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Rapid isolation of biomarkers for compound specific radiocarbon dating using high-performance liquid chromatography and flow injection analysis–atmospheric pressure chemical ionisation mass spectrometry

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Abstract

Repeated semi-preparative normal-phase HPLC was performed to isolate selected biomarkers from sediment extracts for radiocarbon analysis. Flow injection analysis–mass spectrometry was used for rapid analysis of collected fractions to evaluate the separation procedure, taking only 1 min per fraction. In this way 100–1000 μg of glycerol dialkyl glycerol tetraethers, sterol fractions and chlorophyll-derived phytol were isolated from typically 100 g of marine sediment, i.e., in sufficient quantities for radiocarbon analysis, without significant carbon isotopic fractionation or contamination.

Keywords: Radiocarbon dating; Sediments; Glycerol dialkyl glycerol tetraethers; Sterols; Phytols

1. Introduction

Radiocarbon analysis has been used since the 1950s for dating purposes, but only since the development of accelerated mass spectrometry (AMS) in the 1990s, has the required sample size been reduced to such an extent that compound-specific radiocarbon analysis has become possible [1]. Several scientific disciplines could potentially benefit from this development, ranging from earth sciences to archaeology and environmental research. For instance, radiocarbon analysis of specific sedimentary organic compounds of known origin (so-called biomarkers) can be used as an alternative dating method for carbonate-poor, but organic carbon-rich sediments. Dating based on total organic matter is not possible since this organic matter is a complex mixture derived from different sources with different radiocarbon ages [2–4]. In addition, a greater insight in carbon cycling processes can be gained by measuring the age range of different organic matter fractions or organic compounds in sediments or soils [2,5,6]. Compound-specific radiocarbon analysis mainly focuses on lipids because other compounds like proteins and carbohydrates generally degrade much faster and are in general virtually absent in the sedimentary record [7].

To measure the radiocarbon contents of individual

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lipids, substantial amounts (>30 μg) are needed [1]. Furthermore, carbon isotopic fractionation effects during the isolation procedure should be avoided as much as possible as this will influence the ^{14}C content of the compounds, although the final determination of the radiocarbon age is corrected for fractionation using the $\delta^{13}C$ value of the measured compound [8]. Measuring original ^{14}C contents is, however, always better than measuring ^{14}C contents which are heavily influenced by method induced fractionation effects.

So far, isolation of specific lipids for radiocarbon dating from complex mixtures of organic compounds has only been done successfully using preparative capillary gas chromatography [1,9]. However, this method is time-consuming and can only be used for relatively apolar, low-molecular-mass compounds, typically hydrocarbons and derivatized alcohols or alkanoates with less than 30 carbon atoms. Furthermore, if derivatized compounds are measured, an additional measurement error is introduced by the added carbon atoms of the derivitazing agent, for which has to be corrected.

Here we present a relatively fast method to isolate higher molecular mass ($>C_{30}$) and more polar lipid biomarkers out of sedimentary lipid extracts using semi-preparative high-performance liquid chromatography (HPLC) and flow injection analysis–mass spectrometry (FIA–MS). The method enables the isolation of glycerol dialkyl glycerol tetraethers (GTGTs), specifically GDGT-0 and crenarchaeol (Fig. 1, I and II, respectively), which are produced by archaea, two sterol fractions (Fig. 1, III and IV), derived from eukaryotes, and chlorophyll-derived phytol (Fig. 1, V) derived from photosynthetic organisms, without fractionation and without detectable contamination, in quantities sufficient for radiocarbon dating.

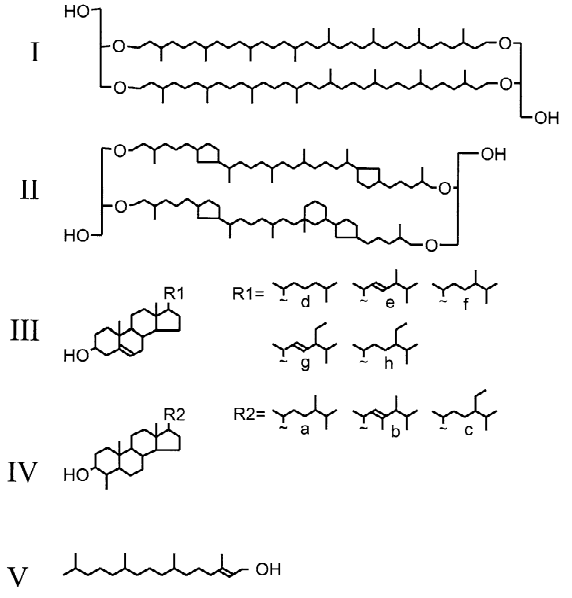

Fig. 1. Structures of isolated compounds. (I) GDGT-0. (II) Crenarchaeol. (III) Δ5-Sterols. (IV) 4-Methylsterols. (V) Phytol. Different sterols are defined by different groups R1 and R2: (a) 4,24-dimethyl-5α(H)-cholest-3β-ol; (b) 4,23,24-trimethyl-5α(H)-cholest-22-en-3β-ol (dinosterol); (c) 4-methyl, 24-ethyl-5α(H)-cholest-3β-ol; (d) cholest-5-ene-3β-ol; (e) 24-methyl-cholest-5,22-diene-3β-ol; (f) 24-methyl-cholest-5-ene-3β-ol; (g) 24-ethyl-cholest-5,22-diene-3β-ol; (h) 24-ethyl-cholest-5-ene-3β-ol.

2. Experimental

2.1. Sediment samples, controls and reference compounds

Sediment sub-samples of different age were obtained from piston cores taken in 1996 at Hole 1034C of Ocean Drilling Project leg 169S, Saanich Inlet, Canada [10]. In addition, a freeze core was taken in the Saanich Inlet in 1998, from which two subsamples were taken.

To ensure that no contamination of allochthonous carbon was introduced during the isolation procedure, both a radiocarbon dead sample and a modern sample were subjected to the isolation procedure. Radiocarbon dead GDGTs were isolated from a 6-million-year-old Miocene marl from Vena del Gesso basin, Italy (for further information, see Sinninghe Damsté et al. [11]). A cholesterol standard (99+%; Sigma–Aldrich, Steinheim, Germany) with a modern radiocarbon signature served as the modern carbon control, as well as a control on carbon isotopic fractionation. The cholesterol standard also served as a testing and reference compound for HPLC–MS. A phytol standard (90–95%; Fluka, Buchs, Switzerland) was also used as a reference compound.

2.2. Lipid extraction and pre-treatment

Sediment samples (100–150 g dry mass) were freeze dried before lipid extraction. Extraction was performed with a dichloromethane–methanol (DCM–MeOH, 9:1, v/v) mixture using an Automated Solvent Extractor (Dionex, Sunnyvale, CA, USA), deployed over three static cycles of 5 min of 100 °C and 1000 p.s.i., totalling on average 200 ml of solvent (1 p.s.i.=6894.76 Pa). To remove salt, the extracts were washed with 100 ml double-distilled water against DCM in a separatory funnel, and dried over Na_2SO_4. The bulk of the solvent was removed by rotary evaporation and the remaining solvent under a stream of nitrogen. The extracts were subsequently separated into two fractions by column chromatography over activated Al_2O_3 with *n*-hexane (hydrocarbon fractions, 4 column volumes) and DCM–MeOH (1:1, v/v; polar fractions, 3 column volumes) as eluents. Column volumes were 25–30 ml and column height was 13–15 cm. The polar fractions were used for the HPLC isolation procedure (Fig. 2). The solvents of the polar fractions were removed as described above, and the residues dissolved by sonication (10 min) in ca. 2 ml of *n*-hexane–isopropanol (99:1, v/v). The resulting suspensions were centrifuged (1 min, 2300 *g*) and the supernatants were filtered through a 4-mm diameter PTFE filter (0.45 μm pore size) to produce fractions suitable for injection on HPLC. The residues after filtration were recovered by rinsing the filter with DCM, and combined with the pellets that remained after centrifugation. These residues were combined with the backflush fractions collected from the semi-preparative HPLC step (see Section 2.3). The solvents of the combined fractions were removed as described above, and the residues saponified by reflux (1 h) in 10 ml 1 *M* KOH–MeOH (96%) solution. The fractions were acidified to pH 3 with 2 *M* HCl in water–MeOH (1:1, v/v) and washed with DCM and double-distilled water in a separatory funnel. The DCM fractions, containing the saponified polar lipids, were dried over Na_2SO_4. Further preparation for HPLC was performed as described above.

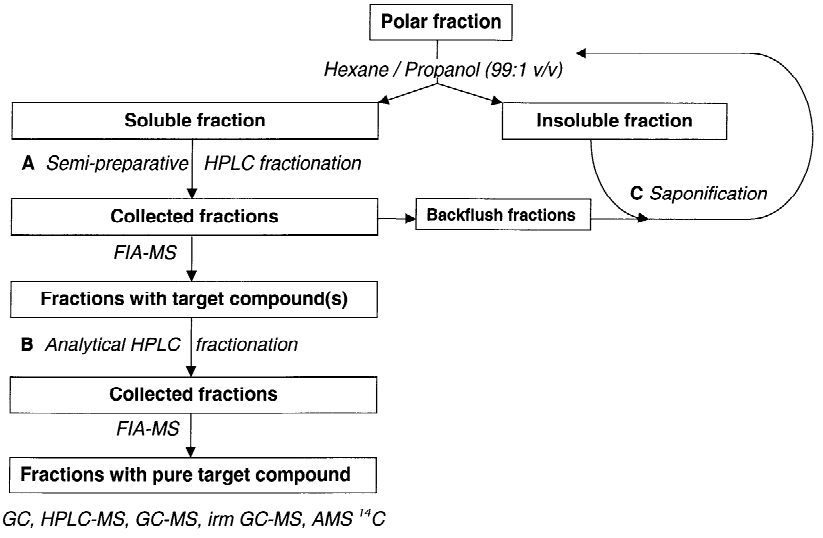

Fig. 2. Flow diagram of the followed isolation procedure. The polar fraction of a total lipid extract is dissolved in *n*-hexane–isopropanol (99:1, v/v) by sonication (10 min). The soluble part of the fraction is subjected to semi-preparative HPLC (step A) to yield 1-min fractions. Identification of target compound containing fractions is performed by FIA–MS. Fractions containing the same compound are combined and these combined fractions are then each subjected to a second preparative HPLC fractionation step (B) to yield 0.5-min fractions. Again FIA–MS is performed to identify which fractions contain the target compound. These are combined and dried to yield isolated compounds. The part not amenable by the followed HPLC technique, i.e., the backflush fraction combined with the fraction insoluble in *n*-hexane–isopropanol (99:1, v/v), is saponified by basic hydrolysis (step C). The resulting in *n*-hexane–isopropanol (99:1, v/v) soluble fraction is then subjected to steps A and B. irm: isotope ratio monitoring; AMS: accelerator mass spectrometry.

2.3. Isolation of compounds by semi-preparative HPLC

For semi-preparative HPLC, a Hewlett-Packard (Palo Alto, CA, USA) 1100 series HPLC equipped with an autoinjector and Chemstation chromatography manager software was used, coupled to an Isco (Lincoln, NE, USA) Foxy Jr. fraction collector. Fractions soluble in *n*-hexane–isopropanol (99:1, v/v) (see Section 2.2) were first separated using a semi-preparative NH_2 column (Econosphere, 250× 10 mm, 10 μm; Alltech Associates), maintained at 30 °C. Injection volumes were typically 100 μl, containing up to 1 mg of material. Compounds were eluted isocratically with 99% *n*-hexane and 1% isopropanol for 5 min, followed by a linear gradient to 1.8% isopropanol in 44 min. Flow rate was 2.5 ml/min. After each run, the column was cleaned by back-flushing *n*-hexane–isopropanol (90:10, v/v) for 10 min. All eluents, including the backflush fraction, was collected in 1-min fractions in pre-combusted (480 °C; 8 h) glass tubes. A second HPLC purification was performed on an analytical NH_2 column (Econosphere, 250×4.6 mm, 5 μm; Alltech Associates) using injection volumes of typically 25 μl out of 200 μl in which the fractions were concentrated. The same conditions as described above were used, except that the flow-rate was reduced to 1 ml/min and that back flushing was performed with *n*-hexane–isopropanol (95:5, v/v). Fractions of 30 s were collected during time windows where target compounds were expected to elute.

After isolation, the fractions containing 4-methylsterols were eluted over a pipette column partly filled with pre-combusted silicalite (PQ Zeolites, The Netherlands) with ethyl acetate, a method similar as described by West et al. [12], to remove *n*-alkanols which were also present in these fractions.

As a reference compound for recovery, purity (contamination) and carbon isotopic effects a known amount (~1 mg) of the cholesterol standard was also subjected to the isolation procedure.

2.4. Flow injection analysis–atmospheric pressure chemical ionization mass spectrometry

Small aliquots of collected fractions (10–30 μl; 0.02–0.1% of the total, 1% in case of phytol) were analyzed by flow injection analysis–atmospheric pressure chemical ionization mass spectrometry (FIA–APCI-MS) using the same HPLC system as described above, coupled to a Hewlett-Packard HP 1100 MSD mass spectrometer using atmospheric pressure chemical ionization (APCI-MS), operated in positive ion mode. Conditions for APCI-MS were as follows: nebulizer pressure 50 p.s.i., vaporizer temperature 400 °C, capillary voltage −4 kV, corona current 4 μA (≈1.8 kV). Injections were made at 1-min intervals into a stream of *n*-hexane–isopropanol (99:1, v/v) with a flow-rate of 1 ml/min. Positive ion spectra were in general generated by scanning *m/z* 300–1450 (stepsize 0.1, 2.04 s/cycle) and in some cases by scanning *m/z* 150–1450 for tetraethers (Fig. 1, I and II), Δ5 sterols (Fig. 1, III) and 4-methylsterols (Fig. 1, IV) and by scanning *m/z* 200–450 (stepsize 0.1, 1.92 s/cycle) for phytol (Fig. 1, V).

2.5. High-performance liquid chromatography–mass spectrometry

HPLC–MS analysis of individual tetraethers was performed on 1% aliquots of the obtained tetraether fractions using the same system as described above, following the method as described by Hopmans et al. [13]. Quantification was performed by integration of peaks in the summed mass chromatograms of $[M+H]^+$ and $[M+H]^+ +1$ ions and comparison with a standard curve obtained using a dilution series of a known amount of a GDGT-0 standard.

2.6. Gas chromatography and gas chromatography–mass spectrometry

To quantify amounts of sterols and chlorophyll-derived phytol, GC analyses were performed on 1 or 2% aliquots of total fractions, using a Fisons 8000 series instrument equipped with an on-column injector and a flame ionisation detection (FID) system. A fused-silica capillary column (25 m×0.32 mm) coated with CP Sil 5 (film thickness 0.12 μm) was used with helium as carrier gas. Known amounts of deuterated ante-iso C_{22}-alkane standard were added to the aliquots for quantification. The fractions were subsequently dissolved in 10 μl pyridine together with 10 μl bis(trimethylsilyl)trifluoroacetamide

(BSTFA). This mixture was heated (60 °C; 20 min) to convert alcohols into their corresponding trimethylsilyl ethers. The derivatized fractions were diluted with 75 μl ethyl acetate and injected at 70 °C. The oven was programmed to 130 °C at 20 °C/min and then at 4 °C/min to 320 °C at which it was held for 10 min. Quantification of the amounts of sterols and phytol was performed by comparing their integrated peak areas with that of the added standard.

GC–MS was performed using a Hewlett-Packard 5890 gas chromatograph interfaced to a VG Autospec Ultima mass spectrometer operated at 70 eV with a mass range of m/z 50–800 and a cycle time of 1.7 s (resolution 1000). Gas chromatography was performed as described above. Compounds were identified by comparison of mass spectra and retention times with those reported in literature.

2.7. Stable carbon and radiocarbon isotopic measurements

To check for unwanted fractionation effects, compound specific stable carbon isotopic compositions of some phytol fractions and of the cholesterol standard were measured before and after isolation. Analyses were performed on a Delta-C or a Delta-plus XL isotope ratio monitoring (IRM) GC–MS system, both in principle similar to the Delta-S system as described previously by Merrit et al. [14]. The gas chromatograph was set up as described above. The carbon isotopic compositions of the compounds were corrected for added carbon molecules by using BSTFA with a known carbon isotopic value. Values reported were determined by two or three replicate analyses, and the results were averaged to obtain a mean value and to evaluate the measurement error.

Radiocarbon analyses were performed at the National Ocean Sciences AMS Facility (NOSAMS), Woods Hole, MA, USA. The isolated compounds were combusted to CO_2 and subsequently reduced to graphite using Cobalt as a catalyst, following the procedure described by Pearson et al. [15]. Stable carbon isotope measurements were also performed on subsamples of the CO_2 gas produced during the graphitization process with a VG Prism [16].

Stable carbon isotopic compositions are reported in standard delta notation relative to the VPDB standard, with a standard error of 0.4‰ in the case of IRM-GC–MS measurement, and 0.1‰ in the case of measurement on CO_2. ^{14}C contents are expressed as fraction modern [16].

2.8. ^{13}C and ^{1}H NMR

To evaluate the purity of isolated fractions, a representative isolated GDGT-0 (Fig. 1, I) fraction was analyzed by high field ^{13}C NMR and a representative isolated phytol (Fig. 1, V) fraction was subjected to ^{1}H NMR. NMR spectrometry was performed on a Bruker ARX400 spectrometer equipped with a dual ^{1}H–^{13}C probe. Spectra were obtained at 300 K in C^2HCl_3. Proton and carbon chemical shifts were referenced to internal C^2HCl_3 (7.24/77.0 ppm).

3. Results and discussion

3.1. Target lipids

Compound specific radiocarbon analysis can only be performed when the desired compounds can be isolated from sediments in quantities large enough for AMS, i.e., >30 μg. For the specific purpose of dating, they need to be derived from a marine organism, as terrestrial sourced compounds can be of considerable age before they enter a marine sediment. In contrast, marine derived compounds need only to be corrected for a, generally known, reservoir effect. A good candidate is crenarchaeol (Fig. 1, II), which is present in high quantities in globally distributed sediments [17], and is derived from ubiquitously occurring marine pelagic crenarchaeota [18]. Also present in high quantities in almost all sediments is GDGT-0 (Fig. 1, I) [17], a lipid produced by a large number of archaea [19].

A third candidate is the class of 4-methylsterols (Fig. 1, IV), which are predominantly produced by dinoflagellates [20] and can therefore also possibly be used as marine biomarkers suitable for compound specific radiocarbon dating. As any single 4-methylsterol, isolation of dinosterol (Fig. 1, IV, b) would not yield enough material, but the isolation of 4-methylsterols as a class would. Pearson et al. [2] showed that the entire compound class of sterols could serve as an excellent proxy for the ^{14}C

concentration of ocean surface waters. This suggests that the isolation of sterols in classes instead of isolation of individual lipids is a valid procedure. Furthermore, all 4-methylsterols are derived from the same source with the same radiocarbon content. For these reasons, it was decided to isolate these compounds as a group.

For the same reasons, it was also decided to isolate Δ5 sterols (Fig. 1, III) as a group. These compounds are produced by nearly all aerobic eukaryotes and are virtually absent in prokaryotes, and can therefore be regarded as biomarkers for phototrophic eukaryotes [21]. Many of them have been attributed to phytoplankton as well as to terrestrial plants [22], but the most abundant sedimentary Δ5 sterols (C_{27} and C_{28}) are generally assumed to be of phytoplanktonic origin. Furthermore, the investigated setting of Saanich Inlet is known to be highly productive [23] and the sedimentary organic carbon to be of a predominant marine origin [24].

A fifth candidate selected for isolation is phytol (Fig. 1, V) that is released upon saponification of the residual polar fraction of the total lipid extract (Fig. 2). This phytol is derived from non- or hardly degraded chlorophyll and has, like the Δ5 sterols, an almost exclusively phytoplanktonic origin [25]. Free sedimentary phytol was only present in low amounts while phytol still bound to chlorophyll moieties was present in quantities sufficient for radiocarbon analysis.

3.2. Isolation

Compounds had to be isolated in substantial amounts compared to amounts generally handled on current analytical chromatographic equipment. Therefore, it was necessary to start with large amounts of lipid extracts (approximately 20 mg of polar material, derived from 100 to 150 g of freeze-dried sediment). To increase throughput, the separation was performed in two steps (Fig. 2). A first purification of the target compounds was performed by semi-preparative HPLC, where 1-min fractions were collected. The use of a semi-prep column allowed for a relatively large throughput of polar fractions (ca. 1 mg/injection). After combination of collected fractions containing the same compounds, they were further purified on an analytical size column. In this step smaller time fractions (0.5 min) were collected to preserve the resolution obtained by the column. Free sedimentary phytol, collected after steps A and B (Fig. 2) coeluted with other compounds and was only present in low amounts. Therefore this phytol was not used. Phytol released upon saponification of the residual polar fraction of the total lipid extract (step C, Fig. 2) was isolated by a second performance of steps A and B. In this second isolation, compounds coeluting with phytol were absent.

A fast detection of target compounds in the collected fractions was performed by FIA–APCI-MS. Lipid detection by UV was not feasible, as the target lipids contain no chromophores. The great advantage of FIA is the (machine) time that can be saved: while GC or HPLC–MS analyses take more than 1 h per isolated fraction, FIA only takes 1 min per sample. To first assess the quality and specificity of the spectra generated by FIA–APCI-MS, the results of the FIA of the first batch of collected fractions were compared with GC, GC–MS and HPLC–MS analyses of these fractions. This made it possible to routinely scan for target compounds in later collected fractions derived from successive extracts. The total ion current (TIC, Fig. 3) generated by this method provides hardly any information due to a high background signal. The base peak chromatogram (BPC, Fig. 3) indicates, however, in which fractions single compounds are dominantly present. By using specific mass chromatograms and peak apex mass spectra (Figs. 3 and 4) the different target compounds could rapidly be traced within the collected fractions.

Fractions containing 4-methylsterols (Fig. 1, IV) were recognizable by m/z 428 generated by its dominant constituent dinosterol (Fig. 1, IV, b). They eluted after approximately 17 min during the semi-preparative step (Fig. 3), but coeluted with *n*-alkanols. The *n*-alkanols could, however, easily be removed using silicalite, a molecular sieve (Fig. 5) [12]. The mass spectrum and retention time of phytol (around the 19th min) was determined by analysis of a standard, but the generation of detectable positive ions by APCI turned out to be inefficient resulting in low intensities of specific ions (Fig. 4; m/z 277, 293, 295 and 361). Only by studying specific mass chromatograms and injection of sufficient amounts of

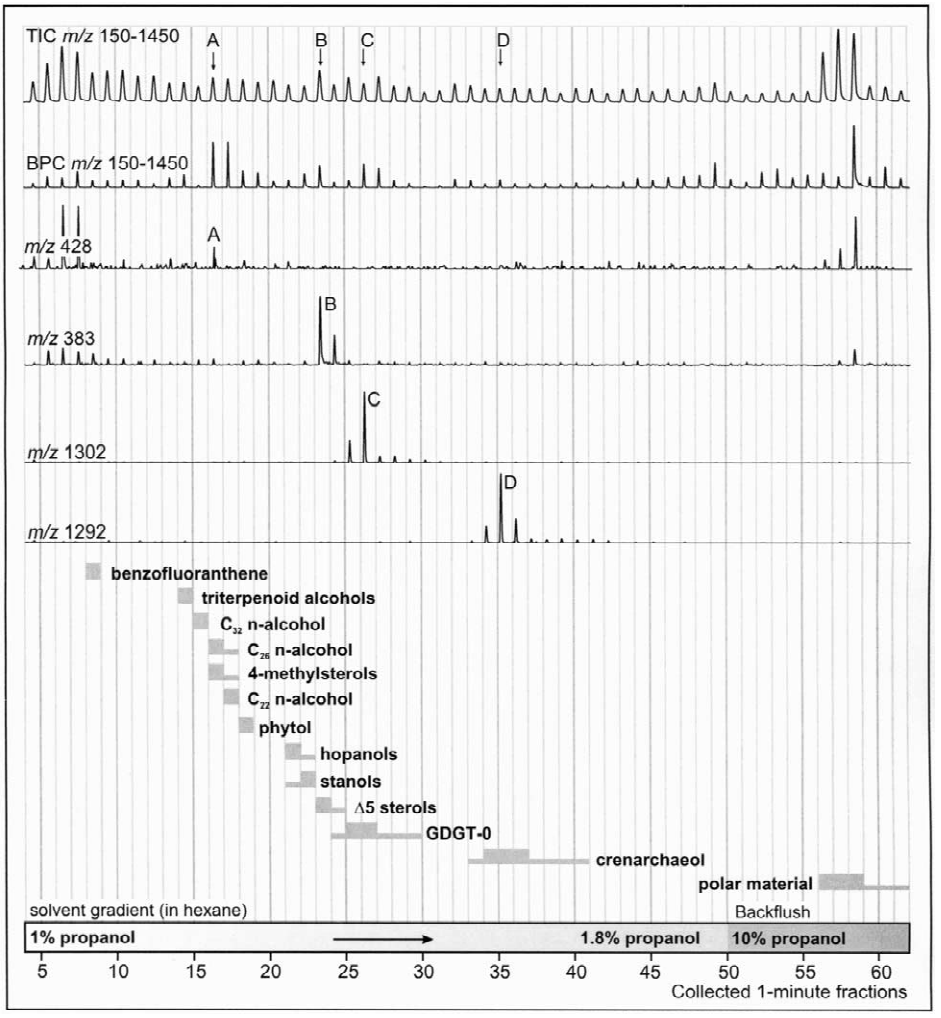

Fig. 3. Total ion current (TIC, m/z 150–1450), base peak chromatogram (BPC) and mass chromatograms of a flow injection analysis of 1-min fractions obtained with semi-preparative HPLC of one of the samples, with an overview of the major lipids present in the collected fractions. Each peak in the TIC corresponds to one collected fraction. The first peak in the chromatograms correspond to the fraction collected between 4 and 5 min. The base peak chromatogram (BPC) indicates in which fractions single compounds are dominantly present. m/z 383 is characteristic for Δ5-sterols, m/z 1302 is indicative for GDGT-0, m/z 1292 is indicative for crenarchaeol and m/z 428 indicates the presence of dinosterol and other 4-methylsterols. Below the mass chromatograms, gray areas indicate the presence of major compounds or compound classes in the different fractions, as detected by GC or HPLC–MS. Fractions containing only a small part of the total amount of a compound (class), i.e., collected at the start or end of elution, are indicated by smaller grey areas. Fractions collected during the backflush phase of the followed method are included in the FIA–MS series. Only after several minutes after the backflush started (at 50 min), the polar material that retained on the column started to elute, as can be derived from the high TIC values in the fractions collected between 56 and 59 min. The fractions indicated by the letters A, B, C and D contain the highest amounts of 4-methylsterols, Δ5-sterols, GDGT-0 and crenarchaeol, respectively. The mass spectra of fractions B, C and D are presented in Fig. 4.

the analyte (100–200 ng, 1% of the total), was it possible to recognize phytol-generated ions above background level during FIA–APCI-MS. Therefore, all fractions which could possibly contain phytol, based on retention time, were also screened by GC analysis. Analysis of the cholesterol standard provided a reference spectrum and retention time for Δ5-sterols, which yield base peaks at m/z $[M+H]^+$-

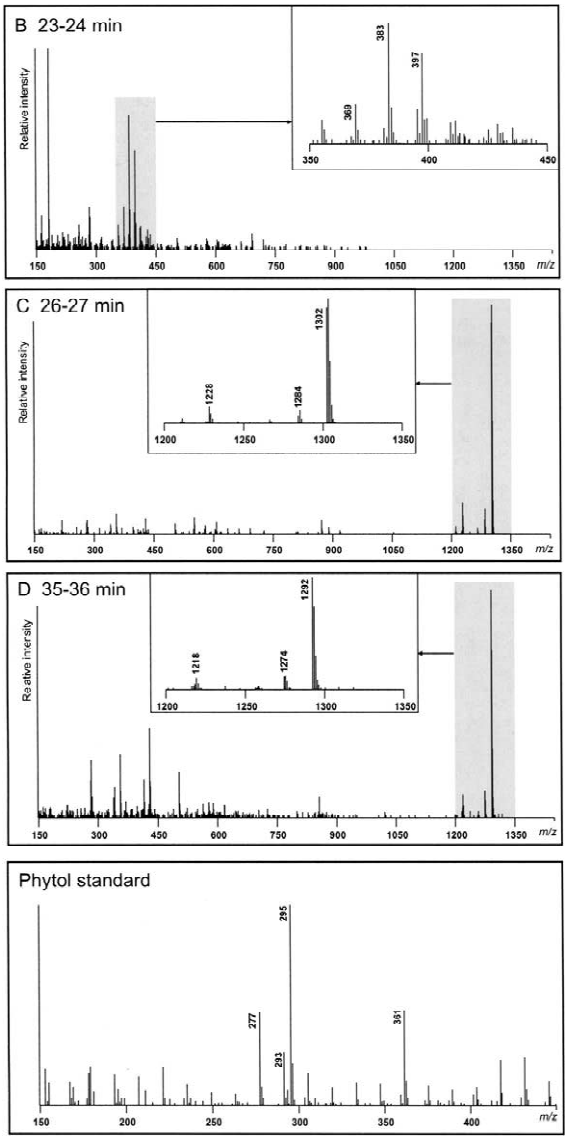

Fig. 4. Mass spectra of the fractions collected at 23–24 min (B), 26–27 min (C) and 35–36 min (D) (see Fig. 3), and the mass spectrum of a phytol standard. The upper spectrum (B) clearly shows the $[M+H]^+$-18 ions generated from C_{27}, C_{28} and C_{29} Δ5-sterols, namely m/z 369, 383 and 397, respectively. The mass spectra C and D are dominated by the ions $[M+H]^+$, $[M+H]^+ + 1$, $[M+H]^+$-18 (loss of H_2O) and $[M+H]^+$-74 (loss of glycerol), indicative for tetraethers. The mass spectrum of phytol is characterized by m/z 277, 293, 295 and 361. Other peaks in the mass spectra are background ions and mainly derived from the septa on the vials used for FIA–MS analysis.

18, generated by the loss of their hydroxyl-group (Fig. 4). Δ5-Sterols (Fig. 1, III) eluted at 23–25 min and coeluted partly with GDGT-0 (Fig. 1, I), which started to elute after 24 min (Fig. 3). GDGTs give very clear and distinctive mass spectra that are easy to recognize, characterized by m/z $[M+H]^+$, $[M+H]^+ + 1$, $[M+H]^+$-18 and $[M+H]^+$-74 (Fig. 4) [13]. During the second purification step using the analytical size column and smaller time fractions, the Δ5-sterols and GDGT-0 could, however, be collected separately.

The amounts (Table 1) and purity of the different fractions were determined by HPLC–MS, GC and GC–MS analysis. The quantities obtained were more than sufficient for ^{14}C analysis (Table 1). More importantly, integration of chromatograms produced by GC and compound identification using GC–MS of the sterol and phytol fractions indicated that they consisted at least for 95% of the desired compounds (Fig. 5). HPLC–MS analysis of the isolated GDGT fractions showed that GDGT-0 and crenarchaeol were also >95% pure. However, material non-amenable to APCI-MS or GC, especially column bleed, could still remain undetected. To ascertain that no major undetectable contamination was present, high field ^{13}C NMR was performed on a representative isolated GDGT-0 fraction. The spectrum compared favorably with literature data, i.e., all significant resonances could be attributed to carbons from GDGT-0. This strongly suggests that no other compounds were present in significant amounts. A representative phytol fraction was subjected to ^{1}H NMR analysis and all significant proton signals could be attributed to phytol, indicating >95% purity. Sterol fractions were not subjected to NMR, but because these fractions were isolated under the same conditions as phytol and the GDGTs, and because they had intermediate retention times during HPLC, absence of significant contamination could also be inferred for the sterol fractions. However, these fractions were not totally transparent but exhibited still a faint greenish lush, most likely derived from chlorophyll-derived moieties. Future isolations of sterols using the described method should therefore be performed on fractions pretreated with base hydrolysis, as was performed on the phytol fractions. This will break down and remove chlorophyll-derived moieties to such an extent that they do not coelute with the sterols.

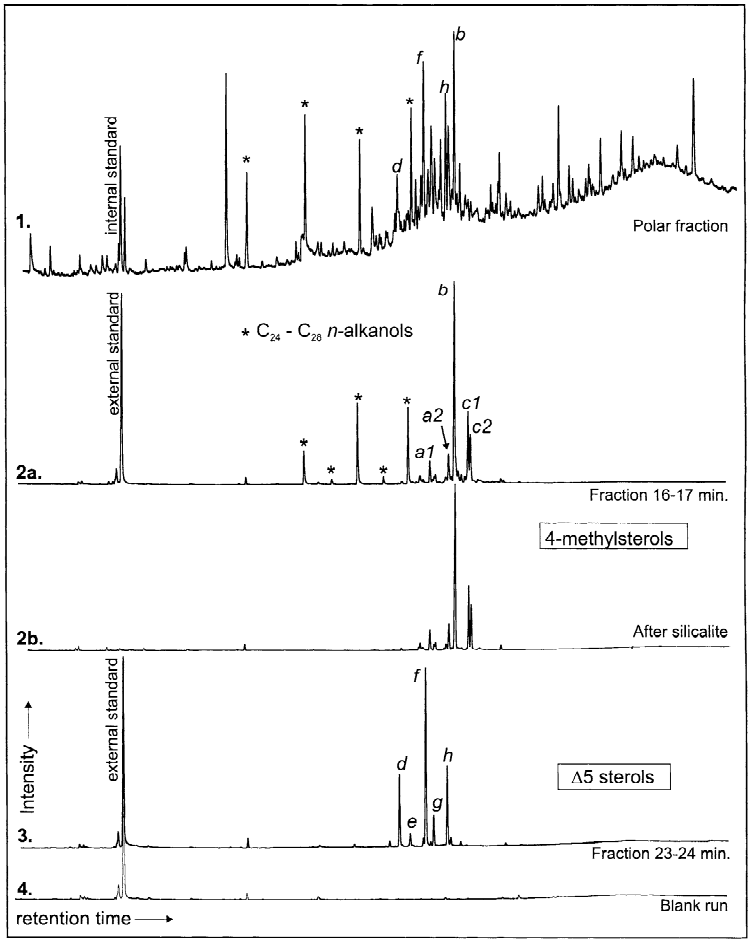

Fig. 5. Gas chromatograms of the polar fraction of a total lipid extract and of the fractions containing Δ5-sterols and 4-methylsterols at different stages of the isolation procedure. (1) The polar fraction shows the complexity of the mixture before separation. (2a) The fraction collected between 16 and 17 min contained a number of straight-chain alkanols, together with 4-methylsterols. (2b) Silicate treatment removed the straight-chain alkanols quantitatively. (3) The fraction collected between 23 and 24 min contained Δ5-sterols. (4) A blank run including the external standard is shown as a reference. Indicated peaks (a)–(h) refer to structures given in Fig. 1, and c1 and c2 are stereo-isomers.

3.3. Isotopic fractionation during isolation

Chromatographic separation leads to (carbon) isotopic fractionation of the compound within an eluting peak [26]. To avoid carbon isotopic fractionation, care was taken to include collected fractions containing the front and tail of eluting peaks into the combined fractions. The $\delta^{13}C$ values of the untreated cholesterol standard, of the cholesterol standard subjected to the isolation procedure and of the CO_2 produced upon combustion of the cholesterol for radiocarbon analysis, were all identical well within the analytical error (Table 1), showing that the HPLC procedure did not result in any significant fractionation.

Chlorophyll-derived phytol did not coelute with other compounds during gas chromatography and therefore it was possible to determine the $\delta^{13}C$ values of phytol before the isolation procedure and to compare those values with the $\delta^{13}C$ values after isolation. This was done for three samples (Table 1). The differences in the $\delta^{13}C$ values ($+0.5$‰; $+0.7$‰ and -0.6‰) are within the measurement error (twice 0.4‰). This points to the absence of a

Table 1
Amounts, stable carbon isotopic values and radiocarbon contents and ages of targeted sedimentary compounds

Compound	Sample	Isolated amount (GC–HPLC–MS) [μg C (±5%)]	CO_2 recovery (pressure) [μg C (±1%)]	$\delta^{13}C$ Value before isolation [‰ (±0.4)]	$\delta^{13}C$ Value after isolation [‰ (±0.4)]	$\delta^{13}C$ Value CO_2 [‰ (±0.1)]	^{14}C Content [F modern (±0.003)]	Radiocarbon age [year BP (±50)]
Cholesterol	Direct	1000	937	−25.9		−25.87	1.129	Modern
	"Isolated"	1000	986		−25.8	−25.87	1.127	Modern
GDGT-0	Vena del Gesso	1100	130			−24.73	0.05 (±0.01)	23 000 (±1400)
Crenarchaeol	Vena del Gesso	1200	150			−22.15	0.049	24 100 (±450)
TOC[a]	Saanich Inlet A					−22.22	0.780	1990
GDGT-0	Saanich Inlet A	1015	982			−21.44	0.677	1560
Crenarchaeol	Saanich Inlet A	990	938			−21.68	0.832	1470
Δ5-Sterols	Saanich Inlet A	480	431			−21.50	0.787	1920
4-Methyl sterols	Saanich Inlet A	400	373			−21.77	0.857	1240
Phytol[b]	Saanich Inlet B	100	95	−21.1	−21.7	−21.80	0.775	2050

[a] Total sedimentary organic carbon.
[b] Phytol fraction of Saanich Inlet A lost.

substantial carbon isotopic fractionation effect (<0.7‰), and thus the procedure does not affect the radiocarbon age determination. Apparent negative and positive shifts indicate furthermore that even if any fractionation is induced by the isolation procedure, this is not biased towards a certain direction.

Overall, the isolation method did not result in any significant carbon isotopic fractionation.

3.4. Radiocarbon contents

The ^{14}C contents of the modern cholesterol standard before and after the isolation procedure were identical within measurement error (Table 1), indicating that no significant contamination of old or fossil carbon was introduced during the isolation procedure. The fossil tetraethers isolated from the 6-million-year-old Vena del Gesso marl were almost radiocarbon-dead (Table 1). The measured values of ca. −950 ‰ indicate a contamination of ca. 5% modern carbon. However, this apparent contamination may also be caused by handling in the last stage of preparation for radiocarbon analysis or by analytical problems in the radiocarbon measurement. For unknown reasons the CO_2 recoveries of these samples were much lower than was expected from the amount measured by HPLC–MS. The eventual small sample size implied a greater error in the ^{14}C analysis due to the background signal and detection limits. Earlier measurements of small, radiocarbon-dead samples isolated by the established method of preparative capillary gas chromatography produced approximately the same results (data not published). Therefore, we conclude that no significant contamination of modern carbon was introduced during the HPLC isolation procedure. The radiocarbon contents of the different isolated compounds or compound classes, as given for one sediment sample (Table 1) are within the range of expected values, when compared to the independently determined sediment age (420 years calBP) and taken a reservoir effect of 800 years into account. This indicates that specific compounds and compound classes were indeed successfully and rapidly isolated for radiocarbon dating. The results of these and other ^{14}C measurements, as well as the implications for research in earth sciences and (palaeo)oceanography will further be discussed in a forthcoming paper.

4. Conclusions

Normal-phase HPLC was used successfully to isolate lipid biomarkers with neglectable carbon isotopic fractionation or contamination involved. The application of FIA–APCI-MS greatly increased the speed of scanning the collected fractions for the presence of target compounds. With an average work-up time of 1 week, the followed procedure proved to be a relatively fast and easy way to isolate higher molecular mass ($>C_{30}$) and polar (non-derivatized alcohols) lipid biomarkers for compound-specific ^{14}C analysis, thereby increasing the number and type of organic lipids for which this is possible.

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