

## Comparison of Spectrofluorometric and HPLC Methods for the Characterization of Fecal Porphyrins in River Otters

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A spectrofluorometric method (B. Grandchamp *et al.*, 1980, *Biochem. Biophys. Acta* 629, 577–586) developed for the determination of amounts of uroporphyrin I (Uro I), coproporphyrin III (Copro III), and protoporphyrin IX (Proto IX) in skin fibroblasts was compared with a high-performance liquid chromatography (HPLC) method for the analysis of porphyrins in fecal samples of river otters (*Lutra canadensis*). Heptacarboxylate porphyrin I and coproporphyrin I, two porphyrins determined to be critical in defining the porphyrin profile in fecal samples of river otters with the HPLC method, contributed substantially to the calculation of the concentrations of Uro I and Copro III, respectively, in standard solutions of porphyrins with the spectrofluorometric method. Fluorescent components of the fecal matrix complicated the determination of the concentrations of Uro I, Copro III, and Proto IX with the spectrofluorometric method and resulted in erroneous values for the concentrations of these porphyrins compared with values determined with the HPLC method. These results indicate that the complexity of the sample, particularly with regard to the potential presence of interfering fluorescent compounds, as well as porphyrins additional to Uro I, Copro III, and Proto IX, should be considered prior to the application of the spectrofluorometric method. An alternative HPLC method developed for the rapid characterization of porphyrin profiles in fecal samples of river otters is described. © 2000

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### INTRODUCTION

The use of changes in the profile of fecal porphyrins as a biomarker of effects of crude oil

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exposure in river otters (*Lutra canadensis*) following the *Exxon Valdez* oil spill in Prince William Sound, Alaska has been under investigation in our laboratory. River otters are ubiquitous in the Gulf of Alaska and occupy large home ranges within Prince William Sound (Bowyer *et al.*, 1995). Furthermore, river otters occupy a critical niche in the coastal ecosystem linking the nutrient dynamics of the aquatic and terrestrial environments (Bowyer *et al.*, 1994; Ben-David *et al.*, 1998) and therefore serve as an important indicator species for the evaluation of the chronic effects of exposure to crude oil in biomonitoring and restoration programs.

Heme biosynthesis and porphyrin excretion have been shown to be sensitive to a number of chemicals in the environment (DeMatteis and Lim, 1994). Elevation of porphyrins in blood, urine, and feces due to exposures to heavy metals and halogenated hydrocarbons has been measured in laboratory animals and humans (Daniell *et al.*, 1997). Porphyrin levels also have been used as an indicator of chemical exposure in studies of wildlife. Elevated levels of highly carboxylated porphyrins occurred in livers of herring gull chicks (*Larus argentatus*) collected from sites within the Great Lakes contaminated with polyhalogenated aromatic hydrocarbons (Kennedy and Fox, 1990). Measurements of total porphyrin levels were higher in fecal samples of river otters inhabiting oiled areas in comparison with nonoiled areas of Prince William Sound, Alaska, following the *Exxon Valdez* oil spill (Blajeski *et al.*, 1996).

Field monitoring programs, designed around the use of changes in the profile of fecal porphyrins as a biomarker of contaminant exposure, involve the collection and analysis of large numbers of fecal samples necessitating the development of analytical methods that are rapid and efficient. The application of a spectrofluorometric method for rapid determination of the ratios of (Uro I), uroporphyrin I coproporphyrin III (Copro III), and Protoporphyrin

IX (Proto IX) in skin fibroblasts (Grandchamp *et al.*, 1980) has been reported more recently for the evaluation of porphyrin levels in both laboratory and wild marine organisms (Fossi *et al.*, 1996, 1997a,b). This spectrofluorometric method is based on the measurement of the relative fluorescence signals obtained at each of three discriminating pairs of excitation-emission wavelengths to determine the ratios of Uro I, Copro III, and Proto IX present in biological samples. The purpose of this study was to test the effectiveness of the spectrofluorometric method for the characterization of porphyrin profiles in fecal samples of river otters through comparison with HPLC results.

## METHODS

### *Sample Collection and Preparation*

Fecal samples were obtained from the frozen archive at the Institute of Arctic Biology at the University of Alaska Fairbanks, Fairbanks, Alaska. Fecal samples had been collected from field locations within Prince William Sound during 1990, placed in plastic bags, and stored in a freezer at  $-70^{\circ}\text{C}$ . The samples were lyophilized for 24 h prior to porphyrin extraction and analysis.

### *Standards*

Porphyrin standards used for HPLC analysis included a chromatographic marker kit containing the number I isomers of 8, 7, 6, 5, 4 carboxylate porphyrins and mesoporphyrin IX, Copro III, and Proto IX. The porphyrin standards used for the spectrofluorometric analysis included Uro I, Copro III, and Proto IX fluorescence marker kits, heptacarboxylate porphyrin I (Hepta I), and coproporphyrin I (Copro I). All porphyrin standards were dissolved in 6 N HCl. The concentrations of Hepta I, Copro I, and Proto IX were determined by measuring the absorbance at the Soret peak and applying the appropriate molar absorptivity. All porphyrin standards were purchased from Porphyrin Products, Logan, Utah.

### *Reagents*

Reagents used in extraction procedures included ACS-grade hydrochloric acid, sodium phosphate (monobasic), acetonitrile, and acetone. HPLC reagents included ACS-grade ammonium acetate and HPLC-grade methanol. Milli-Q 18 water was used for all solvent preparations.

### *Equipment*

Porphyrins were concentrated on disposable, 500-mg, trifunctional, C-18 (tC-18). Sep-Pak cartridges (Waters Corp.). Separation of porphyrins was facilitated by a high-performance liquid chromatography system consisting of two Waters 510 pumps, a Rheodyne 7125 injector valve equipped with a  $5\ \mu\text{L}$  injection loop, and a 3-cm-long by 0.46-cm-wide Luna C-18 column equipped with a Security Guard Cartridge System (Phenomenex Corp.). Porphyrins were detected with a McPherson FL-748 fluorescence detector equipped with a 405-nm excitation (Ex) cutoff filter, a 620-nm interference emission (Em) filter with a 10-nm bandwidth, and a red-sensitive photomultiplier tube. Baseline 810 and Maxima 820 chromatographic software (Waters Corp.) were used for system control and peak integration.

Spectrofluorometric analysis was accomplished with an SLM Aminco spectrofluorometer Model SPF 500C equipped with an LX 300 UV lamp and a Hamamatsu R928P red-sensitive photomultiplier tube. A 1.0-ml quartz cuvette was used for spectrofluorometric measurements.

### *Porphyrin Extraction*

Extraction and isolation of porphyrins from the fecal samples was accomplished with a modification of a procedure described by Bowers *et al.* (1992). Porphyrins were extracted with HCl and concentrated on trifunctional C-18 Sep-Pak cartridges. Five milliliters of 6 N HCl was added to 1.0 g of dried fecal material and the sample was macerated with a glass rod for 1 min. The sample was then mixed with a bench-top vortex for 1 min, sonicated in a water bath for 5 min, and mixed again for 1 min. Five milliliters of sodium phosphate buffer (0.01 M, pH 3.5) was added to the sample, which was mixed for 1 min. The sample was then centrifuged for 10 min at 4000 rpm. Following this step, the coarse pellet was removed and the sample was centrifuged again for 10 min at 4000 rpm. Eight milliliters of the supernatant was delivered to the Sep-Pak cartridges.

Trifunctional C-18 Sep-Pak cartridges were used for porphyrin concentration to avoid stripping of the alkyl chain from the support under acidic conditions. The tC-18 Sep-Paks were connected to a Supelco Visiprep vacuum manifold and washed with 7 ml of acetonitrile, followed by 7 ml of sodium phosphate buffer (0.01 M, pH 3.5). Eight milliliters of fecal supernatant was delivered to the Sep-Paks and allowed to gravity feed. The Sep-Paks were washed

with 3 ml of sodium phosphate buffer (0.01 M, pH 3.5) to facilitate complete delivery of the supernatant to the Sep-Pak, followed by 7 ml of sodium phosphate buffer (0.01 M, pH 7.5) to adjust the pH of the column and enhance recovery of porphyrins. Residual sodium phosphate buffer was removed from the column with vacuum set at 5 mmHg. The concentrate at the top of the Sep-Pak containing the porphyrins was eluted into 5-ml cryogenic centrifuge tubes with 1 ml of acetonitrile, followed by 0.5 ml of acetonitrile:1.0 N hydrochloric acid (1:1, v/v), followed by 1 ml of acetone under vacuum set at 15 mmHg. The tubes containing the eluates were placed in a water bath at 55°C and evaporated under a stream of air. The dried residues containing the porphyrins were stored at -70°C. For HPLC analysis, the dried residues were reconstituted in 0.5 ml of 6 N hydrochloric acid and injected directly into the HPLC system.

#### *HPLC Analysis*

The HPLC method used was a modification of a procedure described by Kennedy *et al.* (1986). Separation of porphyrins was facilitated by a 6-min gradient elution and a two-component mobile phase consisting of ammonium acetate (1.0 M, pH 5.16) as solvent A and 100% methanol as solvent B. Gradient elution commenced upon injection at 25% B, increased to 50% B in 1 min, then to 95% B in 3 min, remained at 95% B for 1.5 min, and returned to 25% B in 0.5 min. The column was allowed to reequilibrate for 5 min at 25% B before the next injection.

The concentrations of porphyrins in each fecal sample were calculated with a seven-point calibration curve ranging from 0.0 to 3.0  $\mu\text{M}$ . Calibration standards for porphyrins were dissolved in 6 N hydrochloric acid and remained stable for at least 24 h.

#### *Spectrofluorometric Analysis*

Spectral measurements were made with the excitation bandwidth set at 0.25 nm and the emission bandwidth set at 20 nm. Maintaining a very narrow excitation bandwidth was necessary to discriminate between Uro I and Copro III. The emission bandwidth was set at 20 nm to obtain an adequate fluorescence signal from such a narrow range of excitation energy.

To determine the three pairs of excitation-emission wavelengths necessary to discriminate between Uro I, Copro III, and Proto IX, standard solutions of the three porphyrins were scanned individually at 90 different pairs of excitation-emission

wavelengths from 395–595 nm Ex to 410–610 nm Em, and the fluorescence signal for each porphyrin at each wavelength pair was recorded. The choice of the wavelength pairs was based on those that produced the greatest fluorescent signal for each porphyrin of interest relative to the other two porphyrins (Wehry, 1981).

#### *HPLC Spectrofluorometric Method Comparison*

Fecal samples were applied to the extraction procedure and aliquots of the same resultant extracts representing each sample and containing the porphyrins were applied to both the HPLC and the spectrofluorometric methods within 6 h for analytical comparison. Porphyrins separated and identified with the HPLC method were quantified with the seven-point calibration curve.

To test the accuracy of the spectrofluorometric method, fluorescence signals were measured at each of the three pairs of wavelengths for four different mixtures of Uro I, Copro III, and Proto IX, and the relative concentrations of the porphyrins were calculated. The actual concentrations were then compared with the calculated concentrations.

To determine the contribution of Hepta I and Copro I to the calculation of the concentrations of Uro I, Copro III, and Proto IX, Hepta I and Copro I were added to three different mixtures of porphyrin standards. The concentrations of Uro I, Copro III, and Proto IX were determined with Hepta I and Copro I present in the standard mixtures and compared with the actual concentrations.

The concentrations of Uro I, Copro III, and Proto IX in 10 different fecal extracts also were calculated with the spectrofluorometric method and compared with concentrations calculated with the HPLC method. Fecal extracts prepared for HPLC analysis were diluted 100-fold prior to spectrofluorometric analysis.

#### *Statistical Analysis*

A multivariate analysis of variance model (PROC GLM, MANOVA; SAS) was used to evaluate the contribution of the addition of Hepta I and Copro I to the standard porphyrin mixtures on the calculation of the concentrations of Uro I, Copro III, and Proto IX. One-tailed contrasts (PROC GLM, CONTRAST; SAS) were used to evaluate how Hepta I and Copro I contributed to the calculated concentrations of Uro I, Copro III, and Proto IX independently. Differences in concentrations of the porphyrins in the fecal extracts determined with the HPLC and spectro-

fluorometric methods were evaluated with paired *t* tests. Results were determined to be significant at  $P < 0.05$ . Statistical analyses were accomplished with SAS Statistical Software, SAS Institute, Cary, North Carolina (MANOVA) and Microsoft Excel (Microsoft Corp., paired *t* tests).

## RESULTS

Measurement accuracy with our HPLC system was evaluated and monitored by running porphyrin standards prior to and during sample analysis. An aliquot of a chromatographic marker kit solution (6 N HCl) was used for each porphyrin standard. Differences in porphyrin recoveries obtained from standards run prior to and during sample analysis were within 11% for all porphyrins of the chromatographic marker kit.

Extraction efficiencies were evaluated by processing aliquots of porphyrin standard solutions and fecal samples spiked with aliquots of porphyrin standard solutions through the extraction procedure. Mean recoveries for all porphyrins for the methods standards tests ranged from 70 to 82% (Table 1a). Mean recoveries for all porphyrins for the sample spike tests ranged from 62 to 189% (Table 1b).

The pairs of excitation–emission wavelengths chosen for this study were 407–592 nm for Uro I, 397–591 nm for Copro III, and 410–610 nm for Proto IX. These wavelength pairs provided the most discriminating fluorescence signals for each of the three

porphyrins. The percentage differences between the actual and the measured concentrations of Uro I and Copro III were within 18% for all four trials, however, the concentration of Proto IX was consistently underestimated, with the percentage differences ranging from 27 to 60% (Table 2). In the two mixtures containing no Uro I and Copro III, erroneous detection of these two porphyrins occurred. The percentage differences between the actual and the measured concentrations for Uro I with the wavelength pairs reported by Grandchamp *et al.* (1980) were within 18% for all four trials, however, the percentage differences between the actual and the measured concentrations of Copro III and Proto IX ranged from 3 to 110% and from 20 to 90%, respectively.

The addition of Hepta I and Copro I contributed substantially to the calculated concentrations of Uro I and Copro III with the wavelength pairs chosen for this study (Table 3). A MANOVA model incorporating addition of Hepta I and Copro I as treatment effects (no addition, treatment 1; addition of Hepta I, treatment 2; addition of Copro I, treatment 3) and concentrations of Uro I, Copro III, and Proto IX as dependent variables was significant for treatment effects (Wilk's Lambda,  $F = 7.67$ ,  $P = 0.0056$ ,  $df = 6,8$ ). Independent contrasts revealed that Hepta I contributed significantly to the calculated concentration of Uro I ( $F = 6.16$ ,  $P = 0.048$ ,  $df = 1,2$ ). The contribution of Copro I to the calculated concentration of Copro III was non-significant ( $F = 0.47$ ,  $P = 0.512$ ,  $df = 1,2$ ). Hepta I had minimal effect on the calculated concentration of Copro III ( $P = 0.822$ ) and, likewise, Copro I had no effect on the calculated concentration of Uro I ( $P = 0.726$ ). Also, addition of Hepta I and Copro I to the porphyrin mixtures had no effect on the calculated concentrations of Proto IX ( $P = 0.765$  and  $P = 0.929$ , respectively). Qualitative assessment of the spectral scans of all five porphyrins also confirmed these results. Both the excitation and the emission spectra of Hepta I and Copro I were nearly identical in position to the those of Uro I and Copro III, respectively, (Figs. 1 and 2).

Ten fecal extracts prepared for HPLC analysis were diluted 100-fold and the concentrations of Uro I, Copro III, and Proto IX were calculated with the spectrofluorometric method. For all samples, except for one value for Uro I, the concentrations of the three porphyrins were overestimated significantly by the spectrofluorometric method (Uro I,  $P = 0.0011$ ; Copro III,  $P = 0.000002$ ; Proto IX,  $P \sim 0.0010$ ), (Table 4). Values for Proto IX were calculated in eight samples in which Proto IX was not

TABLE 1

Mean Extraction Efficiencies Determined by (a) Processing an Aliquot of a Standard Chromatographic Marker Kit Solution (Porphyrin Products, Logan, UT) through the Extraction Procedure and (b) Applying an Aliquot of a Standard Chromatographic Marker Kit Solution to a Fecal Sample Prior to Processing through the Extraction Procedure

	Nanomoles applied	Mean percentage nanomoles recovered <sup>a</sup>				
		Uro I	Hepta I	Hexa I	Penta I	Copro I
a <sup>b</sup>	1	77	70	82	74	76
b <sup>c</sup>	9	69	76	77	68	66
	6	72	118	79	70	71
	3	62	189	123	84	140

<sup>a</sup> $n = 2$  for both method standards (a) and sample spike (b) tests.

<sup>b</sup>1.0 nmol porphyrins in an aliquot of 6.0 N HCl treated as a sample and processed through the extraction procedure.

<sup>c</sup>9.0, 6.0, and 3.0 nmol porphyrins in an aliquot of 6.0 N HCl added to each 1.0-g portion of fecal sample prior to processing through the extraction procedure.

**TABLE 2**  
Accuracy of the Spectrofluorometric Method with the Wavelength Pairs Chosen for This Study

Mixture	Actual (nM)			Measured (nM)			Percentage difference		
	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX
1	25	50	75	24	41	55	4	18	27
2	60	20	40	67	22	28	12	10	30
3	40	60	20	41	60	8	3	0	60
4	80	120	40	72	106	20	10	12	50

detected with the HPLC method. Values for Uro I and Copro III also were calculated for samples in which these porphyrins were not detected with the HPLC method. Ratios for the measured concentrations of porphyrins in the 10 samples with the spectrofluorometric method were different from the ratios of the concentrations determined with the HPLC method.

The contribution of fluorescent matrix constituents in the fecal extracts to the measured concentrations of the porphyrins was qualitatively assessed by comparing spectral scans of whole fecal extracts with chromatographic results from HPLC. The spectrofluorometric emission scan of a whole fecal extract revealed a broad fluorescence signal that merged with the porphyrin peak. All of the HPLC chromatograms for the fecal samples analyzed for this study contained material that eluted prior to the porphyrins. This component of the eluate was fraction-collected during HPLC analysis, dried under a stream of air, and reconstituted in 1.0 N HCl for spectrofluorometric analysis. The spectrofluorometric emission scan of the fractionated component revealed a peak similar to the broad fluorescence signal observed in the whole fecal extract emission scan.

## DISCUSSION

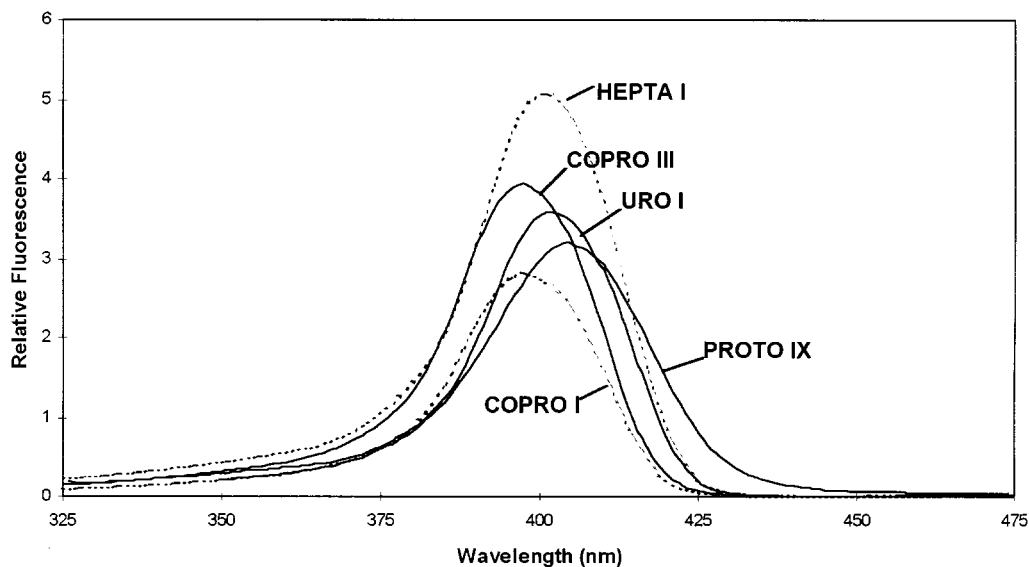
The use of the spectrofluorometric method with the pairs of excitation–emission wavelengths chosen for this study resulted in relatively high accuracy for the determination of Uro I and Copro III in mixtures of porphyrin standards at concentrations ranging from 20 to 120 nM; differences between the actual and the measured concentrations were within 18%. Consistently lower accuracy was observed for Proto IX at concentrations ranging from 8 to 55 nM, with differences between actual and measured concentrations ranging from 27 to 60%. Although use of the wavelength pairs chosen for this study resulted in higher accuracy on average than use of the wavelength pairs reported by Grandchamp *et al.* (1980), the low accuracy associated with the determination of the concentration of Proto IX precludes the use of the method for sensitive evaluation of the concentration of Proto IX relative to the concentrations of Uro I and Copro III. Furthermore, because of the lower accuracy associated with Proto IX, estimation of total porphyrins (Uro I, Copro III, and Proto IX combined) also would be suspect.

HPLC analysis showed that Hepta I and Copro I, as well as Uro I, Copro III, and Proto IX, were critical

**TABLE 3**

Effect of the Addition of Heptacarboxylate Porphyrin I (a) and Coproporphyrin I (b) to Mixtures of Porphyrin Standards on the Calculated Concentrations of Uroporphyrin I, Coproporphyrin III, and Protoporphyrin IX with the Wavelength Pairs Chosen for This Study

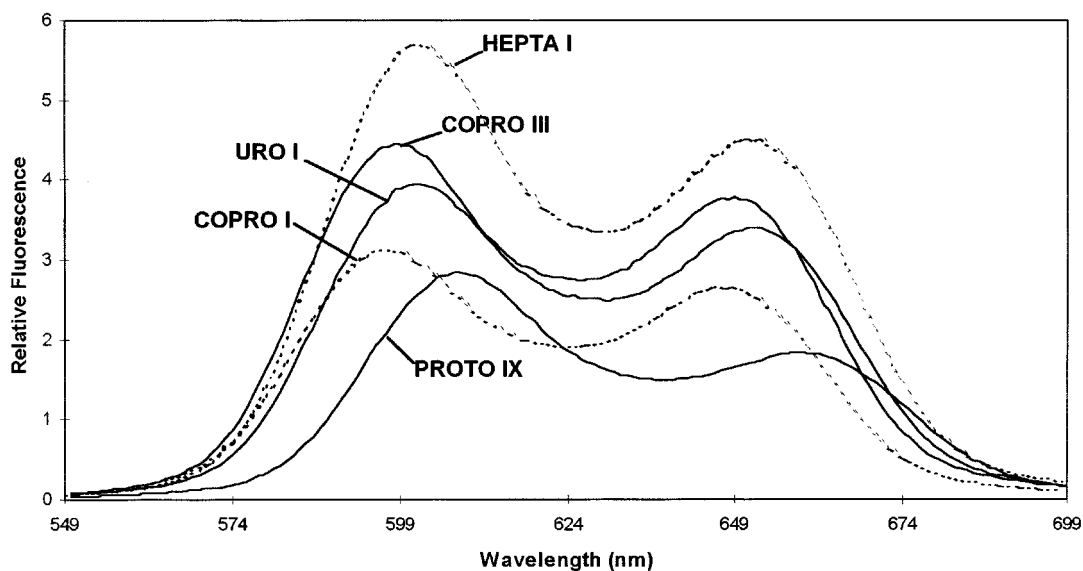
Mixture	Actual (nM)				Measured (nM)			
	Uro I	Copro III	Proto IX	Hepta I	Uro I	Copro III	Proto IX	Hepta I
a	1	0	83	333	83	154	129	271
	2	125	0	292	83	264	50	226
	3	104	313	0	83	243	307	0
b	Mixture	Uro I	Copro III	Proto IX	Copro I	Uro I	Copro III	Proto IX
	1	0	83	333	83	1	169	323
	2	125	0	292	83	162	78	264
	3	104	313	0	83	130	413	0



**FIG. 1.** Overlay of the excitation spectral scans of uroporphyrin I, heptacarboxylate porphyrin I, coproporphyrin I, coproporphyrin III, and protoporphyrin IX. The excitation peaks of heptacarboxylate I and coproporphyrin III are nearly identical in position to those of uroporphyrin I and coproporphyrin I, respectively.

in defining the porphyrin profile in fecal samples of river otters. The results of this study demonstrate that the presence of Hepta I and Copro I contribute substantially to the calculated concentrations of Uro I and Copro III, respectively, using the spectrofluorometric method. Furthermore, because the emission spectra of Hepta I and Copro I are nearly

identical in position to the emission spectra of Uro I and Copro III, respectively, this method lacks the selectivity necessary for more diagnostic studies in which determination of the individual concentrations of Uro I, Hepta I, Copro I, and Copro III may be critical in identifying changes in the porphyrin profile. For example, heptacarboxylate porphyrin



**FIG. 2.** Overlay of the emission spectral scans of uroporphyrin I, heptacarboxylate porphyrin I, coproporphyrin I, coproporphyrin III, and protoporphyrin IX. The emission peaks of heptacarboxylate I and coproporphyrin III are nearly identical in position to those of uroporphyrin I and coproporphyrin III, respectively.

TABLE 4

Comparison of HPLC and Spectrofluorometric Methods for the Determination of Porphyrin Concentrations in 10 Fecal Samples of River Otters from Prince William Sound, Alaska

Sample	HPLC method ( $\mu\text{M}$ )			Spectrofluorometric method ( $\mu\text{M}$ )		
	Uro I	Copro III	Proto IX	Uro I	Copro III	Proto IX
C004	0	0.400	0	167	1144	811
C108	0.134	0.716	0	359	981	85
C031	0	0	0	148	409	168
C011	0.460	0.290	0	144	830	408
C063	0.120	2.052	0	63	1153	738
HB23	0.974	2.122	0	0	1100	1100
KI12	0	0.824	0	78	1132	944
HB04	0.406	1.022	4.441	193	937	874
HB01	0.176	0.318	1.264	267	647	213
DI02	0	0	0	126	595	265

Note. Fecal sample extracts prepared for HPLC analysis (500  $\mu\text{L}$ ) were diluted 100-fold prior to spectrofluorometric analysis. The detection limit for the HPLC method was approximately 10 pmol/500  $\mu\text{L}$  (0.020  $\mu\text{M}$ ).

contributed significantly to the porphyrin profiles in embryo hepatocytes of chickens after exposure to PCBs (Lorenzen *et al.*, 1997). Also, in instances when uroporphyrinogen synthetase is deficient or inhibited, the type I isomer of uroporphyrin may be produced, which, in turn, may result in the accumulation of Copro I (DeMatteis and Lim, 1994).

The spectrofluorometric assay was not appropriate for the determination of the amounts of porphyrins in the fecal samples of river otters. Fluorescent components of the fecal matrix resulted in elevated and erroneous values for the concentrations of Uro I, Copro III, and Proto IX. Qualitative analysis of the emission spectra of whole fecal extracts showed a fluorescence signal extending from 430 to 600 nm, which merged with the porphyrin peak and most likely contributed to erroneous values. That peak corresponded to the emission spectrum of a matrix compound evident as a peak on the HPLC chromatogram that was fraction-collected and analyzed on the spectrofluorometer. This compound could be partially removed by addition of a dilute methanol wash through the C-18 Sep Pak cartridges to the solid-phase extraction-isolation procedure (results not shown). Although the development of more extensive extraction procedures may facilitate the separation of porphyrins from other matrix components, this would preclude the use of the spectrofluorometric assay for rapid and cost-efficient analysis of porphyrins.

The HPLC method described in this study is an alternative to the spectrofluorometric assay and is relatively rapid and sensitive for quantifying the

porphyrins that typically define the porphyrin profile of biological samples. The solid-phase extraction-isolation step to isolate porphyrins from the river otter fecal samples was developed primarily for sample concentration. Attempts to separate porphyrins from all of the matrix components with the C-18 Sep-Pak cartridges were problematical because the same solvent systems necessary for eluting all of the porphyrins from the cartridges also were those that facilitated the removal of the fecal matrix components. The fluorescent matrix components present in the final eluate, however, did not interfere with HPLC separation and quantification of porphyrins.

The HPLC conditions described in this study, incorporating the use of an inexpensive 3-cm-long column and short (6-min) run time with 5-min column reequilibration, facilitates rapid separation and identification of porphyrins. The type I and type III isomers of coproporphyrin were discriminated readily by this method. This HPLC method may serve as a rapid alternative to the spectrofluorometric assay for samples from wildlife containing significant interfering, fluorescent constituents and porphyrins other than Uro I, Copro III, and Proto IX, which contribute to the porphyrin profile.

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