Rapid Procedure for Fecal Porphyrin Assay

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Hydrochloric acid extraction of feces in the presence of ether yields an extract suitable for spectrophotometric estimation of total porphyrin and for further separation by "high-performance" liquid chromatography (HPLC) or thin-layer chromatography. A total porphyrin reference interval of less than 200 nmol/g dry weight of feces was established from data on 106 normal subjects on an unrestricted diet. Total fecal porphyrin values in 11 porphyria cutanea tarda patients were considerably higher than given by the widely used Rimington method (respective means, 652 and 239 nmol/g dry weight). Our HPLC method for separation of porphyrin methyl esters on a silica column, with quantification by fluorescence, is described. HPLC separations performed on 23 porphyria cutanea tarda patients gave the following mean proportions of total fecal porphyrins: dicarboxylics 21%, coproporphyrin 9%, isocoproporphyrins 28%, pentacarboxylporphyrin 9%, hexacarboxylporphyrin 11%, heptacarboxylporphyrin 18%, and uroporphyrin 4%.

Additional Keyphrases: *liquid chromatography · porphyria cutanea tarda · screening · fluorometry · Rimington method · compared · reference interval*

Determination of fecal porphyrins is important in the diagnosis and differential diagnosis of porphyria. In the most widely used methods porphyrins are extracted from the fecal sample with diethyl ether and glacial acetic acid, then differentially extracted into "protoporphyrin" and "coproporphyrin" fractions with various concentrations of hydrochloric acid (*1, 2*). The porphyrin fractions obtained are significantly contaminated, and up to 30% of the protoporphyrin may actually be in the coproporphyrin extract (*3*). The procedure is time consuming and ether-insoluble porphyrins such as heptacarboxylporphyrin and uroporphyrin found in porphyria cutanea tarda are not included.

The following method gives a rapid estimate of total porphyrin and can be used to exclude many specimens from further investigation. Hydrochloric acid is used to extract the fecal porphyrins in the presence of ether. The porphyrins remain in the acid layer, and interfering chlorophyll and carotenoid pigments are extracted and removed by the ether. The value for total fecal porphyrin is calculated from Soret region absorbance measurements of the aqueous acid layer. The subsequent separation of fecal porphyrins is facilitated by using the aqueous acid extract, which is substantially purified and free of solid residues. We describe the use of the acid extract in a "high-performance" liquid chromatographic (HPLC) method based on the formation of porphyrin methyl esters, which are separated on a column of silica by use of ethyl acetate and heptane and quantified fluorimetrically. Quantitative HPLC porphyrin separation results are presented for normal subjects and patients with porphyria cutanea tarda (PCT).

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Materials and Methods

Reagents

Hydrochloric acid (354 g/L, rel. density 1.18), methanol, chloroform, dry potassium carbonate, and sulfuric acid (980 g/L, rel. density 1.84) were all of AR grade. Methanol/sulfuric acid (90:5 by vol) was prepared by slowly adding 25 mL of sulfuric acid to 450 mL of methanol. Diethyl ether, AR grade, was always shown to be free of peroxides before use, as these can destroy porphyrins. Ethyl acetate and heptane were of "HPLC" grade (Lichrosolv; Merck, F.R.G.). The methyl esters of protoporphyrin IX, coproporphyrin III, and uroporphyrin I were obtained from Sigma Chemical Co., St. Louis, MO, for use as HPLC standards.

Equipment

A recording spectrophotometer capable of producing an absorbance scan in the visible region (wavelengths between 390 and 425 nm) is required. We used a Perkin-Elmer Model 320 with scan speed set to 60 nm/min and spectral bandpass to 1 nm.

For the HPLC we used a Hewlett-Packard Model 1084B liquid chromatograph incorporating an integrating plotter giving peak areas and two pumps for gradient elution. The detector was a Schoeffel Model FS970 fluorescence detector set to 400 nm excitation wavelength with a 590-nm filter on the emission side. The porphyrins were separated on a 25 cm × 0.5 cm column packed with 10-μm spherical silica (Hewlett-Packard). The flow rate used throughout was 1.5 mL/min.

Total Fecal Porphyrin Determination

Fecal samples were collected as 10–20 g portions of untimed fecal specimens from patients on unrestricted diets. The samples were not homogenized or mixed. Two samples of feces were accurately weighed: 25–50 mg into a graduated centrifuge tube for analysis and a larger sample (about 250 mg) into a vessel suitable for drying. The larger sample was either dried in a vacuum desiccator over concentrated sulfuric acid or lyophilized. Concentrated hydrochloric acid (1.0 mL) was added to the centrifuge tube and vortex-mixed until all particles disintegrated. After standing for 5 min, the mixture was again vortex-mixed. If necessary, any remaining particles were broken up with a glass rod. Diethyl ether (3.0 mL) was added and thoroughly mixed to give an emulsion, followed by 3.0 mL of distilled water and further mixing. The water was added within 10 min of adding the acid to the feces, to avoid undue alteration of protoporphyrin. The mixture was centrifuged, resulting in an ether layer, a pad of insoluble material at the interface, and a layer of aqueous acid. Chlorophyll derivatives and carotenoid pigments are partitioned into the ether phase and the fecal porphyrins remain in the aqueous acid (lower) layer. The volume of the aqueous acid layer may be considered to be 4.5 mL, or measured if desired. A sufficient volume of the acid layer was transferred with a Pasteur pipette to a cuvette, for spectrophotometry.

Using a scanning spectrophotometer equipped with a chart recorder, we scanned the absorbance over the Soret

region including the wavelengths between 390 and 425 nm. The absorbance difference was obtained graphically by joining the absorption at 390 nm to that at 425 nm. The tangent to the curve of the scan between these wavelengths parallel to this line was drawn. The height of the point of contact of the tangent to the curve above the joining line is the corrected absorbance difference (ΔA) required for the calculation (4). The graphical construction is illustrated in Figure 1, which shows scans obtained for fecal samples with normal and above-normal porphyrin content.

The total fecal porphyrin (nmol/g dry weight) was calculated as:

$$\Delta A \times 3.3 \times \frac{4.5}{t} \times \frac{ww}{dw}$$

where ΔA = corrected absorbance difference
3.3 = factor converting absorbance to concentration in micromoles per liter
4.5 = volume of acid extract in milliliters
t = weight of feces taken for analysis, in grams
ww = wet weight of the larger sample, in grams
dw = dry weight of the larger sample, in grams

HPLC Separation

The residue-free aqueous acid extract containing the mixed fecal porphyrins may be injected directly into some HPLC systems, notably those developed by Lim et al. (5, 6). The HPLC results described here were obtained by using porphyrin methyl esters. The procedure:

Add 1.0 mL of aqueous acid extract to 9.0 mL of methanol–sulfuric acid and allow to stand overnight at room temperature. Add 3.0 mL of chloroform and isolate it by pouring the mixture into a separating funnel containing about 40 mL of distilled water. Neutralize the chloroform immediately by vortex-mixing with about 50 mg of potassium carbonate and then washing with 10 mL of distilled water. Evaporate the chloroform containing the porphyrin methyl esters to a final volume of about 0.5 mL, ready for injection onto the HPLC column.

Equilibrate the column with ethyl acetate/heptane (35/65 by vol) before injecting the sample. Separate porphyrins

with two successive 6-min linear gradients: firstly from 35% to 45% ethyl acetate (in heptane) followed by 45% to 85% ethyl acetate. Maintain isocratic elution with ethyl acetate/heptane (85/15 by vol) for a further 6 min. With this system, void volume 4 mL, all the different carboxyl groups from protoporphyrin to uroporphyrin are resolved within 15 min.

Porphyrin methyl esters dissolved in chloroform are injected, separated, and the areas under the fluorescence peaks calculated and displayed as a percentage of the total peak area.

Standard solutions containing porphyrin methyl esters are used to compare the proportions given by fluorescence peak areas with the known molar proportions of the standard.

We separately dissolved the methyl esters of protoporphyrin IX, coproporphyrin III, and uroporphyrin I in three chloroform solutions and standardized them spectrophotometrically. Four standard solutions were prepared by mixing known volumes of the three solutions together so that each porphyrin would cover a range of concentrations up to 400 nmol/L. Each porphyrin showed a linear relation of peak area to concentration. Moreover, an equimolar mixture of the methyl esters of protoporphyrin, coproporphyrin, and uroporphyrin gave fluorescence peak areas in the molar ratio 1.00/0.95/1.05. The proportion of each porphyrin in the HPLC traces was reported as a percentage of the total area of the fluorescence peaks.

Results

The present method was compared to the widely used Rimington method (2), in which the porphyrins are extracted from the fecal sample with ether and glacial acetic acid. Fecal samples from 52 general-hospital outpatients who were on unrestricted diets were assayed by both methods. The resulting regression line (mean ± SD) for the plotted results (where y is the present method) was $y = (1.07 \pm 0.15)x + (6.4 \pm 0.4)$, $r = 0.901$, S_{yx}27 nmol/g dry wt. Thus, results by the two methods correlated well, although a systematic error was observed, higher readings being given by the present method. This may be due to improved extraction of fecal porphyrins by concentrated hydrochloric acid as compared with glacial acetic acid, or to some residual contamination of the aqueous acid extract by dietary chlorophyll derivatives.

We tested 11 patients with overt clinical and biochemical PCT by both methods, and the results are shown in Table 1. PCT was diagnosed according to the criteria described by
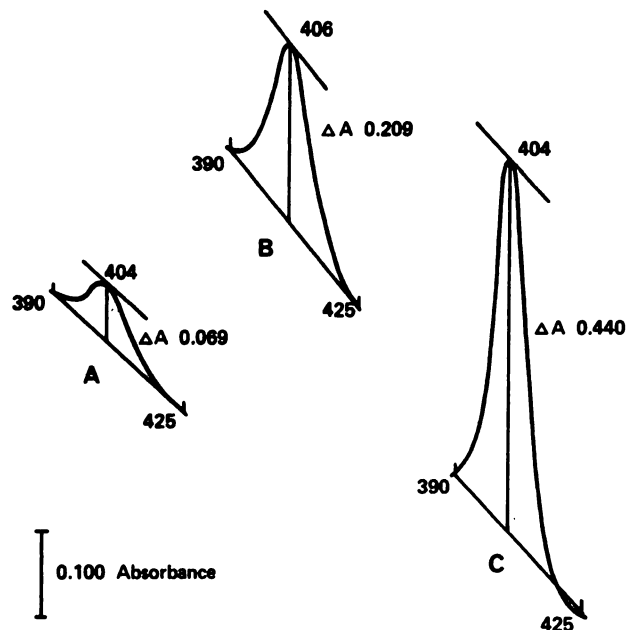

Fig. 1. Spectrophotometric scans of absorbance vs wavelength for three acid extracts, A, B, and C

The absorbance difference required for the calculation is shown as ΔA. Each scan displays the correcting wavelengths, in nanometers. Total fecal porphyrin was 170, 440, and 1770 nmol/g dry weight in extracts A, B, and C, respectively

Table 1. Total Fecal Porphyrin in 11 PCT Patients[a]

	Present method	Rimington method		
	Total fecal porphyrin	Copro.	Proto.	Total
Reference intervals	<200	<30	<135	<165
	714	39	90	129
	843	130	147	277
	900	115	130	245
	380	77	52	129
	688	76	327	403
	191	26	57	83
	480	133	127	260
	682	88	108	196
	568	86	210	296
	735	122	116	238
	991	124	245	369
Mean	652	92	158	239
Range	191–991	39–133	52–327	83–403

[a] All values nmol/g dry weight.

Gray et al. (7), i.e., the typical urine and fecal porphyrin excretion pattern seen with HPLC. The improved extraction with the present method is evident, with significantly more porphyrin extracted as compared with the Rimington method (respective means: 652 and 239 nmol/g dry weight). Because the Rimington method measures only ether-soluble porphyrins, it fails to include the ether-insoluble hexa- and heptacarboxylporphyrin and uroporphyrin fractions found in PCT feces (see Table 2). The Rimington method was found to underestimate the fecal porphyrins in PCT patients and, as Table 1 shows, could represent the excretion as either normal or only a little above normal.

A reference interval was obtained by testing fecal specimens from 106 general-hospital outpatients (65 men and 41 women) who were on unrestricted diets. The data are shown in Figure 2. This non-gaussian distribution could not be easily transformed to a normal distribution. These results probably reflect the highly diet-dependent nature of fecal porphyrin excretion. Inspection of the distribution shows that 101 of the 106 subjects had results less than 200 nmol/g dry weight, with the remaining five patients ranging from 216 to 245 nmol/g dry weight. We decided to investigate further any fecal sample for which the porphyrin content exceeded 200 nmol/g dry weight by separating the porphyrins with our HPLC technique.

Fecal porphyrin separation by HPLC was carried out on 53 samples from patients whose urinary and erythrocyte porphyrin values were within normal limits and with total fecal porphyrin between 200 and 300 nmol/g dry weight. The main fraction of fecal porphyrin excretion invariably was found to elute at the retention time shown by dicarboxylic porphyrin, but was not identical to protoporphyrin. These related dicarboxylic porphyrins were almost certainly deuteroporphyrin, mesoporphyrin, and related porphyrins derived from dietary heme and normal blood loss into the gut.

HPLC separation is being routinely undertaken whenever the fecal porphyrin exceeds 200 nmol/g dry weight, and in some cases where it is less if either the clinical circumstances warrant porphyrin separation or the urine analysis shows above-normal porphyrin values. A total fecal porphyrin upper limit of 200 nmol/g dry weight for normal subjects

is similar to that used by Eales (8), who reported that 95% of his 127 subjects had a total porphyrin value of <174 nmol/g dry weight.

Table 2 shows results of porphyrin separations carried out on fecal samples from 23 normal subjects and 23 patients with overt clinical and biochemical PCT. All PCT patients were diagnosed according to the criteria used by Gray et al. (7). The dicarboxylic porphyrin fraction was not entirely protoporphyrin but was a mixture of porphyrins with retention times similar to protoporphyrin. If this mixed dicarboxylic fraction is excluded, the main fractions of fecal porphyrin excretion in the PCT patients were the isocoproporphyrin group (including de-ethylisocoproporphyrin), with a mean of 28% of the total, and heptacarboxylporphyrin, with a mean of 18%.

An important feature of the fecal porphyrin excretion pattern in PCT is that the proportion of heptacarboxylporphyrin always exceeded that of uroporphyrin, whereas the reverse was always observed in the case of the urinary porphyrins.

The importance of the isocoproporphyrin series in the diagnosis and differential diagnosis of PCT has been previously noted (9, 10) but the presence of heptacarboxylporphyrin has been neglected. Very few measurements of the amounts of individual porphyrins in the feces of patients with PCT have been reported by newer separation methods that can identify the isocoproporphyrin series and heptacarboxylporphyrin. For the purpose of comparing the data shown in Table 2 with the published values of Elder (10), the results were re-calculated so as to exclude the mixed dicarboxylic fraction (Table 3). Our results agree well with those of Elder.

Discussion

The present method for total fecal porphyrin has been developed for routine use in clinical laboratories to exclude fecal samples low in porphyrin that require no further analysis. The fecal samples used are small portions of an untimed collection, which are not homogenized or mixed, so there is a sampling error associated with the method. Although measures such as drying and pulverizing the feces would give a more-homogeneous sample, there would be no advantage unless the collections were made over a timed period, possibly with the use of a meat-free diet several days before initiating collection. The inconvenience to outpatients of timed fecal collections and the difficulty in handling large fecal collections led to the adoption of the same
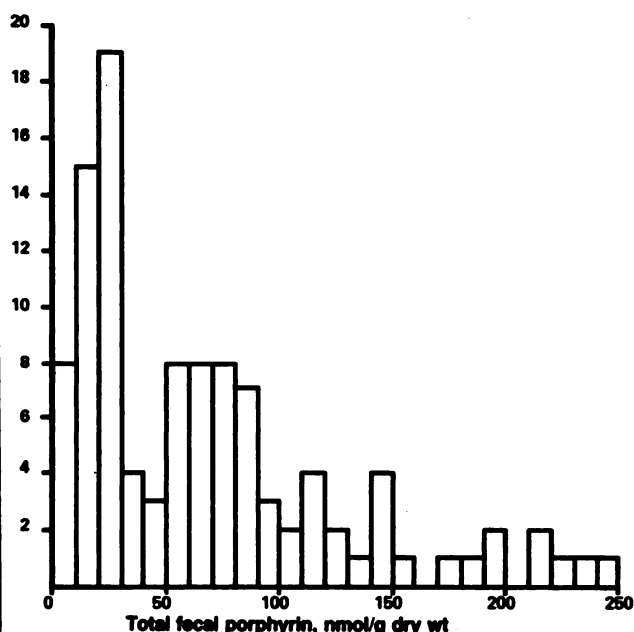

Fig. 2. Frequency distribution of results for total fecal porphyrin
Results are for 106 subjects on unrestricted diets. The reference interval is 200 nmol/g dry weight or less

Table 2. Mean Values, and Ranges, for Fecal Porphyrin Excretion in Porphyrin Cutanea Tarda

	PCT patients n = 23	Normals n = 23
Total fecal porphyrins, nmol/g dry wt.	535 190–1268	110 10–230
HPLC separation results, % of total porphyrin		
Dicarboxylics	21 10–50	85 60–98
Copro	9 2–21	13 2–33
Isocopro series	28 10–51	—
Pentacarboxyl	9 1–14	—
Hexacarboxyl	11 2–21	—
Heptacarboxyl	18 9–30	—
Uro	4 1–9	<1 0–2

	Present study 23 cases	Elder (10) 13 cases
	% of total porphyrin	
Copro	12	17
	2–30	6–55
Isocopro series	34	36
	11–51	30–44
Pentacarboxyl	11	17
	2–17	9–28
Hexacarboxyl	13	11
	4–23	8–16
Heptacarboxyl	24	17
	15–31	12–25
Uro	5	4
	1–11	3–7

sampling method used by other workers in clinical porphyrin laboratories (8). The scatter of values (S_{yx}) that we saw on comparing results by the Rimington method with those by the present method is due at least in part to the sampling error in taking small samples of non-homogeneous material for analysis. This error can be minimized by a little experience in avoiding the sampling of obviously non-homogeneous areas of the specimen.

The quantification of porphyrins in the aqueous acid extract was based on the correction described by Rimington and Sveinsson (11). The presence of impurities gave a large background absorbance, and we made two modifications to allow the correction to be used for fecal extracts. Firstly, the error due to the nonlinear nature of the spectral absorption of the impurities was decreased by choosing the correcting wavelengths, 390 and 425 nm, closer together than the originally described 380 and 430 nm. The second modification avoids the error that occurs when the Soret maximum does not lie at the midpoint of the correcting wavelengths. The graphical construction described above ensures that the wavelength chosen for the measurement of the corrected absorbance measurement will give the maximum absorbance difference. This is not the same as the wavelength giving the maximum Soret absorption, although the difference between the two is only measurable when the porphyrin concentration is very low.

The choice of factor for conversion of the absorbance difference to concentration is a compromise, because any fecal sample contains several different porphyrins. For 1 μmol/L solutions of pure porphyrin in 2.8 mol/L hydrochloric acid, the absorption coefficients corrected in the described manner are: protoporphyrin 0.200, coproporphyrin 0.330, and uroporphyrin 0.405 (12). Taking reciprocals to give factors converting absorbance to concentrations in μmol/L gives: protoporphyrin 5.00, coproporphyrin 3.05, and uroporphyrin 2.47. Dicarboxylic porphyrins form the main fraction of normal excretion. These porphyrins include metabolic protoporphyrin and a complex mixture of protoporphyrin, deuteroporphyrin, mesoporphyrin, and other related porphyrins derived from diet or normal blood loss into the gut. The absorption coefficients for deuteroporphyrin and mesoporphyrin are greater than for protoporphyrin but slightly less than for coproporphyrin (13). The value of 3.3 we chose for the calculation will therefore give too low a value if the sample consists mainly of protoporphyrin (as occurs in variegate porphyria) or one that is too high if it consists mainly of uroporphyrin.

Although position isomers (e.g., coproporphyrin type I and type III) have identical spectrophotometric characteristics,

more than 20 different porphyrins can occur in feces, each with individual optical characteristics. Thus there is no unique value for the molar absorption difference obtained in the calculation, because it varies according to the composition of the sample.

Complex mixtures of fecal porphyrins are found in some forms of porphyria; for example, the isocoproporphyrin group that forms the main fraction of fecal excretion in PCT is a mixture of dehydroisocoproporphyrin, isocoproporphyrin, hydroxyisocoproporphyrin, and de-ethylisocoproporphyrin. These porphyrins, and the five principal porphyrins of urinary excretion—coproporphyrin, uroporphyrin, and the three partly decarboxylated intermediates—can all occur in feces commonly analyzed, in amounts exceeding 10% of the total. Precise absorption coefficients for such variable mixtures are not possible.

This problem is common to all current clinical methods for quantitative fecal porphyrin determination. For example, the "protoporphyrin" fraction given by the Rimington method (2) varies from mainly protoporphyrin, absorption maximum 408 nm, to a mixture of mesoporphyrin and deuteroporphyrin with absorption maximum 401 nm, but the factor used to convert absorbance to porphyrin holds only for protoporphyrin.

The Rimington method produces two substantially purified extracts but measures only ether-soluble porphyrins. These are mainly protoporphyrin, mesoporphyrin, and deuteroporphyrin in one extract and coproporphyrin in the other. The present method gives an aqueous acid extract that contains these ether-soluble porphyrins as well as the ether-insoluble porphyrins found in PCT (see Tables 1 and 2). Feces from variegate porphyria patients also contain ether-insoluble porphyrins; these are the porphyrin-peptide conjugates known as X-porphyrins (14). We tested fecal specimens from three variegate porphyria patients by both the Rimington method and the present method and then submitted the fecal residues left by both methods to repeated extraction. As expected, in both cases the residues still contained much X-porphyrin, which is known to be resistant to complete extraction by all the usual methods (14) and which was therefore not further examined.

The use of the acid extract as a purified source of total fecal porphyrins for separation methods such as HPLC has several advantages.

Firstly, interfering derivatives of chlorophyll and carotene are removed by partition into the ether layer. The value of this was shown by making methyl ester preparations of eight fecal samples from normal individuals by direct esterification of bulk fecal material (7) and by esterification of the acid extract by the present method. We then compared the HPLC traces obtained on using the described conditions. We found that the proportion of non-protoporphyrin compounds eluting in the same area as dicarboxylic porphyrins was decreased from a mean of 67% of the total peak area of the chromatogram in the direct-esterification samples to 22% with the present method. This decrease in these spurious peaks makes the chromatograms easier to interpret, because the peak due to metabolic protoporphyrin is more readily identified, by comparison with the retention time for authentic protoporphyrin ester. The present technique gives a clearer and more easily interpretable chromatogram than do methods based on the esterification of bulk fecal material (3, 4, 7).

The second advantage of using the acid extract for HPLC is that it is free of solid residues and can be used successfully by direct injection into the HPLC systems developed by Lim (5, 6). A novel reversed-phase system with which there is simultaneous separation of type I and type III isomers of

porphyrin free acids is described, and the profiles obtained by injection of the hydrochloric acid extract obtained on using our method are given for all the major categories of porphyria (6). A major advantage of using the porphyrin free acids is in avoiding the derivatization and extraction steps necessary in the preparation of methyl esters.

In the present method the porphyrins are separated by HPLC of their methyl esters on silica. Three methods were considered for the formation of the methyl esters. Lyophilizing the aqueous acid extract would produce a dry residue that could then be reacted with methanol–sulfuric acid. This approach was not adopted for routine use because of the possibility of damage to the freeze-drying apparatus by hydrochloric acid. Talc has been widely used to adsorb porphyrin free acids at pH 3.5 before methyl esterification (15, 16), but certain batches of talc have shown irreversible binding of porphyrins, particularly uroporphyrin (17). Because the use of talc also requires several added manipulations (pH adjustment, washing, and centrifuging) with a concomitant increased potential for losses, we did not use it.

Our use of 1 mL of acid extract and 9 mL of methanol–sulfuric acid is based on the finding of With (18), who showed that the presence of up to 10% by volume of water did not seriously interfere with porphyrin esterification. Other workers have successfully prepared porphyrin methyl esters in the presence of about 10% water. Lim et al. (7) reacted 2 mL of urine with 15 mL of methanol–sulfuric acid; and Day et al. (19) used one volume of plasma to 10 volumes of methanol–sulfuric acid.

We found our method of preparing methyl esters to be simple and reliable and avoided the problems associated with adsorbing with talc.

The methods described were developed for routine use in clinical analysis. The method for total fecal porphyrin gives a result more quickly than the previous differential-extraction methods, and it allows the analyst to eliminate those samples that prove to be low in porphyrin and thus require no further analysis. Where porphyrin separation is necessary, the method provides a residue-free extract suitable for thin-layer chromatographic or HPLC separation techniques.

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