High-Performance Liquid Chromatography-Mass Spectrometry of Porphyrins by Using an Atmospheric Pressure Interface

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A method is described for the high-performance liquid chromatography (HPLC) mass spectrometry analysis of porphyrin mixtures by using an atmospheric pressure interface, which can operate in two modes: pneumatically assisted electrospray and atmospheric pressure chemical ionization (APCI). Optimization of the conditions and evaluation of spectral information has been carried out by using direct injection of free-base and metallo porphyrin standards. The most effective results were obtained using APCI. HPLC-APCI mass spectrometry analysis of the demetallated vanadyl porphyrin fraction from the Triassic Serpiano oil shale has allowed rapid characterization of the distribution; more than 50 significant components are present. The presence of trace amounts of high molecular weight ($> C_{33}$) cycloalkano porphyrins indicates the occurrence of photic zone anoxia in the ancient water column. This example illustrates the potential of the approach for studies of porphyrin mixtures of environmental or biological significance, which should be applicable to other types of metallo and free-base components that can be separated by HPLC under normal or reversed-phase conditions. *(J Am Soc Mass Spectrom 1996, 7, 965–971)*

Red metallo porphyrin pigments, which occur almost ubiquitously as complex mixtures of Ni and/or VO complexes in organic-rich marine and lacustrine sediments and in petroleums, result mainly from modification of the chlorophylls of phototrophic organisms originally present in the water column at the time of deposition [1–5]. Porphyrins also play a variety of important biochemical roles, for example, as iron complexes (e.g., **1** in Figure 1) in oxygen-carrying proteins (hemoglobin, myoglobin). Hence, qualitative and quantitative determination of porphyrin distributions has application in biological, biomedical, and earth sciences, such as in the diagnosis of metabolic abnormalities [6] or in the assessment of palaeoenvironments [4]. As an example of the latter, the presence in sedimentary organic matter of specific high molecular weight ($\geq C_{33}$) components (e.g., **2a** as the Ni or VO complex), derived from the chlorophylls of anaerobic phototrophic bacteria (e.g., **3**), indicates the occurrence of anoxygenic photosynthesis in the original water column [7–9]. These and other key components most often are identified from the ^{1}H nuclear magnetic resonance (NMR) spectra of the individual compounds isolated by preparative high-performance liquid chromatography (HPLC), with the selection of components for structure determination based on their electron ionization (EI) mass spectra. However, the complexity of the mixtures (which also contain structural isomers) makes it time-consuming to obtain mass spectral information. Other assignments have relied on HPLC coinjection of the mixtures with synthesized or isolated components (e.g. [8]), but this approach lacks precision in the absence of mass spectral information. There is a need, therefore, for a routine combined chromatography–mass spectrometry approach for the analysis of porphyrin mixtures. Some success has been achieved by using gas chromatography–mass spectrometry (GC-MS) for alkyl porphyrins, which are derivatized to improve their volatility by using a silylation procedure [10–13]. However, HPLC has proved to be more effective than GC-MS for component separation, and both metallo complexes and their demetallated counterparts, the free-base (FB) species, routinely can be examined as can less volatile components that contain functional groups [14–18].

The distributions of chlorophylls and other green chlorin pigments (i.e., dihydroporphyrins) that contain functional groups can be obtained readily by HPLC mass spectrometry by using, for example, a thermospray interface [19] or continuous flow fast-atom bombardment (FAB) [20]. These techniques have not proved to be as suitable for the analysis of porphyrins. In our laboratory the use of a thermospray interface [19] re-

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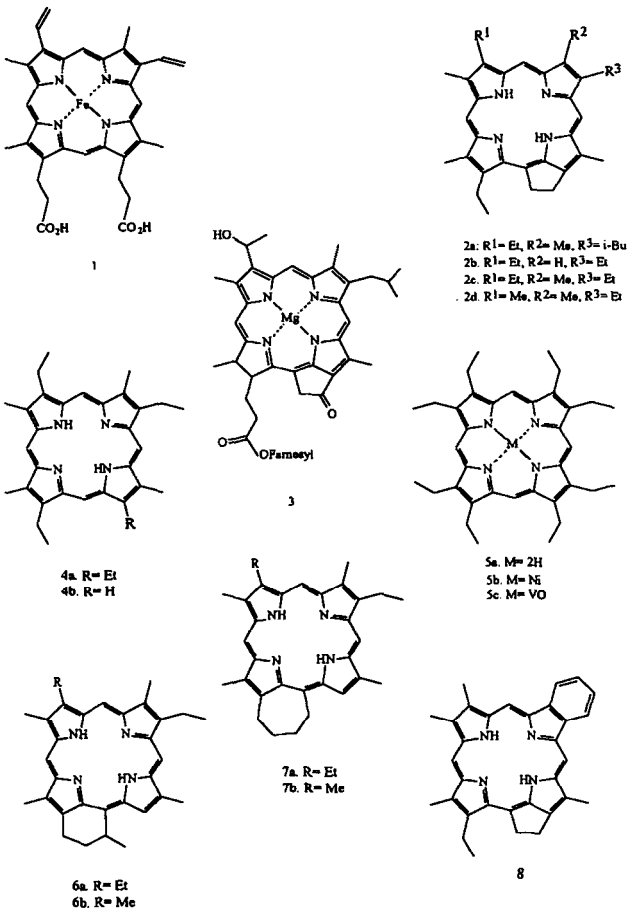

Figure 1. Structures of tetrapyrroles discussed in the text.

sulted in the precipitation of components at the tip of the interface probe, despite a high ion source temperature, so the sensitivity of detection was low. Also, continuous flow FAB only accepts low eluent flows, so microbore HPLC columns or flow splitting have to be used. There is one report of the use of a particle beam interface for vanadyl porphyrins, but sensitivity was relatively low, although still three times higher than using a thermospray interface [21]. Unpublished work in our laboratory that used a particle beam interface for FB alkyl porphyrins showed a detection limit of only 10 ng.

We report here the application of HPLC–atmospheric pressure ionization (API) mass spectrometry to the characterization of porphyrin mixtures. Our aim is to develop a routine procedure to obtain component distributions with high sensitivity of detection. The interface used can operate in two modes: pneumatically assisted electrospray ionization (ESI) and atmospheric presssure chemical ionization (APCI). In ESI the solution forms a dispersion of charged droplets to yield gas-phase ions of the solute [22–24]; droplet formation is assisted by a flow of nitrogen (sheath and auxiliary gas) [25, 26]. Such a system (also known as ion spray) may improve the stability of electrospray ionization and allows higher flow rates at the cost of reduced sensitivity [23]. APCI has received less attention, because ionization depends on the formation of gas-phase ions from external ionizing electrons, typically from a corona discharge [26, 27]. When coupled to an HPLC column APCI is less dependent on solvent composition, although the chemical background may be high at low masses due to ion cluster formation from the HPLC eluent, and operation at high temperatures has been reported to cause decomposition of thermally labile compounds [26]. APCI has been recommended for applications that involve relatively apolar analytes [26], and it has been noted that prior chromatographic separation may improve the detection of components of low volatility or in low concentration [27]. ESI is usually recommended for applications that involve analytes in ionic form in solution or that are very polar or thermally labile, and when optimum sensitivity can be achieved by using microbore HPLC columns [26].

Experimental

General

Etioporphyrin III (**4a**) was supplied by Dr. J. G. Erdman. Octaethylporphyrin (OEP; **5a**) was obtained from Porphyrin Products Inc. (Logan, UT). The isolation and separation of porphyrins from Serpiano oil shale, demetallation of porphyrin fractions, metallation of standards, as well as the normal phase HPLC conditions (mobile phase contains hexane, acetone, dichloromethane, pyridine, and acetic acid) for the analysis of FB porphyrins have been described elsewhere [12, 17, 18, 28].

Instrumentation

Analyses were performed by using a Waters (Milford, MA) 600-MS high-performance liquid chromatograph coupled to a Finnigan-MAT (San Jose, CA) TSQ 700 triple quadrupole mass spectrometer via a Finnigan-MAT atmospheric pressure ionization source. A two-way valve allowed the chromatographic column to be by-passed for the direct introduction through a 20-μL loop of dichloromethane solutions of the standards, which were eluted with dichloromethane or hexane. The eluent was passed through a photodiode array detector (PDA; Waters 991) and introduced to the spectrometer through either an unheated, pneumatically assisted electrospray probe (ESI) or a heated, nebulized spray into a point corona discharge region (APCI). In both systems nitrogen was used to nebulize and sheath the liquid inlet. Ions were extracted via a heated capillary to a skimmer lens arrangement at reduced pressure and transferred by an octapole to the main analytical quadrupole assembly.

Interface Conditions

Conditions were optimized by direct injection of 10-μL aliquots of FB (**5a**; 5 ng/μL), Ni (**5b**; 15 ng/μL), and VO (**5c**; 13 ng/μL) OEP solutions under different conditions to obtain the highest abundance of the protonated molecule MH^+ (Figure 2), at a flow rate of 1 mL/min—the optimum flow rate for the HPLC conditions used.

APCI mode: Nitrogen pressure 50 psi (sheath) and 10 psi (auxiliary), vaporizer 550 °C, ion transfer capillary 250 °C and 30-V offset, and 5 μA to the corona electrode;

ESI mode: Nitrogen pressure 50 psi (sheath) and 30 psi (auxiliary), 7 kV (FB) or 4 kV (metallo species) to the electrospray capillary, and ion transfer capillary 300 °C and 30-V offset.

In the APCI mode the intensities of MH^+ and the fragment ions are strongly dependent on the temperatures of the ion-transfer capillary and the vaporizer spray; high temperatures presumably improve the vaporization of the porphyrins and the efficiency of their transfer to the spectrometer. The extent of fragmentation was, however, a complex function of the temperatures, pressures, and voltages within the interface. In the ESI mode the abundance of MH^+ is primarily dependent on the sheath gas pressure. This is consistent with reports that droplet formation almost entirely may be due to the turbulent flow of the nitrogen gas [22]. Again, the appearance of the spectra changed in a complex manner with instrument conditions.

Results and Discussion

Mass Spectra of Standards

In both ESI and APCI modes, the spectra (Figure 2) of etioporphyrin III (**4a**) and of octaethylporphyrin (**5a**) and its Ni and VO complexes (**5b**, **5c**) show MH^+ as the base peak. In the spectra of the FBs, MH^+ is particularly abundant and accounts for more than 90% of the total ion current. In contrast to the behavior under EI conditions [29], the VO complex loses the metallo ligand in both modes, with the addition of two protons to give an ion that corresponds to the FB species. This process occurred with all the solvents used. The more ready loss of the ligand from the VO complex compared with the Ni complex may relate to differences in the position of the metal relative to the planar macrocyclic ring. Nickel forms a type A square planar complex with the metal sitting within the ring, whereas the VO ligand (coordination type B) sits above it [6]. Work is underway with a larger suite of metallo complexes to investigate further their fragmentation behavior.

Free-base porphyrins have a high proton affinity, and ionization occurs in both ESI and APCI modes by gas-phase ion–molecule reactions [22]. This explains in part the similarity of their ESI and APCI spectra (Figure 2). The APCI spectra of the FB species show, however, an ion at $[MH + 53]^+$ with all the solvents used and sometimes at $[MH + 32]^+$. These ions are not observed in the spectra of the metallo complexes or in the ESI mode and may be the result of the formation of adducts or complexes during the APCI ionization pro-

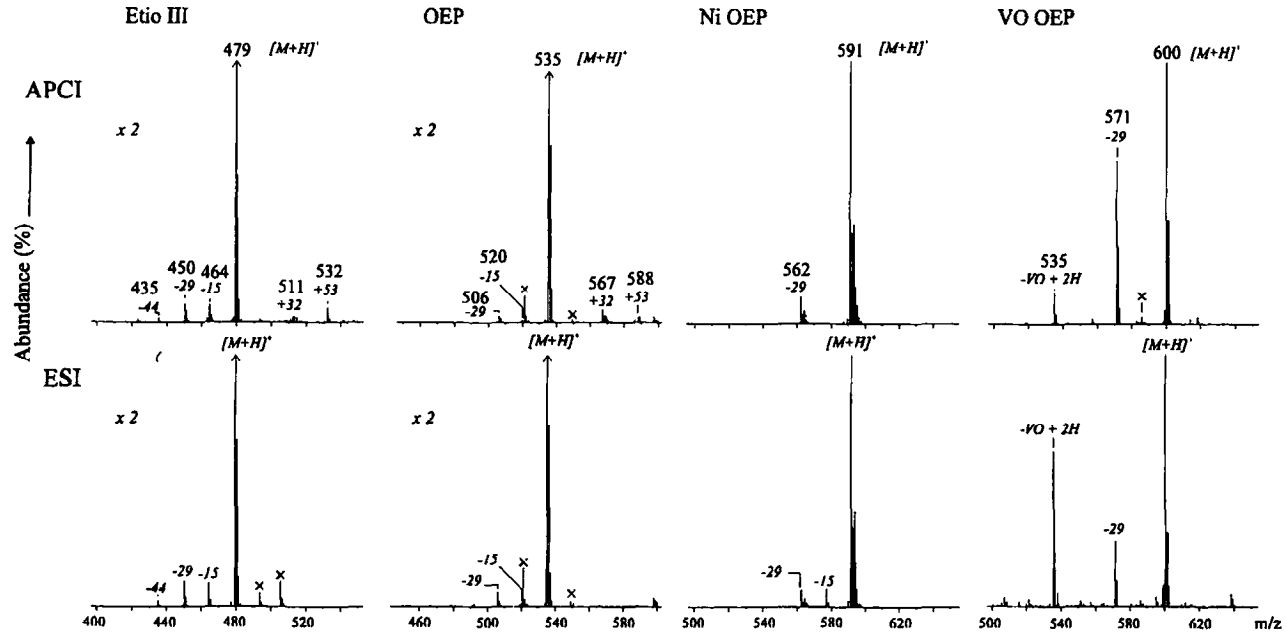

Figure 2. APCI and ESI mass spectra of etioporphyrin III, octaethylporphyrin (OEP), and nickel and vanadyl OEP (× = porphyrin impurities).

cess. Although accurate mass measurements were not obtained, the $[MH + 53]^+$ ion probably results from formation of the iron complex (i.e., it corresponds to $[M - 2H + {}^{56}Fe]^{+\cdot}$); the iron perhaps arises from vaporization from the corona discharge electrode or from liquid-phase ion exchange processes, by analogy with the EI spectra in which $[M - 2H + Fe]^{+\cdot}$ and $[M - 2H + Cu]^{+\cdot}$ ions are commonly observed.

Fragmentation of MH^+ occurs in the alkyl substituents with α and β cleavage; both odd and even mass fragments are formed (cf. Figure 2). For OEP these fragmentations correspond to loss of methyl, methane, ethyl, and ethane. Collision-induced decomposition (CID) can be promoted, for example, by increasing the potential between the spray or vaporizer cone and the capillary–skimmer region, which accelerates the ions and leads to CID [22, 26]. High temperatures and elevated flows of nitrogen also promote fragmentation. To obtain further structural information about the substituents from the fragment ions, CID also can be brought about by increasing the offset voltage applied to the octapole, which can be quickly modified (e.g., at alternate scans). An increase in the offset voltage (i.e., an increase in the collision energy) results in an increase in the number of substituents cleaved. Hence, the fragmentation pattern obtained by this method should be able to provide information on the type and number of substituents on the macrocycle, and further studies are in progress.

Linearity and Sensitivity

In both the APCI and ESI modes the abundance of MH^+ increased with the amount of FB OEP injected over the concentration range studied (Figure 3), although the relative abundances of MH^+ and the fragment ions remained essentially constant. However, the response was directly proportional only to concentration in the APCI mode. At any given concentration of standard injected in the APCI mode, reproducibility was higher than in the ESI mode and the increase in the signal with concentration was greater. In the ESI mode, under the optimized conditions, scanning from m/z 500 to 650 at 2 s/scan, 32 pg (60 fmol) of FB OEP were detected with a signal-to-noise ratio greater than 10 for MH^+, whereas 64 pg were required to obtain a similar response in the APCI mode. Therefore, ESI appears to be slightly more sensitive with respect to limit of detection. For both Ni and VO OEP in the two modes the response always was less than for FB OEP for the same amount injected (Figure 3).

Natural Mixtures

The most effective conditions for the characterization of complex mixtures of porphyrins in terms of chromatographic resolution, sensitivity, and reproducibility relate to FB components in the APCI mode for the following reasons:

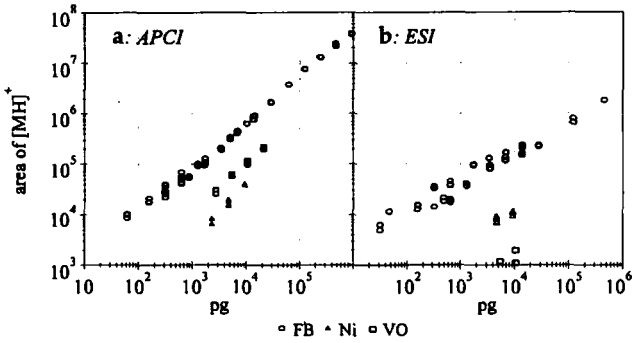

Figure 3. Intensity (peak area) of MH^+ versus amount (picograms) of OEP as free-base (FB), Ni, or VO complex injected off-column in (**a**) APCI and (**b**) ESI. Scales are logarithmic.

1. HPLC of FB alkyl porphyrins under normal phase conditions affords higher chromatographic resolution than for the metallo species under reversed-phase conditions [14–18].
2. The sensitivity attainable for the FBs is greater than for the metallo species (Figure 3).
3. The selectivity of the analysis is poorer for the Ni species because the contributions of the metal isotopes give rise to more abundant isobaric ions.
4. With our system APCI was found to give a more stable and reproducible response than ESI, and ESI operation led to slight peak tailing and some loss of chromatographic resolution, presumably due to adsorption of the porphyrins in the unheated silica capillary that connects the HPLC outlet with the interface. In contrast, in APCI operation the silica capillary is maintained at high temperature.

A typical application of HPLC-APCI mass spectrometry to the characterization of complex mixtures is illustrated in Figures 4–7, which relate to the demetallated vanadyl porphyrins from Serpiano shale (Triassic; Switzerland). The overall distribution (Figure 4) is a composite plot based on the intensity of the base peak in the spectra (i.e., MH^+). Table 1 lists the most abundant component (or components if in similar abundance) in each peak. Mass chromatograms are shown for one of the major series of component types, the cycloalkanoporphyrin (CAP) type (Figure 5) that contains an exocyclic ring that may be five-, six-, or seven-membered (e.g., **2**, **6**, and **7**) [1, 4, 30]. The fraction contains > 50 significant components (> 150 in toto) and comparison with the on-line PDA chromatogram (monitored at 498 nm) showed no loss of chromatographic resolution. Previously some of the major components of this sample have been characterized fully after HPLC isolation and ^1H NMR analysis [31–35] (Table 1). The mixture is dominated by CAP compounds with a range of carbon numbers and structural isomers (Figure 5); the major component is desoxophylloerythroetioporphyrin (C_{32} DPEP **2c**; 38 in

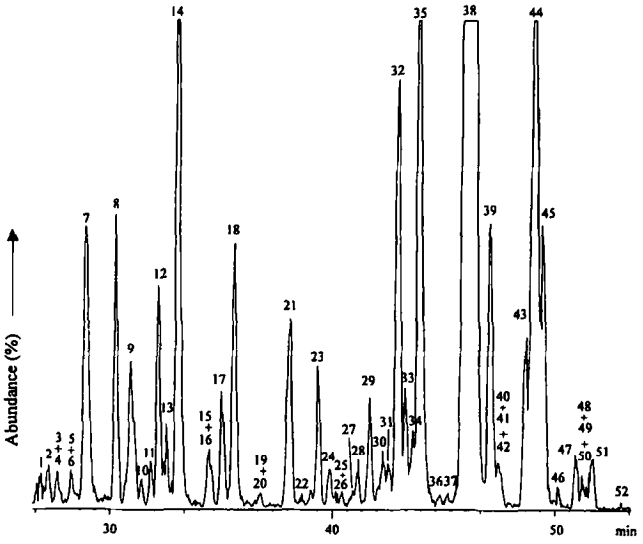

Figure 4. Normal phase HPLC-APCI mass spectrometry base peak chromatogram (abundance of the most intense ion in each scan) of demetallated VO porphyrins from Serpiano oil shale. Identities of significant components in each peak are given in Table 1.

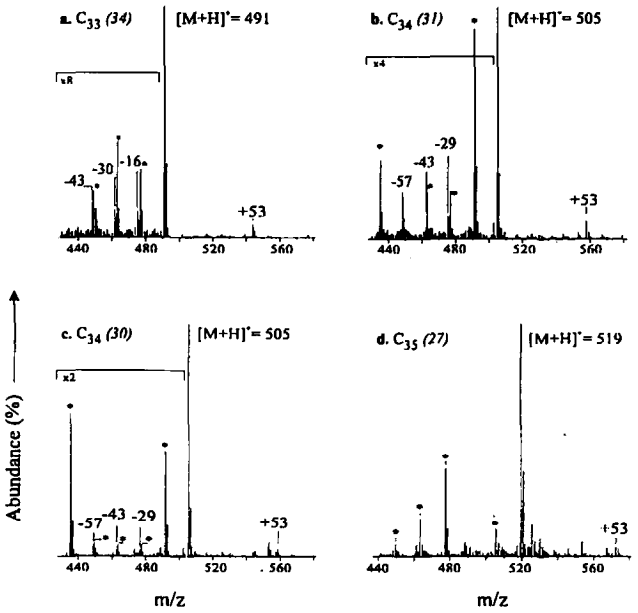

Figure 6. Mass spectra of selected C_{33}-C_{35} cycloalkanoporphyrin components from Serpiano oil shale. See Figures 4 and 5 and Table 1 for peak identities. The asterisks (*) indicate ions from coeluting porphyrins.

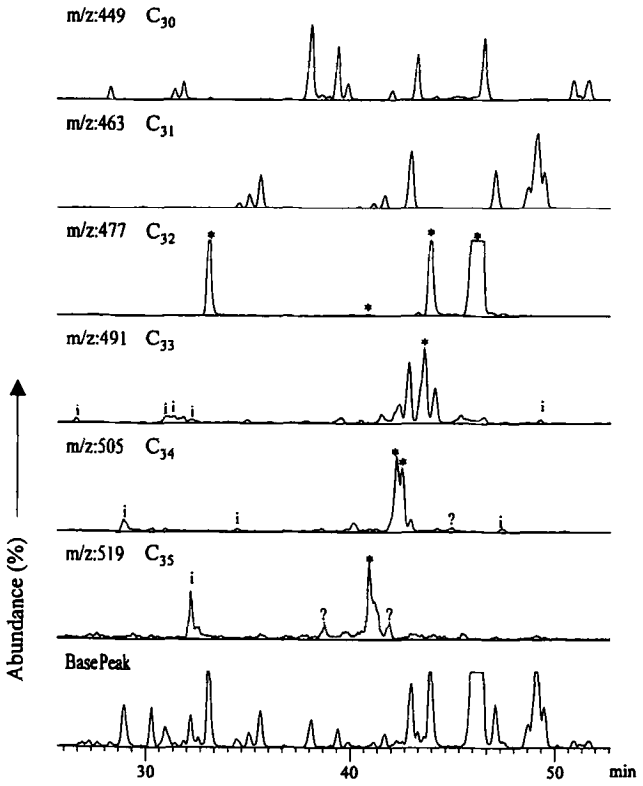

Figure 5. Normal phase HPLC-APCI mass spectrometry base peak chromatogram (abundance of the most intense ion in each scan) of demetallated VO porphyrins from Serpiano oil shale and mass chromatograms of C_{30}-C_{35} cycloalkanoporphyrin (CAP) components. For the asterisk (*), see mass spectra in Figures 6 and 7; i denotes peaks that correspond to isotopic contributions from non-CAP components; ? indicates unknown.

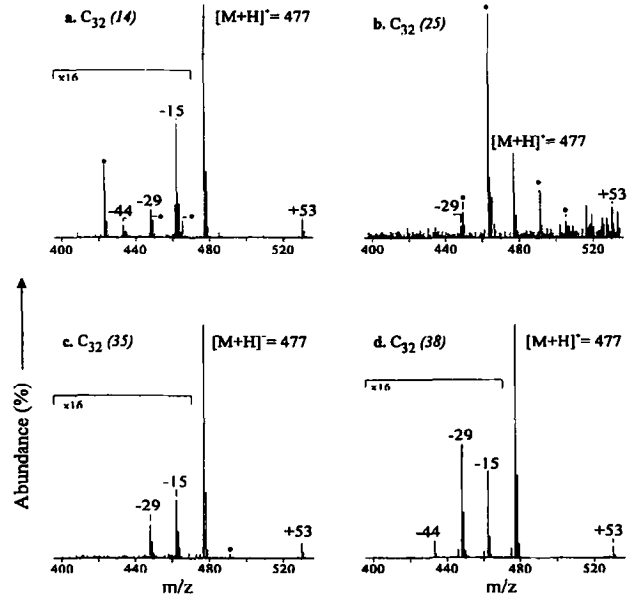

Figure 7. Mass spectra of selected C_{32} cycloalkanoporphyrins from Serpiano oil shale. See Figures 4 and 5 and Table 1 for peak identities. The asterisks (*) indicate ions from coeluting porphyrins.

Figure 4). Components without an exocyclic ring, that is, etioporphyrins (e.g. etioporphyrin III; **4a**; 8 in Figure 4), with two exocyclic rings (bi CAPs) and a benzo CAP (**8**; 48 in Figure 4) are also present. Component 18 (Figure 4), previously assigned as a C_{31} etioporphyrin [31, 33] by HPLC coinjection with a standard isolated

from an oil, is seen to be a C_{31} CAP from the MH^+ at m/z 463. This emphasizes the greater specificity of HPLC mass spectrometry for coinjection studies.

The spectra provide only limited structural information (Figures 6 and 7), because fragmentation is limited to losses within alkyl substituents. However, some preliminary observations can be made. The presence of ions at $[MH^+ - 43]$ and/or $[MH^+ - 57]$ in the spectra that correspond to C_{33}-C_{34} CAPs (Figure 6) indicates that these are components with "extended" alkyl chains ($> C_2$). These ions, which cannot be assigned to isotopic contributions from other components, can be attributed to the presence of C_3 and/or C_4 alkyl substituents, by analogy with fragmentation in ethyl ($[MH^+ - 15]$ and $[MH^+ - 29]$) and methyl ($[MH^+ - 15]$) substituents. The spectra of the C_{32} CAPs in Figure 7, which include three components of known structure (Table 1), reveal the presence of only methyl and ethyl substituents, with apparently no fragmentation in the exocyclic ring. There are, however, variations in the relative abundance of the $[MH^+ - 15]$ and $[MH^+ - 29]$ ions, which provide an indication of differences in the number of methyl and ethyl substituents. Thus, component 14 (Figure 4; **6a**), with five methyl and two ethyl substituents, shows a more intense $[MH^+ - 15]$ ion than component 38 (Figure 4; **2c**), which has four methyls and three ethyls and shows a more intense $[MH^+ - 29]$ ion. Further studies with standards, which include tandem mass spectrometry, are in progress.

The CAP series provides a good example of the potential of HPLC-APCI mass spectrometry to provide information relevant to palaeoenvironmental assessment because $> C_{33}$ components are present (Figure 6). Such components with extended alkylation are diagenetic products of the chlorophylls (e.g., **3**) of green sulfur bacteria [5, 8, 9]. Therefore, their presence provides evidence for anoxygenic photosynthesis in the original water column and hence for anoxia that extends into the zone of light penetration of a highly stratified water column.

Conclusions

HPLC-API mass spectrometry provides a means for the rapid characterization of the components of porphyrin mixtures. We have demonstrated its application to alkyl porphyrins, but the method also should be applicable to other types of metallo and free-base porphyrins, which include functionalized components and porphyrins of biological origin, that can be separated by HPLC under normal or reversed-phase conditions. For alkyl porphyrins the spectra can provide information about the nature of the alkyl substituents on the macrocycle and of the ligand in the complexes. Evidence has been obtained for the occurrence of anoxygenic photosynthesis during deposition of the Serpiano oil shale.

Table 1. Carbon number and porphyrin type of components from Figure 4

Component[a]	MH^+ (m/z)	Component type (structure)[b]
1	479	C_{32} etio
2	423	C_{28} etio
3	465	C_{31} etio
4	423	C_{28} etio
5	449	C_{30} cap
6	423	C_{28} etio
7	451	C_{30} etio (**4b**)[c]
8	479	C_{32} etio (**4a**)[d]
9	437	C_{29} etio
10	449	C_{30} CAP
11	449	C_{30} CAP
12	465	C_{31} etio
13	465	C_{31} etio
14	477	C_{32} CAP (**6a**)[e]
15	463	C_{31} CAP (**6b**)[e]
16	451	C_{30} etio
17	463	C_{31} CAP
18	463	C_{31} CAP
19	503	C_{34} bi CAP
20	435	C_{29} CAP
21	449	C_{30} CAP
22	449	C_{30} CAP
23	449	C_{30} CAP
24	449	C_{30} CAP
25	477	C_{32} CAP
26	463	C_{31} CAP
27	519	C_{35} CAP
28	463	C_{31} CAP
29	463	C_{31} CAP
30	505	C_{34} CAP
31	505	C_{34} CAP
32	463	C_{31} CAP (**2b**)[e]
33	449	C_{30} CAP
34	491	C_{33} CAP
35	477	C_{32} CAP (**7a**)[e]
36	477	C_{32} CAP
37	477	C_{32} CAP
38	477	C_{32} CAP (**2c**)[e]
39	463	C_{31} CAP (**7b**)[e]
40	489	C_{33} bi CAP
41	503	C_{34} bi CAP
42	477	C_{32} CAP
43	463	C_{31} CAP
44	463	C_{31} CAP (**2d**)[e]
45	463	C_{31} CAP
46	489	C_{33} bi CAP
47	449	C_{30} CAP
48	485	C_{33} benzo CAP (**8**)[f]
49	475	C_{32} bi CAP
50	449	C_{30} CAP
51	449	C_{30} CAP
52	491	C_{33} CAP

[a] See Figure 4. [b] See Figure 1. [c] Reference 34. [d] Reference 33. [e] Reference 32. [f] Reference 35.

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