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Isolation of neutral and acidic lipid biomarker classes for compound-specific-carbon isotope analysis by means of solvent extraction and normal-phase high-performance liquid chromatography☆

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Abstract

An analytical method for the quantitative determination of neutral and acidic lipid biomarkers in particulate and sediment samples has been developed. The method involves a first step with solvent extraction to isolate the neutral from the acidic compounds and a second step using normal-phase HPLC on a Nucleosil silica column to separate four different classes of neutral compounds: (1) aliphatic hydrocarbons, (2) aromatic hydrocarbons, (3) ketone compounds and (4) sterol and alcohol compounds. Recoveries of the individual spiked lipid biomarkers for the whole analytical procedure ranged from 88 to 106% for fatty acids, from 50 to 60% for aliphatic hydrocarbons ($\geq$ *n*-C17), from 50 to 60% for polycyclic aromatic hydrocarbons (PAHs) ($\geq$3 rings), 83% for friedelin and 60–80% for the sterol and alcohol compounds. The isolated compound classes were analysed by gas chromatography–combustion–isotope ratio mass spectrometry to measure the stable carbon isotope ratios in the individual compounds. The method enables the isolation of compound classes without fractionation for compound-specific carbon isotope analysis (δ^{13}C). This analytical protocol has been applied, and proved suitable, for the determination of lipid biomarkers (sterols, fatty alcohols and fatty acids) in marine particulate material and for the determination of PAHs in sediment samples.
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1. Introduction

Lipid biomarkers in the marine environment are derived from several sources, such as bacteria, plankton and terrestrial higher plants [1–3]. Studies of their distribution in the environment help elucidate biogeochemical processes [4–7], but recent findings have significantly reduced the specificity of some biomarkers [8]. The analytical development of gas chromatography–combustion–isotope ratio mass spectrometry (GC–C–IRMS) allows the determination of the δ^{13}C of specific biomarkers, thereby improving the veracity of source apportionment. This technique is rapidly becoming an important tool in a variety of fields, including molecular organic geochemistry and applied analytical chemistry. However, to be useful for quantitative and qualitative analysis, the complex mixtures of organic compounds are best separated into compound classes of different polarity.

Isolation of lipid classes from complex mixtures of organic compounds has usually been done by column liquid or adsorption chromatography on inorganic normal phases, such as silica-gel and alumina [9–13]. However, this method is time-consuming and tedious. Other separation techniques based on thin-layer chromatography (TLC) are well-developed and partly automated, but they have still the disadvantages of being time-consuming and limited in terms of separation efficiency [14]. High performance liquid chromatographic (HPLC) offers a range of advantages over the traditional methods, including more rapid sample preparation, increased automation, higher separation capacity and lower solvent consumption. These factors make

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HPLC very attractive to organic chemists and, in the future, this technique will certainly became a standard separation process. However, when $\delta^{13}C$ analyses are envisaged, care should be taken during the isolation of the compounds from the complex sample matrix in order to ensure that no isotopic alteration of the products occurs that would otherwise influence the $\delta^{13}C$ content of the compounds [15–16].

Potential isotopic fractionation can occur at any step of the analytical procedure, including sample preparation and derivatisation. Fractionation effects during derivatisation have been investigated, at least theoretically [17]. Silylation does not cause carbon kinetic isotopic fractionation because no carbon-containing bond is involved in the derivatisation reaction. In esterification, a carbon centre is involved in the final product and, hence, a carbon kinetic isotope effect is possible. However, since the reactions usually employ an excess of derivatisation agent, the reaction is quantitative and rapid with no carbon kinetic isotope effect observed [17].

Here, we develop and present a method to isolate lipid biomarker classes out of particulate lipid extracts using a solvent extraction to separate neutral and acidic lipids, followed by a normal-phase HPLC to isolate the different classes of neutral lipids. The method enables the isolation of fatty acids, aliphatic hydrocarbons, aromatic hydrocarbons, ketones and sterols-fatty alcohols without fractionation for compound-specific carbon isotope analysis (CSIA on $\delta^{13}C$) by IRMS following on-line combustion of compounds separated by GC.

2. Experimental section

2.1. Reagents and analytical standards

Solvents used were of high purity pesticide quality (Burdick and Jackson Labs., Muskegon, MI, USA). Sodium sulfate anhydrous, sodium chloride, *bis*-(trimethylsilyl)-trifluoroacetamide (BSTFA), BF_3 in methanol (20%) and 32% HCl were supplied by Merck (France). KOH pellets were purchased from Sigma (France). Sodium sulphate used for drying organic extracts was baked at 450 °C overnight before use in order to remove any traces of organic contaminants.

All standards were of the highest purity commercially available: *n*-alkanes, pristane, phytane and polycyclic aromatic hydrocarbons (PAHs) were obtained from Baker J.T. (Noisy le Sec, France), perdeuterated alkanes and perdeuterated PAHs from Cambridge Isotope Laboratories (USA), friedelin tech from Aldrich (France), squalane, 5α-androstane, 5β(H) cholanic acid, cholest-5-en-3β-ol (cholesterol) and 24-ethylcholest-5-en-3β-ol (sitosterol) were purchased from Sigma (L'Isle d'Abeau, France), 5β-cholestan-3β-ol (coprostanol) from Research Plus (Bayonne, NJ, USA), 5α-androstan-3β-ol, 24α-methylcholest-5-en-3β-ol (campesterol) and 5α-cholestan-3β-ol (cholestanol) from Steraloids (New Port, USA), fatty acid standards and fatty acid methyl esters (FAMEs) from Larodan Fine Chemicals (Malmö, Sweden), lycopane, 5β(*H*) cholane, 17β(*H*),21β(*H*) hopane, hop-22(29)-ene (diploptene), β,β-carotene, from Chiron (Trondheim, Norway).

Stock individual standard solutions (0.8–1 mg ml^{-1}) were prepared by dissolving accurate amounts of standards in isooctane and were stored at −4 °C. Working standard mixtures were obtained by further dilution of stock solutions with methanol for spiking or in isooctane for GC analysis.

2.2. Isolation

The analytical protocol used for the isolation of the different lipid classes involves a first step with solvent extraction to isolate the neutral from the acidic compounds, and a second step using normal-phase HPLC on a Nucleosil silica column to separate four different classes of neutral compounds: (1) aliphatic hydrocarbons, (2) aromatic hydrocarbons, (3) ketone compounds and (4) sterol and alcohol compounds. Standard mixtures were processed through all steps of the analytical protocol to monitor the recoveries and potential stable isotope fractionation during the isolation procedure. Each step of the analytical protocol was developed and optimised by using ∼2 to 5 μg of individual standards, and this approach was tested using marine particulate material collected in sediment traps from Almeria-Oran frontal zone and a sediment-certified reference material (IAEA-383).

Freeze–dried particulate material (100 mg dry mass) and sediment (2 g dry mass) were spiked with internal standards (n-$C_{24}{}^2H_{50}$, [$^2H_{10}$] anthracene (anthracene-d_{10}), [$^2H_{10}$] pyrene (pyrene -d_{10}), [$^2H_{12}$] perylene (perylene-d_{12}), friedelin, 5α-androstan-3β-ol and cholanic acid) prior to the ultrasonic extraction with a mixture of MeOH–CH_2Cl_2 (1:1) as described elsewhere [18,19].

2.2.1. Isolation of neutral lipids from acidic lipids

A volume of 250 μl methanol containing a mixture of 2–5 μg of individual aliphatic, PAHs and fatty acids was spiked into 8 ml MeOH–CH_2Cl_2 (1:1). Particulate samples, previously spiked with the internal standards, were ultrasonically extracted with 8 ml MeOH–CH_2Cl_2 (1:1) for 20 min. The extracts were centrifuged at 2000 × g for 5 min and filtered through glass wool. The procedure was repeated twice with 8 ml MeOH–CH_2Cl_2 (1:1) and the pooled extracts were evaporated under N_2 to 3 ml. The total extracts were saponified using 1 ml KOH 6% in methanol–water (80:20) plus 1 ml of Milli-Q water (80 °C, 1 h). Neutral lipids were recovered from the basic solution (pH > 13) by partitioning into *n*-hexane (5:2, v/v, three aliquots of 2 ml). The residual water phase was acidified to pH < 2 with 1 ml 6 M HCl and acidic lipids were extracted with hexane:ethyl acetate 9:1 (2 ml, three times). The neutral organic extract was rinsed with 3 ml of Milli-Q water to remove any KOH residue, dried with sodium sulfate and evaporated to 500 μl. The acidic ex-

tract sample was then evaporated to dryness under a gentle stream of nitrogen before the derivatisation.

2.2.2. Isolation of neutral lipid classes by normal-phase HPLC

2.2.2.1. Filtration through membranes and filter syringes. The neutral organic extract of about 500 μl was filtered through a pre-washed (three times with 2 ml of hexane) 13-mm diameter PTFE membrane filter of 0.2 μm pore size (Lida-Interchim, France or Sartorius, France) using a 13 mm Swinny stainless steel syringe filter holder (Advantec, MFS, by Interchim, France) and a 2 ml glass syringe with Luer-Lock tip (Micromate, Popper and Sons). The tube containing the extract was rinsed twice with 200 μl hexane and the solvent washings were recovered together with the extract filtrate through the same membrane filter. Two different filter syringes with a polypropylene housing were also tested for recoveries and blanks: a GHP Acrodisc of 25 mm and 0.45 μm equipped with a glass fibre pre-filter and a polypropylene hydrophilic membrane (Merck, France) and a Minisart RC15 of 15 mm and 0.2 μm equipped with a membrane of cellulose (Sartorius, France).

Blank tests of the filters were obtained by filtering 500 μl of hexane solvent and evaporating the total eluate to 50 μl before injection into the GC.

2.2.2.2. HPLC separation. The filtrate was concentrated to 200 μl in conical tapered vials and subject to fractionation by HPLC on a normal-phase column (Interchrom Nucleosil® 100 Å silica column, 25 cm × 0.4 cm i.d., 5 μm; Macherey–Nagel, Interchim, France) with a 2 cm guard column of the same packing material. Samples were injected through an HP 1100 autosampler (Agilent, Palo Alto, CA, USA) equipped with a 500 μl loop, a quaternary LC pump, a thermostated column compartment and coupled to an SF-2120 fraction collector (Advantec, Bioblock, France). An elution gradient with three solvents was used: (A) hexane, (B) methylene chloride, (C) methanol. The program elution was adapted from Ref. [20]. It comprised isocratic hold at 100% hexane for 10 min followed by a gradient from 0 to 20% methylene chloride over 5 min, then to 100% methylene chloride over 5 min, isocratic hold at 100% methylene chloride for 10 min, followed by a gradient from 0 to 5% methanol over 5 min and isocratic hold for 25 min. The column temperature was set at 20 °C and the flow rate during the fraction collection period was 0.5 ml min^{-1}. After collecting the fractions, the flow rate was increased to 1 ml min^{-1} and the column was conditioned to 100% methylene chloride over 5 min, followed by a gradient to 100% hexane over 5 min and back-flushing with hexane for 5 min. Before the next run started, the column was re-equilibrated with hexane for 5 min, thereby giving a total conditioning time of 20 min.

The elution of polycyclic aromatic compounds, which were the only chromophoric target compounds, was monitored using diode-array detection (DAD) and measuring the absorbance at 254 and 235 nm.

Successive small fractions of the mobile phase were collected and then concentrated under nitrogen flow for analysis by GC–flame ionisation defection (FID).

2.3. Derivatisation

The fraction containing the sterols was treated with 200 μl BSTFA for 1 h at 70 °C to convert the alcohols and sterols to their corresponding trimethylsilyl ethers. The derivatised extract was then evaporated to dryness under a nitrogen stream and redissolved in isooctane for injection on the gas chromatograph.

The methyl esters were prepared by transesterifying the lipid extract with 500 μl of 20% BF_3 in methanol at 80 °C for 1 h. The solution was allowed to cool and 2 ml of saturated sodium chloride was added before the fatty acid methyl esters were extracted with hexane (2 ml, three times). The total organic extract was rinsed with 3 ml of saturated sodium chloride, dried with sodium sulfate, transferred to a vial with isooctane rinses and evaporated to 250 μl for the GC analysis.

2.4. GC and GC–MS analyses

GC was performed with a Hewlett-Packard HP 5890 series II equipped with a FID system, split/splitless injector and an HP 7673 autoinjector. A 5% phenylmethylpolysiloxane fused silica capillary column was used (DB5, 30 m × 0.25 mm i.d.; film thickness 0.25 μm; Agilent). Helium was the carrier gas (1.2 ml min^{-1}). The oven temperature was programmed from 60 °C (0.5 min hold) to 290 °C at 6 °C min^{-1}. The injector and detector temperatures were 270 °C and 320 °C, respectively. Peak identify was confirmed using GC–MS (Hewlett-Packard 5889B MS "Engine") operated in the electron impact mode at 70 eV. The operating conditions were: mass range 50–550 U; electron energy 70 eV, transfer line temperature 280 °C; 0.9 scan s^{-1}. The polycyclic aromatic hydrocarbons in sediment samples were analysed using selective ion monitoring (SIM) to enhance sensitivity [20].

Recovery yields of the different spiked standards and internal standards of environmental samples were quantified relative to GC internal standards: n-$C_{19}{}^{2}H_{40}$ for the aliphatic fraction (F1); $[^{2}H_{12}]$chrysene (chrysene-d_{12}) for the aromatic fraction (F2); 5α-androstane for the ketone (F3), and acid fraction; friedelin for the sterol–alcohol fraction (F4).

2.4.1. GC–C–IRMS

The lipid biomarkers were analysed for their stable carbon isotope composition using a HP 5890 GC equipped with a HP 7673 autoinjector and interfaced through a combustion furnace with a Finnigan MAT Delta C isotope-ratio mass spectrometer. The Delta-C GC–C–IRMS system is basically similar to the Delta-S system as described previously

[21,22]. Briefly, the effluent from the capillary GC column enters a combustion furnace operated at 940 °C and is combusted quantitatively to CO_2 and water. Water of combustion is removed from the effluent stream using a tubular Nafion membrane and the analyte-derived CO_2 enters the electron impact ion source of the high-precision IRMS system.

The GC–C–IRMS system was equipped with a 100% methylpolysiloxane fused silica column (Ultra-1, 50 m × 0.32 mm i.d.; 0.5 μm film thickness) pre-connected with a press-fit connector (Supelco, France) to a 0.32 mm i.d.. deactivated fused silica capillary retention gap of 5 m. Injections of 2 μl for standards or samples in isooctane were made via an on-column injector. The GC oven was programmed from 60 to 100 °C at 10 °C min^{-1}, then to 310 °C at 4 °C min^{-1} and maintained at 310 °C for 40 min. Values reported were determined by at least three replicates to calculate the average and standard deviation. All $\delta^{13}C$ values are reported in the delta notation relative to the Pee Dee Belemnite (PDB) standard as follows:

$$\delta^{13}C(‰) = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{PDB}} - 1\right] 10^3$$

Corrections for the isotopic change introduced in the derivatisation of sterols, fatty alcohols, and fatty acids were determined through derivatisation of standards of known isotopic composition and applying the equation of Jones et al. [23]. Cholesterol, methanol, 18:0 fatty acid and 18:0 fatty acid methyl ester (FAME) of known isotopic carbon composition (measured by elemental analyser coupled to isotope ratio mass spectrometer) were used to calibrate the GC–C–IRMS and correct the isotopic change introduced by the derivatisation. The surrogate standards, 5α-androstan-3β-ol, cholanic acid and the GC internal standards, friedelin and 5α-androstane, of known isotopic composition served as internal isotopic standards.

The series of standards and samples was measured in triplicate by a cyclic sequence, rather than measuring three times immediately one after the other, to eliminate errors caused by a temporal drift during the long measurement time of a sequence. The combustion unit was regularly oxidised by flushing oxygen through the oven for at least 1 h. After this oxidation phase, the instrument was left overnight to remove the excess oxygen before continuing with new measurements. The precision (standard deviation) for most analytes with GC–C–IRMS signals higher than 0.5 V (*m/z* 44) was comparable to the instrument specifications (0.5‰).

3. Results and discussion

To ensure accuracy for high-precision compound-specific isotope analysis (CSIA), it is necessary that any pretreatment step, including separations, derivatisation, drying of sample solutions under a stream of nitrogen, etc. preserves the isotopic composition of the individual components. Thus, every step in the sample preparation protocol must be scrutinised for potential mass discriminatory effects which might adulterate the measurement of isotopic abundance by isotopic fractionation processes. For tests here, the $\delta^{13}C$ values of untreated standards are compared with the $\delta^{13}C$ values of the standards subjected to the isolation procedure.

Table 1
Recovery efficiencies (REC, %) with relative standard deviation (R.S.D., %) of fatty acids and fatty acid methyl esters after the derivatization protocol

Compound	Fatty acids, derivatization		Fatty acid methyl esters, derivatization	
	REC (%)	R.S.D. (%)	REC (%)	R.S.D. (%)
C_{12}	81	6	56	21
$C_{14:1}$	89	6	66	20
C_{14}	96	6	69	20
$C_{16:1}$	94	8	70	19
C_{16}	100	8	73	19
$C_{18:2}$	97	9	71	18
$C_{18:3} + C_{18:1}$	97	9	71	18
C_{18}	101	9	74	17
$C_{20:4}$	95	9	71	16
$C_{20:1}$	102	9	72	17
C_{20}	100	9	74	16
C_{21}	104	9	75	16
$C_{22:1}$	108	9	72	16
C_{22}	100	10	74	16
C_{23}	102	10	75	15
$C_{24:1}$	100	10	71	13
C_{24}	107	10	77	15
C_{25}	113	8	79	15
C_{26}	113	10	83	15

3.1. Derivatisation of fatty acids

Fatty acids were methylated to their corresponding FAMEs with the commercially available BF_3–methanol complex, which is a common and rapid standard derivatisation method [24–25]. Only one carbon atom is added, thus minimising changes to the $\delta^{13}C$ value of the parent compound. Since the reaction usually employs an excess of derivatisation agent, the esterification is quantitative and rapid with no carbon kinetic isotope effect [17]. The drawback is that the reaction takes place in aqueous phase and therefore the target compounds must be back extracted to an organic phase before GC analysis.

Table 1 summarises the experimentally determined recoveries of fatty acid standard compounds after derivatisation and extraction into hexane. Recoveries were obtained by external calibration against FAME standards. High yields were measured for all fatty acids (81–113%) with relative standard deviations <10%.

Some derivatized acids, such as the FAMEs, are sufficiently volatile to be lost during routine concentration procedures, especially if taken to dryness. The potential losses of FAMEs were assessed by taking the FAME standards to dryness, followed by the treatment with BF_3–MeOH reagent

Table 2
Mean recoveries (REC, %) and R.S.D. (%) from triplicate extractions on individual hydrocarbon compounds after saponification and solvent extraction with hexane

	Abbreviation code	REC (%)	R.S.D. (%)
Naphthalene	N	73	7
n-C_{12}	12	70	17
1-Methylnaphthalene	C1-N	78	7
2,6-Di-methylnaphthalene	C2-N	79	6
n-C_{14}	14	73	9
Acenaphthylene	AC	75	7
Acenaphthene	APH	78	6
Fluorene	F	79	7
n-C_{16}	16	78	8
n-C_{17}	17	81	8
Pristane	Pr	79	7
Phenanthrene	Ph	81	7
Anthracene	A	84	9
n-$C_{18:1}$	18:1	81	9
n-C_{18}	18	82	8
Phytane	Phy	82	8
2-Methylphenanthrene	C1-Ph	84	8
1-Methylphenanthrene	C1-Ph	86	7
3,6-Dimethylphenanthrene	C2-Ph	86	8
n-C_{20}	20	82	8
Fl	Fl	84	8
Py	Py	84	8
n-C_{21}	21	83	9
n-C_{22}	22	82	9
1-Methylpyrene	C1-Py	86	8
n-C_{24}	24	83	9
Chry	Chr	86	9
n-C_{26}	26	83	8
Squalane	Sq	84	8
n-C_{28}	28	82	8
Perylene	Per	88	9
n-C_{30}	30	82	8
n-C_{32}	32	81	7
n-C_{34}	34	81	7

and re-extraction with hexane solvent. The recoveries were slightly lower that those obtained with fatty acids, in particular for the more volatile compounds (n-C_{12} to n-C_{14}) (Table 1). These results highlight the importance of not taking the final derivatized extract to dryness.

3.2. Separation of neutral from acid lipids by solvent extraction

The neutral fraction of the total lipid extract was obtained by extracting the saponified total lipid extract with hexane [26–27]. Table 2 summarises the mean recoveries and repeatability from triplicate extractions of 2–5 μg for individual hydrocarbon compounds spiked into 3 ml methanol after saponification with 6% KOH at 80 °C for 1 h, and extracting with three aliquots of 2 ml hexane. Recoveries were all >70% with R.S.D. <9%, except for the most volatile alkane n-C_{12} for which a R.S.D. of 17% was obtained.

The acidic fraction was recovered by extracting the remaining solution after acidification to pH < 2. Two different solvent mixtures, hexane [26,27] and the hexane–ethyl acetate (9:1) [28] were tested for recovery of the acidic lipids from the aqueous samples. The recoveries, repeatability (R.S.D.) and carbon isotopic compositions for each target compound both before and after isolation are shown in Table 3. Extraction with hexane–ethyl acetate (9:1) proved to be more effective as regards the recovery yields. Recoveries of the acidic compounds from the aqueous phase with hexane–ethyl acetate (9:1) were all >82% with good repeatability (R.S.D. < 8%).

3.3. Isolation of the different neutral lipid classes by normal-phase HPLC

3.3.1. Filtration of the neutral extract before HPLC

One of the main requirements for preparative HPLC is to prevent unwanted particles from the sample extract reaching the in-line frits of the injector and detector. Accordingly, the sample extracts must be filtered through a filter or membrane of small pore size (0.45–0.2 μm). Table 4 illustrates the recoveries of hydrocarbons after filtration using filter syringes of polypropylene and cellulose, and two different brands of PTFE membrane filters (Lida and Sartorius). Recoveries for all aliphatic and aromatic hydrocarbons ranged from 65 to 94% for all PTFE membrane filters and cellulose filter syringe. Lower recoveries, from 52 to 80%, were obtained for the polypropylene filter syringe, which is probably related to the higher dead volume (<100 μl) of this 25 mm diameter filter compared to that of 15 mm diameter (<10 μl).

Blanks have been another aspect addressed in the analysis of lipids by GC techniques. The FID chromatograms (Fig. 1) of the blanks illustrate that both PTFE membranes contain very few extra peaks, whereas the filter syringes contain significant interferences, probably derived from the polyethylene housing. According to these results, PTFE membrane filters with the Swinny stainless syringe filter holder were selected as the most appropriate procedure for filtering the neutral organic extracts before HPLC fractionation.

3.3.2. HPLC separation

The separation of compounds ranging from the non-polar hydrocarbons to the more polar sterol and alcohol lipid classes on a stationary phase, like silica, requires a mobile phase gradient. Elution starts with a non-polar solvent, such as hexane, and finishes with a polar solvent mixture, including methanol, to elute the sterol and alcohol compounds. The choice of solvents was based on those typically used in the adsorption chromatography on silica adsorbents [12–13]. A minimal proportion of 5% of methanol was required to elute the sterol and alcohol compounds. Gradient elution in normal-phase HPLC has a notoriously bad reputation for poor repeatability and unpredictable retention because of preferential adsorption of the polar organic solvent and of water onto the column packing. However, the use of a sophisticated gradient-elution chromatograph and working

Table 3
Mean recoveries (REC, %) with relative standard deviation (R.S.D., %) of fatty acids after the separation of neutrals and extracting the acidified remaining extract with two different solvents: hexane and hexane:ethyl acetate (9:1). Stable carbon isotopic values ($^{13}\delta$C values, ‰ with S.D. for $n = 3$) of the derivatized fatty acids standards are compared with the $^{13}\delta$C of fatty acids obtained after their extraction from the acidified extract and derivatization ($\Delta\delta^{13}$C)

	Derivatized standard		Hexane, after extraction and derivatization					Hexane–ethyl acetate (9:1), after extraction and derivatization				
FA	δ^{13}C	S.D.	REC (%)	R.S.D. (%)	δ^{13}C	S.D.	$\Delta\delta^{13}$C	REC	R.S.D.	δ^{13}C	S.D.	$\Delta\delta^{13}$C
C_{12}	−27.97	0.62	66	8	−28.51	0.67	0.54	88	8	−28.27	0.62	0.30
$C_{14:1}$	−23.90	0.68	73	9	−24.43	0.53	0.53	95	6	−23.66	0.52	−0.24
C_{14}	−26.11	0.21	74	8	−25.73	0.51	−0.38	99	5	−26.57	0.33	0.46
$C_{16:1}$	−30.50	0.42	77	9	−31.00	0.58	0.50	99	5	−29.74	0.57	−0.64
C_{16}	−29.03	0.48	83	5	−29.08	0.29	0.05	106	1	−29.07	0.58	0.04
$C_{18:2}$	−28.32	0.37	78	10	−28.34	0.31	0.02	103	4	−28.63	0.44	0.30
$C_{18:3} + C_{18:1}$	−29.82	0.48	76	9	−30.44	0.23	0.61	100	4	−30.01	0.48	0.19
C_{18}	−26.61	0.44	79	10	−26.72	0.35	0.11	101	1	−26.92	0.65	0.31
$C_{20:4}$	−15.59	0.45	66	9	−16.28	0.54	0.69	95	4	−15.53	0.74	−0.06
$C_{20:1}$	−26.31	0.62	74	9	−26.72	0.48	0.41	96	5	−26.63	0.34	0.31
C_{20}	−27.82	0.61	80	5	−27.97	0.59	0.14	97	2	−27.97	0.63	0.14
C_{21}	−26.46	0.62	76	10	−27.13	0.37	0.67	96	2	−26.24	0.77	−0.23
$C_{22:1}$	−29.89	0.60	73	9	−30.54	0.36	0.65	94	5	−29.95	0.33	0.06
C_{22}	−27.72	0.62	69	14	−28.30	0.49	0.59	96	1	−28.13	0.68	0.42
C_{23}	−29.23	0.52	61	15	−29.08	0.42	−0.15	94	1	−29.17	0.69	−0.06
$C_{24:1}$	−29.25	0.54	70	5	−29.26	0.57	0.00	93	5	−29.21	0.58	−0.05
C_{24}	−28.57	0.58	51	15	−29.09	0.68	0.52	92	1	−28.67	0.47	0.10
C_{25}	−28.04	0.58	48	16	−28.55	0.55	0.51	88	1	−27.99	0.68	−0.05
C_{26}	−26.43	0.59	43	15	−26.92	0.60	0.49	82	1	−27.04	0.52	0.61
Cholanic	−16.16	0.62	88	9	−16.83	0.36	0.67	103	5	−16.69	0.43	0.52

with dry solvents at controlled constant temperature does allow repeatable results [29].

A critical parameter was the separation of aliphatic and aromatic hydrocarbons. In this case, the HPLC was calibrated by monitoring the absorbance of PAHs with the diode-array detector at 254 nm. A typical HPLC chromatogram is illustrated in Fig. 2 where the PAHs eluted between 9 and 17 min. According to the absorbance of PAHs

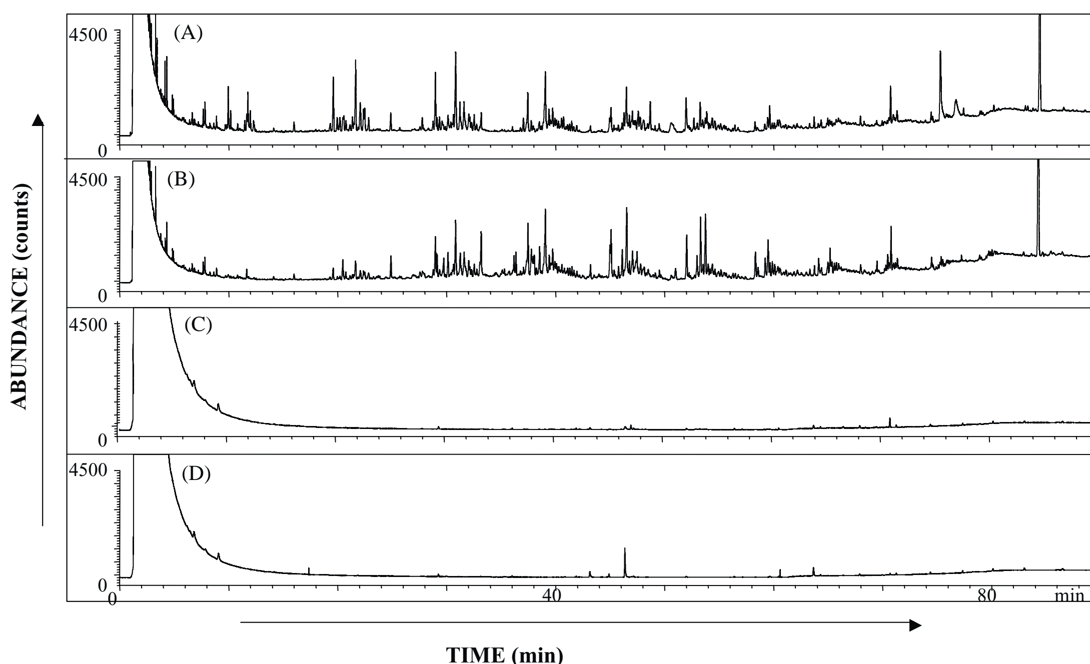

Fig. 1. FID chromatograms of the blank tests for four filtration systems. (A) GHP Acrodisc of 25 mm and 0.45 μm equipped with a glass fiber pre-filter and a polypropylene hydrophilic membrane. (B) Minisart RC-15 of 15 mm and 0.2 μm equipped with a membrane of cellulose. (C) Lida PTFE membrane filter of 0.2 μm pore size. (D) Sartorius PTFE membrane filter of 0.2 μm pore size.

Table 4
Recovery efficiencies (REC, %) and relative standard deviation (R.S.D., %, $n = 3$) from 500 μl solution containing 2 μg of individual hydrocarbons after filtration on different filter syringe (F-syringe) and membranes

	F-syringe Acrodisc GHP-25 mm polypropylene		F-syringe Minisast RC-15 mm cellulose		Membrane Lida 13 mm PTFE		Membrane Sartorius 13 mm PTFE	
	REC (%)	R.S.D. (%)	REC (%)	R.S.D. (%)	REC (%)	R.S.D. (%)	REC (%)	R.S.D. (%)
Naphtalene	52	4	65	1	66	12	73	5
n-C_{12}	54	1	67	5	67	14	74	4
C1-N	54	1	68	5	67	12	75	5
C2-N	53	1	68	6	67	14	75	4
n-C_{14}	57	2	73	7	67	15	75	2
AC	53	2	68	6	67	15	74	4
Acenaphthene	53	2	69	7	68	16	75	4
Fluorene	55	2	72	8	70	17	75	3
n-C_{16}	59	4	81	9	70	16	76	1
n-C_{17}	80	21	78	8	70	18	76	2
Pristane	74	23	86	7	70	17	76	2
Phenanthrene	68	4	93	10	72	16	76	2
Anthracene	67	1	91	7	71	19	77	3
$C_{18:1}$	64	7	76	12	71	17	77	2
C_{18}	61	6	81	6	71	17	77	3
Phytane	61	6	83	8	71	17	77	3
C1-Ph	57	5	78	7	77	18	77	3
C1-Ph	75	3	83	13	72	8	77	4
C2-Ph	62	7	89	7	71	17	77	4
n-C_{20}	63	7	83	6	71	17	76	3
Fluoranthene	57	5	78	6	71	19	76	3
Pyrene	62	8	81	7	71	19	77	4
n-C_{21}	57	5	73	5	71	19	76	4
n-C_{22}	67	8	87	2	71	17	77	5
C1-Py	60	5	81	5	70	19	76	5
n-C_{24}	62	8	83	4	70	19	76	6
Chrysene	62	6	82	4	72	18	76	6
n-C_{26}	60	8	82	4	69	21	77	6
Squalane	59	8	82	4	69	17	77	7
n-C_{28}	58	7	78	3	68	17	77	8
Perylene	59	6	80	4	68	17	77	9
n-C_{30}	59	5	79	3	68	18	76	9
n-C_{32}	59	2	79	3	68	15	77	10
n-C_{34}	65	1	84	3	69	12	77	13
$C_{24}{}^{2}H_{50}$	80	4	94	6	71	8	76	16

Abbreviation codes as those from Table 2.

and the GC–FID analysis of the successive small fractions collected from the HPLC separation, the aliphatic hydrocarbon fraction was collected between 4.30 and 9.30 min, PAHs from 9.30 to 20 min, ketone derived compounds from 20 to 30 min, and sterol and alcohol biomarkers from 30 to 60 min. Table 5 illustrates the recovery rates obtained for each compound class when standard compounds were used for the assessment of the efficiency of the HPLC separation. Recoveries of aliphatic hydrocarbons within the first fraction (4.3–9 min) ranged from 62 to 87% with repeatability <14%, except for the more volatile n-C_{12} (R.S.D. of 20%). Minor amounts of the aliphatic hydrocarbons were eluted in the second fraction containing the polyaromatic hydrocarbons. The amount was about 2% of the total spike amount of ~3 to 4 μg. Examples of chromatograms of the first and second fraction obtained using GC–FID are shown in Fig. 3. Recoveries of polycyclic aromatic hydrocarbons in the second fraction ranged from 69 to 95% with a R.S.D. <14%. Ketones, represented by friedelin, eluted in the third fraction with a recovery of 83% and a R.S.D. of 8%. Sterol and alcohol compounds were recovered in the fourth fraction with values ranging from 86 to 93% and R.S.D. <11%.

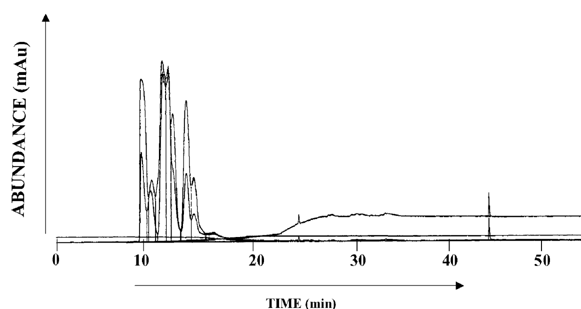

Fig. 2. Representative HPLC–DAD chromatogram (254 and 235 nm DAD absorption) of total neutral lipids. PAHs eluted between 9 and 17 min.

Table 5
Recovery efficiencies (REC, %) and relative standard deviation (R.S.D., %) from 200 μl solution containing ∼3–4 μg of each individual neutral compound after HPLC separation

F1: 4.3–9.3 min			F2: 9.3–20 min			F3: 20–30 min			F4: 30–60 min		
	REC (%)	R.S.D. (%)		REC (%)	R.S.D. (%)		REC (%)	R.S.D. (%)		REC (%)	R.S.D. (%)
n-C_{12}	62	20	Naphtalene	69	3	Friedeline	83	8	n-C_{14}-OH	93	1
n-C_{14}	69	3	C1-N	73	3				n-C_{16}-OH	88	3
n-C_{16}	74	9	C2-N	74	3				n-C_{18}-OH	86	5
n-C_{17}	75	10	AC	74	3				n-C_{20}-OH	87	4
Pristane	76	10	Acenaphthene	75	3				n-C_{22}-OH	87	5
$C_{18:1}$	77	9	Fluorene	79	8				n-C24-OH	87	4
C_{18}	78	9	Phenanthrene	84	11				Coprostanol[1]	87	7
Phytane	78	10	Anthracene	88	14				Cholesterol[2]	91	10
n-C_{20}	80	9	C1-Ph	86	11				Cholestanol[3]	91	11
n-C_{21}	81	9	C1-Ph	90	13				Campesterol[4]	91	10
n-C_{22}	81	9	C2-Ph	86	11				Sitosterol[5]	92	9
n-C_{24}	82	10	Fluoranthene	88	12				Androstanol[6]	88	3
n-C_{26}	83	12	Pyrene	88	12						
Squalane	83	12	C1-Py	88	12						
n-C_{28}	83	14	Chrysene	95	11						
n-C_{30}	83	14	Perylene	89	8						
n-C_{32}	83	14									
n-C_{34}	87	10									
$C_{24}{}^{2}H_{50}$	82	10									

Abbreviation codes as those from Table 2. 1: coprostanol, 5β-cholestan-3β-ol; 2: cholesterol, cholest-5-en-3β-ol; 3: cholestanol, 5α-cholestan-3β-ol; 4: campesterol, 24α-methylcholest-5-en-3β-ol, 5: sitosterol, 24-ethylcholest-5-en-3β-ol; 6: androstanol, 5α-androstan-3β-ol.

With the HPLC gradient settings, a total neutral lipid extract was analysed in 60 min followed by a reverse gradient of 10 min at 1 ml min^{-1} to speed up the column re-equilibration, a 5 min time of flushing-out the potential impurities, and a 5 min isocratic equilibration time with the starting mobile phase to re-equilibrate the column before the next injection. This resulted in a time between injections of 80 min, but the automatic system allowed operation overnight without supervision. The retention time of the PAH compounds proved to be highly repeatable, showing a standard deviation of 0.09 min after passing 70 samples during a 3-month period time.

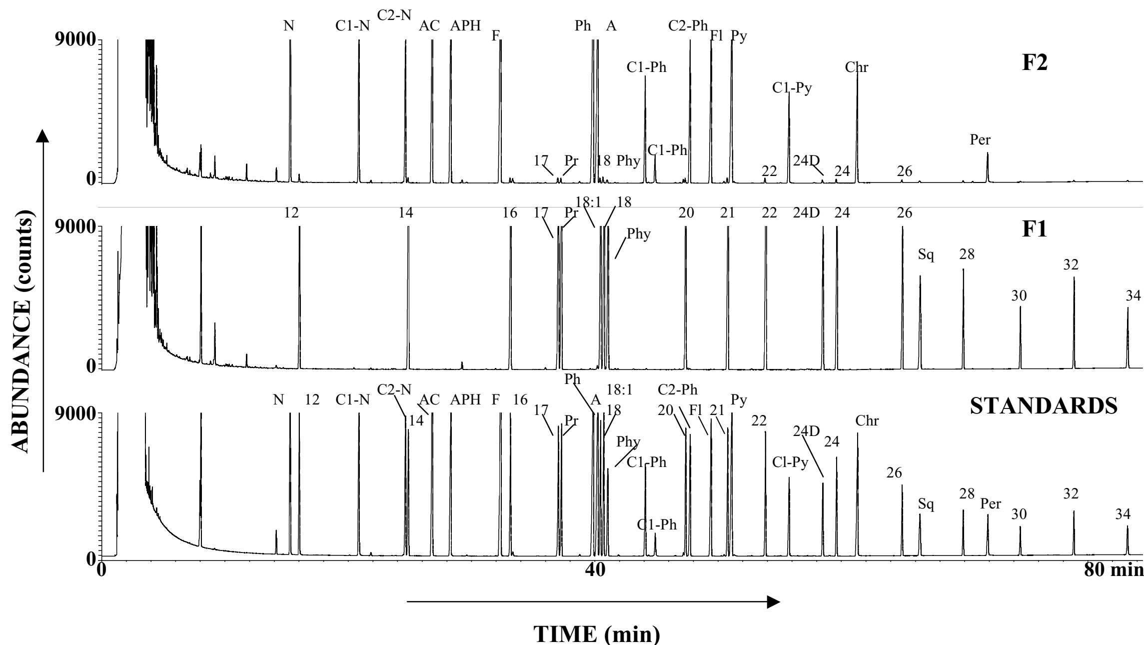

Fig. 3. FID chromatograms of the hydrocarbon standards and of the first (F1) and second (F2) fractions after the HPLC separation of the hydrocarbon standards. Abbreviation codes as those from Table 2.

Table 6
Recovery yields (REC, %) of aliphatic hydrocarbons when compounds are eluted between two fractions (F1 and F2) and differences ($\Delta\delta^{13}C$) of the carbon isotope ratios before and after HPLC isolation for F1

	F1, REC (%)	F2, REC (%)	Standards		F1		$\Delta\delta^{13}C$
			$\delta^{13}C$	S.D.	$\delta^{13}C$	S.D.	
n-C_{12}	62	19	−30.44	0.59	−30.73	0.28	0.29
n-C_{14}	64	17	−31.99	0.03	−32.45	0.17	0.47
n-C_{16}	70	17	−32.60	0.55	−32.71	0.10	0.12
n-C_{17}	73	18	−25.73	0.40	−25.83	0.14	0.11
Pristane	72	26	−24.93	0.17	−25.12	0.03	0.19
$C_{18:1}$	65	17	−31.69	0.68	−32.15	0.19	0.47
C_{18}	76	18	−34.14	0.36	−34.74	0.47	0.60
Phytane	74	25	−28.43	0.23	−28.83	0.10	0.41
n-C_{20}	68	16	n.d.[a]	–	−25.14	0.50	–
n-C_{21}	80	16	−26.16	0.19	−26.57	0.19	0.42
n-C_{22}	80	15	−25.79	0.34	−26.40	0.28	0.62
$C_{24}{}^2H_{50}$	81	15	−30.21	0.16	−30.58	0.23	0.37
n-C_{24}	82	14	−25.71	0.34	−26.04	0.25	0.33
n-C_{26}	83	15	−29.20	0.24	−29.31	0.25	0.12
Squalane	82	13	−21.91	0.22	−21.88	0.19	−0.03
n-C_{28}	85	13	−26.63	0.23	−26.56	0.56	−0.07
n-C_{30}	89	13	−28.57	0.57	−28.28	0.35	−0.30
n-C_{32}	91	13	−26.75	0.60	−26.27	0.64	−0.47
n-C_{34}	90	13	−29.60	0.63	−29.21	0.92	−0.39

[a] n.d., not determined.

3.3.3. Isotopic fractionation during HPLC isolation

Since chromatographic separation leads to carbon isotopic fractionation of the compound within an eluting peak [15–16], an initial concern with respect to the HPLC approach was the potential for isotopic fractionation as a result of the isolation procedure. Potential carbon isotopic fractionation effects were assessed in circumstances when analytes, such as aliphatic hydrocarbons, were separated into two fractions. This is illustrated in Table 6 with recoveries of aliphatic hydrocarbons for the first and second fraction, as well as the carbon isotope ratios before and after the isolation procedure for F1. The differences in the $\delta^{13}C$ values of the untreated standard mixture and those isolated by HPLC ($\Delta\delta^{13}C < 0.62‰$) fall within the range of uncertainty of the $\delta^{13}C$ values of the compounds in the original mixture. The important result from this series of experiments is that no measurable isotopic fractionation occurred when these compounds were fractionated into two fractions using silica column. This aspect likely reflects the absence of sorption/desorption interactions between non-polar compounds, e.g. aliphatic hydrocarbons and the silica normal phase. Similarly, other preparative HPLC using the molecular sieve properties of zeolite [30] and gel permeation chromatography on gel [31] showed no isotopic fractionation of the steroids and hopanoids, even if they were analysed in separate fractions. Molecular sieving techniques also have no observable fractionation effect on the measurement of $\delta^{13}C$ in partially adducted n-alkanes [32]. In contrast, isotopic chromatographic effects have been observed during reverse phase HPLC or GC separations as a result of different solute-stationary phase interactions dominated by Van der Waals dispersion forces [15–16,33].

4. Overall method and isotope fractionation

Tables 3, 7, 8 and 9 shows the percentage recoveries (REC) and repeatability (R.S.D.), respectively, for fatty acids, aliphatic hydrocarbons, aromatic hydrocarbons, and fatty alcohol and sterol compounds, when standard compounds were used for the assessment of the efficiency of the overall analytical protocol including the extraction, fractionation and derivatisation steps. They also show the differences in $\delta^{13}C$ values of the standards before and after the analytical protocol.

Recoveries of fatty acids from the acidified extract using hexane:ethyl acetate (9:1) solvent ranged from 88 to 106% with R.S.D. $<8\%$ (Table 3). The $\delta^{13}C$ values of the derivatized fatty acid standards and those obtained after extracting and derivatisating the fatty acids were all within the analytical error (± 2S.D. $= 1‰$), showing that the isolation of fatty acids by solvent extraction did not result in any significant thermodynamic isotope effect.

Recoveries of the aliphatic hydrocarbons (Table 7), including n-alkanes of carbon chain length $>n$-C_{16}, isoprenoids and terpenoids ranged from 50 to 60% with R.S.D. $<12\%$. Recoveries yield $<40\%$ and R.S.D. $>40\%$ were obtained for the more volatile n-alkanes (n-C_{12} and n-C_{14}). The $\delta^{13}C$ values of standards before and after the isolation procedure fall well within the analytical error (± 2S.D.), showing that the isolation of aliphatic hydrocarbons by solvent extraction and HPLC procedure did not result in any measurable isotope effect. Although the differences in the $\delta^{13}C$ values ($<1‰$) are within the measurement error (twice 0.5‰), a subtle bias in isotope ratio determinations of the more volatile compounds was observed with a slight

Table 7
Mean recoveries (REC, %) with relative standard deviation (R.S.D., %) of aliphatic hydrocarbons after the overall analytical protocol and differences ($\Delta\delta^{13}C$) of the carbon isotope ratios ($\delta^{13}C$ with standard deviation) before and after the analytical protocol

	F1		Standards		F1		$\Delta\delta^{13}C$
	REC (%)	R.S.D. (%)	$\delta^{13}C$	S.D.	$\delta^{13}C$	S.D.	
n-C_{12}	29	76	−33.51	0.62	−32.77	0.54	−0.73
n-C_{14}	41	39	−32.24	0.45	−31.24	0.51	−1.00
n-C_{16}	49	19	−31.79	0.41	−31.15	0.26	−0.64
n-C_{17}	51	12	−27.73	0.20	−27.82	0.20	0.09
Pristane	52	12	−25.76	0.47	−25.76	0.36	−0.01
n-$C_{18:1}$	52	10	−31.88	0.60	−31.96	0.51	0.08
n-C_{18}	54	9	−31.50	0.37	−31.49	0.54	−0.02
Phytane	54	9	−31.05	0.18	−30.79	0.53	−0.26
n-C_{20}	57	6	−34.22	0.26	−33.79	0.36	−0.43
n-C_{21}	57	6	−34.75	0.12	−34.87	0.17	0.12
n-C_{22}	57	6	−24.86	0.71	−24.71	0.43	−0.16
n-C_{24}	58	7	−25.97	0.42	−25.91	0.26	−0.06
n-C_{26}	58	5	−32.92	0.66	−32.28	0.43	−0.64
Squalane	58	5	−22.12	0.64	−22.49	0.52	0.37
n-C_{28}	57	5	−26.44	0.68	−26.52	0.58	0.08
n-C_{30}	58	4	−28.40	0.60	−28.23	0.52	−0.18
n-C_{32}	60	6	−26.91	0.64	−27.28	0.38	0.38
n-C_{34}	59	4	−29.44	0.59	−29.44	0.32	0.00
5β-Cholane	60	7	−18.29	0.14	−18.48	0.49	0.19
Hopane[a]	58	6	−27.67	0.27	−28.29	0.37	0.62
Diploptene[b]	55	6	−27.88	0.51	−28.45	0.39	0.57
Lycopane	59	5	−27.79	0.07	−27.85	0.19	0.06
β,β-Carotene	58	5	−28.37	0.23	−28.60	0.33	0.23
$C_{24}{}^{2}H_{50}$	57	6	−30.90	0.49	−30.45	0.55	−0.45

[a] 17β(H),21β(H)hopane.
[b] hop-22(29)-ene.

deviation toward heavier isotopic values. These results are consistent with the thermodynamic isotope effect caused by slight differences in vapour pressure between the heavier and lighter isotopomer.

Recovery yield values from 50 to 60% and R.S.D. <12% were obtained for PAHs containing more than two rings, whereas lower recoveries and higher R.S.D. were measured for the more volatile PAHs, naphthalene to acenaphthene (Table 8). Similar to the aliphatic hydrocarbons, the $\delta^{13}C$ differences values ($\Delta\delta^{13}C$) of standards before and after the isolation procedure were lower than the analytical error, with the highest differences for the more volatile com-

Table 8
Mean recoveries (REC, %) with relative standard deviation (R.S.D., %) of aromatic hydrocarbons after the overall analytical protocol and differences ($\Delta\delta^{13}C$) of the carbon isotope ratios ($\delta^{13}C$ with standard deviation) before and after the analytical protocol

	F2		Standards		F2		$\Delta\delta^{13}C$
	REC (%)	R.S.D. (%)	$\delta^{13}C$	S.D.	$\delta^{13}C$	S.D.	
Naphthalene	36	49	−24.44	0.37	−23.54	0.53	−0.90
C1-naphthalene	43	28	−29.60	0.61	−28.63	0.61	−0.97
C2-naphthalene	47	22	−24.01	0.46	−23.40	0.45	−0.61
Acenapthylene	45	18	−22.15	0.45	−21.80	0.38	−0.35
Acenapthene	46	17	−23.25	0.45	−22.85	0.47	−0.40
Fluorene	51	12	−23.91	0.50	−23.50	0.70	−0.41
Phenanthrene	54	9	−24.28	0.65	−23.90	0.65	−0.38
Anthracene	56	8	−22.22	0.52	−21.85	0.62	−0.37
C1-Phenanthrene	56	10	−23.50	0.74	−23.00	0.70	−0.50
C1-Phenantrene	56	19	−24.77	0.73	−24.00	0.70	−0.77
C2-Phenanthrene	59	9	n.d.[a]	–	n.d.	–	–
Fluoranthene	58	8	−23.81	0.29	−23.50	0.40	−0.31
Pyrene	62	9	−24.54	0.26	−24.72	0.38	0.18
1-Methylpyrene	59	8	−25.95	0.60	−26.25	0.50	0.30
Chrysene	63	9	−22.88	0.19	−23.40	0.52	0.52
Perylene	60	10	−23.3	0.59	−23.80	0.65	0.50

[a] n.d., not determined.

Table 9
Mean recoveries (REC, %) with relative standard deviation (R.S.D., %) of fatty alcohol and sterols after the overall analytical protocol and differences ($\Delta\delta^{13}C$) of the carbon isotope ratios ($\delta^{13}C$ with standard deviation) before and after the analytical protocol

	F4		Standards		F4		$\Delta\delta^{13}C$
	REC (%)	R.S.D. (%)	$\delta^{13}C$	S.D.	$\delta^{13}C$	S.D.	
n-C_{14}-OH	80	5	−29.30	0.19	−29.59	0.34	0.28
n-C_{16}-OH	76	6	−29.27	0.24	−29.41	0.53	0.14
n-C_{18}-OH	76	7	−30.33	0.44	−30.55	0.37	0.21
n-C_{20}-OH	73	7	−28.34	0.57	n.d.[a]	–	–
n-C_{22}-OH	72	7	−29.23	0.60	−29.40	0.61	0.17
n-C_{24}-OH	72	6	−28.40	0.61	−28.20	0.36	−0.20
Coprostanol[1]	65	8	−26.54	0.18	−26.79	0.21	0.26
Cholesterol[2]	60	6	−24.81	0.38	−25.07	0.25	0.26
Cholestanol[3]	69	2	−25.05	0.26	−25.34	0.15	0.29
Campesterol[4]	60	5	−29.11	0.24	−29.39	0.07	0.28
Sitosterol[5]	60	6	−28.93	0.10	−29.20	0.16	0.26
Androstanol[6]	66	4	−32.23	0.41	n.d.	–	–

1: Coprostanol, 5β-cholestan-3β-ol; 2: cholesterol, cholest-5-en-3β-ol; 3: cholestanol, 5α-cholestan-3β-ol; 4: campesterol, 24α-methylcholest-5-en-3β-ol; 5: sitosterol, 24-ethylcholest-5-en-3β-ol; 6: androstanol, 5α-androstan-3β-ol.
[a] n.d., not determined.

pounds, which showed a similar enrichment in the heavier isotopomer.

Recovery yields for fatty alcohols and sterol compounds ranged from 60 to 80% with R.S.D. <8% (Table 9). The $\delta^{13}C$ values of standards before and after the isolation procedure agreed within 0.3‰, a value considered well within the instrument precision. This close agreement shows that no measurable mass discrimination or isotopic fractionation effect occurs during the isolation of these compounds by solvent extraction and HPLC fractionation.

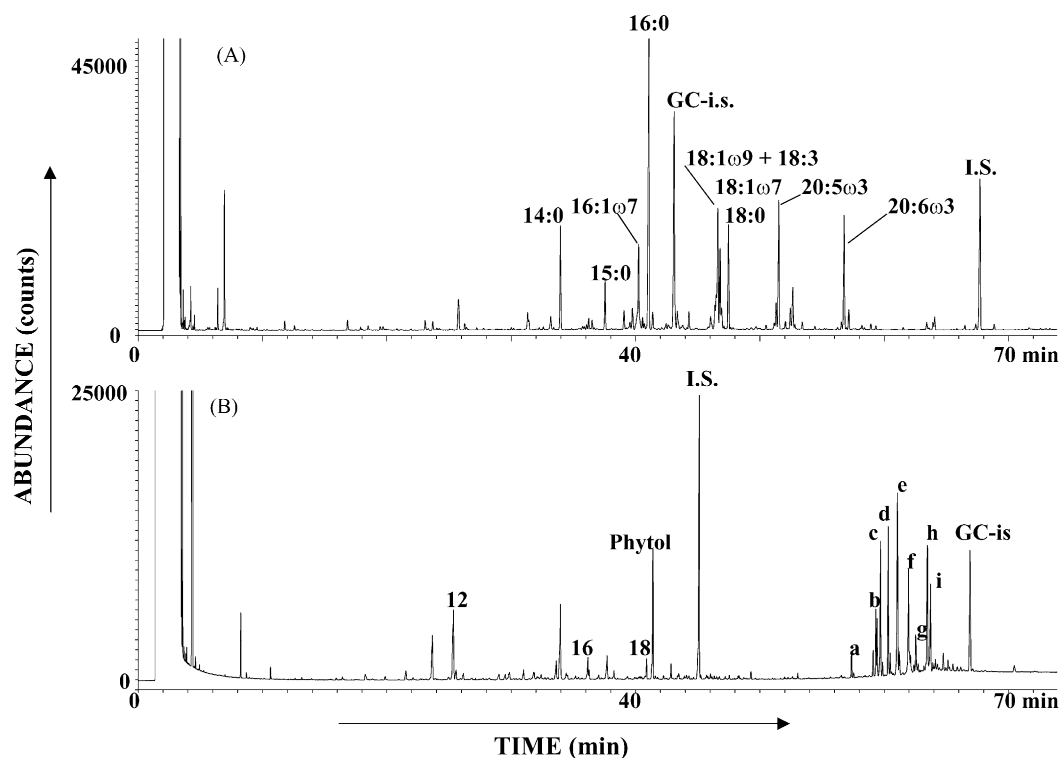

Fig. 4. FID chromatograms of the acid fraction (A) and the sterol-alcohol fraction (B) of marine particulate material from sediment traps in Almeria-Oran frontal zone after the overall isolation procedure. Compound codes for the acid fraction: n:pωx are fatty acids with n the number of carbon atoms, p the number of double bonds, the first double bond being located between x and $x + 1$ relative to the terminal methyl group; GC–is and IS are, respectively, 5α-androstane and cholanic acid. Compound codes for the sterol-alcohol fraction are: number, carbon atoms in n-alkanols: (a) 24-Norcholesta-5,22(E)-dien-3β-ol; (b) 27-Nor-24-methylcholesta-5,22(E)-dien-3β-ol; (c) cholesta-5,22(E)-dien-3β-ol; (d) cholest-5-en-3β-ol (cholesterol); (e) 24-methylcholesta-5,22(E)-dien-3β-ol (brassicasterol); (f) 24-methylcholesta-5,24(28)-dien-3β-ol; (g) 24-ethylcholesta-5,22(E)-dien-3β-ol (stigmasterol); (h) 24-ethylcholest-5-en-3β-ol (sitosterol); (i) 24-etylcholesta-5,24(28)(Z)-dien-3β-ol (fucosterol); (IS) 5α-androstan-3b-ol; (GC-is) friedelin.

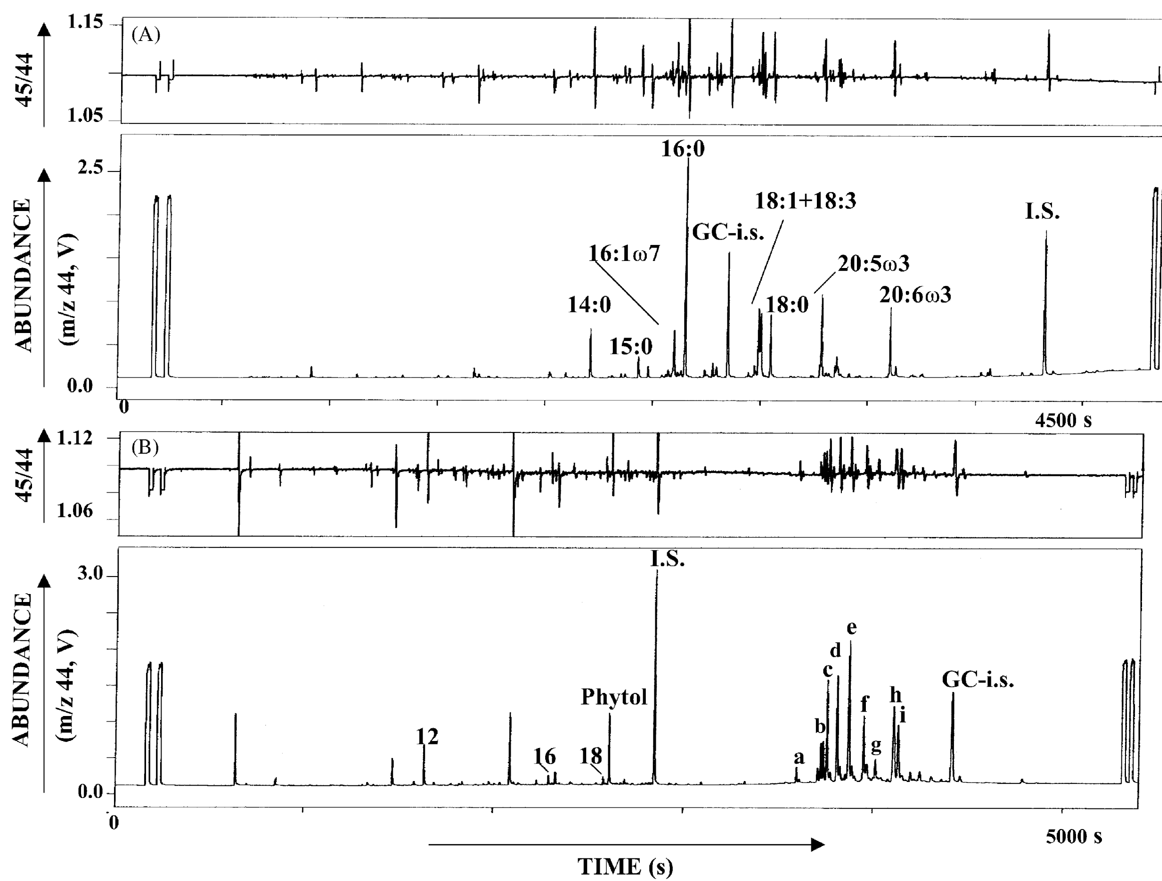

Fig. 5. GC–C–IRMS chromatograms of the acid fraction (A) and the sterol-alcohol fraction (B) of marine particulate material from sediment traps in Almeria-Oran frontal zone after overall isolation procedure. The first and last pair of peaks correspond to the reference CO_2 gas. For abbreviation codes see legend from Fig. 4.

Table 10
Certified and measured PAHs concentrations ($ng\,g^{-1}$) in certified reference material IAEA-383 (marine sediment)

Compound	*m/z*	Measured values	Certified values	
			Median value	95% Confidence interval
[$^2H_8$]Naphthalene (REC, %)	136	32		–
[$^2H_{10}$]Antracene (REC, %)	188	54		–
[$^2H_{10}$]Pyrene (REC, %)	212	60		–
[$^2H_{12}$]Perylene (REC, %)	264	70		–
Phenanthrene ($ng\,g^{-1}$)	178	179	160	140–190
Anthracene ($ng\,g^{-1}$)	178	22	30	25–34
Fluorene ($ng\,g^{-1}$)	166	23	27	24–34
Naphthalene ($ng\,g^{-1}$)	128	125	96	52–110
Fluoranthene ($ng\,g^{-1}$)	202	328	290	260–350
Pyrene ($ng\,g^{-1}$)	202	220	280	210–350
Benzo[*a*]anthracene ($ng\,g^{-1}$)	228	64	105	83–130
Chrysene ($ng\,g^{-1}$)	228	129	170	120–220
Benzo[*e*]pyrene ($ng\,g^{-1}$)	252	194	160	120–210
Benzo[*a*]pyrene ($ng\,g^{-1}$)	252	141	120	77–140
Perylene ($ng\,g^{-1}$)	252	61	58	41–130
Indeno[1,2,3-*cd*]pyrene ($ng\,g^{-1}$)	276	148	150	130–160
Benzo [*ghi*]perylene ($ng\,g^{-1}$)	276	224	110	69–230

Overall, this study demonstrates that losses during the solvent removal step by nitrogen blow-down of the more volatile compounds (n-C_{12}, n-C_{14}, naphthalene, methylnaphthalene) resulted in lower recoveries and a slightly more enriched $\delta^{13}C$ values.

5. Application to marine particulate samples

The described method for separation of lipid classes was successfully applied to determine the molecular abundance and carbon isotope ratios of individual sterol, alcohol and fatty acid compounds in marine particulate material collected in the Almeria-Oran frontal zone [34,35]. Figs. 4 and 5 show, respectively, a typical GC–FID and GC–C–IRMS of the sterol and fatty acid fractions using capillary columns coated with a non-polar phase. The detection limits of these lipid biomarkers were estimated to range between 6 and 15 ng g^{-1} for individual compound based on a typical FID instrumental signal to noise of 3, a sample weight of 2 g and a final extract volume of 100 μl.

The $\delta^{13}C$ values of 24-ethylcholest-5-en-3β-ol in sinking particles from Almeria-Oran frontal zone fell in the range −24.9 to −27.6‰ [34]. These values were somewhat intermediary between typical terrestrial (−35 to −27.5‰; [36,37]) and marine levels (−26.8 to −18.7‰; [38,39]). However, the isotopic signature of 24-ethylcholest-5-en-3β-ol agreed with that of several other related compounds, some of which are known to be derived from marine algae, such as methylcholesta-5,22(*E*)-dien-3β-ol and 24-methylcholesta-5,24(28)-dien-3β-ol. Thus, it was inferred that 24-ethylcholest-5-en-3β-ol in large particles from Almeria-Oran frontal zone was derived from marine algae rather than terrestrial sources. The low abundance (<5.8%) of long-chain fatty acids (LCFAs > C_{22}) and their relative enriched $\delta^{13}C$ values (−24 to −30‰) compared to the terrestrial LCFAs (−30 to 35‰; [40,26,41]) also demonstrated that the contribution of organic matter from continental inputs was negligible in the area [35]. Fatty acid distribution and compound-specific isotope analysis of $\delta^{13}C$ ranging from −22 to −32‰ suggested that the acids were totally marine in origin. The $\delta^{13}C$ of the bacterial FA (e.g. *iso*-and *anteiso*-C_{15} and C_{17} FAs) were comparable to those typical of phytoplankton (16:1ω7, 20:5ω3) which likely indicated recycling and resynthesis of organic material with no significant isotope fractionation by a bacterial population associated with the planktonic biomass.

The efficiency of the protocol described here was also demonstrated for analyses of hydrocarbons in a certified reference material from IAEA (IAEA-383) [42]. Fig. 6 shows the representative GC–MS total scan chromatogram of the non-polar aliphatic hydrocarbon fraction (Fig. 6A) and the corresponding semipolar PAH fraction (Fig. 6B). The detection limits of these individual hydrocarbons were estimated

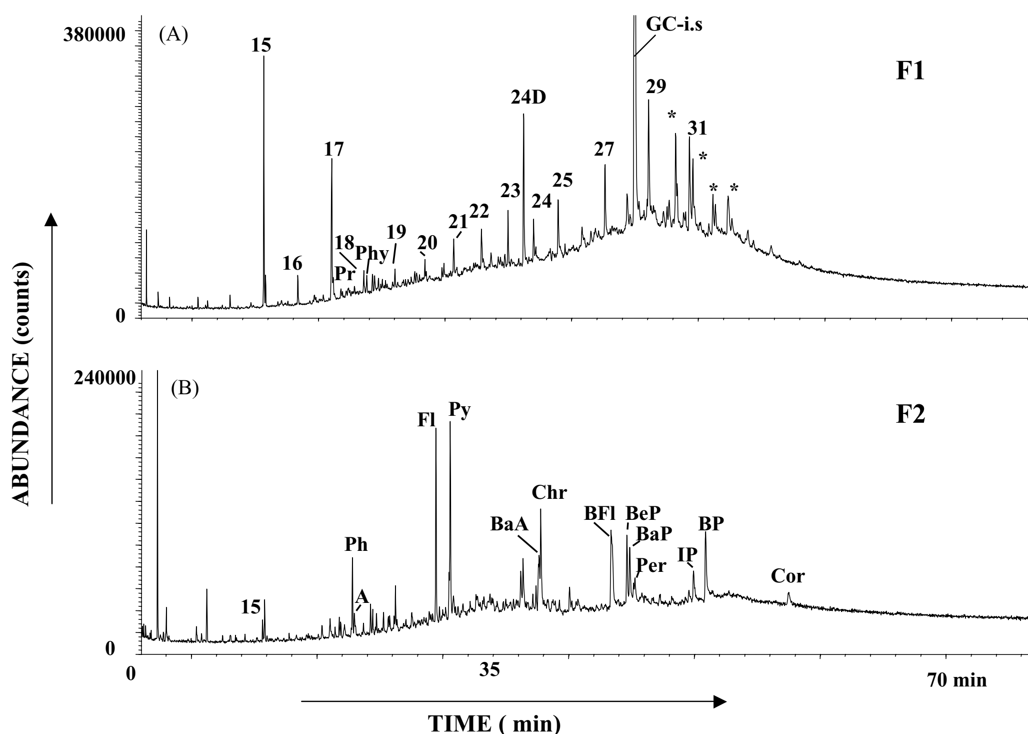

Fig. 6. GC–MS total scan chromatograms of the aliphatic hydrocarbon fraction (F1) and aromatic hydrocarbon (F2) fraction of the IAEA-383 certified reference sediment after all isolation procedure. Abbreviation codes as those from Table 2; GC-is: 5α-cholestane; asterisk (*) indicates terpenoid compounds; 24D: n-$C_{24}{}^{2}H_{50}$, internal standard; BaA, benzo[*a*]anthracene; BFl, benzo[*b*]fluoranthene + benzo[*j*]fluoranthene + benzo[*k*]fluoranthene + benzo[*a*]fluoranthene; BeP, benzo[*e*]pyrene; BaP, benzo[*a*]pyrene; IP, indeno[1,2,3-*cd*]pyrene; BP, benzo[*ghi*]perylene; Cor, Coronene.

to be ~10 ng g^{-1} for the typical FID and ~1 ng g^{-1} for the GC–MS–SIM instrument, based on the detection limit of each instrument, a sample weight of 2 g and a final extract volume of 100 μl. Reliable fractionation of the hydrocarbons and quantitative values of PAHs by GC–MS–SIM are shown in Table 10. Recoveries of deuterated internal PAH standards from certified reference sediment compared well with those obtained from spiked samples (Table 8). Although low recoveries were obtained for the more volatile compounds ([$^2H_8$]naphthalene, 32%), quantification by deuterated internal standards obtained PAH concentrations in agreement with the certified values. These results emphasise the importance of quantification by internal standardisation using internal standards of similar physico-chemical characteristics to the compounds being quantified (e.g. perdeuterated compounds).

6. Conclusions

An analytical method for the isolation of neutral and acid lipid classes with negligible carbon isotopic fractionation has been developed and optimised. The proposed method includes an HPLC step to isolate neutral lipid classes that offers a combination of simplicity and automation. Overall, the isolation method did not result in any significant carbon isotopic fractionation. The highest differences of $\delta^{13}C$ were obtained for the more volatile compounds (*n*-alkanes<*n*-C_{16} and PAHs with two rings), which also showed the lowest extraction efficiencies (<40%). A slight deviation toward heavier isotopic values (up to l‰) was consistent with the thermodynamic isotope effect caused by slight differences in vapour pressure between heavier and lighter isotopomers. This study demonstrates the presence of a subtle bias in isotope ratio determinations of volatile compounds, but good accuracy for the concentration levels of these volatile components might be achieved using internal standards of similar chemical structure.

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