Journal of Chromatography, 115 (1975) 325–333

CHROM. 8601

APPLICATIONS OF HIGH-PRESSURE LIQUID CHROMATOGRAPHY AND FIELD DESORPTION MASS SPECTROMETRY IN STUDIES OF NATURAL PORPHYRINS AND CHLOROPHYLL DERIVATIVES

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(Received July 9th, 1975)

SUMMARY

Mixtures of porphyrins derived from natural sources can be readily separated by high-pressure liquid chromatography both analytically and on a preparative scale. A variety of procedures have been developed not only for the esters but also for free acids, and on the analytical scale quantitation is easily achieved by visible absorption. The retention times are largely characteristic of the number of carboxylic acid side chains (or other polar groups) but further information can be obtained by mass spectrometric studies of the various fractions. Field desorption mass spectrometry is particularly useful for this purpose because the emitter wire can be dipped directly into the eluates. The field desorption spectra of porphyrin free acids and esters as well as their metal complexes give essentially molecular ions with little or no fragmentation in most cases, whereas electron-impact mass spectrometry, particularly of free acids, is impeded by the low volatility of porphyrins. Mixtures can also be analysed by field desorption mass spectrometry, and this provides not only a rapid qualitative assessment of the components of a mixture, but also a check on the subsequent chromatographic separations.

INTRODUCTION

Hitherto the analysis and characterisation of the components of a mixture of porphyrins has relied on the use of paper, thin-layer or column chromatography and countercurrent distribution techniques[1], followed by spectroscopic studies (UV, mass spectrometry (MS) and NMR in particular). High-pressure liquid chromatography (HPLC) now seems likely to supersede the former methods in view of the many advantages it has in speed, quantitation and resolution, whilst field desorption MS is particularly suited to handle relatively involatile substances like the porphyrins and their metal complexes. In this paper we wish to indicate how these two new techniques can be used either independently or in combination to separate and analyse a

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mixture of porphyrins and to characterise partially the individual components.

Only a few applications of HPLC to the analysis of porphyrins have been reported previously, and these have been largely concerned with separations of isomeric substances. Thus, hardero- and isoharderoporphyrin have been resolved in a silica gel column[2] and the separation of isomeric coproporphyrins has been described by Battersby[3]. In the related field of vitamin B_{12} chemistry, preparative HPLC has played an important role in the total synthesis of cobyric acid derivatives[4,5].

MS together with other spectral methods has had extensive application in structural studies of natural tetrapyrrolic compounds[6]. However, the porphyrins involved in haem metabolism are relatively involatile and in most cases satisfactory mass spectra may only be obtained by conversion into their more volatile methyl esters[7]. The preparation of bis-(trimethylsiloxy)-silicon complexes of alkylporphyrins enhanced their volatility considerably and these derivatives have been used in gas chromatography (GC)[8,9] and GC–MS[10] studies. Attempts to extend this study to porphyrins with polar substituents in their side chains resulted in the preparation of the bis-(diethoxy)-silicon complex of mesoporphyrin IX dimethyl ester, which was considerably more volatile than its parent porphyrin[10]. Copper complexes of porphyrin esters have also been studied as a means of achieving enhanced volatility for mass spectral study; however, they were found to be less volatile than the parent porphyrin esters[11].

A solution of the problem of the involatility of porphyrins in MS has now been provided by the application of the so-called "soft ionisation" technique —field desorption. In this method the ions are produced by direct desorption from a tungsten filament coated with fine carbon needles (the emitter), and its worth has already been proved with a variety of thermally labile or involatile organic compounds including metallic salts[12,13]. Furthermore, field desorption MS is a particularly useful technique to use in combination with HPLC because the emitter wire can be dipped directly into the column eluates; the utility of the combination has already been shown in studies of steroids[14] and vitamins[15].

We now wish to describe the application of these techniques to porphyrins obtained from various biological sources, especially the urine and faeces of porphyric subjects, as well as to chlorophyll derivatives.

EXPERIMENTAL

HPLC was carried out with a system consisting of a Waters Model 6000 pump and a septum injector, with $\frac{1}{8}$ in. O.D. × 0.085 in. I.D. analytical columns of 316 stainless steel, and a Cecil variable-wavelength detector fitted with a 10-μl flow cell. For maximum sensitivity the detector was set at about 400 nm and used to monitor the porphyrin Soret band. With complex mixtures containing several porphyrins, two different techniques were found suitable for obtaining rapid and complete separations, *viz.* (i) flow programming and (ii) gradient elution.

Flow programming. Using a solvent mixture of intermediate polarity the less polar components in the mixture were eluted at a low flow-rate, and the more polar components then eluted by a step-wise increase in flow-rate to shorten retention times and sharpen the peaks.

Gradient elution. As the pump in use had a very small internal volume it was possible to employ a simple glass mix chamber to generate an exponential solvent

gradient which could be delivered to the column for elution of complex mixtures[16].

Details of the solvent systems, columns, etc. are given in the text and in the legends to the figures. For preparative-scale separations a 4 ft. × 1 in. O.D. stainless-steel column was used.

Field desorption mass spectra were determined with a Varian CH5D double-focussing high-resolution mass spectrometer equipped with a combined field desorption–field ion–electron impact source, a SS100 data system and a Statos fast recorder. Samples were prepared as chloroform solutions (approximately 10 μg/μl) and transferred to the conditioned emitter by dipping[17] or direct loading from a 5-μl syringe. 10-μm lengths of tungsten wire spot-welded on supporting posts and conditioned in a Varian apparatus in a manner similar to that described by Schulten and Beckey[18] were used as anodes. The HPLC fractions were concentrated before transfer to the emitter. Spectra were obtained at a source temperature of 80° and a resolution of 1200; a 3-kV accelerating potential with −7 kV on the cathode and wire currents between 18 and 22 mA were used.

RESULTS AND DISCUSSION

HPLC analysis of porphyrins (methyl esters in the case of carboxylic ester derivatives) is readily performed on silica columns, the packing material being either of the pellicular type (*e.g.*, Corasil II) or the microparticulate type (5–10 μm porous silica, *e.g.*, Merckosorb SI 60 or Partisil). Examples of some typical separations and conditions are shown in Table I.

TABLE I

ANALYTICAL SEPARATIONS OF CLOSELY RELATED PORPHYRINS BY HPLC

Compound	*Column*	*Solvent: ethyl acetate in light petroleum (b.p. 60–80°) (% by volume)*	*Flow-rate (ml/min)*	*Retention time (min)*
Octamethyl porphin	6-ft. Corasil II	2.5	1.0	5.8
Octaethyl porphin				4.4
Harderoporphyrin trimethyl ester	6-ft. Corasil II	25	1.0	13.0
Isoharderoporphyrin trimethyl ester				11.2
Protoporphyrin IX dimethyl ester	6-ft. Corasil II	30	1.0	7.1
Mesoporphyrin IX dimethyl ester				6.3

As described in Experimental, a variable-wavelength detector set at about 400 nm was normally used to monitor the porphyrin Soret band. In the case of phaeophytins *a* and *b*, whose UV maxima differ significantly (*a*, 412 nm; *b*, 434 nm), it was found advantageous to use two detectors coupled in series and set at different wavelengths. The results are shown in Fig. 1.

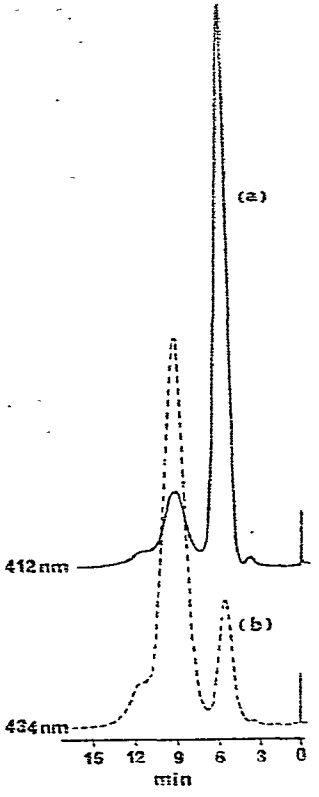

Fig. 1. Separation of phaeophytins *a* and *b* by HPLC. Column: 2 × 3 ft. × ⅛-in. O.D. Corasil II. Mobile phase: 20% ethyl acetate in light petroleum (b.p. 60–80°); flow-rate, 0.7 ml/min. Detector: two UV detectors in series.

Whereas isocratic elution at constant flow-rate is adequate for the resolution of closely related porphyrins or mixtures containing relatively few components, more complex mixtures generally require the application of flow programming or gradient elution. An example of the use of flow programming is shown in Fig. 2. The principal disadvantage of this technique is that the use of a higher flow-rate results in a corresponding loss in sensitivity of detection. An example of the use of a solvent gradient in the separation of a mixture of natural porphyrin polycarboxylic esters on silica is shown in Fig. 3.

In all the elution techniques employed, the high reproducibility of retention data provides for facile identification of peaks by comparison with standards, and quantitative analysis is readily achieved by comparison of peak areas. Mesoporphyrin IX is an excellent internal standard for quantitation, since it is stable, has an extinction coefficient of the same order of magnitude as porphyrins of biological origin and, having only two carboxylic ester residues, generally elutes in advance of the latter.

Whilst the above examples illustrate the use of liquid–solid adsorption chromatography for the separation of homologous series of porphyrin esters, a number of other chromatographic systems may also be employed. Copro- and isocoproporphyrin methyl esters were separated by reversed-phase partition chromatography on

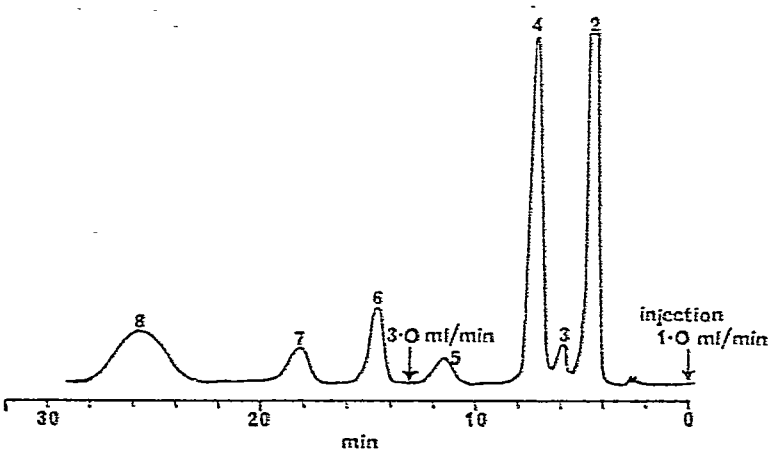

Fig. 2. Separation of porphyrin methyl esters (2–8 carboxyl groups) by HPLC using isocratic elution with flow programming. Column: 2 × 3 ft. × ⅛ in. O.D. Corasil II. Mobile phase: 45% ethyl acetate in light petroleum ether (b.p. 60–80°). Detector: UV, 400 nm.

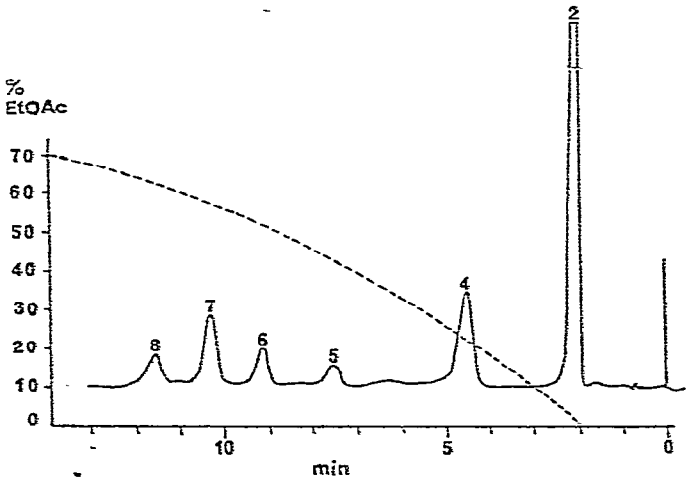

Fig. 3. Separation of porphyrin methyl esters (2–8 carboxyl groups) by HPLC using gradient elution. Column: 2 ft. × ⅛ in. O.D. Merckosorb SI 60 (10-μm silica). Mobile phase: gradient (———), hexane to ethyl acetate; flow-rate, 3.0 ml/min. Detector: UV, 400 nm.

a column packed with Corasil-C_{18} (pellicular silica with a chemically bonded octadecylsilane coating) using 20% chloroform in light petroleum (b.p. 60–80°) as the mobile phase. A partition column was prepared containing silica gel with a chemically bonded benzoylbenzylsilane coating[19] and this gave a good separation of the methyl esters of coproporphyrin and "S411" (a dehydrocoproporphyrin).

Although porphyrins are readily analysed as their methyl esters, their widespread occurrence in nature as free acids makes it desirable to have available a chromatographic method of analysis which does not require prior derivatisation. Such a method has been found in the ion-exchange technique, in which the free acids may be applied directly in aqueous solution, and an example of the use of this method is shown in Fig. 4.

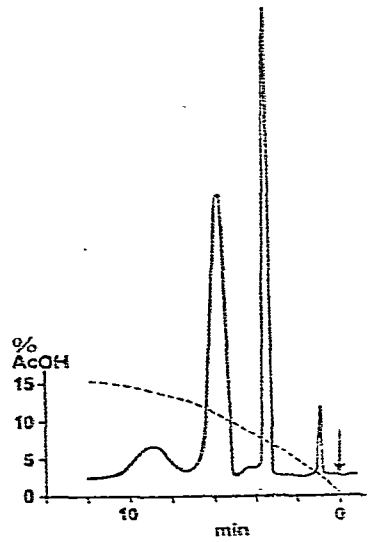

Fig. 4. Ion-exchange separation of porphyrin di-, tetra- and pentacarboxylic acids by HPLC. Column: 3 ft. × ⅛ in. O.D. Pellionex SAX. Mobile phase: gradient (— — —) methanol to acetic acid; flow-rate, 2.0 ml/min. Detector: UV 400 nm.

HPLC is particularly useful for the analysis of very small quantities of porphyrins. In a high-sensitivity run (UV setting 0.1 a.u.f.s.), 1–2 pmoles of coproporphyrin were readily detected by monitoring at 400 nm. Analyses were routinely carried out on 10–20 pmoles of components and collection of the material from a single peak generally gave a sufficient quantity for a field desorption mass spectrum to be obtained.

As the field desorption spectra of the pure porphyrins only gave significant ions in the molecular ion and the doubly charged molecular ion regions, it seemed likely that this technique could provide a fingerprint of the molecular species present in natural porphyrin mixtures. Fig. 5 shows the field desorption spectrum obtained from the methylated extract of the urine of a human porphyric. The five groups of ions at *m/e* 942, 884, 826, 768 and 710 are assigned to the methyl esters of the octa-, hepta-,

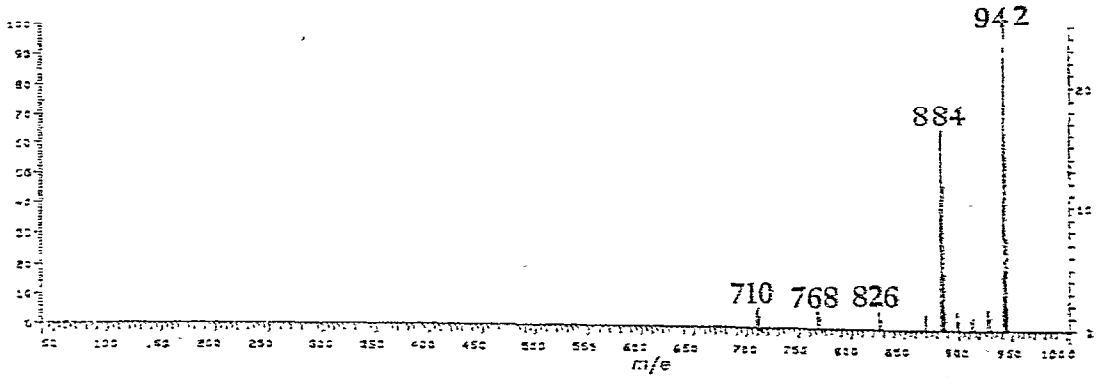

Fig. 5. Field desorption spectrum of a mixture of porphyrin methyl esters from a human porphyric.

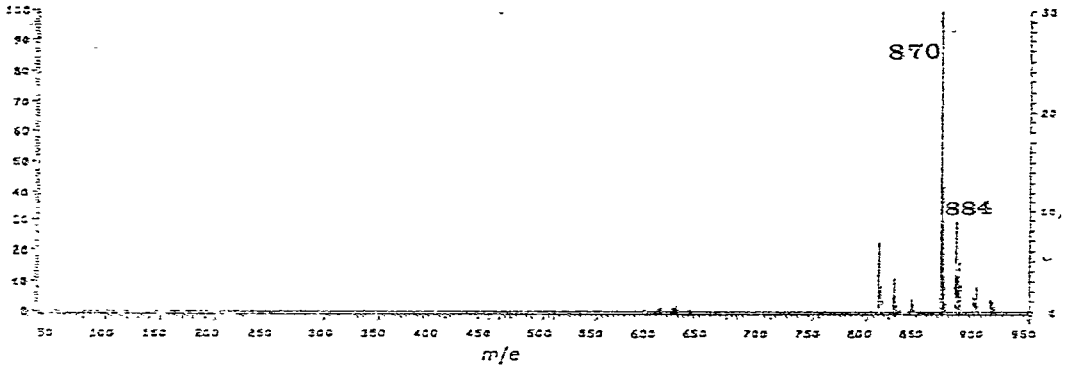

Fig. 6. Field desorption spectrum of a mixture of phaeophytins *a* and *b*.

hexa- and pentacarboxylic porphyrins, respectively. Fig. 6 shows the field desorption spectrum obtained from a mixture of phaeophytins *a* and *b*, the ions at *m/e* 870 and 884 being assigned to the molecular ions of phaeophytins *a* and *b*, respectively.

For the complete identification of a complex mixture of porphyrins, a combined HPLC–MS technique was employed. A field desorption spectrum of the crude mixture was taken first, providing information about the number and type of components. These were then separated by HPLC, and further mass spectra of the individual, isolated fractions, together with retention data from the chromatograms, allowed a complete characterisation of the constituents to be made, wherever possible, by direct comparisons with authentic samples.

The field desorption spectra of the various tetrapyrrolic compounds are given in Table II. With the exception of a haematoporphyrin and haemin, M^+ or

TABLE II

FIELD DESORPTION MASS SPECTRA OF PORPHYRINS

Compound	*Mol. wt.*	*m/e (% relative intensity)*
Mesoporphyrin IX	566	568(45) 567(100) 566(76)
Hematoporphyrin	598	564(39) 563(73) 562(100)
Coproporphyrin III	654	(a) 658(2) 657(9) 656(32) 655(100) 653(1) 609(2) (b)* 658(1) 657(10) 656(32) 655(100) 654(2) 653(3) 611(2) 609(3) 541(3)
Hemin	652	618(17) 617(50) 616(100)
Aetioporphyrin	478	480(11) 479(35) 478(100)
Octaethylporphyrin	534	536(13) 535(40) 534(100)
Protoporphyrin IX dimethyl ester	590	591(41) 590(100)
Mesoporphyrin IX dimethyl ester	594	597(1) 596(10) 595(45) 594(100) 297.5(8) 297(10)
Coproporphyrin III tetramethyl ester	710	713(5) 712(19) 711(52) 710(100) 651(3)
Pentacarboxylic porphyrin pentamethyl ester	768	770(14) 769(53) 768(100) 709(8)
Hexacarboxylic porphyrin hexamethyl ester	826	830(3) 829(5) 828(18) 827(36) 826(100) 767(1) 725(1) 413(0.5)
Heptacarboxylic porphyrin heptamethyl ester	884	885(41) 884(100) 882(8)
Uroporphyrin III octamethyl ester	942	945(2) 944(6) 943(12) 942(100) 941(8) 940(8) 913(10) 912(7) 884(6) 883(13)

* Higher emitter current.

$(M + 1)^-$ ions are the base peaks in the spectra and there is little or no fragmentation. Haematoporphyrin has as its base peak an ion at *m/e* 562 attributed to the loss of two molecules of water from the parent compound, and in the case of haemin the base peak in its spectrum is at *m/e* 616, consistent with the chlorine-free ligand.

The relative intensities of the $(M)^+$ and $(M + 1)^+$ ions are dependent on the nature of the porphyrin. Porphyrin-free acids and octaphenyltetraazaporphyrin undergo cationisation and $(M + 1)^+$ ions are the base peaks in their spectra; this effect is particularly marked in the case of coproporphyrin III, where the M^+ ion is only 2% of the $(M + 1)^+$ ion. With alkylporphyrins and the porphyrin methyl esters, little or no cationisation takes place.

The HPLC technique is also valuable in the preparative-scale separation of porphyrins. An application of this is the isolation of porphyrins found in the faeces of rats poisoned with hexachlorobenzene[20]. Fig. 7a shows an analytical gradient separation of the crude porphyrins as their methyl esters, using a 2 ft. × $\frac{1}{8}$ in. O.D. col-

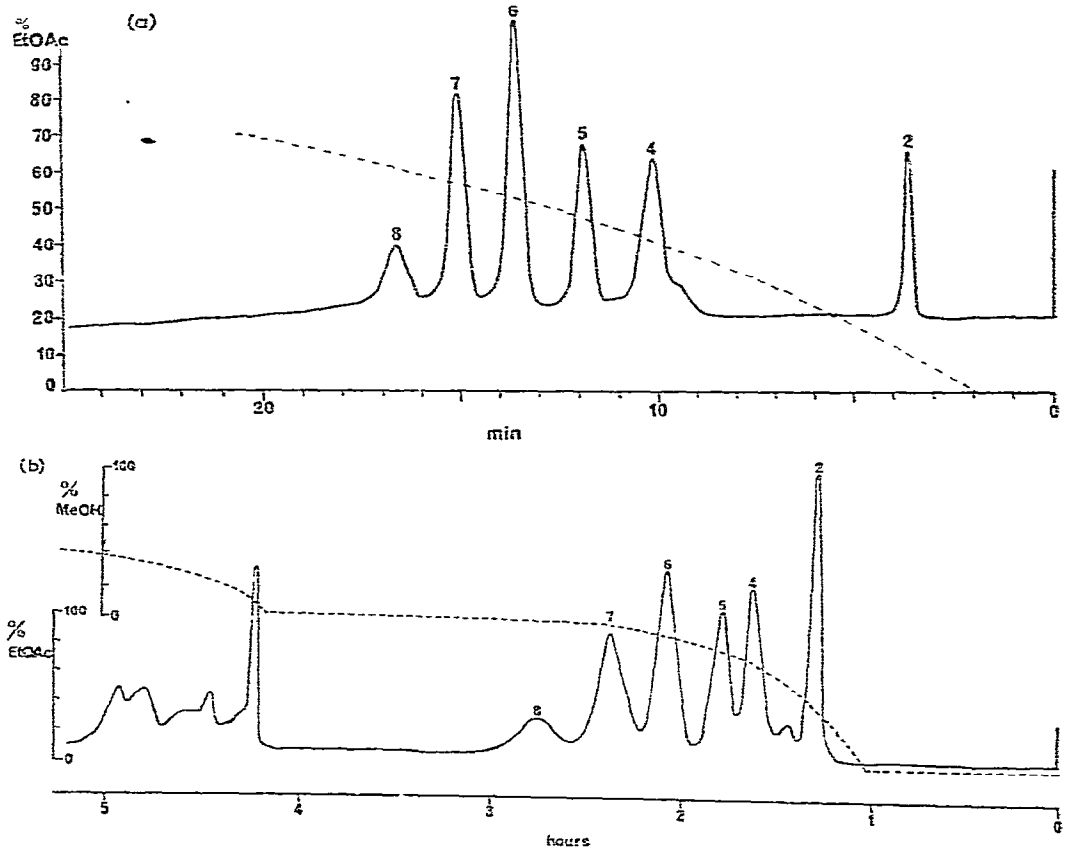

Fig. 7. HPLC separation of porphyrin methyl esters (2–8 carboxyl groups) obtained from the faeces of rats poisoned with hexachlorobenzene. (a) Analytical run. Column: 2 ft. × $\frac{1}{8}$ in. O.D. Merckosorb SI 60. Mobile phase: gradient (— — —) hexane + 0.5% isopropanol to ethyl acetate + 0.5% isopropanol; flow-rate, 2.0 ml/min. Detector: UV, 400 nm. (b) Preparative run. Column: 4 ft. × 1 in. O.D. Porasil A(60). Mobile phase: gradient (— — —) hexane to ethyl acetate to methanol; flow-rate, 9.9 ml/min. Detector: UV, 440 nm.

umn packed with 10-μm Merckosorb SI 60. For the separation on a preparative scale, a 4 ft. $\times$ 1 in. O.D. column packed with 37–75-μm Porasil A(60) was employed (Fig. 7b). It will be noted that, although the elution time is greatly increased, resolution is maintained. The use of a variable-wavelength detector is especially advantageous in that, by tuning to a wavelength at which the response is lowered, overloading of the detector response can be avoided and the peaks can all be kept on scale. A number of the components in this mixture have already been identified[20] and further work in this field is currently in progress.

ACKNOWLEDGEMENTS

We thank the Science Research Council for financial assistance toward the purchase of the mass spectrometer and the Royal Society for similar help with the combined field desorption–field ion–electron impact source and the high-pressure liquid chromatograph. N.E. thanks the Medical Research Council for financial support. We are indebted to Dr. G. H. Elder (Welsh National School of Medicine) for the natural porphyrin mixtures.

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