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RAPID SEMI-PREPARATIVE CLASS SEPARATION OF ORGANIC COMPOUNDS FROM MARINE LIPID EXTRACTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND SUBSEQUENT QUANTITATIVE ANALYSIS BY GAS CHROMATOGRAPHY

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SUMMARY

Quantitative analysis of biogenic and anthropogenic organic markers in the atmospheric and marine environment is difficult, owing to the low concentration of these compounds in air and water.

In the present study, a rapid semi-preparative separation of lipids extracted from a recent marine sediment is proposed by liquid chromatography on a silica column. The basic idea of this method is to carry out a precisely controlled gradient elution (with emphasis on solvent purity, water content of the mobile phase, etc.) in order to obtain reproducible retention times, which would allow separation without detection. Each column is first calibrated with standards at detectable concentration using UV and RI detection. The saponified extract is directly injected on the column and then eluted by a mobile phase of isooctane containing from 0.5% to 10% of 2-propanol. In a single injection, the following classes can be separated with good resolution: alkanes, aromatic hydrocarbons, fatty acids, alcohols, sterols and hydroxy-fatty acids. The total time of 40 min, includes a recycling time of 10 min. Each fraction is then derivatized and analyzed by gas chromatography or by gas chromatography coupled to mass spectrometry. Quantitative analysis is carried out with standards.

INTRODUCTION

Environmental organic chemistry has made tremendous progress during the past ten years owing to the development of separative chromatographic techniques coupled with spectroscopic analysis, mainly gas chromatography–mass spectrometry (GC–MS).

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Both organic geochemistry and marine organic chemistry use lipids as markers of biogeochemical processes in sedimentary[1–3] or marine environments[4,5]. Each class of markers brings its own information to the understanding of these processes. Qualitative and quantitative analysis of the various chemical compounds in a sample is in general required to reach conclusive data. One of the difficulties in this work consists in running as far as possible a complete molecular analysis of complex mixtures on trace amounts of organic matter (less than 5 mg of total lipid extract). So an analytical method is needed which can rapidly separate the lipid extract into the various main chemical classes with sufficient resolution and allow subsequent quantitative analysis.

High-performance liquid chromatography (HPLC) on silica columns is a convenient method of separation according to functional groups with a good resolution. It looks suitable to improve the separation procedure that is generally used in geochemistry and marine chemistry. Lipids are extracted by organic solvents and usually subjected to potassium hydroxide–methanol saponification prior to chromatographic separation[2,4]. Unsaponified and saponified compounds are separated by successive liquid–liquid extractions in basic and acidic media. Silica gel chromatography (on column or on thin-layer plates) is generally used for separation of the different classes of lipids: aliphatic hydrocarbons, polycyclic aromatic hydrocarbons, fatty acids, fatty alcohols and sterols (*cf.* procedures A and B, Fig. 1). Unfortunately, liquid–liquid extraction in basic media becomes quantitatively unworkable for low-concentration samples.

A procedure for low-concentration samples consisting in a separation on a silica gel column directly after saponification has been successfully used[4] (*cf.* procedure B, Fig. 1). The difficulty in this procedure is the poor resolution of alcohols,

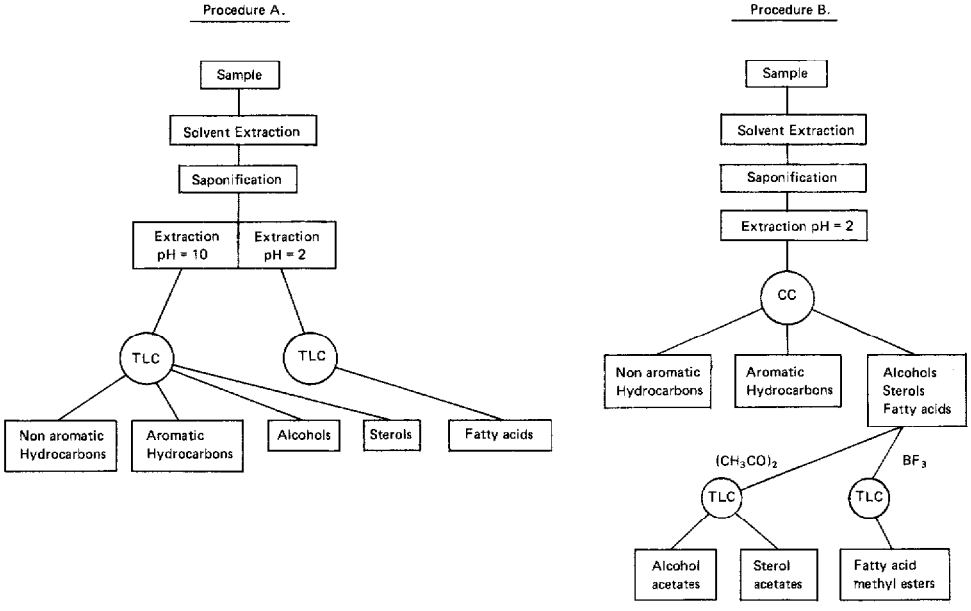

Fig. 1. Procedures used to separate lipid extracts into the various main chemical classes; CC, silica gel column chromatography.

sterols and fatty acids, which need further separation after derivatization.

These two methods give rise to the problem of a long analysis time owing to the several extraction and separation steps and, for thin-layer chromatography, owing to the delicate quantitative extractions of the compounds from silica. In addition, for procedure B, it is necessary to fractionate the samples into aliquots, which is not convenient for very low-concentration extracts.

In this paper, we present an HPLC separation which considerably shortens the time of analysis and solves most of the aforementioned problems.

EXPERIMENTAL

Lipid sample preparation

We have chosen intertidal sediment sampled in Birlot bay, Brehat Island, Brittany, France, as representative of a natural environment. The sandy sediment was scraped from an area of 0.25 m^2 to a depth of *ca.* 2 cm. Wet sediment was stored in aluminium foil jars at room temperature and frozen immediately in the laboratory. An amount of 500 g of the sediment previously freeze-dried was extracted twice with 500 cm^3 of methanol–toluene (7:3) for 24 h. The extract was then evaporated and taken up into a solution of methanol–water (5:1, 20 cm^3) 1 *N* in potassium hydroxide and refluxed for 2 h under argon. Saponified and unsaponified lipids were extracted with heptane–diethyl ether (1:1) after acidification of the methanolic solution to pH 2 with concentrated hydrochloric acid. The total extract (24.5 mg) was evaporated and dissolved in 20 cm^3 of chloroform and divided into ten aliquots.

Liquid chromatography

Separations were performed on a 20 cm × 4.1 mm I.D. column packed with 5 μm silica, Spherosil XOA 600 normaton (Rhone Poulenc Industry, France) using a 5060 Varian pump (Varian, Palo Alto, CA, U.S.A.) at a flow-rate of 2 cm^3 min^{-1}. All injections were made through a 1.115-cm^3 sample loop with a conventional injection valve (Rheodyne, Berkeley, CA, U.S.A., Model 7010). Detection was performed with a UV spectrophotometer (Pye Unicam, Cambridge, U.K.) at 210 nm or with a R401 refractometer (Waters Assoc., Milford, MA, U.S.A.). Mobile phases were mixtures of 2-propanol and isooctane from SDS (Valdone, France).

Gas chromatography

Alkanes and alcohols were directly analysed by GC respectively on a 25 m × 0.32 mm I.D. glass WCOT SE 52 column, temperature-programmed from 100 to 250°C at 2°C min^{-1}, and on a 25 cm × 0.32 mm I.D. fused-silica CP Sil 5 column, temperature-programmed from 120 to 300°C at 2°C min^{-1}. Carrier gas was helium at a flow-rate of 4 cm^3 min^{-1}. Field ionization detectors were used with a flash vaporization injector.

Fatty acids were esterified by heating with 14% BF_3 in methanol (2 cm^3, 50°C) for 20 min and their methyl esters were chromatographed on a 25 m × 0.32 mm I.D. fused silica CP Sil 5 column programmed from 120 to 300°C at 2°C min^{-1} or a 25 cm × 0.32 mm I.D. glass WCOT FFAP column programmed from 100 to 190°C at 2°C min^{-1}.

Alkanes, alcohols and fatty acid methyl esters were identified by comparison

of their retention times with authentic standards and spiked with internal or external standards for quantitative determinations.

Gas chromatography–mass spectrometry

Sterols were converted into acetate derivatives and analyzed using a Ribermag R 10-10 capillary GC–quadrupole MS system fitted with a 25 m × 0.32 mm I.D. fused-silica WCOT CP Sil 5 column coupled to the source. The GC oven was programmed from 100 to 300°C at 3°C min^{-1} with helium as carrier gas (2 cm^3 min^{-1}). Typical MS operating conditions were used and mass spectra were obtained by scanning from 100 to 500. Data acquisition limits for the various lipid classes are *ca.* 6 ng per g of sediment. Blanks levels for each class were less than 100 times the sample concentration.

RESULTS AND DISCUSSION

HPLC separation

A preliminary study with simulated samples containing alkanes, alcohols, fatty acids, and sterols was carried out and each class was separately examinated.

Fatty acids. Some authors prepared derivatives that could be detected by UV spectrophotometers[6,7] or spectrofluorimetric detectors[8], but these derivatives are not convenient for further GC–MS analysis. Refractometric detection is not sensitive enough[9,10]. We chose UV detection at 210 nm without derivatization. In this case, the minimal detectable injected amount of palmitic acid is *ca.* 5 μg.

Fig. 2 shows the variation of the logarithmic capacity factor (log k') as a function of the number of carbon atoms of the *n*-alkyl chain for various fatty acids (saturated and unsaturated). Long-chain acids are eluted first and log k' increases

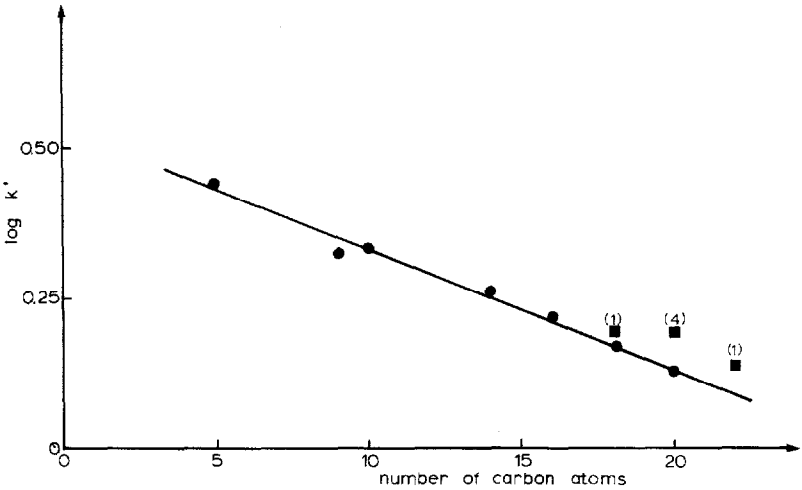

Fig. 2. Variation of the capacity factor (log k') with the number of carbon atoms in the *n*-alkyl chain of the fatty acids. ●, Saturated fatty acids; ■, unsaturated fatty acids (numbers in parentheses indicate the number of double bonds in the carbon chain). Column, Spherosil XOA 600, 5 μm, 20 cm × 4.1 mm I.D.; flow-rate, 2 cm^3 min^{-1}; pressure, 80 bar; mobile phase, 99% isooctane and 1% 2-propanol; detection, UV at 210 nm.

linearly with carbon number. This figure shows also that the retention time of an unsaturated acid is a little longer than that of the corresponding saturated acid. These results were observed for various compositions of the mobile phase. It is possible to extrapolate the retention times of fatty acids from the retention measurements of two or three.

Hydroxy fatty acids. Results were similar to those obtained for fatty acids.

Sterols. As they are detectable at 210 nm, the separation of sterols is easy, even at low concentration.

Alkanes and alcohols. They are practically undetectable by UV or RI detection at a concentration of 1 g l^{-1}. As the mobile phase is isooctane containing less than 5% of 2-propanol, it is impossible to obtain concentrated solutions. The only way to detect them is to fractionate the column effluent and to analyse it by GC without derivatization. The influence of the alkyl chain length and the number of carbon–carbon double bonds is supposed to be the same as those observed for fatty acids.

Separation programme

A sample containing *n*-alkanes (C_{10}, C_{12} and C_{16}), *n*-alkanols (from C_8 to C_{22}), *n*-fatty acids (from $C_{10:0}$ to $C_{23:0}$ as saturated fatty acids and $C_{18:1}$ as unsaturated), hydroxy fatty acids ($C_{11:0}$, $C_{18:0}$, $C_{18:1}$), and sterols (cholestanol, stigmasterol, 7-dehydrocholesterol and coprostanol), was prepared in a solution of isooctane with 0.5% of 2-propanol. The concentration of each compound is *ca.* 40 mg l^{-1}. The chromatogram obtained and the elution gradient are shown in Fig. 3. Mobile-phase composition varies from 0.5% of 2-propanol in isooctane to 10%. The fraction

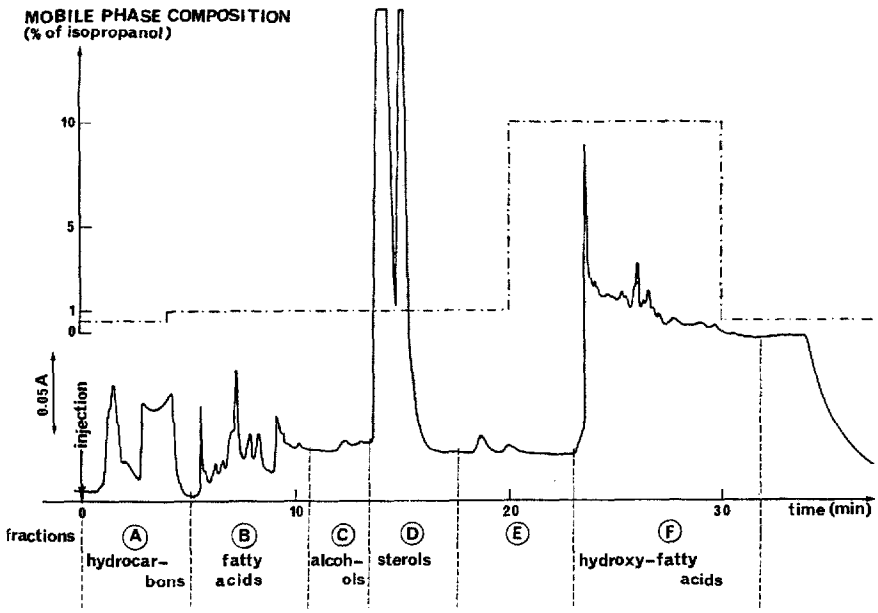

Fig. 3. Chromatogram obtained from 1.115 cm^3 of the standard solution: alkanes, alcohols, fatty acids, sterols and hydroxy fatty acids at a concentration of 40 mg l^{-1} for each compound. Operating conditions as in Fig. 2, except the mobile phase which was a mixture of isooctane and 2-propanol with 0.7% of water. The elution gradient is indicated in dotted line. The fractions are represented by A to D.

corresponding to each class of compounds is denoted by the letters A–F.

The changes in baseline are due to a difference in UV absorbance at 210 nm between isooctane and 2-propanol. One can observe also that D is the only fraction easily detected, corresponding to the sterols. Alkanes, fatty acids, alcohols and hydroxy fatty acids are respectively recovered in the fractions A, B, C and F (Fig. 3).

This separation according to main classes of lipids is accomplished in 30 min. In order to obtain a rapid equilibration of the silica column for a new injection (recycling time), an isohydric mobile phase[11–13] is used. Reproducible separations need a constant activity of the silica, and the water content of the solvents must be adjusted to obtain the same equilibrium with the silica. 2-Propanol and isooctane are isohydric when their water contents are respectively 0.7% and 0.004%. An isohydric mixture of 0.5% 2-propanol in isooctane contains 0.0039% of water. In practice, the water content of dry pure isooctane was ignored and 0.7% of water was added to pure 2-propanol. Under these conditions, the water content of the mixture containing 0.5% of 2-propanol in isooctane is 0.0035%.

Accurately reproducible separations are obtained with a recycling time of 10 min. So, in 40 min one can separate lipid extracts in five main classes: alkanes, aromatic hydrocarbons, fatty acids, alcohols, sterols, hydroxy fatty acids. Separation between non-aromatic and aromatic hydrocarbons could be achieved on this column, but the starting mobile phase should be pure isooctane and the recycling time must be longer. It seems better to separate fraction A on another column as it is a very easy separation.

As shown in Table I, satisfactory resolution between each fraction is obtained as far as short-chain acids ($C_{12:0}$) and short-chain alcohols ($C_{14:0}$) are not expected in natural samples mainly because the solvent extraction of the sediment was followed

TABLE I

OVERLAP BETWEEN THE COMPOUNDS ANALYSED IN THE VARIOUS FRACTIONS: PERCENTAGE OF RECOVERIES FOR EACH COMPONENT OF THE SIMULATED SOLUTION

Compounds	*Fraction*				
	A	*B*	*C*	*D*	*F*
Alkanes	100% of each alkane				
Fatty acids		100% $C_{23:0}$ 100% $C_{18:1}$ 100% $C_{16:0}$ 90% $C_{12:0}$ 30% $C_{10:0}$	10% $C_{12:0}$ 70% $C_{10:0}$		
Alcohols			100% C_{22} 80% C_{14} 30% C_{12}	20% C_{14} 70% C_{12}	
Sterols				100% of each sterol	
Hydroxy fatty acids (HFA)					100% of each HFA

TABLE II

TOTAL AMOUNT OF EACH FRACTION OF IDENTIFIED COMPOUNDS

The amount indicated is the average value obtained for three injections of 2.45 mg of extract.

	Fraction				
	A *Alkanes*	*B* *Fatty acid methyl esters*	*C* *Alcohols*	*D* *Sterols*	*F* *Polar compounds*
Total amount (in μg per g of dry sediment)	0.38 ± 0.02	23.3 ± 1.0	0.15 ± 0.05	0.077 ± 0.005	1.76 ± 0.3

by rotary evaporation. Any time lipids undergo liquid–liquid extraction, and evaporation, one cannot expect any reasonable quantification for fatty acids $< C_{14:0}$ and aliphatic alcohols $< C_{12:0}$[14].

Preliminary studies indicate that it is easy to improve the resolution between fractions B, C and D by using less eluting mobile phases, *i.e.* by choosing eluting conditions that are a function of the range of the chain carbon numbers for fatty acids and fatty alcohols.

Separation of natural samples is carried out with the same eluting programme as the standard simulated sample, which must be injected from time to time to check the reproducibility of the separation.

TABLE III

PROPORTIONS OF *n*-ALKANES IN FRACTION A

n-Alkane	*Percentage in fraction A*
C_{18}	0.5
C_{19}	0.5
C_{20}	0.8
C_{21}	1.1
C_{22}	1.3
C_{23}	2.1
C_{24}	1.3
C_{25}	5.3
C_{26}	1.3
C_{27}	8.7
C_{28}	2.9
C_{29}	12.7
C_{30}	5.3
C_{31}	32.4
C_{32}	3.7
C_{33}	13.0
C_{34}	1.3
C_{35}	2.1
C_{36}	1.3
C_{37}	1.3
C_{38}	1.1

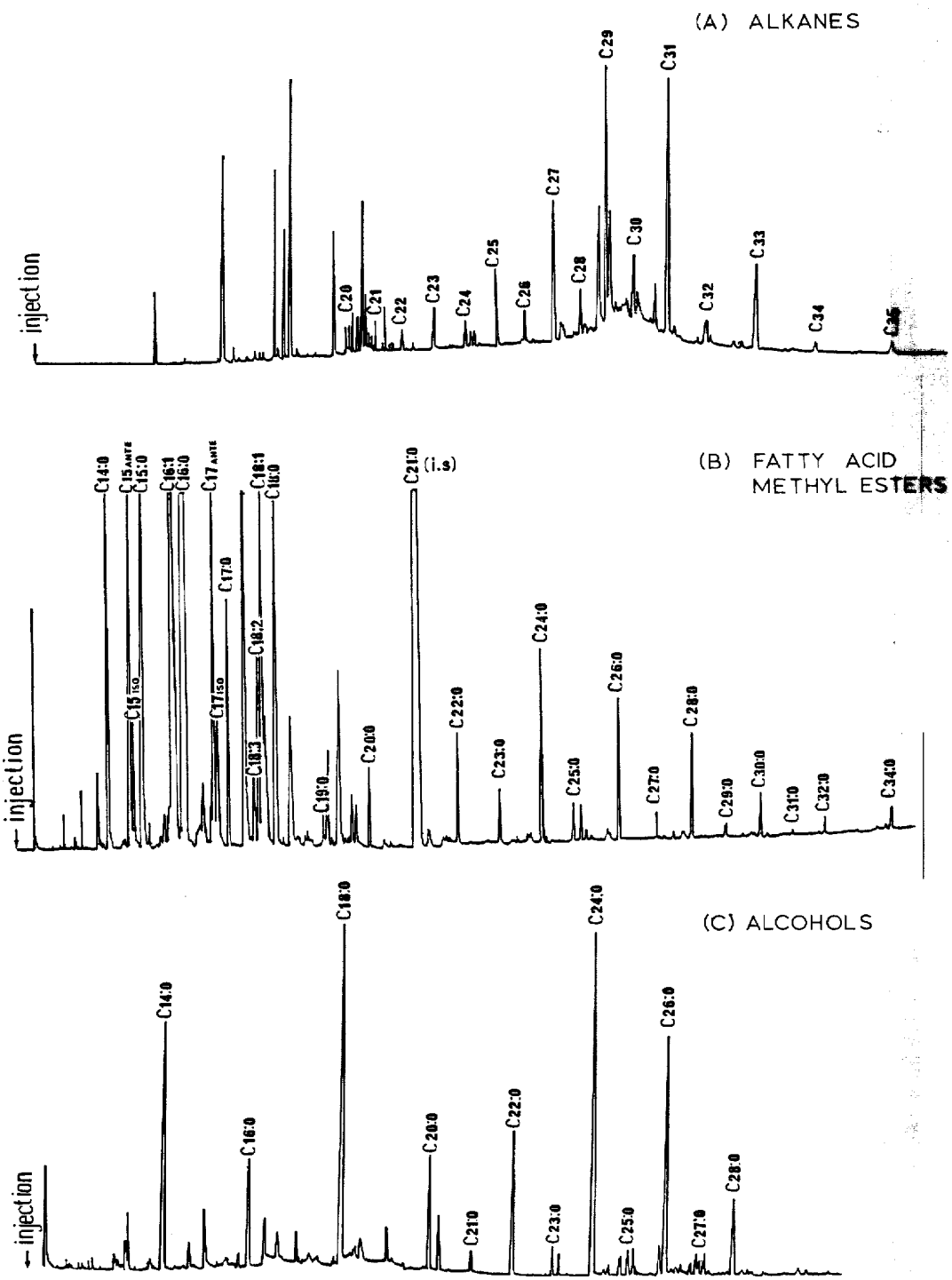

Fig. 4. Selected chromatograms. (A) Alkanes, SE 52 column temperature-programmed from 100 to 250°C at 2°C min^{-1}. (B) Fatty acid methyl esters and (C) alcohols, CP Sil 5 column temperature-programmed from 120 to 300°C at 2°C min^{-1}.

Application to a sedimentary lipid extract

The 24.5 mg of sediment extract is divided into ten fractions in order to check the reproducibility of the method directly on natural samples and to compare our results with those obtained by procedure B (Fig. 1). Table II indicates the total amount of each fraction related to 1 g of dry sediment.

Fraction A: total hydrocarbons. This fraction is again eluted on a silica column to separate non-aromatic and aromatic hydrocarbons. *n*-Alkanes are directly identified by GC (Fig. 4A). Table III indicates the percentage distribution of *n*-alkanes between C_{18} and C_{38}. Odd-number *n*-alkanes are predominant, as is usual in intertidal marine sediments, and 1 $\mu g\ g^{-1}$ of unresolved and unidentified hydrocarbons was found (Fig. 4A). The aromatic compounds are analysed by liquid chromatography on an amino-bonded silica column. The chromatogram (Fig. 5) shows a separation by increasing aromaticity beyond perylene.

Fraction B: fatty acid methyl esters. The fatty acids are derivatized to methyl esters and quantified by GC with $C_{21:0}$ as internal standard (Fig. 4B). Fatty acids are identified from $C_{14:0}$ to $C_{34:0}$. The fatty acids from C_{14} to C_{21} represent *ca.* 85%

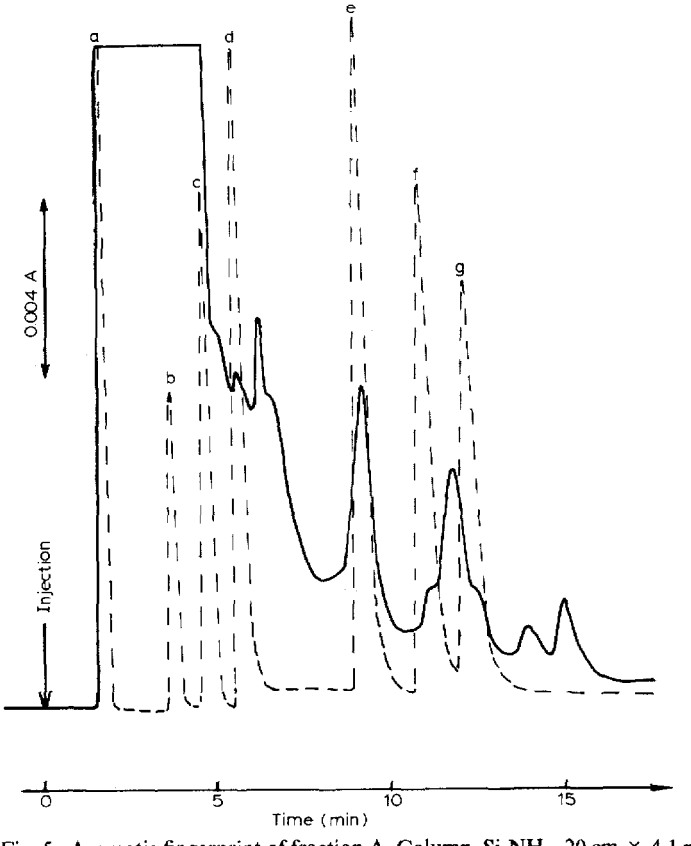

Fig. 5. Aromatic fingerprint of fraction A. Column, Si-NH_2, 20 cm × 4.1 mm I.D.; mobile phase, hexane; flow-rate, 1.8 $cm^3\ min^{-1}$; pressure, 62 bar; detection, UV at 254 nm, S = 0.02 DO. Dotted line: standards, a = benzene; b = fluorene; c = phenanthrene; d = pyrene; e = chrysene; f = benzopyrene; g = perylene. Solid line: fraction A, evaporated and dissolved in 200 μl of hexane; injection, 10 μl.

TABLE IV

PROPORTIONS OF FATTY ACID METHYL ESTERS IN FRACTION B

Fatty acid methyl ester		*Percentage in fraction B*
n-$C_{14:0}$		3.0
$C_{14:0}$	(*iso*)	0.3
n-$C_{15:0}$		9.0
$C_{15:0}$	(*ante*)	1.9
$C_{15:0}$	(*iso*)	1.1
n-$C_{16:0}$		31.0
n-$C_{16:1}$ +	*n*-$C_{16:2}$	18.9
$C_{16:0}$	(*iso*)	0.9
n-$C_{17:0}$		2.6
$C_{17:0}$	(*ante*)	4.3
$C_{17:0}$	(*iso*)	0.8
n-$C_{18:0}$		4.3
n-$C_{18:1}$		2.6
n-$C_{18:2}$		5.1
n-$C_{18:3}$		3.0
n-$C_{19:0}$		0.3
n-$C_{20:0}$		0.9
n-$C_{21:0}$		0.5
n-$C_{22:0}$		1.2
n-$C_{23:0}$		0.6
n-$C_{24:0}$		2.4
n-$C_{25:0}$		0.5
n-$C_{26:0}$		1.7
n-$C_{27:0}$		0.3
n-$C_{28:0}$		1.2
n-$C_{29:0}$		0.3
n-$C_{30:0}$		0.5
n-$C_{31:0}$		0.2
n-$C_{32:0}$		0.2

of the total acids (Table IV) and are predominantly of marine origin with a balance of terrestrial input (C_{22}–C_{32} = 9.1%)[15].

Fraction C: alcohols. Fraction C is directly analysed by GC and spiked with external standard for quantitative determination (Fig. 4C). Even-number *n*-alkanols are predominant, with a maximum at C_{24} (Table V). Other alcohols are present but were not studied in detail.

Fraction D: sterols. The most abundant of them were identified by GC–MS as shown in Table VI. But fraction D contains a complex mixture of about twenty compounds and requires additional GC–MS analysis to achieve structural elucidation, which is not our purpose in this paper.

Fraction F: polar compounds. A number of polar compounds were found in fraction F. This fraction was methyl-esterified with diazomethane and derivatized to the tetramethylsilyl ether. Electron-impact GC–MS spectra show that most of them are hydroxylated compounds, as indicated by an intense peak m/e = 73, but no evidence for known α,β- or ω-hydroxy fatty acid methyl ester structures was found. Structural elucidation of these compounds is in progress.

TABLE V

PROPORTIONS OF *n*-ALKANOLS IN FRACTION C

n-Alkanol	*Percentage in fraction C*
$C_{14:0}$	10.3
$C_{16:0}$	5.1
$C_{18:0}$	21.7
$C_{20:0}$	5.6
$C_{21:0}$	1.1
$C_{22:0}$	7.9
$C_{23:0}$	1.5
$C_{24:0}$	23.9
$C_{25:0}$	1.5
$C_{26:0}$	15.1
$C_{27:0}$	1.5
$C_{28:0}$	4.8

These data have been compared qualitatively for all the fractions and quantitatively for *n*-alkanes and fatty acid methyl esters and are in good agreement with those obtained with separation procedure B (Fig. 1).

The minimal analysable amount has not yet been determined. Nevertheless, one aliquot was diluted ten times (corresponding to 0.24 mg of lipid extract) and separated by HPLC. The analytical data are identical with those obtained from the initial aliquot. This allows us to perform a separation on particulate or dissolved organic matter from a 20-l sample of natural water, because the concentrations of both dissolved and particulate total lipid extracts in seawater usually range from 20 to 200 $\mu g\ l^{-1}$ (refs. 16 and 17).

CONCLUSION

Several points of general interest arise from the semi-preparative HPLC sep-

TABLE VI

CONCENTRATION OF STEROLS IDENTIFIED IN FRACTION D

Sterols	*Concentration (ng g^{-1} of sediment)*
Cholest-5-en-3β-ol (C_{27}, cholesterol)	46 ± 2
5α-Cholestan-3β-ol (C_{27}, cholestanol)	5.8 ± 0.6
24-Methylcholest-5-en-3β-ol (C_{28}, campesterol)	6.0 ± 0.1
24-Ethylcholest-5-en-3β-ol* (C_{29}, β-sitosterol)	19.3 ± 0.5
24-Ethyl-5α-cholestan-3β-ol* (C_{29}, sitostanol)	

* Peaks unresolved on GC CP Sil 5 column.

aration of extracted lipids presented here. First, a great variety of compounds with a large range of polarity could be separated in a single injection with good reproducibility. Second, the method is appropriate for samples present in trace amounts, such as in fresh and marine water. Third, satisfactory resolution is obtained in 40 min, allowing convenient quantitative analysis by GC–MS.

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