CHARACTERISATION OF CHLORIN STERYL ESTERS IN SEDIMENTS BY DESORPTION/MASS SPECTROMETRY AND GEOCHEMICAL SIGNIFICATION

BY

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ABSTRACT

Desorption mass spectrometry of sedimentary chlorin steryl esters. – High molecular weight chlorins may be desorbed intact into the ion source of a conventional mass spectrometer from an extended desorption probe. In conjunction with electron impact ionisation, this technique yields mass spectra which are characteristic of the macrocycle-type as well as of the side chain structure. The potential of the method is demonstrated by the characterisation of chlorin steryl esters extracted from a recent lacustrine sediment and from the overlying water column. Results suggest that these compounds are synthesised by biological mediation in the water column. Although the potential of this biomarker class to describe the hydrobiological and the geochemical conditions is still to assess, some geochemical implications based on ours results are discussed.

Key-words: chlorins, chlorin steryl esters, desorption mass spectrometry, lacustrine sediments, geochemistry.

INTRODUCTION

Since the Treibs' hypothesis (Treibs, 1936) on the biological origin of the organic matter in ancient sediments and petroleum's, tetrapyrrole pigments are one of the most studied classes of sedimentary organic molecules (Callot *et al.*, 1990; Keely *et al.*, 1990). Because of their low volatility they are difficult to analyse by classical electron-impact mass spectrometry. Mass spectra of functionalised tetrapyrroles obtained after conventional solid probe introduction show generally only pyrolitic fragments. Special ionisation techniques such as field desorption (Dougherty *et al.*, 1980), fast atom bombardment (Keely *et al.*, 1988), and more recently thermospray or electrospray (Van Berkel *et al.*, 1991) have been used to obtain molecular weight information and, in

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favourable cases, to determine the tetrapyrrole macrocycle structure. However, the above techniques are not widely accessible. Moreover, with chlorins bearing a side-chain, like those found in recent sediments as early degradation products of chlorophylls or chlorin steryl esters, they generally do not give fragments informative of the side-chain structure (Eckardt *et al.*, 1992). We show here that using an extended desorption probe on a conventional EI/MS instrument (D/EI/MS), it is possible to obtain interpretable mass spectra for reference chlorin molecules including a synthetic pyropheophorbide-a stigmasteryl ester (structures in Fig. 1). The presence of the molecular ions and fragments arising both from the mocrocycle and from the side-cain, allows an unambiguous characterisation of these compounds. We then demonstrate the potential of the technique by applying it to the identification of a series of chlorin steryl esters (CSE) in a fraction isolated from a lacustrine sediment. The results obtained from the D/EI/MS analysis of the sedimentary CSE are confirmed by the GC/MS analysis of the sterols released by hydrolysis of the CSE fraction. Finally, geocemical implications of these sedimentary biomarkers are discussed on the basis of a comparison between the distributions of free sedimentary sterols, water column free sterols and sterols bonded to chlorins.

EXPERIMENTAL

Solvent extraction and TLC separation: The extraction was performed according to a method described by King & Repeta (1991). Approximately 500 g of wet freshwater sediment were extracted by sonication (5 min.) with 4 portions of 250 ml acetone followed by 3 portions of 250 ml methylene chloride. The combined extracts were reduced to 200 ml in a Rotavapor and an equal volume of hexane/diethylether, 3/7 (v/v), was added. The organic phase was separated, dried over Na_2SO_4 and evaporated, yielding about 100 mg of a dark-brown residue. The residue was dissolved in acetone and submitted to chromatography on a preparative plate (Merck, Kieselgel 60) using hexane/acetone, 3/1 (v/v). We observed 4 green bands with Rf values greater or equal to 0,2. After dissolution in acetone and filtration in order to recover the green substances from SiO_2, we obtained about 12 mg of dried organic matter.

HPLC isolation of chlorin steryl esters: CSE were recovered from the above material by semi-preparative HPLC using a reverse phase column RP-18 (Merck, lichrosolv 250 mm x 10 mm i.d.). Isocratic elution with acetone/methanol, 1/1 (v/v), at a flow rate of 3 ml/min, was monitored by a UV detector (366 nm). CSE were collected between 33-51 minutes (Figure 2).

UV/VIS spectroscopy: The electronic absorption spectrum was recorded from 350 to 700 nm in acetone, with a Perkin-Elmer lambda 5 spectrophotometer at a scan speed of 120 nm/min, a slit width of 2 nm and a 1 cm long cell. Spectral data for chlorin steryl esters fraction (ex: 5) are: λ max [nm] = 663, 605, 532, 503, 407 (407/663 = 2,33).
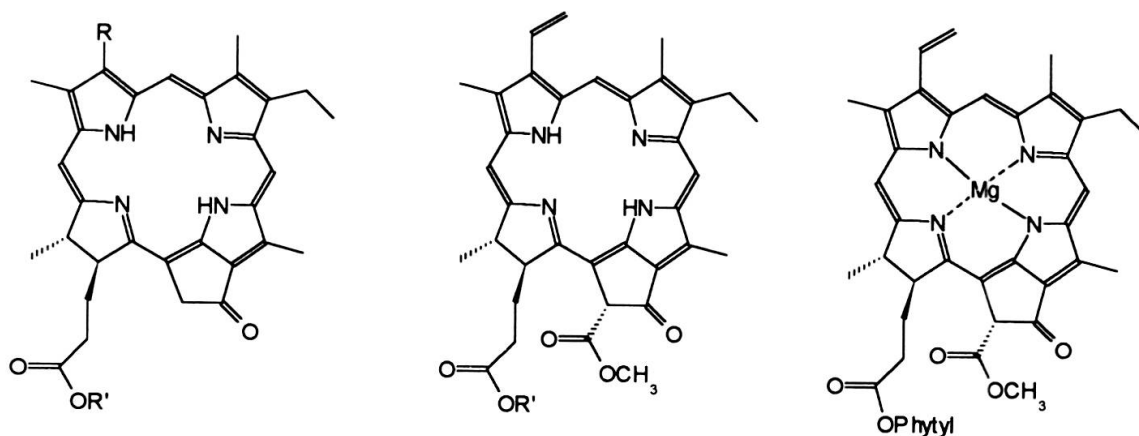
(1) R = vinyl, R' = H ; *pyropheophorbide-a*

(1a) R = ethyl, R' = H; *mesopyropheophorbide-a*

(1b) R = vinyl, R' = CH₃ ; *pyropheophorbide-a methyl ester*

(1c) R = vinyl, R' = phytyl ; *pyropheophytin-a*

(2) R' = H ; *pheophorbide-a*

(2a) R' = phytyl ; *pheophytin-a*

(3) *chlorophyll-a*
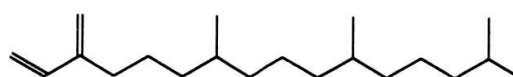
(4) *Neophytadiene*
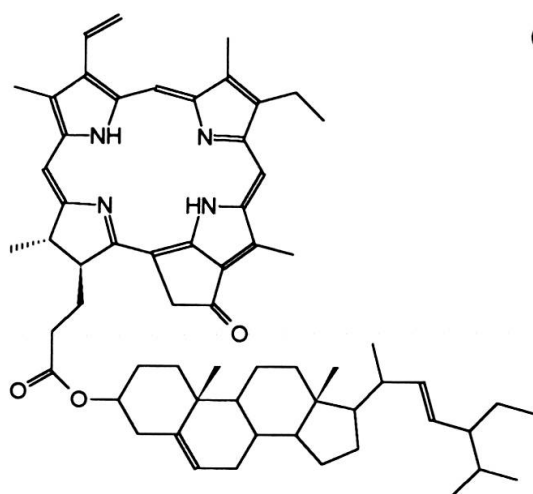
(5) *pyropheophorbide-a stigmasteryl ester*

Fig. 1

Structures.

Reference compound: pyropheophorbide-a methyl ester (1b) (Sigma chemicals) l max [nm] = 663, 605, 532, 503, 407 (407/663 = 2,36).

Desorption mass spectrometry in EI mode: The mass spectra were recorded on a VG Mass Lab, Trio-2 quadrupole mass spectrometer using an extended desorption probe (Cotter, 1980). The sample, dissolved in acetone, was deposited on a platinum wire (d =
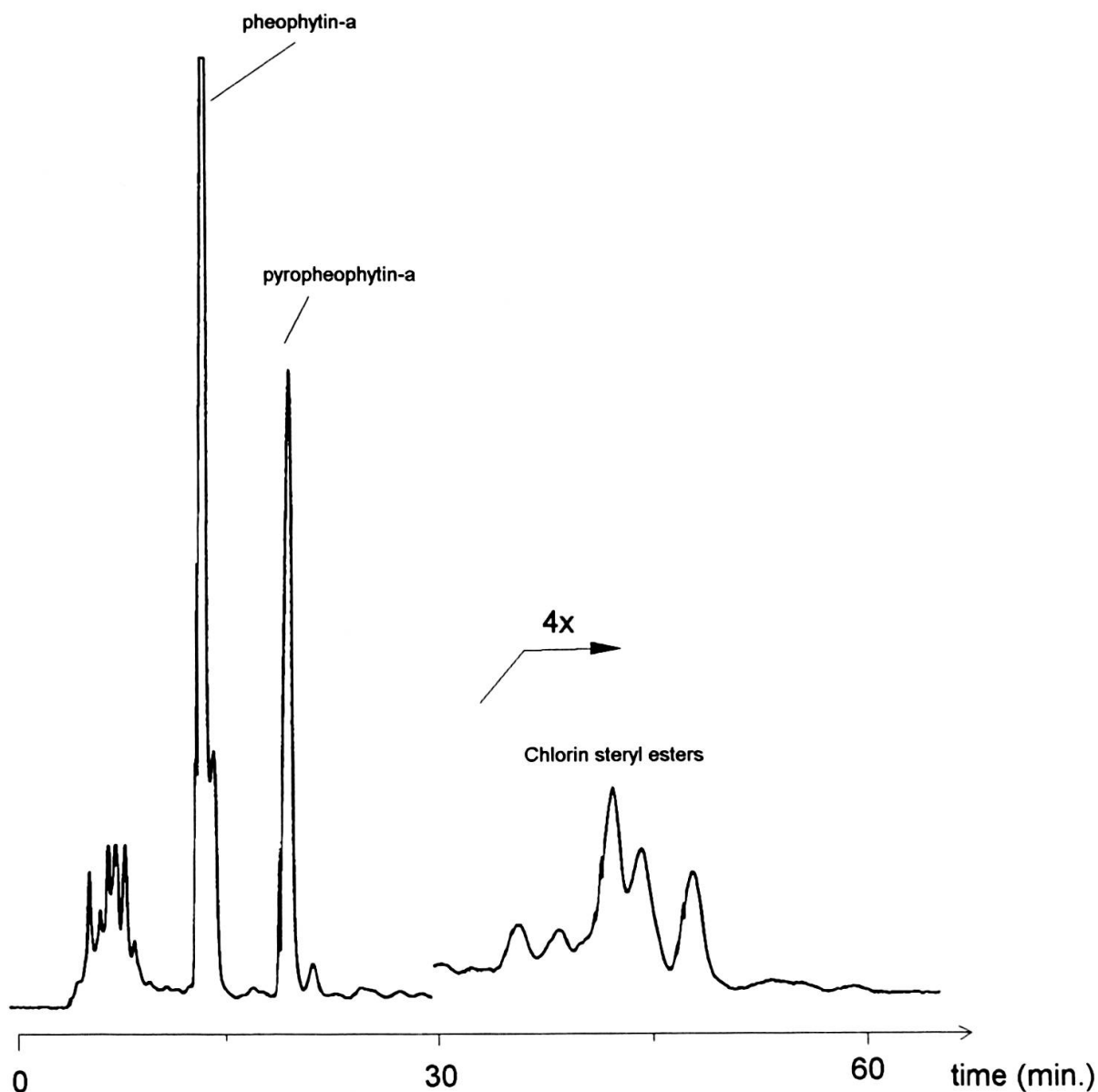
FIG. 2

Reversed-phase HPLC chromatogram, conditions given in the text.

0,127 mm). After evaporation of the solvent, the probe was inserted into the spectro-meter and heated according to the following program: no heating during 0,5 min, then up to 1200 mA at a rate of 10 000 mA/min, and finally, 0,7 min at 1200 mA. The scan rate was 1 s/scan from 50 to 1000 Daltons. The electron impact ion source was operated at 70 eV (nominal) and 220° C.

Hydrolysis of chlorin steryl esters: The fraction containing the CSE was refluxed with a mixture of THF/NaOH 4M, 1/1 (v/v), for 6 h. After adding diethylether in order to obtain a phase separation, the organic layer was separated and washed with 3 portions

of 5% acetic acid followed by 5 portions of distilled water. The organic extract was dried with Na_2SO_4 and evaporated under N_2.

Derivatisation and gas chromatography-mass spectrometry (GC-MS): Before analysis, free sterols resulting from the hydrolysis were converted to their trimethylsilyl ethers with N, O-bis-trimethylsilyl trifluoroacetamide (BSTFA, Fluka) at 80° for 20 min. Separations were obtained on an Altech SE-54 fused silica capillary column (30 m x 0,32 mm, 0,25 µm film thickness, 50 cm retention gap) using helium as the carrier gas (50 kPa). The derivatised sterols were injected at 40° C and the temperature program was 1 min at 40° C, 10° C/min to 200° C, 3° C/min to 280° C and finally 30 min at 280° C.

Typical operating conditions of the mass spectrometer were: electron impact mode with a nominal electron energy of 70 eV; source temperature of 220° C; mass range of 50-650 Daltons at 1 scan/s.

Preparation of pyropheophorbide-a stigmasteryl ester: Pyropheophorbide-a (<u>1</u>) was obtained by hydrolysis of the commercial methyl ester (<u>1b</u>) as described above for CSE hydrolysis. The esterification of <u>1</u> with stigmasterol was adapted from Hassner *et al.* (1978) as following. A solution of <u>1</u> in anhydrous THF with 1 equiv. of dicyclo-hexylcarbodiimide (DCC) and 0,12 equiv. of 4-pyrrolidinopyridin (4-P-P) was heated to reflux for 6 h. After cooling, diethylether was added to obtain a distinct phase separation and the mixture worked up as described for CSE hydrolysis.

RESULTS AND DISCUSSION

Mass spectrometry: The mass spectra of the reference compounds, obtained by D/EI/MS, are reproduced in Figure 3. The mass spectrum of pyropheophorbide-a methyl ester (<u>1b</u>) is characterised by its abundant molecular ion at m/z 548, and a few fragment ions at m/z 533 [M-CH_3], 461 [M-CH_2-CH_2COOCH_3], 447 [M-($C_2H_3COOCH_3$ + CH_3)], 433 [m/z 461-C_2H_4; presumably from the ethyl substituent at position C-8 of the macrocycle] and some doubly charged ions in the low mass range *i.e.*, m/z 274 [M^{++}], 237 [M-$CH_2C(OH)OCH_3$]$^{++}$ (reverse McLafferty rearrangement). The absence of significant fragments in the low mass range shows the stability of the macrocycle due to its aromatic character.

The mass spectrum of pyropheophytin-a (<u>1c</u>) shows the molecular ion at m/z 812. The fragment at m/z 534 is produced by the loss of the phytyl moiety with migration of hydrogen atom; it formally corresponds to the molecular ion of the free acid (<u>1</u>). Except slight differences in their relative abundances, the fragments in the mass range 400-534 are the same as for <u>1b</u> and are produced by the same mechanisms. However, the low mass range of the spectrum is now completely different and contains abundant hydro-carbon fragments. In fact, it is quite similar to the mass spectrum of neophytadiene (<u>4</u>) as given by Enzell *et al.* (1984). The neophytadiene molecular ion may be either produced by the same mechanism that gives rise to the m/z 534 ion, but with charge

compound **1b**

compound **1c**

compound **2a**
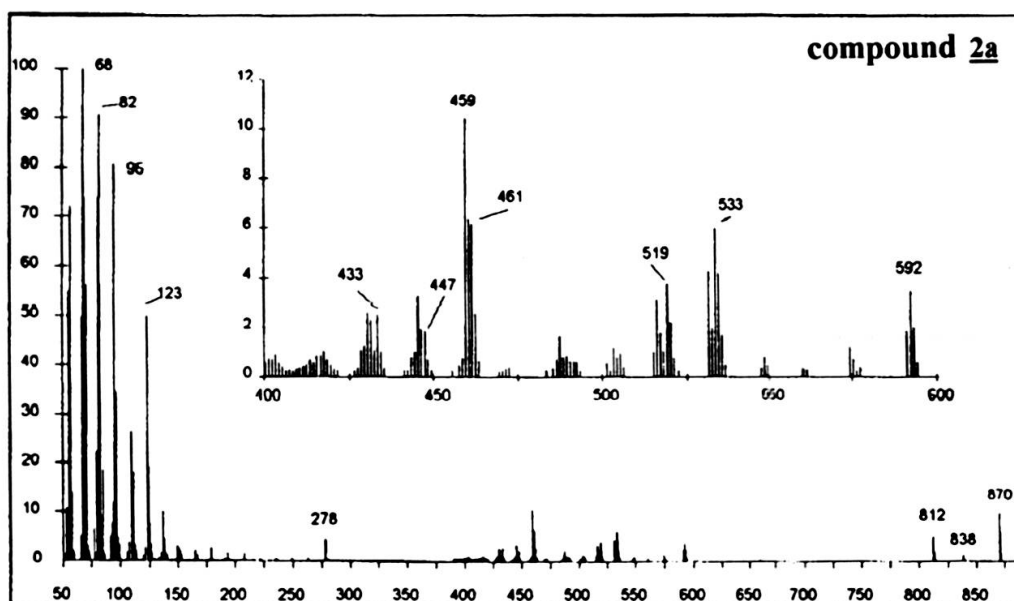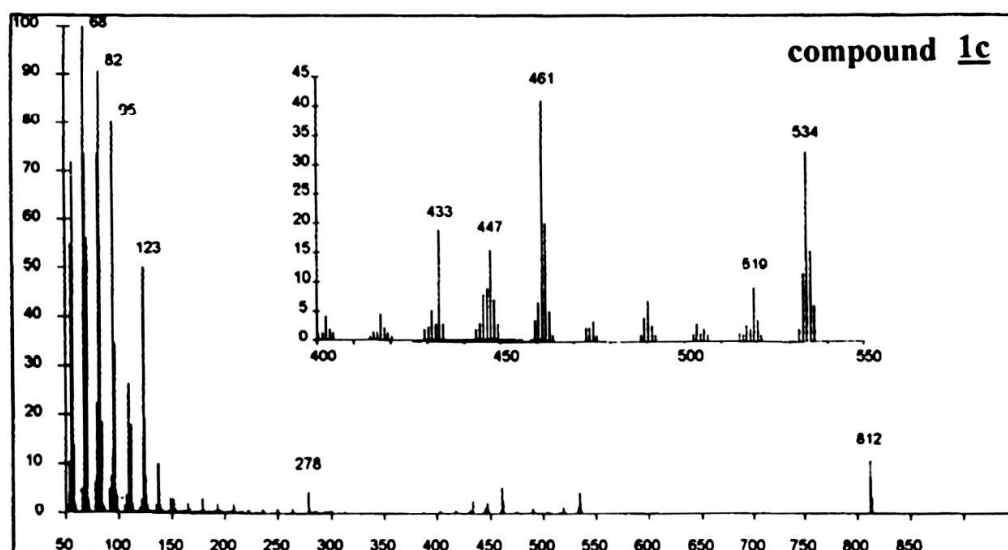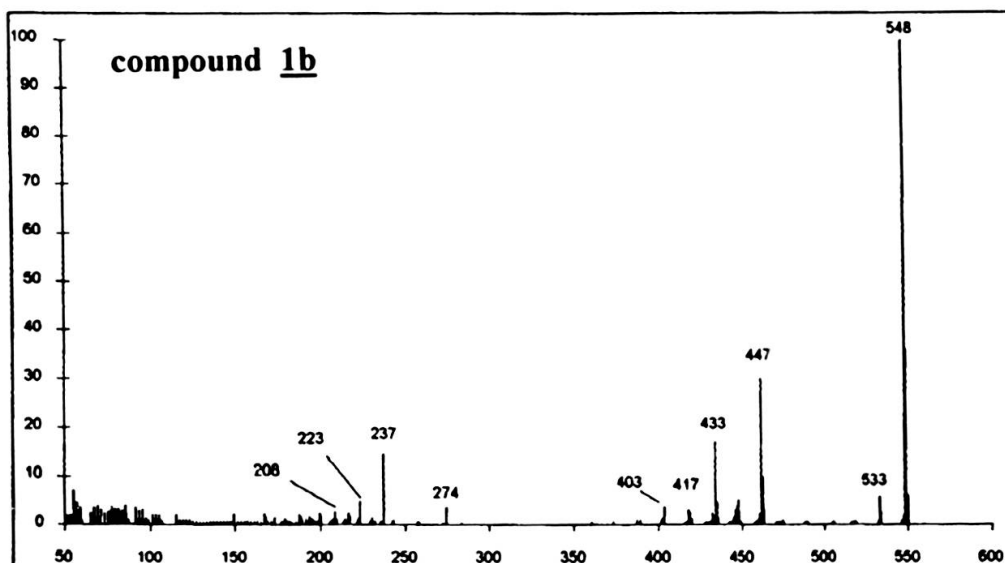
retention on the olefin, or from a partial thermal decomposition of <u>1c</u> during desorption. The evolution of the relative abundances of the ions in the mass spectra recorded at different stages of desorption (not documented here) suggests that the two above processes are superposed.

The mass spectrum of pheophytin-a (<u>2a</u>), compared to that of <u>1c</u>, shows the influence of the supplementary carbomethoxy substituent on the exocycle. Ions at m/z 838 (M-CH$_3$OH) and m/z 812 (M-CH$_2$CO) arise from this substituent, the last one corresponding to the molecular ion of <u>1c</u>. On the other hand, loss of phytadiene proceeds either directly from the molecular ion (m/z 592) or after the initial loss of the carbomethoxy substituent (m/z 534). Although most of the ions in the mass range 400-534 are the same as for <u>1b</u> and <u>1c</u> and may be rationalised in the same way, they are accompanied by ions shifted two and three Daltons towards the lower masses (for ex. m/z 431, m/z 445, m/z 459), indicating that the elimination of the carbomethoxy substituent from the macrocycle may also operate by loss of HCOOCH$_3$ and (HCOOCH$_3$ + H). The low mass end of the spectrum (i.e., m/z 45 to 300; not shown here) is quite identical to that of <u>1b</u>, confirming the same structure for the alcohol esterifying the propionate substituent.

Finally, one can see (Fig. 4A) that the mass spectrum of the synthetic pyro-pheophorbide-a stigmasteryl ester (<u>5</u>) is almost superimposable to that of the pyropheophytin-a (<u>1c</u>) at the m/z 400-550 range. This is easily rationalised by analogy with the fragmentation of <u>1c</u>: the loss of the steroidal alkyl from the molecular ion at m/z 928, with migration of an hydrogen atom to give the ionised free acid at m/z 534 which then fragments the same way as for <u>1c</u>. The low mass range of the spectrum (m/z 45-400) allows again to characterize the structure of the side chain since it is quite similar to that of the synthetic stigmasta-3,5,22-triene.

In conclusion, D/EI/MS mass spectra of above reference compounds indicated that this technique gives easily interpretable data with information on the chlorin macrocycle type and on the structure of the substituents.

The potential of the technique for the identification of an unknown series of chlorin steryl esters (CSE) can be seen from the mass spectrum shown in Figure 4B. It was obtained with D/EI/MS of a complex mixture isolated from a lacustrine sediment as described in the experimental part. About 2 μg of mixture was used to obtain this spectrum. Molecular ions at m/z 930, m/z 928, m/z 916, and m/z 902 indicates the presence of several components. However, the fragments in the mass range 400-600, characteristic of the macrocycle type, are quite similar to those observed in the spectra of <u>1c</u> and <u>5</u> indicating that all the components in the mixture have the same macrocycle, i.e., pyropheophorbide-a. This is also confirmed by the electronic absorption spectrum of the mixture, which is quasi identical with that of <u>1b</u> and <u>5</u>. Combined with the masses

FIG. 3

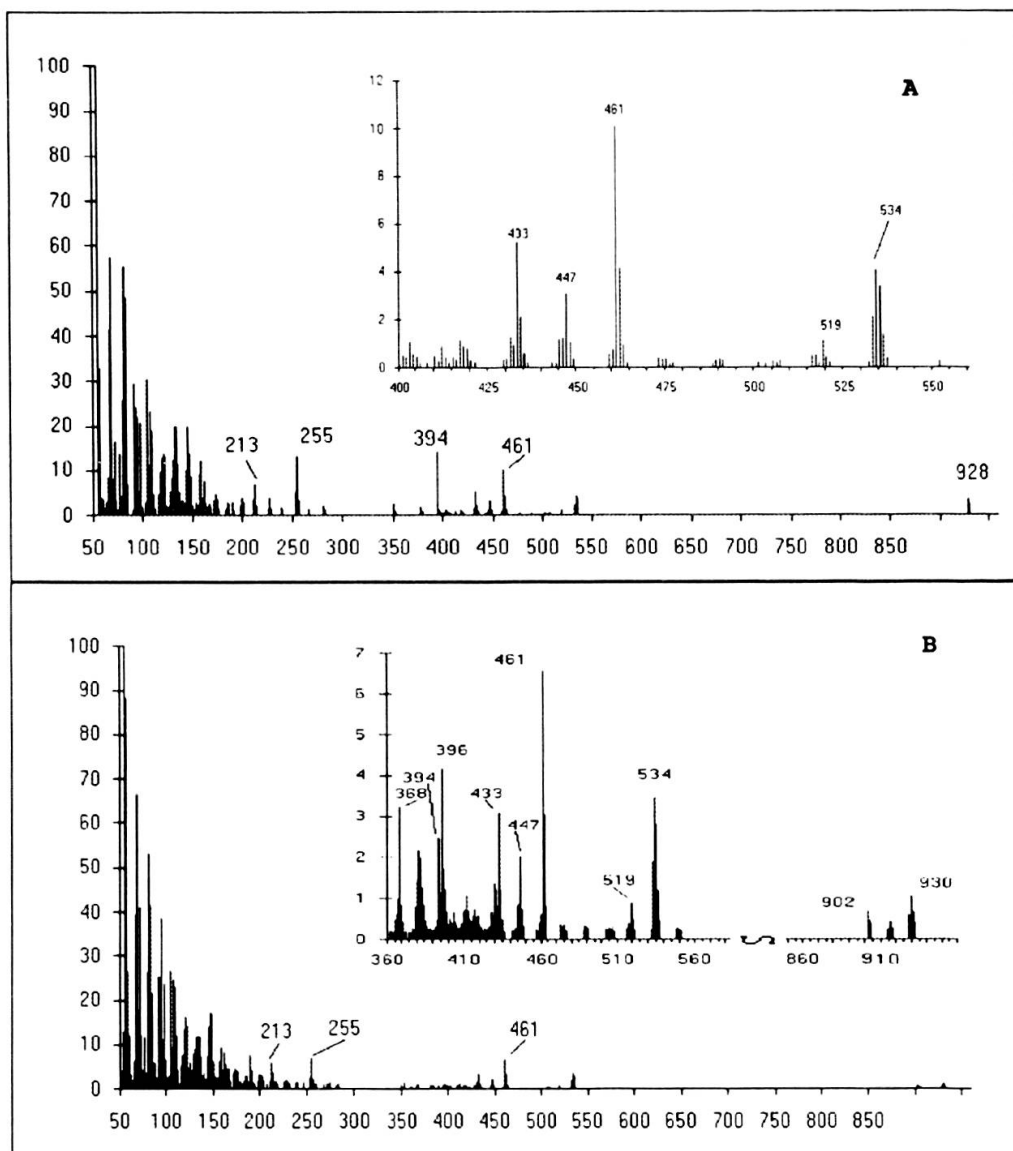D/EI mass spectra of compounds <u>1b</u>, <u>1c</u> and <u>2a</u>.

FIG. 4

(A) D/EI mass spectrum of synthesised standard of pyropheophorbide-a stigmasteryl ester. (B) D/EI mass spectrum of surface sediment chlorins steryl ester fraction isolated by HPLC.

of diverse molecular ions, these data allow to infer the molecular weights of the esterifying alcohols as 414, 412, 400, and 386. This is confirmed by the low mass range of the spectrum, 45-400 Daltons, containing the mass spectra of the ionised olefins produced from the side chain. Ions at m/z 396, 394, 382, and 368 corresponding to the olefins of the above alcohols are all observed. Moreover, the occurrence of an olefin ion at m/z 380 suggests also the presence of an alcohol with MW = 398, at low proportion however, since the corresponding molecular ion at m/z 914 is not visible. The steroidal character of these olefins may be easily deduced from the significant ions at m/z 255 (steroidal olefin-steroid side chain) and m/z 213 (steroidal olefin-side chain-steroid ring D). In conclusion, the unknown sedimentary mixture is composed of pyropheophorbide-

TABLE 1

Sterols mentioned in the next or referred to in Fig. 5.

Ref. N° in Figure 5	Trivial Name	IUPAC Name
1	cholesterol	cholest-5en-3β-ol
2	brassicasterol	24-methyl-cholesta-5,22-dien-3β-ol
3	campesterol	24-methyl-cholest-5en-3β-ol
4	stigmasterol	24-ethyl-cholesta-5,22-dien-3β-ol
5	β-sitosterol	24-ethyl-cholest-5en-3β-ol
6	dinosterol	4a,23,24-tri methyl-cholesta-5,22 dien-3β-ol
7	citrostanol	4a-methyl-24-ethyl-cholestan-3β-ol

a steryl esters including $C_{29:1}$, $C_{29:2}$, $C_{28:1}$, $C_{28:2}$, and $C_{27:1}$ sterols, all having a double bond in the cyclic skeleton, the second unsaturation being in the side-chain. The absence of a m/z 257 fragment excludes the occurrence of any stanol as esterifying alcohol.

To verify the above informations deduced from D/EI/MS, the mixture was hydrolysed and the released sterols were analysed by gas chromatography-mass spectrometry after derivatisation to TMS-ethers (see exp. part). Figure 5A shows the total ion chromatogram obtained from this experiment. The mass spectra (not shown here) of the five most intense peaks allows to identify them as cholesterol (MW = 386), brassicasterol (MW = 398), campesterol (MW = 400), stigmasterol (MW = 412), and β-sitosterol (MW = 414), corroborating the results from D/EI/MS.

Geochemical aspects: The GC-MS chromatogram of Figure 5B shows the distribution of free sterols found in the surface sediment. Their distribution in the water column was obtained from the analysis of the particulate matter collected in a sediment trap during 9 months and is shown in Figure 5C. The peak numbers in Figure 5 refer to sterols in Table 1.

A comparison of the three chromatograms in Figure 5 shows that stanols and 4-methylstanols, which are predominant components of sedimentary free sterols (Fig. 5B), are quasi absent in the water column and in the sedimentary CSE. On the other hand, cholesterol is the main sterol in the water column and in the sedimentary CSE but it is only present in trace in sediment sterols. The resemblance of the distribution of water column sterols with that of sedimentary CSE is remarkable. It is obvious that the sedimentary CSE are not produced in the sediment but in the water column. In fact, we could detect trace amounts of CSE in the water column material by HPLC/UV. We can therefore conclude that the formation of chlorin steryl esters happens in the water column and the reaction is certainly biologically mediated by water column organism(s), as already suggested (Eckardt *et al.*, 1992; Chillier, 1994; King & Repeta, 1994). Whatever the reaction mechanism, it is essential to note that CSE seem to include only sterols produced by the microplanktonic biomass of the basin and not those from allochthonous sources (like C_{29} sterols) or from macroorganisms (like 4-methylstanols) (Klink *et al.*, 1992).
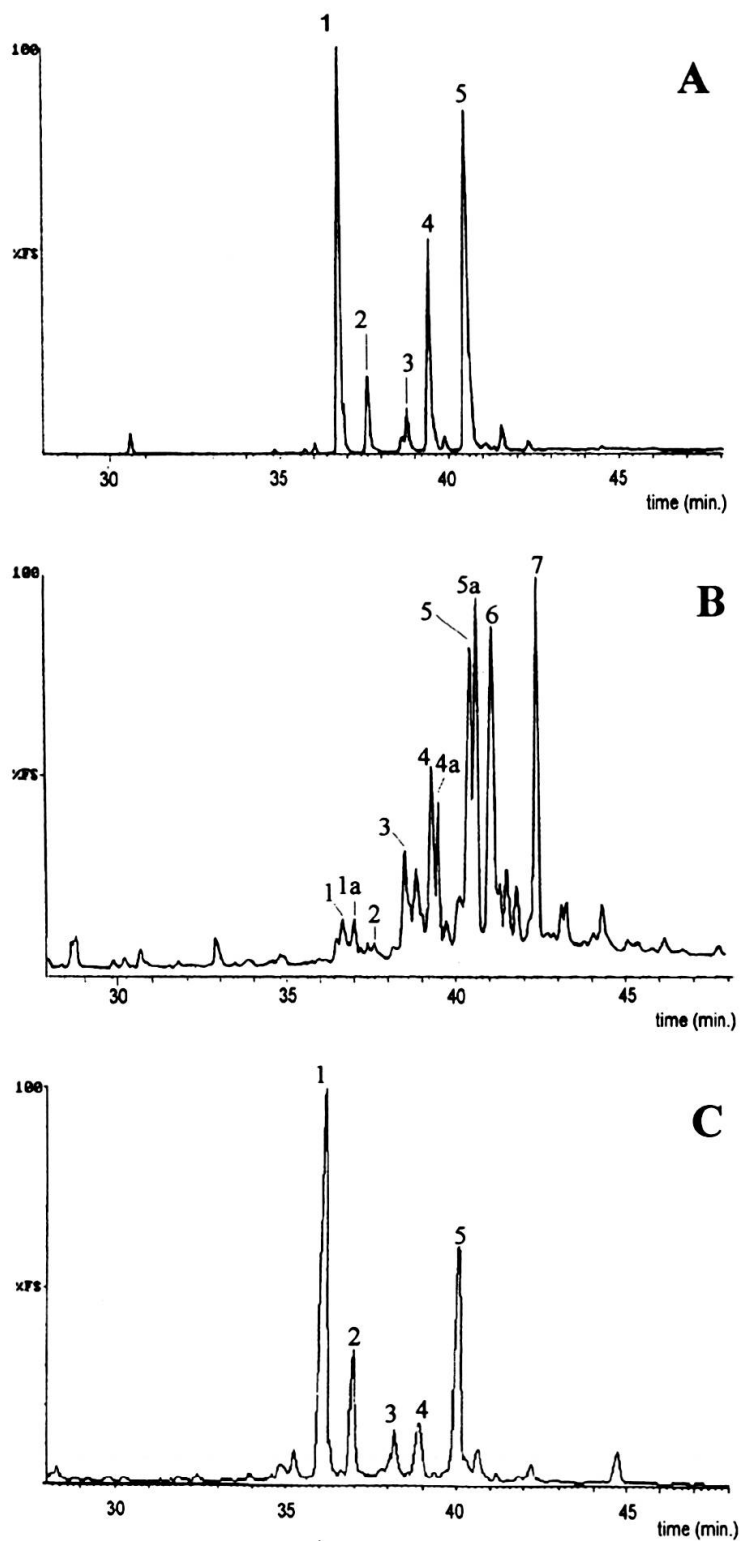
FIG. 5

GC/MS of (A) the sterols bound to pyropheophorbide-a in the surface sediment of Motte lake. (B) free sedimentary sterols (C) water column free sterols. The identification of the various sterols is given in Table I. Xa designs the stanol corresponding to the sterol X.

According King & Repeta (1994), the distribution of sterols in the sedimentary CSE should reflect the autochthonous biomass input at the time of deposition more precisely than free sterols do. Because sterols incorporated in CSE are protected towards preferential degradation or other diagenetic reactions, such as reduction of sterols to stanols. These post-depositional reactions can modify the original distributions of free sterols incorporated into the sediment. For example, the high proportions of 4-methylsterols in the sediment, as compared to the water column, would result from a preferential degradation of 4-desmethylsterols relative to 4-methylsterols. In fact, we have shown by heating experiments in laboratory, that the degradation rate of desmethylsterols versus 4-methylsterols is somewhat higher (Dreier et al., 1988). The differences in the rate constants can explain some preferential conservation of 4-methyl-sterols in ancient sediments but certainly not the predominance of 4-methyl-sterols in a surface sediment like the one we studied. On the other hand, King and Repeta's hypothesis would only be true if the sedimentary chlorin steryl esters were stable towards further diagenesis or if the post-depositional hydrolysis rates of different chlorin steryl esters were similar, which is yet to prove. In fact, in laboratory, we have been able to observe that the chlorin steryl esters are more stable than the chlorin phytyl esters towards alkaline hydrolysis. This observation suggests that the steric hindrance of the alcohol moiety plays a role in the hydrolysis rate of these compounds. Therefore, some reserve should be made about King and Repeta's hypothesis, when the CSE distributions of ancient sediments are to be used to infer paleobiological conditions of the water column.

CONCLUSION

The D/EI/MS technique described in this paper presents a clear advantage over other mass spectrometric techniques used in demetallated chlorin analysis by the fact that it gives information as well on the molecular ion than on the macrocycle type and the side chain structure. It allows to identify such a complex molecule as a chlorin steryl ester without ambiguity.

Studies of the distributions of chlorin steryl esters and free sterols in the surface sediment and in the water column shows that the formation of chlorin steryl esters happens certainly in the water column and concerns only sterols biosynthesised by the microplankton; sterols from allochthonous sources or from aquatic macro-organisms are not incorporated into CSE fraction. Therefore, although with some reserves, the studies of CSE in ancient sediments should provide paleoecological information on planktonic populations of the depositional environments.

ACKNOWLEDGEMENTS

RÉSUMÉ

En utilisant une sonde d'introduction étendue il est possible de désorber des chlorines de haut poids moléculaire dans la source d'ionisation d'un spectromètre de masse conventionnel. Couplée avec l'impact électronique, la technique permet d'obtenir des spectres de masse contenant des ions caractéristiques de la structure, à savoir des ions moléculaires ainsi que des ions typiques du macrocycle et de ses substituants. Cette technique a été utilisée avec succès pour l'identification des chlorine stérylesters dans les sédiments de surface et dans la colonne d'eau d'un lac du bassin lémanique. La comparaison des distributions des stérols incorporés dans ces esters avec celles des stérols libres trouvés dans la colonne d'eau et dans le sédiment permet de répondre à certaines questions relatives à la formation et à la signification géochimique de cette nouvelle classe de marqueurs biologiques.

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