Analysis of Petroporphyrins by Chemical Ionisation Mass Spectrometry

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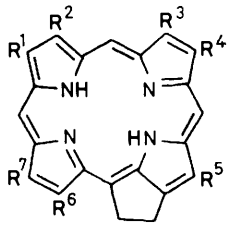
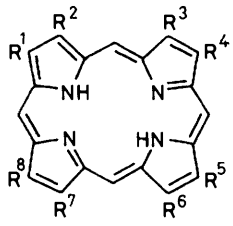
The CH_4 chemical ionisation (c.i.) mass spectra of three standard porphyrins and two petroporphyrin mixtures have been examined and compared with their respective electron impact (e.i.) mass spectra. In the e.i. mode $(M - 15)^+$ fragments are observed, the isotope peaks of which coincide with homologue molecular ions. Much reduced fragmentation is observed in the c.i. mass spectra, resulting in an easier analysis of complex mixtures of petroporphyrins. This occurs with only a slight loss in sensitivity compared with low voltage e.i. or field desorption (f.d.) mass spectrometry.

SINCE the initial report of porphyrins in a Triassic sediment by Treibs,[1] petroporphyrins have been shown [2-5] to be complex mixtures of two major homologous series, deoxophylloerythroetiopetroporphyrin (1) and the aetiopetroporphyrins (2), together with contributions



(1)



(2)

(3) $R^1 = R^3 = R^5 = R^7 = Me$, $R^2 = R^4 = R^6 = R^8 = Et$

(4) $R^1 = R^4 = R^5 = R^8 = Me$, $R^2 = R^3 = R^6 = R^7 = Et$

(5) $R^1 = R^2 = R^3 = R^4 = R^5 = R^6 = R^7 = R^8 = Et$

from other minor series. The petroporphyrins occur throughout the geosphere, where their composition and distribution are illustrative of the action of the biochemical and geochemical processes occurring therein.[3]

The ability to identify and quantitate accurately the constituents of such petroporphyrin mixtures would be an important asset in natural product chemistry. A designated petroporphyrin (*e.g.* C_{30} aetiopetroporphyrin) may well be composed of a number of isomers. Indeed, the aetiopetroporphyrins are present in at least two series which are separable by t.l.c.

Mass spectrometry provides a powerful method for the analysis of petroporphyrins since these compounds give distinctive molecular ions [6,7] thus indicating the degree of alkylation of the porphyrin nucleus. Nevertheless, accurate quantitation is hindered in the electron impact (e.i.) mode because of interference from isotopic contributions of fragment ions which may coincide with the molecular ions of lower members of the homologous series.

Chemical ionisation (c.i.) mass spectrometry [8] is increasingly being used as a method of soft ionisation and has found applications in many fields,[9,10] especially drugs and their metabolites, where simple, easily quantitated assays are essential. The c.i. mass spectra of certain reference porphyrins and of two fractions of petroporphyrins have, therefore, been examined with a

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view to determining whether or not the technique would be suitable for similar applications.

EXPERIMENTAL

Mass Spectrometry.—Mass spectra were obtained using a Finnigan 4000 quadrupole mass spectrometer operating in cyclic scanning mode. The scan time was 5 s and the scan range was *m/e* 350—500 for aetioporphyrin-I (aetio-I) aetioporphyrin-II (aetio-II), GB3, and GB5, and *m/e* 400—550 for octaethylporphyrin (OEP), and the OEP–aetio-II standard mixtures. Operating conditions consisted of an indicated source temperature of 300 °C (real temperature *ca.* 250 °C) and emission current of 350 μA. For c.i. mass spectrometry, methane was used as the reagent gas, a source pressure of *ca.* 0.1 Torr being maintained. In both modes the electron energy was 35 eV, this being selected for maximum sensitivity. Data were collected and processed by a DEC PDP-8e computer with an RTS/8 V2B operating system modified to allow for simultaneous data acquisition and processing.[11]

Samples were dissolved in methylene chloride and then transferred (*ca.* 100 ng—1 μg) into a clean glass crucible. After evaporation of solvent, they were introduced into the mass spectrometer by direct insertion probe. The probe temperature was raised slowly to 100 °C to allow the volatilisation of low molecular-weight contaminants, and then programmed to 300 °C at *ca.* 50° min^{-1}. The porphyrins vaporised between 175 and 280 °C. To avoid the effects of differential evaporation the mass spectra of the porphyrin mixtures were averaged over their complete volatility range.

Standard Porphyrins.—Aetio-I (3), aetio-II (4), and OEP (5) were donated by Professors G. W. Kenner and K. M. Smith, and were pure by h.p.l.c. and mass spectrometry. No DPEP standards were available but, considering their similar structure, the spectra are expected to be broadly similar to those of aetioporphyrins.[4]

Three standard mixtures of OEP and aetio-II were prepared, containing 22.4, 51.0, and 79.4% OEP respectively, by determining the concentration of solutions of the standards by u.v. absorption spectrometry and mixing the appropriate amounts.

Petroporphyrins.—The petroporphyrins were isolated from the bitumen Gilsonite found in the Uinta Basin, Utah, U.S.A., using a modification of the method of Dunning and Rabon.[12] Gilsonite (100 g) was extracted ultrasonically, with a mixture of toluene and methanol (5 × 200 ml; 1 : 1 v/v), yielding a crude extract (22 g), which was purified by column chromatography on alumina (400 g B.D.H. grade II) using gradient elution of methylene chloride in hexane. The eluates were monitored by absorption spectrometry, and the petroporphyrin-containing fractions, which were eluted in the range 25—45% methylene chloride in hexane, were combined and evaporated to dryness. These were purified further by column chromatography on silica gel (60 g, Hopkin and Williams, M.F.C. 100—200 mesh) by gradient elution using the same solvent system, the petroporphyrin fractions being eluted in the range 15—35% methylene chloride in hexane. The crude nickel petroporphyrin mixture (0.2 g) was separated into three fractions labelled ' top ' (with R_F 0.51—0.54), ' middle ' (with R_F 0.46—0.49), and ' bottom ' (with R_F 0.40—0.45) by t.l.c. on silica gel G, with toluene and hexane (1 : 1 v/v). The bottom nickel petroporphyrin fraction was then demetallated with methanesulphonic acid by the procedure of Erdman[13] and the resultant free-base petroporphyrins (6 mg) further separated by t.l.c. in silica gel G using toluene–methylene chloride (1 : 1 v/v) as eluant. Six petroporphyrin fractions were obtained and two, labelled GB3 and GB5, which each contained several porphyrinic components as indicated by e.i. mass spectra (Figure 2a and b, respectively), were selected for study. GB3, with R_F 0.22—0.27, had a predominantly DPEP-type absorption spectrum in chloroform, whilst GB5, with R_F 0.38—0.43, displayed an aetio-type absorption spectrum [$\lambda_{max.}$ 400 (Soret), 499 (Band IV), 529 (Band III), 565 (Band II), and 616 (Band I) nm : ratio of band extinction coefficients 1.00 : 0.31 : 0.37 : 0.44 and 1.00 : 0.71 : 0.50 : 0.32, respectively].

High Pressure Liquid Chromatography (H.P.L.C.).[14] H.p.l.c. analyses were carried out using columns (25 cm × 4.6 mm i.d.) packed with Partisil (5 μm, Whatman). The equipment comprised two solvent delivery pumps (Waters M6000D), a solvent programmer (Waters M660), and a spectrophotometer (Varian Variscan L635M), equipped with Varian flow cells (8 μl capacity), as detector, the system being monitored at 400 nm. Solutions (*ca.* 1 μl) were introduced into the columns *via* a septum inlet port using a 10 μl syringe and the stop-flow technique. Gradient elution was performed with the following solvent mixtures: System A [hexane–toluene (9 : 1 v/v)], and System B [toluene–chloroform (1 : 1 v/v)].

GB5 was eluted with a concave gradient, starting at 27% B and reaching 100% B after 40 min, and a flow rate of 1.5 ml per minute at ambient temperature. The analysis yielded four slightly overlapping peaks with retention times of 10.6, 12.0, 13.0, and 15.1 min, assigned as C_{29} aetio-, C_{31} aetio-, C_{28} aetio-, and C_{30} aetio-petroporphyrins, respectively. Assignments were based on the petroporphyrin study of HajIbrahim,[15] in which components were trapped by preparative h.p.l.c. and their molecular ions identified by e.i. mass spectrometry.

Quantitation (Table 2) was carried out by the measurement of peak areas by triangulation, on the assumption that the components had identical absorption coefficients. The retention data indicate that the C_{28}—C_{31} aetiopetroporphyrins are not a homologous series.

It was not possible to resolve GB3 into its components using the chromatographic conditions described above. Optimum resolution was achieved by eluting with a concave gradient starting at 10% B and reaching 50% B after 25 min, with a flow rate of 1.5 ml per minute at room temperature. A complicated trace of eight partially resolved peaks and shoulders was obtained with retention times of 27.0, 28.0, 29.8, 30.4, 30.8, 31.3, 31.6, and 32.3 min. The peak at 30.4 min was assigned as a C_{29} aetiopetroporphyrin by co-injection with a sample previously isolated by preparative h.p.l.c. and characterised by e.i. mass spectrometry.[16] On the basis of the study of HajIbrahim,[15] the peaks at 30.8, 31.3, 31.6, and 32.3 min were tentatively assigned as C_{33} petroporphyrin, and C_{30} DPEP, C_{31} DPEP,

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[13] U.S.P. 3,190,829/1965.
[14] S. K. HajIbrahim, P. J. C. Tibbetts, C. D. Watts, J. R. Maxwell, G. Eglinton, H. Colin, and G. Guiochon, *Analyt. Chem.*, 1978, **50**, 549.
[15] S. K. HajIbrahim, personal communication.
[16] Unpublished data.

and C_{32} DPEP petroporphyrins, respectively.[15] The peaks at 27.0, 28.0, and 29.8 min could not be assigned.

In order to obtain comparative retention data for GB3 and GB5, the h.p.l.c. analysis of GB5 was repeated under the conditions already employed for GB3 (*i.e.* starting at 10% B and reaching 50% B after 25 min, with a concave gradient and flow rate of 1.5 ml per min). The chromatogram showed the same four components but the resolution was poor. The retention times of the C_{29} aetio-, C_{31} aetio-, C_{28} aetio-, and C_{30} aetio-petroporphyrins were 22.0, 23.5, 24.0, and 25.5 min respectively. From these data the two C_{29} aetiopetroporphyrins detected in GB3 and GB5 are shown to be structural isomers, as indicated by the large difference in their retention times (30.4 and 22.0 min, respectively). Alkylporphyrins exist both as structural (with different alkyl substituents) isomers, which are separable chromatographically, and type (with the same alkyl substituents differently arranged) isomers, which are not.

RESULTS AND DISCUSSION

E.I. Mass Spectrometry.—The e.i. mass spectra for the porphyrin standards (Figure 1a and b) show that most of the ion current is carried by the molecular ion and its isotope peaks, but significant ions occur at $(M - 15)^+$, due to benzylic cleavage, with several smaller ions in the high mass region. Outside the scan range employed, a peak due to double ionisation of the porphyrin nucleus (M^{2+}) may also be observed.[6,7] The mass spectrum of aetio-I was identical to that of aetio-II, as the mode and extent of fragmentation seems to be independent of the substitution patterns on the porphyrin macrocycle.

Due to the large number of carbon atoms in petroporphyrins, the isotope peaks of these compounds are strong. It is apparent, therefore, that the significant ion at $(M - 14)^+$, due to the first isotopic peak of the largest fragment, would coincide with the molecular ion of the next lower homologue. Thus, if these two components were mixed, quantitation by measurement of the molecular ion currents would be invalid. The quantitative analysis of any natural petroporphyrin mixtures is, therefore, complicated by the need to account for the fragmentation observed in the e.i. mode. Attempts have been made [17] to rationalise such mass spectral data by computer analysis, using idealised mass spectra based on that of standard aetio-II. However, the extent of fragmentation is dependent on the type and number of side-chain groups and, therefore, varies over the range of alkyl substituents observed in petroporphyrin samples; hence, the analyses were not quantitative.

It is possible to operate at much lower electron energies,[18] *i.e.* 12 eV, thus reducing fragmentation, but this gives much reduced sensitivity.

C.I. Mass Spectrometry.—The c.i. mass spectra for the aetioporphyrin standards (Figure 1c and d) are very similar in type (aetio-I again being identical to aetio-II), showing an envelope of ions which includes an ion at $M^{+\cdot}$ due to charge transfer and ions at $(M - 1)^+$, $(M + 1)^+$, and above, due to proton-transfer reactions and reductive processes within the methane gas plasma. A small amount of addition to give $(M + C_2H_5)^+$ may also be observed. Since all these are low energy processes, the extent of fragmentation is small. Double ionisation peaks were not observed when an extended scan range was used. Thus, almost all the ion current is concentrated in the quasi-molecular ion region (*ca.* $M - 1$ to $M + 6$) and the relative abundances of aetiopetroporphyrins within a complex mixture may be determined by the summation of the ion currents within that region. Accuracy of quantitation is then limited only by the threshold setting applied in the course of computer-controlled acquisition, and the amounts of high molecular-weight non-porphyrinic material acting as a background. However, where the mixture includes

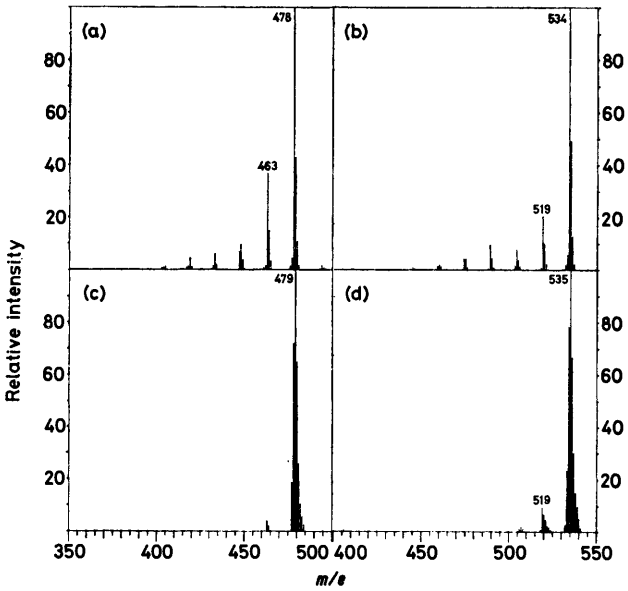

FIGURE 1 E.i. mass spectra of (a) aetio-II and (b) OEP. C.i. mass spectra of (c) aetio-II and (d) OEP

DPEP compounds, superimposition of their ion envelopes with those of the corresponding aetiopetroporphyrins of the same carbon numbers can occur. This coincidence of ions prevents accurate quantitation and limits the effectiveness of c.i. for the analysis of total petroporphyrin mixtures containing both classes of compound. However, the problem can be avoided if prior separation of the two series can be achieved by t.l.c. or other chromatographic procedures.

Analysis of Standard OEP–Aetio Mixtures.—The three prepared standard mixtures were analysed by c.i.m.s. and h.p.l.c., quantitation being by summation of the quasi-molecular ion currents and triangulation of eluting peaks, respectively, in order to determine the comparative analytical capabilities of the two methods. In addition, several analyses were performed such that the reproducibility of each method would be observed. The results for these experiments (Table 1) show that the

[17] L. A. Perry, B.Sc. Thesis, University of Bristol, 1977.
[18] E. W. Baker, T. F. Yen, J. P. Dickie, R. E. Rhodes, and L. F. Clark, *J. Amer. Chem. Soc.*, 1967, **89**, 3631.

quantitations for each method are reasonably consistent, especially in the case of mixture II, where near equimolar quantities are present. However, the c.i.m.s.

TABLE 1

Analysis of the standard OEP–aetio-II mixtures *

	% OEP by u.v.–vis.†	%OEP by c.i.m.s.‡	% OEP by h.l.p.c. §
I	22.4	16.9 ± 2.7	22.6 ± 0.4
II	51.0	51.2 ± 1.0	51.3 ± 0.4
III	79.4	84.2 ± 1.0	80.1 ± 0.9

* Average of three experiments. † Quantitation by extinction coefficients of the porphyrins. ‡ Quantitation by summation of quasi-molecular ions. § Quantitation by triangulation of eluting peaks.

and h.p.l.c. results for the mixtures I and III are in less good agreement, possibly because of a slight prejudice of the c.i.m.s. method against the smaller component in the mixtures, owing to the threshold setting of the computer acquisition system. Such data indicate that the c.i.m.s. technique will afford useful measurements for complex mixtures.

Analysis of Complex Mixtures of Petroporphyrins.— Under the t.l.c. system described, the two major petroporphyrin series separate almost completely. Fractions eluted with an R_F of *ca.* 0.05—0.25 were identified as containing DPEP petroporphyrins by e.i. mass spectrometry, and those with an R_F of *ca.* 0.25—0.55 contained aetiopetroporphyrins. On this basis, two fractions from Gilsonite bitumen were used as examples of complex mixtures.

Fraction GB5 was taken from the ' aetio region ' of the plate and comprised a mixture of aetiopetroporphyrins, as indicated by the absorption spectrum. This fraction was therefore used as a preliminary test of the efficacy of the application of c.i. to petroporphyrin analysis. The e.i. mass spectrum (Figure 2a) shows that the major components in the fraction are a C_{28} and a C_{31} aetiopetroporphyrin, as indicated by the large molecular ions at *m/e* 422 and 464, with a minor C_{29} component ($M^{+\cdot}$ at *m/e* 436). The presence of a C_{30} aetiopetroporphyrin in the fraction is indicated by the intensity of the ion at *m/e* 450, which cannot be due solely to the first isotope peak of the $(M - 15)^+$ fragment of the C_{31} component. Several (C_{29}—C_{32}) DPEP components may be present in trace quantities, as indicated by the weak ions at *m/e* values corresponding to the molecular weights of these compounds. However, in all petroporphyrin fractions examined, background ions are found (*e.g.* *m/e* 367, 369), ascribed to contaminants remaining after the various extraction and separation processes. All ions of less than 2% base peak intensity were designated as trace components and not incorporated into any quantitative calculations.

In the c.i. mode (Figure 2c), all four components were identified as C_{28}—C_{31} aetiopetroporphyrins by their quasi-molecular ion envelopes. In the absence of any significant fragmentation, the process of quantitation by the summation of the quasi-molecular ion currents was facile (Table 2) and the results were in reasonable agreement with the h.p.l.c. peak area measurements.

GB3 is a more complex mixture taken from the junction of the ' aetio ' and ' DPEP ' t.l.c. regions and, although the visible absorption spectrum is of the DPEP

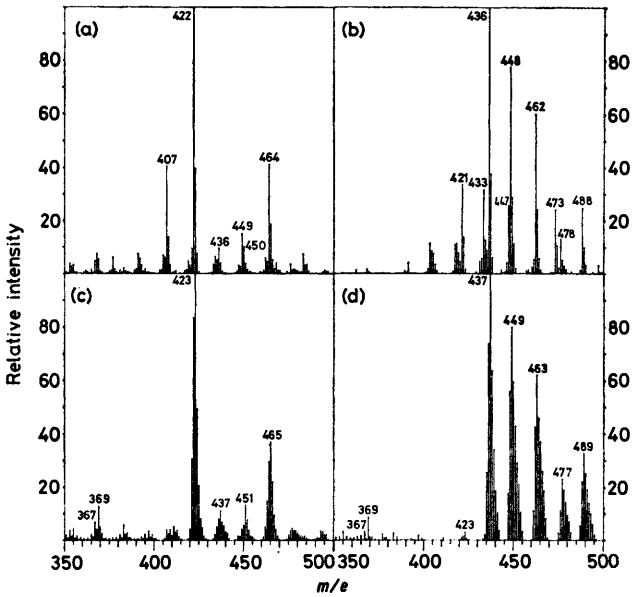

FIGURE 2 E.i. mass spectra of (a) GB5 and (b) GB3. C.i. mass spectra of (c) GB5 and (d) GB3

form,[14] both petroporphyrin types are known to be present. It is a difficult analytical fraction, such as might be encountered in the routine analysis of partially separated petroporphyrin samples. In the e.i. mode (Figure 2b), the major petroporphyrins in the fraction could be identified as C_{29} aetio ($M^{+\cdot}$, *m/e* 436), C_{30} DPEP ($M^{+\cdot}$, *m/e* 448), and C_{31} DPEP ($M^{+\cdot}$, *m/e* 462) petroporphyrins. Minor components due to C_{32} DPEP

TABLE 2

Analysis of the petroporphyrins of fraction GB5

Petroporphyrin	C_{28} aetio	C_{29} aetio	C_{30} aetio	C_{31} aetio
Molecular weight	422	436	450	464
H.p.l.c. retention time *				
I †	13.0	10.6	15.1	12.0
II ‡	24.0	22.0	25.5	23.5
C.I. ion intensities §				
$(M - 2)$	43	23	Tr ¶	54
$(M - 1)$	306	52	44	150
M	835	82	58	297
$(M + 1)$	1 000	112	131	372
$(M + 2)$	495	70	78	221
$(M + 3)$	206	55	40	96
$(M + 4)$	81	33	Tr	4
$(M + 5)$	47	27	Tr	Tr
Σ	3 013	454	351	1 234
% Total petroporphyrins				
By c.i.m.s.	59.6	9.0	6.9	24.4
By h.p.l.c. ‖	65	4	7	25

* Absolute retention time (min.). Reproducibility ±2 min. † Solvent programme 27% B to 100% B in 40 min. ‡ Solvent programme 10% B to 50% B in 25 minutes. § Relative to *m/e* 423 = 1 000. All intensities ±5%. ¶ Tr = Trace ions. ‖ ±10%.

petroporphyrin ($M^{+\cdot}$, *m/e* 476) and a novel C_{33} compound ($M^{+\cdot}$, *m/e* 488) were also evident. The latter is representative of a new series of petroporphyrins recently discovered,[16] which contains either a second isocyclic ring or a double bond in one of the substituent groups on the macrocycle. The presence of strong $(M - 15)^+$ and other fragment ions is again evident, thus rendering accurate quantitation impossible. This is particularly noticeable for the C_{30} DPEP petroporphyrin, whose molecular ion coincides with the first isotope peak of the $(M - 15)^+$ fragment ion of the next higher homologue, the C_{31} DPEP petroporphyrin.

In the c.i. mode (Figure 2d) these fragments are absent, thus allowing quantitation of this complex mixture (Table 3). In addition, a trace component of

TABLE 3

Analysis of the petroporphyrins of fraction GB3

Porphyrin	C_{29} aetio	C_{30} DPEP	C_{31} DPEP	C_{32} DPEP	C_{33} p-p *
Molecular weight	436	448	462	476	488
H.p.l.c. retention time †	30.4	31.3	31.6	32.3	30.8
C.i. ion intensities ‡					
$(M - 2)$	24	N.d. §	N.d.	N.d.	N.d.
$(M - 1)$	256	179	112	28	56
M	739	560	426	111	220
$(M + 1)$	1 000	800	621	230	328
$(M + 2)$	637	594	458	189	252
$(M + 3)$	344	430	372	144	192
$(M + 1)$	187	291	262	95	140
$(M + 5)$	105	211	186	71	102
$(M + 6)$	39	107	83	28	60
$(M + 7)$	N.d.	35	Tr ¶	N.d.	24
Σ	3 331	3 207	2 520	896	1 374
%Total petroporphyrins by c.i.m.s.	29.4	28.3	22.2	7.9	12.1

*p-p = petroporphyrin. † Absolute retention time (min.). Solvent programme: 10% B to 50% B in 25 min. Reproducibility ±3 min. ‡ Relative to *m/e* 437 = 1 000. All intensities ±5%. § N.d. = not detected. ¶ Tr = trace ions.

C_{28} aetiopetroporphyrin may be present, from the small cluster of ions observed at *m/e* 421, 422, and 423. Comparison with quantitation by h.p.l.c. was impossible, due to the complexity of the chromatogram, probably due to the presence of structural isomers. It is impossible to determine the structural differences between isomers by any of the techniques employed.

In a t.l.c. fraction such as GB3, which contains both aetio and DPEP petroporphyrins, the difference in polarity of the two species is at least sufficient to ensure the separation of aetiopetroporphyrins from DPEP compounds of the same carbon number, which is a necessary requirement for quantitation by the c.i. technique. In both classes of compound, polarity is inversely proportional to the carbon number.[16] Thus, low polarity DPEP petroporphyrins (C_{30}—C_{32}) co-elute with the high polarity C_{29} aetiopetroporphyrin.

While e.i. mass spectrometry at normal electron voltages gives the best sensitivity of any ionisation technique, the resultant spectra are not ideal for the qualitative or quantitative analysis of petroporphyrin mixtures, because of the abundant fragmentation which can obscure or distort the peaks of the minor components. If this fragmentation is reduced by lowering the electron voltage,[18] the ionisation efficiency is simultaneously reduced, resulting in poorer sensitivity. The field desorption (f.d.) mass spectra of porphyrins and chlorins have been described [19] and gave analogous results, the bulk of the ion current being carried in the quasi-molecular ion region. Further, the envelope of quasi-molecular ions, observed for c.i., is not apparent, the major ion being the molecular ion with higher peaks being purely isotopic in nature. However, one of the major problems with f.d. mass spectrometry is the inability to quantitate the components of a mixture as each different compound has its own optimal anode temperature.[20] The technique at present requires comparatively large quantities (*ca.* 50 μg) of material. C.i. mass spectrometry therefore represents the best compromise for both the qualitative and the quantitative analysis of partially separated petroporphyrin mixtures, having both good specificity due to the lack of fragmentation and adequate sensitivity.

Preliminary investigations have shown that the behaviour of metalloporphyrins in the c.i. mode is analogous to that of the free-base porphyrins.[16] This technique could be of considerable value for the quantitative analysis of metalloporphyrin mixtures, where the e.i. mode generates fragmentation patterns complicated by contributions from the metal isotopes.[7]

The possibility of interfacing a h.p.l.c. with a mass spectrometer,[21] operated in the c.i. mode, offers further promise for the analysis of petroporphyrin mixtures. H.p.l.c.–m.s. in the c.i. mode should provide a powerful tool for the identification and quantitation of the components in a petroporphyrin mixture.

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