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Determination of porphyrins and metalloporphyrins using liquid chromatography–diode array detection and mass spectrometry

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Abstract

A HPLC procedure has been optimized and successfully applied to porphyrins of environmental interest, such as etio and octaethylporphyrins and their VO and Ni compounds. The use of UV–Vis diode array allowed the detection of the analytes within the 5–15 ng/ml range. In order to achieve greater specificity and some structural information, the coupling of liquid chromatography with mass spectrometry was investigated, and the particle beam interface conditions were optimized. Electron impact (EI) spectra, comparable to those reported in the literature were obtained. The entire procedure has been applied to a real marine sediment, previously spiked with porphyrins to resemble oil-contaminated samples. The results pointed out that the method is suitable for such levels of analytes (5–10 μg/ml), allowing their identification and quantification with no need for purification steps.

Keywords: Porphyrins; Metalloporphyrins

1. Introduction

Metalloporphyrins occur almost ubiquitously as complex mixtures in organic-rich ancient rocks, crude oils, oil shales, coals and sedimentary rocks [1]. In marine sediments they result mainly from modification of the chlorophylls of phototrophic organisms present in the water column at the time of sediment deposition [2].

Porphyrins play a variety of important biochemical roles: they are the active site of many enzymes (cytochromes, catalase, peroxidase, etc.); well-known iron porphyrin complexes are oxygen-carrying proteins (hemoglobin, myoglobin) [3].

They are also important in biomimetic chemistry; in fact they are widely used as models to study oxygen transport [4].

Hence, qualitative and quantitative determination of porphyrin distribution has application in biological, biomedical and earth sciences, such as in the diagnosis of metabolic abnormalities or in the assessment of palaeoenvironments [5]. As an example of the latter, the presence in sedimentary organic matter of specific high-molecular-mass components, deriving from chlorophyll degradation by anaerobic phototrophic bacteria, indicates the occurrence of

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anoxygenic photosynthesis in the original water column [6].

Reversed-phase chromatography, with its ability to separate homologous series of molecules [7], is an attractive method to speciate the metal-containing compounds; in fact, in recent years, the application of high-performance liquid chromatography (HPLC) has expanded to the separation of several metalloporphyrins [8].

Different types of detectors have been used, but higher specificity and structural information would be very useful in extensive investigation of these compounds. Recent studies have focused on the development of liquid chromatography–mass spectrometry (LC–MS) coupled techniques. Several workers have analyzed petroporphyrins using the interfaces based on aerosol formation such as thermospray, particle beam (PB) [9], electrospray [10], atmospheric pressure ionization [11] and laser desorption [12].

The aim of the present work was to develop a new analytical methodology based on LC–MS, for the determination of porphyrins and metalloporphyrins of environmental interest, especially in marine matrices. Recently, the coupling of HPLC to MS by means of a particle beam interface provided good results for other organometallic compounds, such as the alkyltins [13].

In contrast to alkyltins, porphyrins are good chromophores; therefore, in this case UV–Vis diode array detection (DAD) can be employed to develop the chromatographic method. Successful separation of three octaethylporphyrins on a reversed-phase (RP) was obtained in preliminary studies [14]. In order to expand the applicability of HPLC to a variety of metalloporphyrins it was necessary to obtain retention times and other chromatographic data related to other compounds of interest. The mass spectrometer was then coupled at the exit of the DAD system to verify if it was possible to obtain reliable electron impact (EI) spectra and achieve a more specific technique.

2. Experimental

2.1. Reagents and standards

Octaethylporphyrins (OEP, VO OEP, Ni OEP) were obtained from Aldrich (Milan, Italy) while etioporphyrins (Etio, VO Etio, Ni Etio) were obtained from Porphyrin Products (Logan, UT, USA).

Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

The water used to prepare all the solutions and the eluents was obtained from a Milli-Q System apparatus (Millipore).

Stock solutions of porphyrins (100 μg/ml) were prepared by dissolution of pure compounds in CH_3CN or in different CH_3CN–$CHCl_3$ mixtures and stored at 4°C. Standard solutions were then obtained by dilution with CH_3CN.

All reagents and chemicals were of analytical or chromatographic grade.

2.2. Liquid chromatography

A Hewlett-Packard 1090-II liquid chromatograph (Palo Alto, CA, USA) equipped with a UV–Vis diode array detector was used. Separations were carried out at room temperature on a Supershere RP-18 analytical column, 4 μm particle size, 125×2 mm (Merck). Samples were introduced by a 20-μl Rheodyne (Cotati, CA, USA) injector. If not differently indicated, the flow-rate of the mobile phase was 0.4 ml/min. Gradients were formed between two helium degassed solvents. Solvent A was acetonitrile–methanol (50:50, v/v) and solvent B was water. The optimized gradient conditions were: 0–22 min 94–100% A with final hold of 100% A for 2 min.

The following wavelength gradient was utilized to detect the porphyrins: 0–8.0 min: 401 nm; 8.0–11.5 min: 399 nm; 11.5–13.5 min: 391 nm; 13.5–30.0 min: 386 nm.

2.3. Particle beam mass spectrometry

A 59980B Hewlett-Packard particle beam interface coupled to a 5989A Hewlett-Packard quadrupole mass spectrometer was used.

The EI ion source conditions were: electron energy 70 eV, temperature 250°C. The analyzer temperature was set at 100°C.

The PB interface optimization was performed with standard solutions of each porphyrin, in the con-

centration range 5–10 μg/ml, introduced by flow injection. A mixture of acetonitrile–methanol–water (47:47:6) was used as the eluent. Each measurement of ion current was repeated three times and the mean value was considered. The final optimal conditions were: He pressure 324 kPa, capillary tubing position 8, desolvation chamber temperature Maximum, LC flow-rate 0.4 ml/min.

When LC and MS were coupled, the analyses were performed in the selected ion monitoring (SIM) mode as follows: 0.0–9.0 min: 543, 544 (VO Etio); 9.0–12 min: 599, 600 (VO OEP); 478, 479 (Etio); 12–18.3 min: 534, 535 (OEP and Ni Etio); 18.3–30.0 min: 590, 591 (Ni OEP). The dwell time was adjusted to obtain 1 cycle/s.

2.4. Spiked sediment

The proposed method was checked on an Antarctic marine sediment, which was spiked with the porphyrins of interest. The absence of the analytes in the sediment was verified prior to the use. The spiking procedure was as follows: 10 g of the sediment was placed in a glass vessel together with 20 ml of synthetic sea water, in which 10 μg of each porphyrin was dispersed; the vessels were then shaken for 14 days, in order to realize a satisfactory interaction between the porphyrins and the sediment. The sediment was then separated by centrifugation, washed and dried in a oven at 40°C. According to procedures reported in the literature [21] the porphyrins were then extracted by Soxhlet with $CHCl_3$. Finally the extract was dried in a rotary evaporator, redissolved in 1 ml of CH_3CN and analyzed by LC–DAD–MS.

The experiment was repeated on three aliquots of the sediment sample.

3. Results and discussion

3.1. Liquid chromatography

At the beginning of the work we considered only three octaethylporphyrins, modifying the procedure proposed by Sundararaman and Vestal [9], essentially changing the gradient rate. Afterwards, when the three etioporphyrins were added, the gradient had to be further modified, as described in the Experimental section.

For each porphyrin the UV–Vis spectrum was measured, in order to obtain wavelengths of maximum absorption; then, a wavelength gradient was realized to get the best sensitivity for all analytes. Detection limits, calculated as 3σ, were satisfactory for each porphyrin and ranged from 5 to 15 ng/ml. Calibration curves with a wide range of linearity were obtained, enabling the use of the method for real samples analysis. These parameters, together with standard deviation of five replicates, are shown in Table 1.

The chromatogram of a mixture of the six porphyrins is reported in Fig. 1. Separation is very satisfactory, characterized by symmetrical peak shape and good column efficiency, and it is achieved in less than 20 min. We can note that retention times of the different OEPs are always longer than those of the analogous Etio compounds, as expected from the molecular structures. In fact, while OEPs present eight ethyl groups around the porphyne ring, the etio porphyrins have four ethyl and four methyl groups which enhance their polarity.

3.2. Particle beam optimization

The PB interface allows both the coupling of a

Table 1
Figures of merit of the HPLC–DAD separation

Porphyrin	Linearity range (ng/ml)	*R*	Detection limit (ng/ml)	Standard deviation (%)
VO Etio	30–4000	0.9998	5	1.0
VO OEP	40–6500	0.9989	10	1.0
Etio	70–4000	0.9998	10	1.1
OEP	50–8000	0.9997	15	0.7
Ni Etio	40–4000	0.9999	10	0.9
Ni OEP	30–5500	0.9999	10	1.0

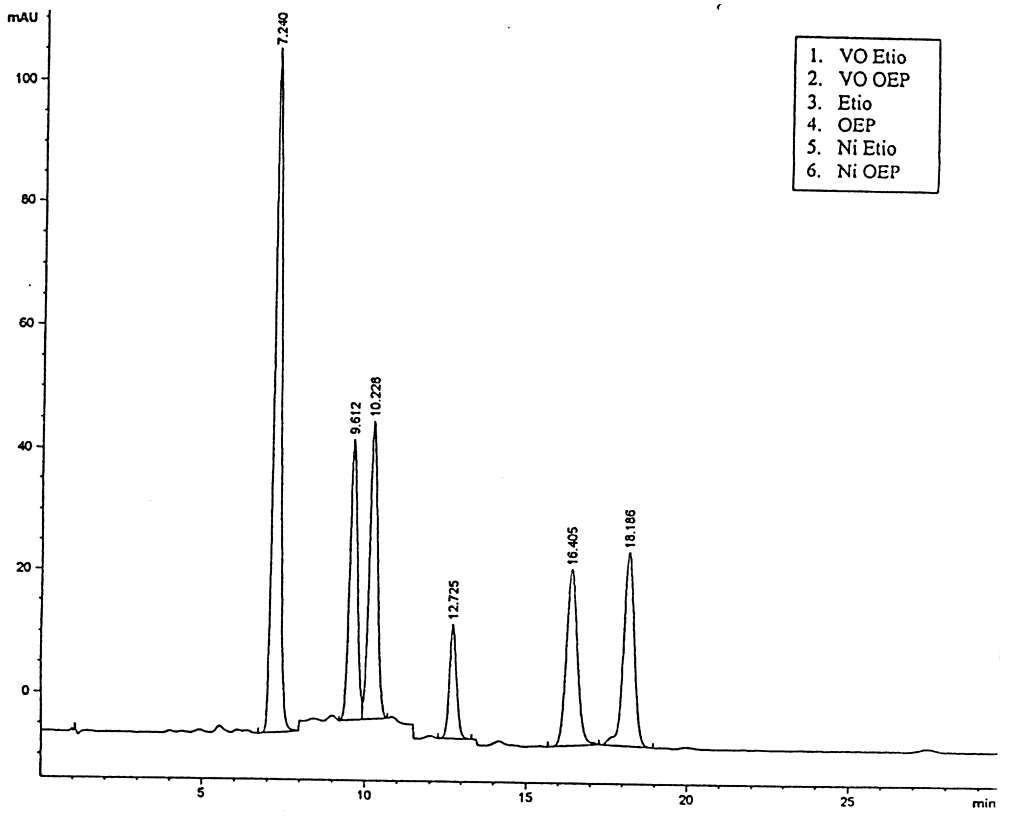

Fig. 1. Chromatogram of a standard mixture of the six porphyrins at 2 μg/ml each.

conventional HPLC system to a mass spectrometer and the acquisition of library searchable EI spectra [15,16], representing a viable alternative for the analysis of small molecules with properties which are not suitable for gas chromatography. The eluent coming from the chromatographic column is converted in aerosol, the solvent vapors are removed and the analyte is introduced into the EI source. The analyte is thus ionized not as a gaseous distribution of single molecules, but as a homogeneous dispersion of molecular aggregates [17,18].

Many variables influence the PB efficiency in delivering the solute to the ionization source, so an accurate optimization of the PB interface is required.

We have considered the influence of the following parameters on the determination of the porphyrins: the position of the fused-silica capillary tubing in the desolvation chamber, the flow pressure of He gas that produces the aerosol, the mobile phase flow-rate and the desolvation chamber temperature. Initially, the MS signal of each compound was measured by integration across the total ion current (TIC); because no effects on the reliability of the spectra during this rough optimization were observed, the PB was then optimized by measuring the molecular ion current in SIM mode. Firstly, the He pressure and the capillary position were optimized: the effect of these parameters was quite similar to that already observed in our previous works [13,19]. The capillary optimal position corresponded to the capillary tube slightly retracted inside the nebulizer tip, while a He pressure value of 324 kPa resulted in the best setting.

Concerning the LC flow-rate we could observe a poor increase of the MS signal while reducing it from 0.4 to 0.2 ml/min with only one exception, octaethylporphyne, that showed an improvement of sensitivity around 15–20%. The reduction of the

flow-rate to obtain such inconsiderable improvements would unacceptably increase the time of analysis, thus the flow-rate of 0.4 ml/min appears the best choice.

The effect of the temperature is critical in the PB interface because it is usually a compromise. In fact a temperature enhancement increases the vapor pressure and the eluent is more easily removed in the desolvation chamber. At the same time, because the analyte volatility increases as well, a temperature enhancement usually increases the analyte losses. In our previous experience, the optimal temperature commonly resulted around 60–70°C: higher temperature caused a decrement of the sensitivity, probably due to the less efficient mass transport of the analyte described above. As shown in Fig. 2, the porphyrins behavior is different: we observe the best sensitivity when the desolvation chamber temperature is set at maximum (this temperature value is not indicated on our PB interface, but it could be estimated around 85–90°C). It is difficult to ascribe such behavior to the eluent since we already used a similar mobile phase when analyzing other compounds, but we were never able to reach such high temperatures. A possible explanation could be found in the strong difference in vapor pressure between the eluent and the analytes. The considered porphyrins are relatively heavy molecules (500–600 u) and they show a very low volatility at 90°C; that allows one to reach a high temperature in the desolvation chamber, removing the eluent more efficiently, without remarkable losses of the analytes.

3.3. Acquisition of EI spectra

The EI spectra were collected by flow injection–PB-MS. In Fig. 3a–c spectra of the etio porphyrins are reported. Comparable spectra were obtained for

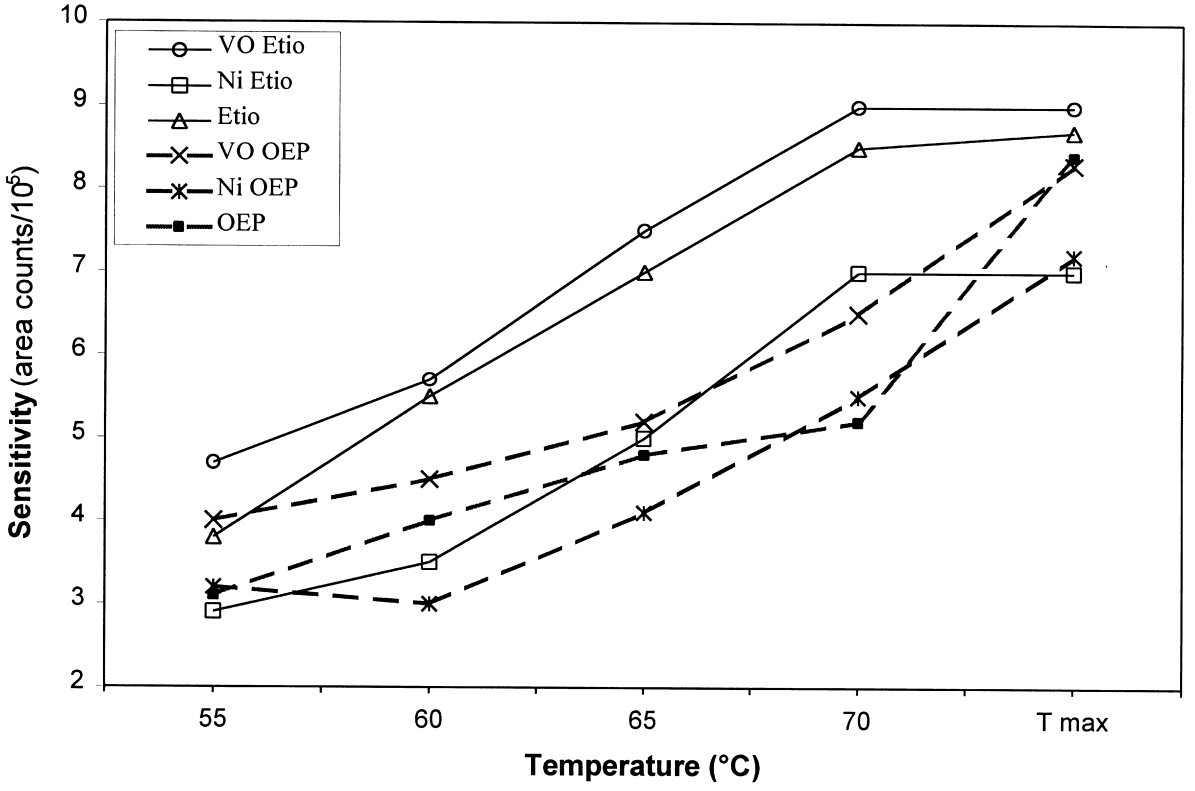

Fig. 2. Effect of the temperature of the desolvation chamber of PB on the mass spectrometer sensitivity. Each point represents the mean value of three injections.

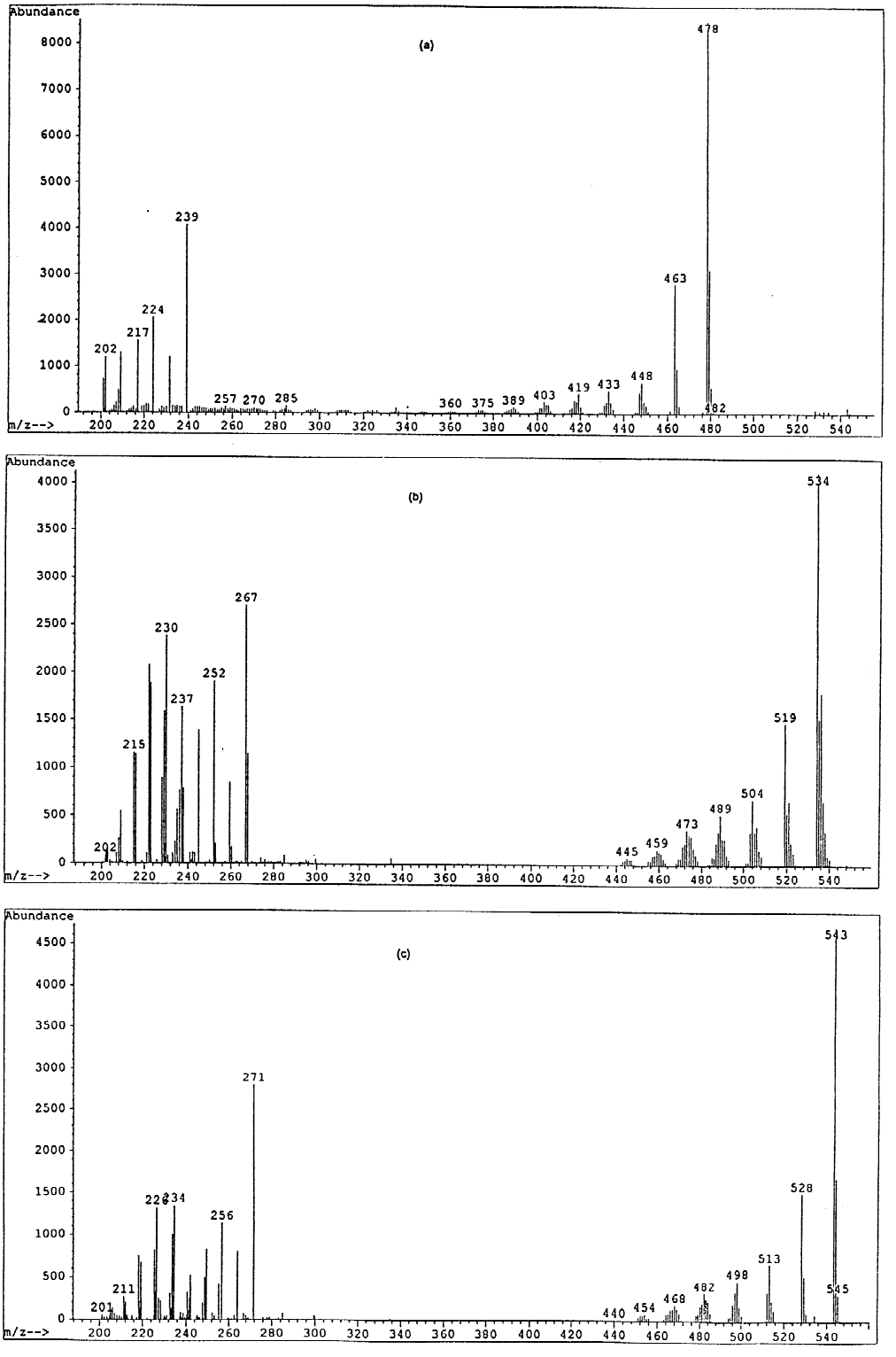

Fig. 3. EI spectra of the etioporphyrins: Etio (a), Ni Etio (b), VO Etio (c).

the octaethylporphyrins. The mass spectra are similar to those reported in the literature [20]; they are characterized by an intense signal of the molecular ion, with fragmentations deriving from the successive methyl losses, and by the doubly charged ion. Moreover, the spectra of the Ni-porphyrins show the isotopic pattern of Ni: it is easy to distinguish the three more abundant isotopes, corresponding to ^{58}Ni (100%), ^{60}Ni (38.5%), ^{62}Ni (5.3%).

All the spectra were collected at different times and at different analyte concentrations: the ratio between the peaks of each EI spectrum never changed significantly.

3.4. Coupling HPLC and MS

The chromatographic column was reconnected and a standard mixture containing the six porphyrins at 2 μg/ml was injected into the LC–MS instrument.

In order to achieve the best sensitivity, the mass spectrometer was programmed in the SIM mode: for each porphyrin the molecular ion $[M]^+$ was chosen as the characteristic ion; its isotopic ion $[M+1]^+$ was also monitored as the qualifier ion.

The obtained MS chromatogram (SIM mode) is reported in Fig. 4. As can be observed by comparing this chromatogram with that of Fig. 1 (DAD), the coupling of LC to MS produces a delay of retention times in the mass chromatogram (about 20 s). Due to the extra-column diffusion that occurs in the capillary connection between the DAD system and the PB and inside the desolvation chamber of the PB itself, a general broadening of the peaks is observed. This broadening determines a partial overlapping of the peaks 2 and 3, corresponding to VO OEP and Etio, respectively, but it is not an important drawback because we determine the single analyte by extracting its molecular ion. By comparing the chromatograms, a better signal/noise ratio for the DAD system can be observed.

3.5. Real sample

In order to verify the applicability of the proposed

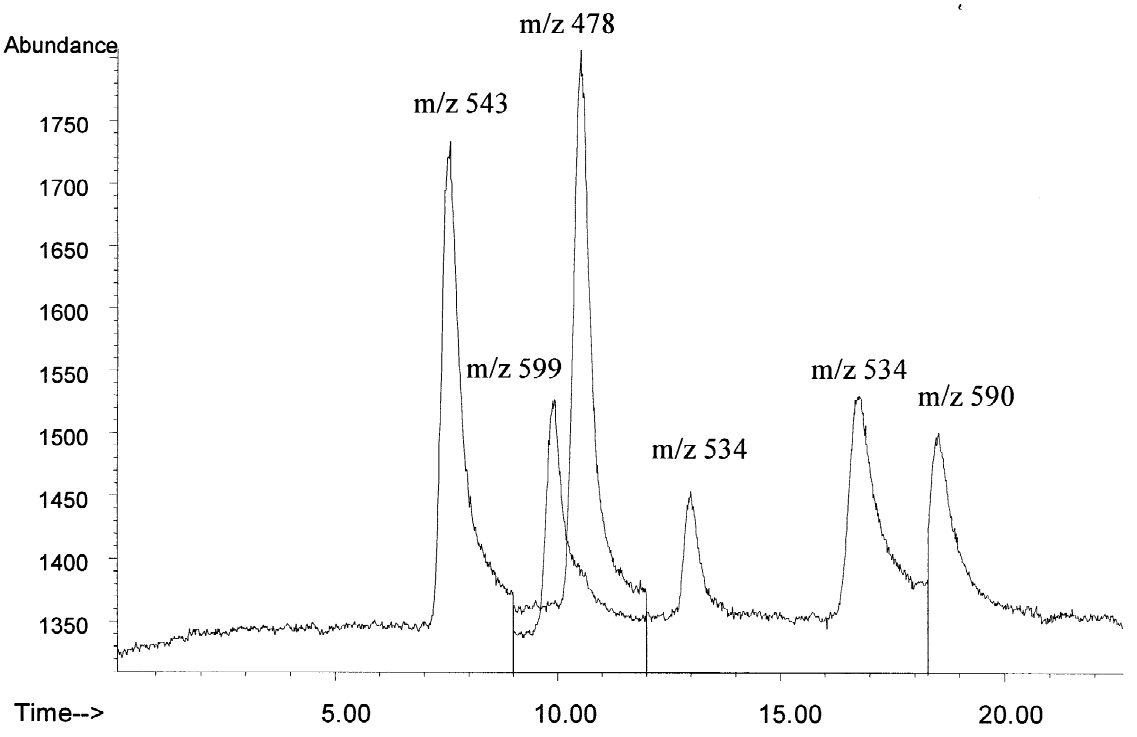

Fig. 4. Mass chromatogram (SIM of the molecular ion) of a standard mixture of the six porphyrins at 2 μg/ml each.

methodology to real samples, we used a marine sediment spiked in our laboratory with the porphyrins of interest. It would be preferable to test the method on a certified standard but, to our knowledge, there are no certified materials available for the porphyrins.

In Fig. 5 both the UV–Vis and the mass chromatograms are reported.

In the UV–Vis chromatogram the presence of co-extracted impurities is evident, but the great difference in retention times allows the quantification of the analytes. On the contrary, while the porphyrins are all clearly detected in the mass chromatogram, quantification for some of them is more difficult. Quantitative analysis, performed by the UV–Vis calibration curves described in Section 2.2, outlines that the porphyrin amounts in the sediments vary from 5.5 μg for OEP to 8.2 μg for VO Etio. The reproducibility among the three experiments is rather good, with a mean standard deviation of 7%.

Assuming that, as reported in the literature, Soxhlet extraction with $CHCl_3$ is practically quantitative,

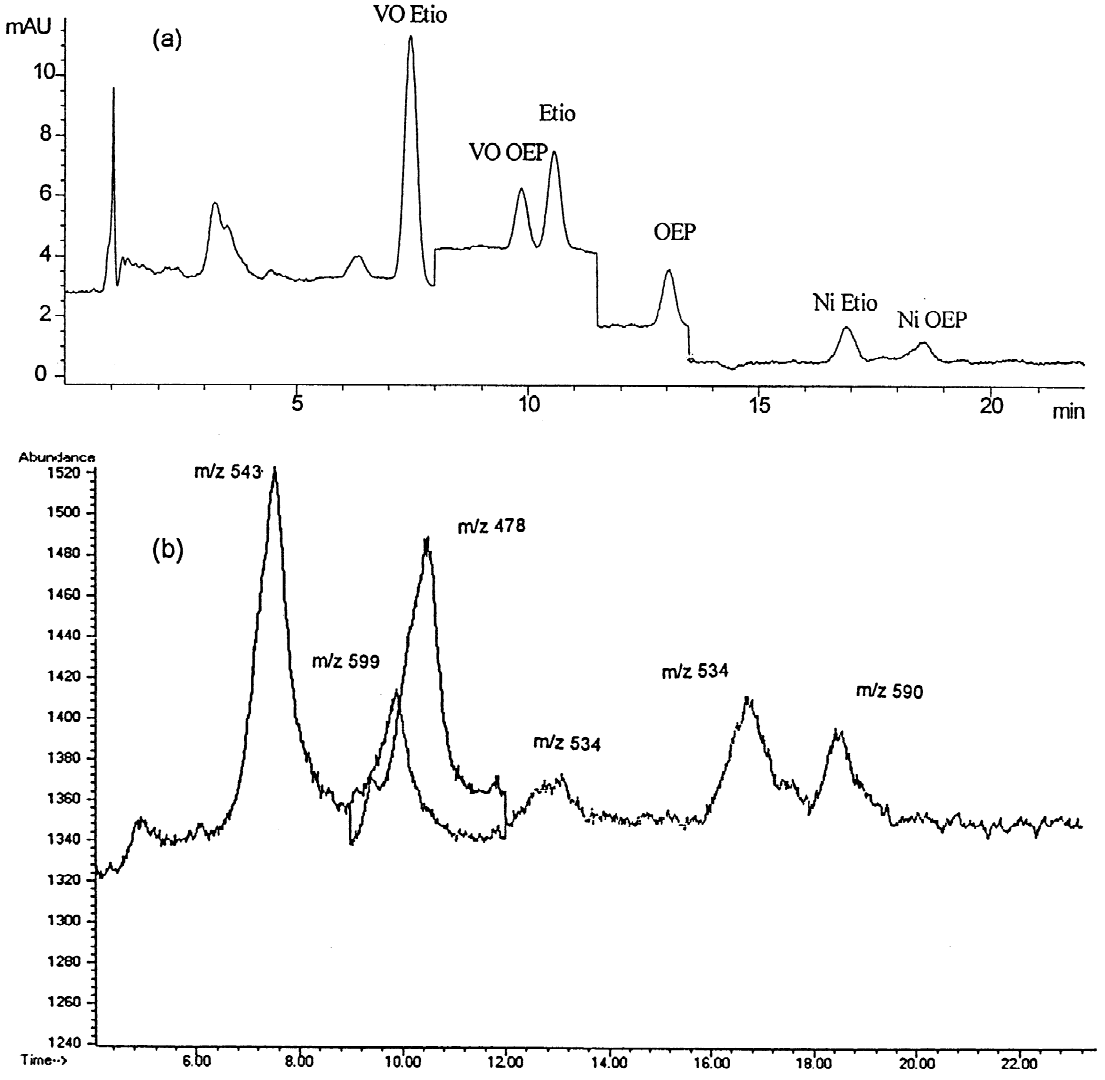

Fig. 5. UV–Vis chromatogram (a) and SIM chromatogram of the molecular ion (b) of the extract of the spiked sediment.

it is evident that absorption of the porphyrins on the sediment is not complete. This is confirmed by the analyses of the surnatant solutions, which show the presence of the analytes, even after 2 weeks of continuous contact. Anyway, the aim of the experiment has been fairly obtained: in fact the proposed method was suitable to analyze porphyrins in real sediments. Although the spiking amount is rather high, the resulting concentrations in the sample are not significantly different from those found in oil contaminated sediments [22].

4. Conclusions

The HPLC–DAD procedure, characterized by an excellent separation, and by good detection limits and linearity, was found to be suitable to analyze the considered classes of porphyrins. The low retention times, together with the good efficiency, allow the extension of the method to other classes of porphyrins or other homologous compounds. This is confirmed by some preliminary experiments effected on Zn, Cu and Fe octaethylporphyrins.

As the PB interface features were suitable for the porphyrins characteristics, it was possible to realize the coupling of LC to MS, achieving a more specific LC procedure.

Preliminary work on spiked real sediments have demonstrated the applicability of the entire procedure to oil-contaminated samples. In case the analyses should involve matrices containing natural levels of metalloporphyrins, the analytical procedure should be improved with a preconcentration step.

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