Determination of porphyrins and biliverdin in bile and excreta of birds by a single liquid chromatography–ultraviolet detection analysis

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Abstract

Methods developed for porphyrin analysis have low recoveries and/or poor precision for the less polar protoporphyrin IX. We describe a simple method of analysis of porphyrins and biliverdin in bile and excreta of birds based on extraction with HCl 3N: acetonitrile and HPLC/UV analyses. Recoveries were good for protoporphyrin IX and other porphyrins (>79%). Applications of this method showed that porphyrins and biliverdin in birds excreta are mainly of biliary-fecal origin rather than urinary origin. Biliverdin and protoporphyrin IX increased proportionately more than the rest of the porphyrins and coproporphyrin III increased more than coproporphyrin I in the bile of Pb-poisoned mallards.
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1. Introduction

Porphyrins are cyclic tetrapyrrolic pigments, formed as intermediates in the biosynthesis of heme. Several toxic compounds, including halogenated hydrocarbons, heavy metals and organophosphorus compounds, are known to be porphyrinogenic due to disturbances of heme synthesis [1,2]. The urinary and fecal porphyrins excretion patterns are important diagnostic tools in the detection of both inherited and chemically induced disorders of porphyrin metabolism in mammals [3,4]. Fecal porphyrins can be interesting biomarkers of exposure to porphyrinogenic compounds in wild animals, because their sampling is completely non-invasive and permits the easy collection of a large sample size without disturbances to protected species [2,5]. Moreover, the exposure to porphyrinogenic contaminants such as halogenated

hydrocarbons and heavy metals can be also monitored in feces [6,7], which in some cases permits linkage of cause and effect within the same non-invasive sample.

Biliverdin is the bile pigment in birds, which is a linear open-chain tetrapyrrol produced from oxidation of heme largely derived from senescent red-blood cells [8]. Unlike most mammals, the amount of excreted bilirubin in birds is very limited because of the low biliverdin reductase activity [9]. The high exposure to toxic compounds producing hemolytic anemia in birds, such as lead, can be manifested by gall-bladder enlargement, viscous bile and green-stained feces [10]. Moreover, biliverdin may offer further criteria to interpret porphyrins in feces due to their common biliary origin.

Several methods have been developed for porphyrin extraction and HPLC analysis in excreta [3,11–18], but most of those methods with published validations for the most polar porphyrins have low recovery or poor precision for the less polar protoporphyrin IX [5,14,18]. We describe

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a simple method to determine simultaneously porphyrins, including protoporphyrin IX, and biliverdin in the excreta and bile of birds. The method has been developed mainly for the analysis of excreta of greylag goose (*Anser anser*), in order to use it in further research into heavy metal exposure in this and other Anatidae species. Bird excreta is formed by intestinal feces and urine and both parts can contribute to porphyrin and biliverdin presence. The pattern of porphyrins and biliverdin analyzed with the described method are compared between intestinal and renal excreta in samples where both parts can be separated, as is the case of the lesser kestrel (*Falco naumanni*). The method is also used to compare the pattern of these pigments in bile of healthy and Pb-poisoned mallards (*Anas platyrhynchos*) to define the changes in porphyrins and biliverdin that should be monitored in bird excreta in the case of exposure to this heavy metal.

2. Experimental

2.1. Chemicals

Porphyrin kits containing 8, 7, 6, 5, 4, 3, 2-carboxyl porphyrins as free acids of the type I series were purchased from Porphyrin Products Inc. (Logan, UT). Coproporphyrin III (4-carboxyl porphyrin) dihydrochloride and protoporphyrin IX were purchased from Frontier Scientific Ltd. (Carnforth, UK). Solvents and reagents for standard solutions and chromatography were methanol (HPLC grade), acetonitrile (HPLC grade) and ammonia solution (25%) from Riedel-de-Haën (Seelze, Germany), and hydrochloric acid (37%) and acetic acid from Panreac (Moncada i Reixac, Spain), and Milli-Q water grade.

2.2. Sample collection

Greylag goose excreta were collected fresh on harvested rice fields of the Guadalquivir marshes, where the birds were feeding in October 2001. Lesser kestrel excreta were collected on sheets of paper distributed the day before in pens of the breeding stock of Torreferrusa Raptor Rehabilitation Center in Barcelona. Only ten samples with clear separation between urinary and intestinal excreta were selected and the parts of the paper containing these excreta were cut and kept in different plastic tubes. Bile samples were collected from mallards found dead with numerous Pb shot pellets in the gizzard or shot by hunters without ingested pellets. All samples were frozen at −20 °C until analysis.

2.3. Sample preparation

Depending on the moisture, 0.02–0.5 g of goose excreta were vortexed in an eppendorf for 15 s with 0.25 ml of HCl 3N and 0.3 ml of acetonitrile, and 0.2–0.3 ml of water were added in those samples with lowest moisture. Concentrations of porphyrins and biliverdin were expressed in dry weight

of goose excreta. Samples were centrifuged for 10 min at 16.060 RCF in a Biofuge Pico Heraeus (Kendro Laboratory Products, Osterode, Germany) and 0.2 ml of supernatant were transferred to glass vials for HPLC analysis. Similarly, kestrel excreta and paper with the adsorbed sample were extracted with 1.25 ml of HCl 3N, 1.5 ml of acetonitrile and 1 ml of distilled water. Bile samples (40–50 mg) were extracted as goose feces, and those from Pb-poisoned mallards were diluted (1:100) before analyses.

2.4. Chromatographic method

The samples were analyzed by an HPLC technique modified from Taylor et al. [5], and with UV–vis spectrometry detection. An HP1100 series quaternary pump, autosampler, column oven and diode array detector were used (Seeltze, Germany). All the chromatographic conditions and quantification were controlled using ChemStation software (ver. A.06.01). A Waters (Milford MA) Spherisorb ODS 2 (5 μm particle size, 4.6 mm $\times$ 100 mm) chromatographic column was used. The flow rate was 1.5 ml/min and a solvent gradient was used. The initial mobile phase composition was methanol 25% and ammonium acetate (1.0 M, pH 5.16) 75%. The solvent gradient consisted in an 8 min linear change to 95% methanol and 5% ammonium acetate, followed by 2 min at these conditions. At this moment the phase composition returned to the initial conditions. The total runtime was 13 min. The column was maintained at 70 °C and the detection was done at 400 nm wavelength.

2.5. Validation of the method

Working solutions (10 μM) of free acid porphyrins and protoporphyrin IX were prepared in 3N HCl, coproporphyrin III was dissolved in methanol, and biliverdin was dissolved in acetonitrile:3N HCl (1:1). The quantification of samples was performed using calibration curves constructed with standard solutions. For the preparation of calibration standards in glass vials, methanol from coproporhyrin III was evaporated before the addition of the other standards. Calibrations were prepared as samples with 0.25 ml of 3N HCl, 0.3 ml of acetonitrile and 0.2 ml of distilled water. Concentrations in the five calibration points were 20.8, 41.6, 83.3, 166.7 and 333.3 nM for each porphyrin and 138.3, 276.7, 553.3, 1107 and 2213 nM for biliverdin. The recovery of the extraction procedure was calculated comparing standard solutions with samples and samples spiked with porphyrins and biliverdin. For the recovery calculation ($n = 5$) the goose excreta was spiked with 312.5 and 40 nmol/g of porphyrins and biliverdin, respectively; and for mallard bile samples spiked with 1250 and 162 nmol/ml, respectively. Precision (coefficient of variation, %), accuracy (mean error, %) and linearity of porphyrins and biliverdin HPLC analyses were calculated from six intra-day and five inter-day calibrations. Calibrations were prepared each day of analysis and injected at the beginning of the analytical sequence and between each group

of 30 samples, recalibrating the method each time, ensuring that the peak retention times did not vary during the in-day analysis.

2.6. Statistical analyses

Linearity was assessed by means of lack of fit tests between observed and expected concentrations in intra- and inter-day calibration curves. Relationships between concentrations or total amounts of porphyrins and biliverdin in the different types of samples were explored by means of Pearson correlations. These analyses were performed with the statistical package SPSS 10.0.6.

3. Results

Recoveries obtained for porphyrins spiked to goose feces were within 79–109% and showed acceptable repeatability (Table 1). Biliverdin recovery from goose feces was low (26%), but the repeatability was high (less than 10% of C.V.). Bile analyses showed good recoveries for porphyrins and biliverdin (90–119%) and coefficients of variation below 15%, except for uroporphyrin (17.1%) and protoporpfyrin IX (27.3%) (Table 1). Precision and accuracy of HPLC analysis obtained from calibrations of porphyrins and biliverdin were similar for intra- and inter-day analyses, with some poor values of precision for uroporphyrin and coproporphyrin III at the highest levels, and for biliverdin, mesoporphyrin and protoporphyrin IX at the lowest levels (Table 2). Linearity of calibrations could be assessed for each compound (Lack of fit test, $p > 0.05$). Calibration curves showed correlation coefficients above 0.995 and the coefficient of variation of their slopes were below 37 and 22% in intra- and inter-day analyses, respectively (Table 3). This variability in the calibrations was compensated by constructing a complete calibration every day of analysis. Moreover, the peak retention times presented considerable variation (0.7 min for

uroporphyrin to 0.1 min for protoporphyrin IX) in the more polar porphyrins between different days of analysis as can be seen in Fig. 1. For this reason the standards were injected every day to ensure the capacity of the method to correctly identify each analyte of the samples.

Comparisons of excreta of different species showed a higher percentage of coproporphyrin III and lower ratio of porphyrins/biliverdin in geese excreta than in intestinal excreta of kestrels (Table 4, Fig. 1). Results were only corrected for the recovery of biliverdin in goose feces. Moreover, the less polar protoporphyrin IX was present at a lower percentage in urates than in intestinal excreta of kestrels; in contrast, uroporphyrin was present at a higher percentage in urates. Biliary concentrations of porphyrins and biliverdin were higher in mallards with Pb poisoning, protoporphyrin IX showing the highest increase (Table 4, Fig. 1). Biliverdin and total porphyrins concentrations were highly correlated in goose excreta ($r = 0.880$), kestrel urinary excreta ($r = 0.816$), and bile of control and Pb-poisoned mallards ($r = 0.865$ and 0.820, respectively), but not in kestrel fecal excreta ($r = 0.19$). However, high correlations were also observed in fecal excreta of kestrels between biliverdin and specific porphyrins such as pentacarboxyl porphyrin ($r = 0.839$) and coproporphyrin III ($r = 0.923$).

4. Discussion

The extraction method used for this study showed good recoveries for the whole range of polarities among porphyrins. This could be accomplished with a single extraction step with an acetonitrile:HCl 3N mixture that permits the solubilization of the less polar porphyrins from biological samples, followed by centrifugation without further purification steps. Reasonably clean extracts could be obtained for HPLC/UV determination of porphyrins and biliverdin for the type of samples analyzed here. Other methods based on HCl extraction and C-18 cartridges clean-up have shown good recoveries for the more polar porphyrins (from uroporphyrin to coproporphyrin), but not for the less polar protoporphyrin IX [4,5,14]. Protoporphyrin IX has been determined in bird excreta after HCl extraction and C-18 clean-up, although no validation details of the method were given by the authors [13]. Another method based on the extraction with an aqueous HCl solution followed by clean-up with diethyl ether has also been employed for porphyrins analysis, including protoporphyrin IX. Although no recovery details were given in most of these studies [3,11,12], some of them describe good recoveries for protoporphyrin IX [17] but with a poor precision [18]. Biliverdin recovery with our extraction method was low in feces, and possibly could have been better extracted with HCl in methanol (1:40) [8]. However, we still consider the method useful because of its high reproducibility, the wide range of polarity among the different analytes quantified and the simplicity of the clean-up procedure and HPLC quantification.

Table 1
Recovery of porphyrins[a] and biliverdin[b] in greylag geese excreta and mallard bile

	Excreta ($n = 5$)			Bile ($n = 5$)		
	Mean	S.D.	CV	Mean	S.D.	CV
Uroporphyrin	105.5	5.8	5.5	90.9	15.5	17.1
Heptacarboxyl porphyrin	106.3	9.5	9.0	99.5	2.5	2.6
Hexacarboxyl porphyrin	79.0	7.1	9.0	104.0	5.1	4.9
Pentacarboxyl porphyrin	90.9	11.9	13.1	115.2	16.5	14.3
Coproporphyrin I	91.3	6.2	6.8	89.8	7.5	8.4
Coproporphyrin III	104.1	5.2	5.0	116.2	6.6	5.7
Mesoporphyrin	79.8	3.8	4.8	118.9	17.7	14.9
Protoporphyrin IX	108.9	16.9	15.5	109.9	30.0	27.3
Biliverdin	26.2	1.93	7.4	92.7	2.7	2.9

[a] Spiked concentration of porphyrins: 312.5 pmol/g WW in excreta and 1250 pmol/ml in bile.
[b] Spiked concentration of biliverdin: 40 nmol/g WW in excreta and 162 nmol/ml in bile.

Table 2
Intra- and inter-day precision (P) and accuracy (Acc) for porphyrins and biliverdin obtained from calibrations

	Level[a]	Intra-day (n = 6)				Inter-day (n = 5)			
		Mean	S.D.	P (%)	Acc (%)	Mean	S.D.	P (%)	Acc (%)
Uroporphyrin	A	28.7	1.9	6.8	37.9	22.6	5.9	26.2	8.5
	B	42.5	2.5	5.9	2.2	38.8	4.0	10.3	−6.7
	C	75.0	2.8	3.7	−10.0	80.1	7.3	9.1	−3.8
	D	134.6	13.1	9.7	−19.3	159.1	21.4	13.5	−4.5
	E	350.8	117.9	33.6	5.3	337.9	11.0	3.3	1.4
Heptacarboxyl porphyrin	A	23.2	1.2	5.2	11.4	21.2	2.5	11.5	2.1
	B	42.0	1.3	3.2	0.9	40.3	1.3	3.1	−3.2
	C	83.4	2.3	2.8	0.1	81.3	6.3	7.7	−2.4
	D	153.0	17.5	11.4	−8.2	163.3	13.3	8.1	−2.0
	E	339.9	28.1	8.3	2.0	335.2	5.7	1.7	0.6
Hexacarboxyl porphyrin	A	22.2	1.5	6.8	6.8	20.2	2.2	10.8	−2.9
	B	42.6	1.8	4.1	2.4	40.4	1.6	3.9	−2.9
	C	81.8	0.9	1.1	−1.8	82.8	5.6	6.8	−0.5
	D	156.8	1.4	0.9	−5.9	169.1	9.3	5.5	1.4
	E	338.4	27.2	8.0	1.5	332.5	4.6	1.4	−0.3
Pentacarboxyl porphyrin	A	22.6	2.2	9.6	8.6	20.2	1.9	9.2	−2.7
	B	43.0	3.1	7.3	3.3	39.8	2.2	5.5	−4.4
	C	81.5	0.8	1.0	−2.2	83.3	5.6	6.7	0.0
	D	158.0	2.7	1.7	−5.2	168.3	8.9	5.3	1.0
	E	337.8	19.3	5.7	1.4	332.7	4.3	1.3	−0.2
Coproporphyrin I	A	21.4	2.5	11.8	2.9	19.9	1.6	8.0	−4.5
	B	42.1	0.6	1.5	1.1	39.7	1.8	4.5	−4.5
	C	82.2	3.7	4.5	−1.3	83.3	6.5	7.8	0.0
	D	160.2	3.8	2.4	−3.9	168.5	8.5	5.0	1.1
	E	336.7	21.0	6.2	1.0	332.7	3.2	1.0	−0.2
Coproporphyrin III	A	26.1	3.9	14.8	25.5	21.0	3.5	16.8	1.0
	B	42.8	6.2	14.4	2.8	38.7	2.5	6.3	−7.0
	C	79.8	8.5	10.7	−4.2	85.3	7.4	8.7	2.4
	D	154.1	17.6	11.4	−7.6	166.0	9.0	5.4	−0.4
	E	340.0	68.9	20.3	2.0	333.5	4.5	1.3	0.1
Mesoporphyrin	A	21.0	3.9	18.4	0.9	20.1	4.9	24.2	−3.2
	B	42.5	4.8	11.4	2.1	37.6	3.7	9.7	−9.6
	C	85.6	12.7	14.8	2.8	85.3	13.1	15.4	2.5
	D	156.1	8.3	5.3	−6.4	169.0	15.2	9.0	1.4
	E	337.9	40.0	11.8	1.4	332.2	5.1	1.5	−0.3
Protoporphyrin IX	A	21.8	5.7	25.9	4.9	22.6	4.2	18.5	8.8
	B	32.5	4.2	12.8	−21.9	38.7	8.5	21.9	−7.0
	C	88.5	8.2	9.3	6.3	79.6	13.4	16.9	−4.4
	D	158.2	13.8	8.7	−5.1	158.2	3.9	2.5	−5.1
	E	337.3	19.8	5.9	1.2	338.3	3.6	1.1	1.5
Biliverdin	A	131.3	43.5	33.1	−5.1	143.1	25.4	17.8	3.5
	B	269.2	16.1	6.0	−2.7	283.3	18.8	6.7	2.4
	C	548.1	73.6	13.4	−0.9	556.8	41.0	7.4	0.6
	D	1005.3	38.2	3.8	−9.2	1019.1	129.5	12.7	−7.9
	E	2266.6	165.3	7.3	2.4	2255.2	70.9	3.1	1.9

[a] Porphyrins levels: A: 20.8, B: 41.6, C: 83.3, D: 166.7, E: 333.3 nM. Biliverdin levels: A: 138.3, B: 276.7, C: 553.3, D: 1107, E: 2213 nM.

Several HPLC methods for porphyrins have been described with UV-detection at 404 nm [3] or fluorescence detection [13,14]. Biliverdin can also be analyzed by HPLC/UV at 374 nm [19]. We employed UV-detection at 400 nm to monitor porphyrins and biliverdin at the same wavelength. Although the peak of absorbance of biliverdin is obtained at a lower wavelength (377 nm), its presence at higher concentration than porphyrins permits detection at 400 nm in most of the samples.

Birds have low activity of biliverdin reductase, the enzyme responsible for the production of bilirubin from biliverdin as the biliary pigment in mammals [8,9]. Biliverdin analysis could give us information about the presence of bile in feces in order to interpret porphyrin concentrations,

Table 3
Linearity[a] of calibrations

Compound	Intra-day ($n = 6$)			Inter-day ($n = 5$)		
	Equation area $= m$ [nM] $+ b$	Slope CV (%)	r	Equation area $= m$ [nM] $+ b$	Slope CV (%)	r
Uroporphyrin	$0.049x - 0.977$	37	0.989	$0.048x - 0.263$	22	0.995
Heptacarboxyl porphyrin	$0.068x - 0.413$	9	0.997	$0.070x - 0.260$	16	0.998
Hexacarboxyl porphyrin	$0.075x - 0.422$	8	0.999	$0.075x - 0.059$	12	0.999
Pentacarboxyl porphyrin	$0.060x - 0.244$	6	0.999	$0.060x - 0.065$	12	0.999
Coproporphyrin I	$0.037x - 0.168$	7	0.999	$0.037x - 0.058$	12	0.999
Coproporphyrin III	$0.048x - 0.203$	21	0.998	$0.043x - 0.051$	11	0.998
Mesoporphyrin	$0.031x - 0.799$	9	0.997	$0.032x - 0.046$	18	0.997
Protoporphyrin IX	$0.029x - 0.118$	13	0.996	$0.027x - 0.189$	16	0.998
Biliverdin	$0.008x - 0.302$	7	0.997	$0.007x - 0.205$	12	0.995

[a] Lack of fit tests with $p > 0.05$ for all the cases.

because correlations between porphyrins and biliverdin in excreta reflect their common biliary origin. According to our results from bile analysis, Pb-poisoned birds would show a higher increase of protoporphyrin IX and biliverdin in feces (53- and 66-fold, respectively), than the rest of porphyrins (7- to 26-fold). Urinary origin of porphyrins and biliverdin in total bird excreta is possibly lower than fecal-biliary origin, although biliverdinuria is a common finding in Pb-poisoned birds [10] and the most polar uroporphyrin is relatively better represented in this part of the excreta than in feces, as can be found in urine of mammals [1,3]. The only sample type where total porphyrins were not correlated with biliverdin
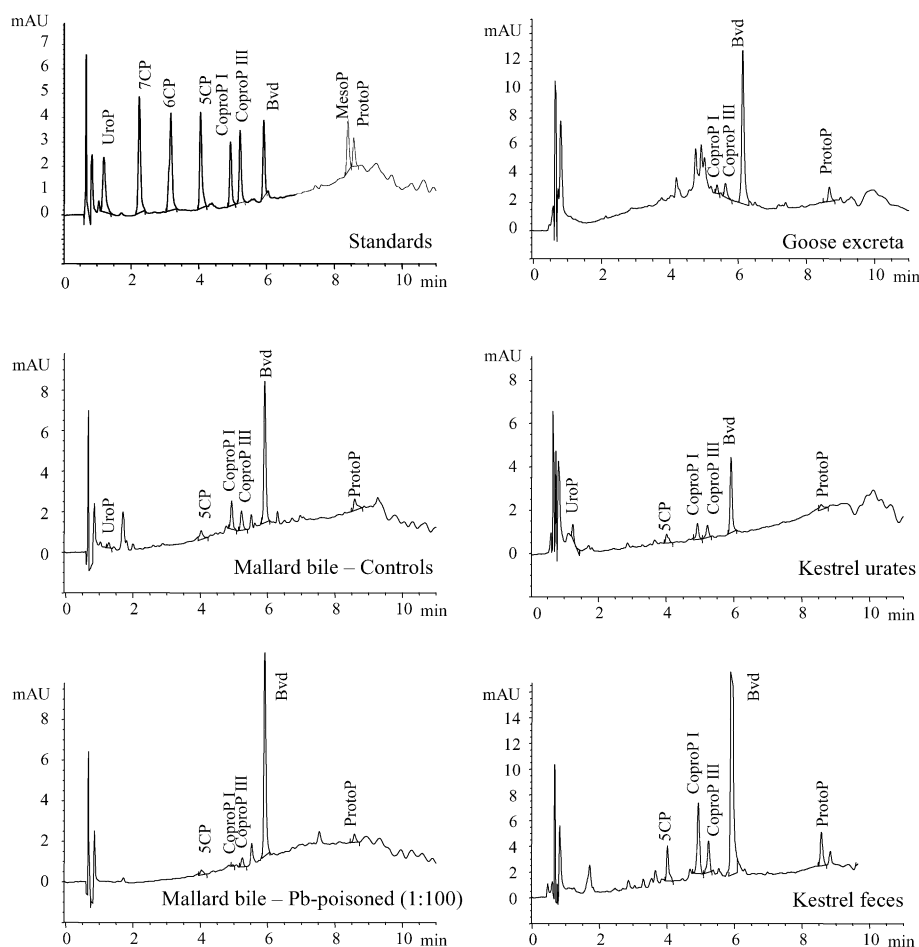

Fig. 1. Chromatograms of extracted porphyrins and biliverdin from bile and excreta of birds. The standard contained 333.3 nM of each porphyrin and 2213 nM of biliverdin, and the injection volume was 10 µl. The chromatogram obtained from goose feces corresponded to a different day of analysis, and consequently retention times differed slightly form the rest of the chromatograms. This variation was compensated by preparing calibration curves every day of analysis. Uro: uroporphyrin, 7CP: heptacarboxyl porphyrin, 6CP: hexacarboxyl porphyrin, 5CP: pentacarboxyl porphyrin, Copro: coproporphyrin, Bvd: biliverdin, MesoP: mesoporphyrin, ProtoP: protoporphyin IX. Note the change in scale on the *y*-axis.

Table 4
Mean (± S.D.) concentrations of porphyrins (%) and biliverdin in bile and excreta of birds

| | Greylag goose excreta (nmol/g DW) $n = 30$ | Lesser kestrel excreta (pmol/excreta[a]) | | Mallard bile (nmol/g) | |
		Urates $n = 10$	Feces $n = 10$	Control $n = 8$	Pb poisoned $n = 7$
Uroporphyrin	ND[b]	25.2 ± 79.5 (7.5)	48.9 ± 36.5 (0.4)	0.11 ± 0.20 (1.6)	ND
Heptacarboxyl porphyrin	ND	ND	18.5 ± 25.6 (0.2)	ND	ND
Hexacarboxyl porphyrin	ND	ND	6.09 ± 12.9 (0.1)	ND	ND
Pentacarboxyl porphyrin	ND	32.8 ± 43.7 (9.8)	686 ± 471 (6.2)	1.45 ± 0.92 (21.3)	20.6 ± 14.2 (12.1)
Coproporphyrin I	0.82 ± 1.34 (18.9)	94.6 ± 123 (28.2)	2,015 ± 1,801 (18.1)	1.50 ± 0.64 (22.1)	10.1 ± 13.3 (5.9)
Coproporphyrin III	1.64 ± 1.76 (38.7)	147 ± 112 (43.7)	1,457 ± 797 (13.1)	1.98 ± 1.24 (29.1)	50.8 ± 33.4 (29.8)
Mesoporphyrin	ND	ND	ND	0.07 ± 0.19 (1.0)	ND
Protoporphyrin IX	1.89 ± 1.13 (43.4)	36.6 ± 64.9 (10.9)	6,903 ± 8,121 (62.0)	1.69 ± 1.00 (25.0)	88.9 ± 119.6 (52.2)
Biliverdin	588 ± 1,165[c]	2,007 ± 2,565	84,786 ± 58,604	144 ± 121	9,519 ± 8,239
Total porphyrins/biliverdin	0.007	0.167	0.131	0.047	0.018

[a] Sample mass was unknown because feces were dropped on desiccant paper in pens.

[b] ND: not detected.

[c] Corrected for the recovery of biliverdin in goose excreta (26%).

was fecal excreta of lesser kestrels. These feces have residues of the carnivorous diet of kestrels, and porphyrins from the diet may be present in amounts that are too high to allow the assessment of alterations in the metabolism of endogenous porphyrins, as observed in humans on diets containing red meat or whole blood [20].

The analysis of protoporphyrin IX was one of the main objectives of this study, in order to use the present method in further studies for monitoring of Pb exposure in greylag geese in the Guadalquivir Marshes (Spain) by a non-invasive analysis of feces. In 1998, the Guadalquivir Marshes suffered an accidental spill of pyrite sludge with high contents of Pb from Aznalcóllar mine [21]. Moreover, Pb shot ingestion is frequent in greylag goose in Guadalquivir Marshes [22]. Zinc protoporphyrin is known to rise in the erythrocytes of Pb-exposed animals due in part to decreased ferrochelatase activity [1,23]. However, its biliary excretion is low [23] and fecal analysis of protoporphyrin has been focused on the metal free form. According to the bile analyses of control and Pb-poisoned mallards (Table 4), the rise of protoporphyrin IX with Pb poisoning may also be expected in feces. Pb also causes the increase of urinary coproporphyrins in mammals [1,4], and more of the isomer III than the isomer I [24]. Coproporphyrin III also increased more than coproporphyrin I in the bile of Pb-poisoned mallards studied here, although to a lower extent than protoporphyrin IX.

The method described here permits the analysis of porphyrins and biliverdin in bile and feces of birds by a simple, fast and inexpensive procedure. We included biliverdin in the analyses of heme precursors for two reasons. First, biliverdin analysis may help in the interpretation of porphyrin concentrations in feces because of their common biliary origin. And second, as biliverdin results from the metabolic breakdown of the heme group, its inclusion in an analysis of the whole heme synthesis and degradation pathway may be useful for the assessment of the exposure to toxic substances affecting both processes, as in the case of Pb. The present method is especially addressed to the analysis of protoporphyrin IX and

biliverdin in feces, both of which are increased in feces of Pb-poisoned birds through disruption of heme synthesis and hemolytic anemia.

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