D. Rinehart, J.R. Montenegro-Burke, B. Hilmers, A. Aisporna, L.T. Hoang, W. Uritboonthai, H.P. Benton, S. D. Richardson, A.J. Williams, G. Siuzdak, Exposome-scale investigations guided by global metabolomics, pathway analysis, and cognitive computing, Anal. Chem. 89 (21) (2017) 11505–11513.

[30] S. Lee, K.M. Lee, S.M. Han, H.J. Lee, C. Sung, H. Min, H. Im, S.B. Han, S. Cha, J. Lee, Comprehensive LC-MS/MS method combined with tandem hybrid hydrolysis for multiple exposure assessment of multiclass environmental pollutants, Environ. Res. 211 (2022), 113053, https://doi.org/10.1016/j.envres.2022.113053.

[31] P. Morales, J.L. Roscales, J. Muñoz-Arnanz, A. Barbosa, B. Jiménez, Evaluation of PCDD/Fs, PCBs and PBDEs in two penguin species from Antarctica, Chemosphere 286 (2022), 131871, https://doi.org/10.1016/j.chemosphere.2021.131871.

[32] M. Argüeso-Mata, S. Bolado, J.J. Jiménez, R. López-Serna, Determination of antibiotics and other veterinary drugs in the solid phase of pig manure, Chemosphere 275 (2021), 130039, https://doi.org/10.1016/j.chemosphere.2021.130039.

[33] I. Simsek, O. Kuzukiran, B. Yurdakok-Dikmen, T. Snoj, A. Filazi, Determination of persistent organic pollutants (POPs) in propolis by solid-phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS), Anal. Lett. 54 (10) (2021) 1668–1682, https://doi.org/10.1080/00032719.2020.1821208.

[34] S. Šturm, A. Škibin, M. Pogačnik, V. Cerkvenik-Flajs, 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, J. Environ. Sci. Health - Part B 55 (7) (2020) 655–668, https://doi.org/10.1080/03601234.2020.1759329.

[35] L. Yao, Y.Z. Lv, L.J. Zhang, W.R. Liu, J.L. Zhao, Y.Y. Yang, Y.W. Jia, Y.S. Liu, L. Y. He, G.G. Ying, Bioaccumulation and risks of 24 personal care products in plasma of wild fish from the Yangtze River, China, Sci. Total Environ. 665 (2019) 810–819, https://doi.org/10.1016/j.scitotenv.2019.02.176.

[36] M. Wang, W. Guo, S. Gardner, M. Petreas, J.S. Park, Per- and polyfluoroalkyl substances in Northern California cats: temporal comparison and a possible link to cat hyperthyroidism, Environ. Toxicol. Chem. 37 (2018) 2523–2529, https://doi.org/10.1002/etc.4239.

[37] K. Makowska, J. Martín, A. Rychlik, I. Aparicio, J.L. Santos, E. Alonso, S. Gonkowski, Hair Sample Analysis as a Method of Monitoring Exposure to Bisphenol A in Dogs, Int J Environ Res. Public Health 19 (8) (2022) 9084600, https://doi.org/10.3390/ijerph19084600.

[38] K. Makowska, J. Martín, A. Rychlik, I. Aparicio, J.L. Santos, E. Alonso, S. Gonkowski, Biomonitoring parabens in dogs using fur sample analysis – Preliminary studies, Sci. Total Environ 807 (2022), https://doi.org/10.1016/j.scitotenv.2021.150757.

[39] K. Makowska, J. Martín, A. Rychlik, I. Aparicio, J.L. Santos, E. Alonso, S. Gonkowski, Assessment of exposure to perfluoroalkyl substances (PFASs) in dogs by fur analysis, Environ. Pollut. 286 (2021), 117435, https://doi.org/10.1016/j.envpol.2021.117435.

[40] J. Ma, H. Zhu, K. Kannan, Fecal excretion of perfluoroalkyl and polyfluoroalkyl substances in pets from New York State, United States, Environ. Sci. Technol. Lett. 7 (3) (2020) 135–142.

[41] C. Abril, J.L. Santos, J.L. Malvar, J. Martín, I. Aparicio, E. Alonso, Determi- nation of perfluorinated compounds, bisphenol A, anionic surfactants and personal care products in digested sludge, compost and soil by liquid- chromatography-tandem mass spectrometry, J. Chromatogr. A (1576 (2018)) 34–41, https://doi.org/10.1016/j.chroma.2018.09.028.

[42] F. Li, C. Zhang, Y. Qu, J. Chen, L. Chen, Y. Liu, Q.i. Zhou, Quantitative characterization of short– and long–chain perfluorinated acids in solid matrices in Shanghai, China, Sci. Total Environ. 408 (3) (2010) 617–623.

[43] A. Luque–Muñoz, J.L. Vílchez, A. Zafra–Gómez, Multiclass method for the determination of pharmaceuticals and personal care products in compost from sewage sludge using ultrasound and salt–assisted liquid–liquid extraction followed by ultra high performance liquid chromatography–tandem mass spectrometry analysis, J. Chromatogr. A 1507 (2017) 72–83.

[44] J.C.W. Lam, J. Lyu, K.Y. Kwok, P.K.S. Lam, Perfluoroalkyl substances (PFASs) in marine mammals from the south China sea and their temporal changes 2002e2014: concern for alternatives of PFOS? Environ. Sci. Technol. 50 (2016) 6728–6736, https://doi.org/10.1021/acs.est.5b06076.

[45] Z. Sang, K.S.Y. Leung, Environmental occurrence and ecological risk assessment of organic UV filters in marine organisms from Hong Kong coastal waters, Sci. Total Environ. 566–567 (2016) 489–498, https://doi.org/10.1016/j.scitotenv.2016.05.120.

[46] Y. Gu, J. Yu, X. Hu, D. Yin, Characteristics of the alkylphenol and bisphenol A distributions in marine organisms and implications for human health: A case study of the East China Sea, Sci. Total Environ. 539 (2016) 460–469, https://doi.org/10.1016/j.scitotenv.2015.09.011.