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Journal of Chromatography A, 1169 (2007) 70–76
JOURNAL OF CHROMATOGRAPHY A
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Purification of dinosterol for hydrogen isotopic analysis using high-performance liquid chromatography–mass spectrometry

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Received 27 July 2007; received in revised form 6 September 2007; accepted 7 September 2007
Available online 14 September 2007

Abstract

A semi-preparative normal-phase high-performance liquid chromatography–mass spectrometry (HPLC–MS) method is presented for the purification of various alcohol fractions from total lipid extracts derived from sediments, for the purpose of hydrogen isotopic measurement by gas chromatography–isotope ratio mass spectrometry (GC–IRMS). 4-Methylsterols, including the dinoflagellate-specific marker dinosterol (4,23,24-trimethylcholestan-22-en-3β-ol), were successfully separated from notoriously co-eluting plant-derived pentacyclic triterpenoid alcohols and alkyl alcohols. We find that substantial hydrogen isotope fractionation occurs during chromatographic separation, demonstrating the importance of recovering the entire peak when subsequent hydrogen isotope analyses are to be performed. This is the first report of such hydrogen isotopic fractionation for a natural unlabelled compound.
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Keywords: Semi-preparative HPLC–MS; Sterols; Hydrogen isotopic fractionation

1. Introduction

Since the first gas chromatograph–isotope ratio mass spectrometer systems (GC–IRMS) became commercially available [1], stable isotope analyses of lipid biomarkers have proven to be a valuable tool in paleoclimate and environmental research [e.g. 2–4]. However, generation of paleoclimate records based on biomarker isotope data is hampered by the substantial time and effort those analyses require. Lipid extracts from marine and lacustrine sediments typically contain thousands of GC-amenable compounds, resulting in densely populated gas chromatograms that are not suitable for analysis by GC–IRMS, which requires baseline separation of analytes. Furthermore, the large quantities of an analyte (50–100 ng for $^{13}C/^{12}C$, 200–400 ng for $^{2}H/^{1}H$, also D/H) that are required for GC–IRMS analyses overload most capillary GC columns, degrading the chromatographic separations by causing wide peaks and diminished resolution. Consequently, total lipid extracts must be substantially pre-purified prior to GC–IRMS analysis. For lipid biomarkers with similar polarity, such as many biological sterols, such pre-purification can be extremely difficult, a problem that has hampered the application of sterol-based isotope analyses. Of no less importance is the requirement that no isotope fractionation be caused by the purification procedure itself.

For some compound classes such as saturated straight-chain alkanes and fatty acids, sufficient purification can sometimes relatively easily be accomplished with hydrolysis and a simple column chromatography step [e.g. 4], although additional steps involving the use of e.g. $AgNO_3$-adducted silica gel and urea adduction may also be required [e.g. 2,3]. Satisfactory purification of other biomarker types can be more elusive. Polycyclic triterpenoid alcohols like sterols, hopanols and higher plant-derived triterpenoid alcohols have specific biological sources and are often present in relatively high quantities, making them good targets for isotopic analysis in paleoenvironmental research. However, due to the wide variety of these compounds, all with a similar molecular weight, polarity and volatility, they are notoriously difficult to separate. Purification typically involves multiple chromatographic steps involving, amongst others, urea adduction and column or thin-layer chromatography with $AgNO_3$-adducted silica gel [e.g. 3,5], and still this may not even give quite satisfactory results. These methods are often complicated, time consuming and difficult to perform in a repro-

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doi:10.1016/j.chroma.2007.09.018

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ducible manner, and consequently can result in low recoveries with the potential for isotope fractionation during workup.

(Semi-) preparative high-performance liquid chromatography (HPLC) provides a substantially higher resolution than column or thin-layer chromatography [6]. Moreover, separations can be performed in a reproducible manner and sample injection and fraction collection can be automated. It is therefore increasingly common for HPLC to be used as part of a procedure to obtain semi-purified fractions of lipids for isotope or structural analysis [7–14]. That such techniques are not yet widespread in organic geochemistry likely results in part from the relative recent introduction of HPLC systems with mass-selective detection (HPLC–MS) that are reliable and affordable and able to ionize the small non-polar molecules common in geological samples.

Here we present a method for purifying various alcohols and sterols from sediment lipid extracts with HPLC–MS to a level suitable for isotope analysis using GC–IRMS. We also present evidence that substantial hydrogen isotope fractionation can occur across an HPLC peak and caution therefore that the entire peak must be collected prior to analysis by GC–IRMS.

2. Experimental

2.1. Sediment samples and reference compounds

Almost purely organic sediment samples containing a complex mixture of lipids, including sterols, alkyl alcohols and pentacyclic triterpenoid alcohols were collected in June 2004 from a saline lake in Palau in the western tropical Pacific (7°09′09″N, 134°21′46″E). A cholesterol standard (+98%, Alfa Aesar, Ward Hill, MA, USA) was used as reference material. Hydrogen isotope standards were obtained from the laboratory of Arndt Schimmelmann, Indiana University, Bloomington, IN, USA.

2.2. Lipid extraction and pre-treatment

Sediment samples (0.5–2 g dry wt.) were freeze dried prior to lipid extraction in a mixture of dichloromethane and methanol (DCM–MeOH, 9:1, v/v) with an Automated Solvent Extractor (ASE-200, Dionex Corp., Sunnyvale, CA, USA), deployed over three static cycles of 5 min at 100 °C and 1500 psi. Solvent was removed under a stream of nitrogen using a Turbovap system (Caliper, Hopkinton, MA, USA). For sterol purification, neutral fractions (NF) were obtained from the total lipid extract by solid-phase extraction (SPE): a glass column (1 cm diameter, 30 cm length), fitted onto a SPE vacuum chamber (Mallinckrodt Baker, Phillipsburg, NJ, USA), was stoppered with pre-extracted cotton wool and filled with *ca*. 15 g silica gel 60 (70–230 mesh, deactivated with 5% (wt.) H_2O). It was then impregnated with 10 ml of KOH-saturated isopropanol [15], and conditioned with *ca*. 3 column volumes (50 ml) of a mixture of diethyl ether and DCM (1:1, v/v). After application of the total lipid extract onto the column using DCM, the NF was recovered with approximately three column volumes (∼50 ml) of the same diethyl ether–DCM mixture. Acids and polar lipids (AF) were recovered from the column using glacial acetic acid and diethyl ether. The mobile phase was drawn through the column using a light vacuum into 60 ml vials. After solvent removal, the neutral fractions were separated into a fraction containing alcohols and less polar lipids (NF1) and a fraction containing more polar neutral lipids (NF2) in a pipette column filled with 5% deactivated silica with a mixture of hexane and ethyl acetate (8:2, v/v) and ethyl acetate, respectively. The majority of the NF1 fractions (typically 90%) were dissolved in 100 μl of a mixture of hexane and DCM (9:1, v/v) prior to injection onto the HPLC–MS.

Table 1
HPLC gradient program

Time (min)	A (%)	B (%)	Flow rate (ml/min)
0	85	15	1.5
27	85	15	1.5
33	65	35	1.5
38	65	35	1.5
38.1	30	70	2.0
48	30	70	2.0
48.1	85	15	2.0
62	85	15	2.0
70	85	15	1.5

A: hexane; B: dichloromethane.

2.3. Isolation of compounds by semi-preparative HPLC and screening by mass spectrometry

An Agilent (Santa Clara, CA, USA) high-performance liquid chromatograph (HPLC) 1100 series, equipped with an autoinjector, a quaternary pump, an integrated fraction collector, and Chemstation chromatography manager software was used. Injection volumes were typically 100 μl and contained up to 1 mg of material. A Prevail Cyano column (250 mm × 4.6 mm, 5 μm; Alltech, Deerfield, CA, USA) maintained at 30 °C was used for separation. Compound classes were separated using programmed mixtures of hexane and DCM as detailed in Table 1. Elution of compounds of interest was monitored using an Agilent 1100 LC/MSD SL Mass spectrometer equipped with a multimode source operated in positive atmospheric pressure chemical ionization (APCI+) mode on *ca*. 2% of the total flow that was split from the eluent using an adjustable flow splitter (ASI, El Sobrante, CA, USA). A Waters (Milford, MA, USA) 510 pump was used to add a flow of 0.3 ml/min hexane to the 2% split in order to supply sufficient solvent to the APCI source to achieve optimal ionization efficiency. Conditions for APCI–MS were as follows: gas temperature: 350 °C; vaporizer temp: 250 °C; drying gas (N_2): 12 l/min; nebulizer pressure: 35 psig; capillary voltage: 2.5 kV; corona current: 6 μA; Corona charge voltage: 2 kV. Mass spectra were obtained in full scan mode (*m/z* 200–700) with the following settings: fragmentor: 140; gain: 1.0; threshold: 150; stepsize: 0.1. The majority of the flow was collected at short time intervals (0.5–1 min) using the fraction collector. Collected fractions were covered with a sheet of aluminum foil and placed in a fume hood to allow the solvents to evaporate overnight. Fractions containing compounds of interest were recombined

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as needed, based on HPLC–MS results, and evaluated for purity using gas chromatography with flame-ionization detection (GC-FID) and/or gas chromatography–mass spectrometry (GC–MS).

2.4. Gas chromatography and gas chromatography–mass spectrometry

To quantify amounts of isolated alcohols, fractions were dissolved in a known amount of DCM and 4% aliquots were taken for GC analysis. Known amounts of 5α-cholestane were added to the aliquots for quantification purposes. After evaporation of the DCM, the aliquots were subsequently dissolved in 10 μl pyridine together with 10 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA, +1% TMCS). This mixture was heated (60 °C; 20 min) to convert alcohols into their corresponding trimethylsilyl ethers. After addition of 10 μl hexane, the derivitized fractions were injected at 60 °C on an Agilent 6890 instrument equipped with a PTV injector operated in splitless mode, and a FID system. A Varian CP Sil 5 fused silica capillary column (60 m × 0.32 mm × 0.25 μm) was used with helium as carrier gas (1.6 ml/min). The oven was programmed to 150 °C at 20 °C/min, then to 280 °C at 10 °C/min and then at 2 °C/min to 300 °C at which it was held for 10 min before a last increase to 320 °C where is was held for 5 min. Quantification of compounds was performed by comparing their integrated peak areas with that of the cholestane standard.

GC–MS was performed on an Agilent 6890 gas chromatograph equipped with a split-splitless injector operated in splitless mode, interfaced to an Agilent 5975 mass-selective detector operated at 70 eV with a mass range of *m/z* 50–700 at 2.28 scans/s. Gas chromatography was performed as described above. Compounds were identified by comparison of mass spectra and retention times with those reported in literature.

2.5. Stable hydrogen isotopic analysis

Alcohol fractions were acetylated by adding 20 μl pyridine and 20 μl acetic anhydride of known hydrogen isotopic composition and heating at 60 °C for 1 h. Acetylated fractions were dried under a stream of nitrogen and dissolved in toluene to reach concentrations of 200–300 mg/ml of the compound of interest, needed to obtain adequate signal intensities on the GC–IRMS system (over 1500 mV). Acetylation was preferred above the somewhat easier method of sylilation, because it adds less additional hydrogen atoms to the derivitized molecules. Stable hydrogen isotope analyses were performed on a DELTA V GC–IRMS system (Thermo Scientific, Waltham, MA, USA) that is similar to that described by Burgoyne and Hayes [16]. The gas chromatograph (Trace Ultra, Thermo) was equipped with a split-splitless injector operated in splitless mode, held under a constant helium flow of 1 ml/min. Samples of 1 μl were co-injected with 1 μl of standard made up of two or three compounds of known isotopic composition, with elution times bracketing the compounds of interest. Isotope values, expressed in the delta notation [1], were calculated with ISODAT software using the co-injection standards. Alcohol δ*D* values were corrected for the hydrogen atoms of the added acetyl group. Instrument performance and the H_3^+ factor were determined on a daily basis using a reference gas and a mixture of *n*-alkanes of known isotopic composition as described in Ref. [1]. Hydrogen isotopic values were typically determined by three replicate analyses, and the results were averaged to obtain a mean value and standard deviation. The precision of lipid δ*D* measurements was 4‰, the root-mean-square (RMS) error of several hundred analyses of a standard sample containing 15 different *n*-alkanes with known δ*D* values.

3. Results and discussion

3.1. Hydrogen isotopic fractionation during normal-phase HPLC

Liquid chromatography can cause fractionation of heavy and light isotopes of hydrogen [17–20], nitrogen [10] and carbon [21]. In reverse phase (RP) isotopically enriched isotopomers elute before depleted ones, while in normal-phase (NP) the isotopically depleted isotopomers elute first [18]. For hydrogen, this effect has only been studied on deuterated compounds, and primarily in RP systems. However, the extent of fractionation over a chromatographic peak of a natural unlabeled compound has never been reported. Of course, to avoid any doubt about

Table 2
Hydrogen isotopic values over the course of an eluting cholesterol peak

Fraction	Amount (μg)	% of total	δ*D* (‰)	SD	Corrected δ*D* (‰)[a]
1st cut	5.0	6.0	−244.0	1.6	−251.4
2nd cut	47.1	55.9	−228.8	0.7	−235.2
3rd cut	28.7	34.1	−177.6	6.0	−180.6
4th cut	3.4	4.0	−100.1	2.4	−97.9
Weighted avg.			−207.1	6.7	−212.0
No HPLC			−209.9	5.9	−215.1
Solid[b]				2.5	−212.0

In the upper four rows the hydrogen isotopic values of four fractions of cholesterol collected over the course of peak elution from HPLC are listed. Furthermore are listed the weighted average of these four fractions, cholesterol that has not been subjected to HPLC and fraction collection, and the cholesterol measured as a solid. SD: Standard deviation of typically three replicate analyses.

[a] Values after correction for the added acetyl group.

[b] The solid cholesterol included an exchangeable hydrogen atom of the hydroxyl group and was not measured acetylated.

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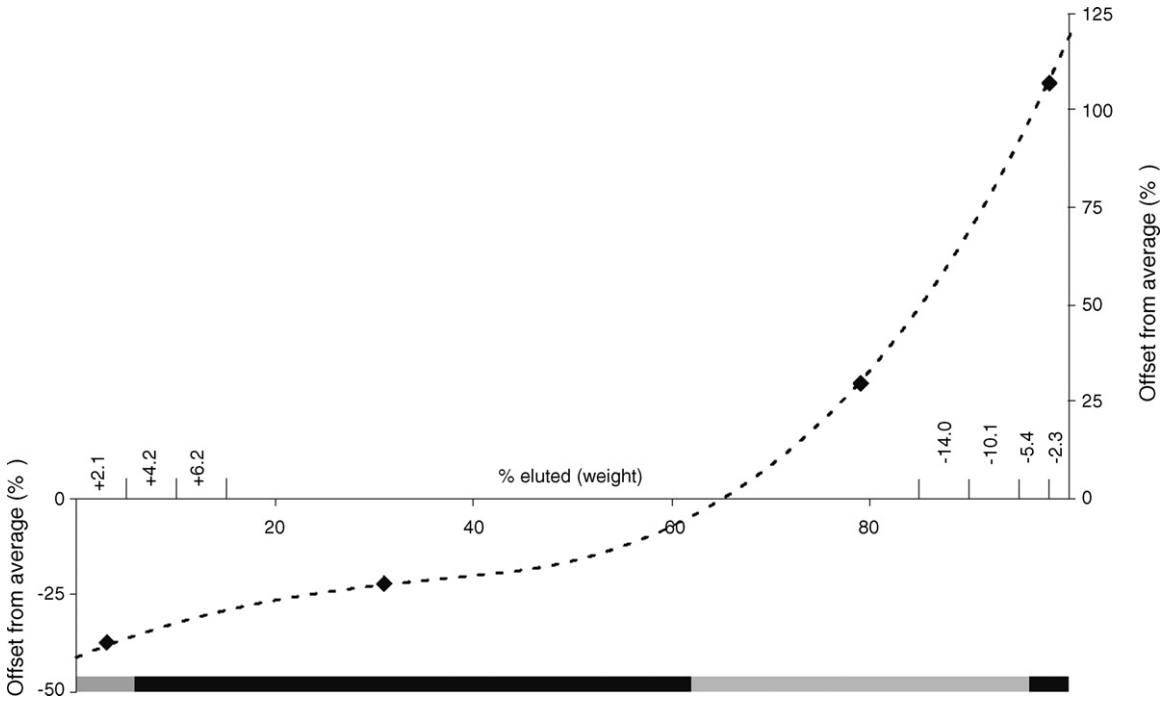

Fig. 1. Graphic representation of the hydrogen isotopic offset of a cholesterol standard over the course of peak elution from normal-phase HPLC. Black diamonds: measured data after subtraction of the weighted mean value (Table 2). The grey and black bars at the bottom of the graph indicate the relative weight of the collected fraction (%). The numbers above the horizontal axis indicate the error in the cholesterol δD value that would have been produced if that part of the peak (first 5, 10, 15, and last 15, 10, 5, 2%) would not have been included in the total collected fraction, based on calculations using the polynomial fitted curve (3rd order, dotted line).

the isotopic integrity of an isolated compound, peaks should be collected quantitatively, and indeed that has been common practice. From the perspective of aiming for a high degree of purity, however, fraction collection centered only around the peak apex, a so-called heart-cut, may sometimes be desired in cases where other compounds elute very closely.

To evaluate the possible effect of incomplete collection of an eluting peak, a cholesterol standard was collected over the course of the eluting peak into four different fractions. The peak displayed good symmetry. Each cut was separately analyzed and its value compared with the hydrogen isotopic value of the standard analyzed directly by GC–IRMS. As an additional reference, the cholesterol was measured as a solid using the same mass spectrometer but coupled to a thermal conversion/element analyzer (TC/EA) [22].

An isotopic fractionation effect can clearly be observed over the course of the peak (Table 2), with deuterium enriched molecules preferentially retained relative to their deuterium depleted counterparts. The weighted average of the four collected fractions gives, within measurement error, the same isotopic values as the reference values (Table 2). If the values of the four collected fractions are plotted against their position (% eluted mass) within the eluting peak, it appears that isotopic fractionation at the front of the peak is less pronounced than at the end of the peak (Fig. 1). Using a 3rd order polynomial fit, an estimate can be made of the effect on the hydrogen isotopic composition of cholesterol were the front or tail of the peak omitted from collection (Fig. 1). For cholesterol and our chromatographic conditions, missing 2% (wt.) of the peak tail, or 5% of the peak front, would result in an error of *ca*. 2‰ or −2‰, respectively, which is within the error of measurement. If the last 5% of the peak tail were to be omitted, however, an error of over −5‰ would be introduced.

The main cause for the isotopic separation lies in the difference in mass of protium and deuterium [20,23]. Because of its greater mass, the vibrational amplitude of deuterium is smaller than protium, causing C–D bonds to be shorter than C–H bonds. This results in slightly lower average volumes and polarizability of bonds involving deuterium compared to those that involve protium. As a result, deuterated compounds exhibit weaker binding to nonpolar moieties than protiated compounds. In RP-HPLC, with a non-polar stationary phase and a polar mobile phase, this results in less retention by the stationary phase and earlier elution, while in NP-HPLC, with a polar stationary phase and a non-polar mobile phase, the deuterated compounds have a smaller affinity for the mobile phase and therefore elute later. The extent of the isotope effect (IE) varies as a function of molecular structure and chromatographic conditions [20], and the observed isotope fractionation of cholesterol reported here is the result of several factors. For instance, the molecular structure of both the analytes and the stationary and mobile phases play a role, with aromatic moieties causing stronger IEs than aliphatic ones, due to stronger non-covalent interactions. A stationary phase with mixed lipophylic and lipophobic properties, combined with a relatively neutral mobile phase, may result in only a weak IE. Furthermore, since the IE is mainly caused by hydrophobic interactions, polar compounds may exhibit smaller IEs than apolar compounds.

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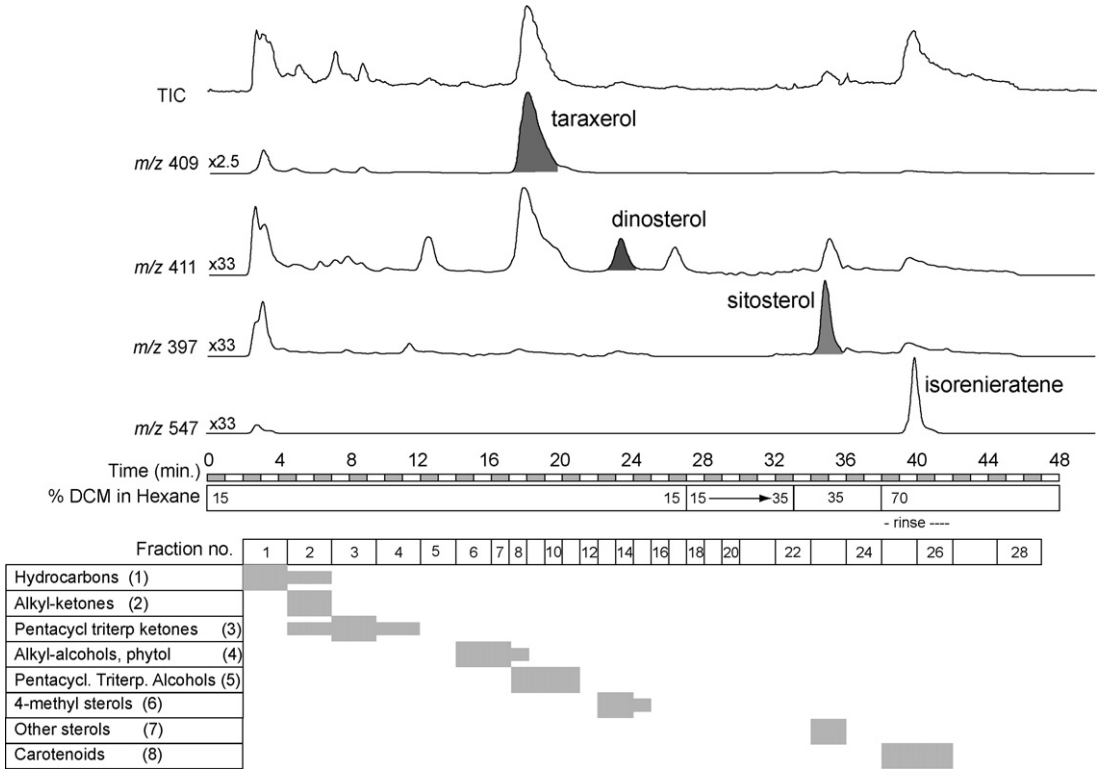

Fig. 2. Total ion current (TIC, *m/z* 50–700) and mass chromatograms of a representative semi-preparative HPLC run. *m/z* 411 represents the ion generated by protonated dinosterol ($M = 428$) after a loss of water ($[M+H-18]^+$). The same is valid for taraxerol ($M = 426$) and sitosterol ($M = 414$). *m/z* 547 indicates the presence of protonated isorenieratene. The (multiplied) vertical scales indicate the relative intensities of the ion signals. Time, the programmed gradient and collected fractions are indicated in the middle bars. The column and capillary volume accounts for a delay time between injection and detection of approximately 2.5 min. (1) Saturated, unsaturated and aromatic hydrocarbons; (2) wax-esters and alkyl-esterified fatty acids are also expected here (not in this sample); (3) e.g. Friedelanone; (4) high molecular weight alkyl alcohols elute earlier than their low molecular weight homologues; (5) oleanol, ursanol and lupanol series, e.g. taraxerol; (6) e.g. dinosterol, see Fig. 3; (7) typically Δ5-sterols with various unsaturations, like cholesterol, campesterol, sitosterol; (8) e.g. β-carotene, isorenieratene, okenone, but together with other neutral polar compounds that are rinsed off.

Nevertheless, it is likely that the IE values reported here for cholesterol on a cyano column would be similar for related compounds using the same mobile phase. Consequently we suggest that at least the middle 95%, but of course ideally 100%, of a chromatographic peak be collected with the NP-HPLC method reported here, if hydrogen isotope analyses are to be performed. We cannot rule out a similar isotope effect for thin-layer or column chromatography at this time, but expect the effect would be smaller given the fewer theoretical plates in those methods.

The relative mass difference between H and D, the underlying cause of the IE, is much larger compared to other environmentally significant stable isotope pairs such as $^{13}C/^{12}C$ or $^{15}N/^{14}N$. In analogy to naturally occurring fractionation effects, which are much smaller for N, C and O than for D/H [24], it can be inferred that the IE during chromatography for these other elements would be much less pronounced than is the case for D/H, but apparently not necessarily negligible for C and N [10,21].

3.2. Separation of alcohols, in particular 4-methyl sterols

The motivation for developing this HPLC–MS method was the desire to find an efficient way to purify dinosterol (4,23,24-trimethylcholestan-22-en-3β-ol), a sterol unique to dinoflagellates [25,26], for hydrogen isotopic analysis by GC–IRMS. That required baseline separation, and preferentially a clean chromatogram for several seconds or more prior to and after the elution of dinosterol on GC. 4-Methyl sterols like dinosterol are less polar than other sterols because the hydroxyl group at the 3 position is partially protected by the neighboring methyl group, and separation from desmethyl sterols has often been performed by column chromatography [13]. However, higher plant-derived pentacyclic triterpenoid alcohols (PTAs) have nearly the same retention characteristics during adsorption chromatographies as the 4-methyl sterols, and separation of these two compound classes is usually difficult. Although often used to separate compounds of similar polarity, even the use of $AgNO_3$-adducted silica would not help much, since the two compound classes exhibit the same amount of double bonds.

Sterols and other compounds of relatively low polarity are regularly separated using reverse phase chromatography, typically with a bonded silica phase (e.g. ODS) and organic solvents such as methanol and acetonitrile [5,13,27]. The retention of compounds in RP is based on the partitioning of the analyte between the polar mobile phase and the non-polar stationary phase, and the hydrophobicity of the analyte plays a major role in this mechanism [20]. Consequently, various homologues of an aliphatic alcohol or acid series, for example, are separable because the short homologues interact less with the stationary

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phase than molecules with larger aliphatic tails [28]. In contrast, separation of compounds with similar hydrophobic tails but different hydrophilic heads is less predictable. Indeed, the retention time of sterols and PTAs in RP-HPLC is primarily a function of molecular weight and the degree of unsaturation [5,13,27], with stereochemistry around the hydroxyl group playing a smaller role. As a result, RP-HPLC is not the first method of choice for separation of complex mixtures into compound classes. In NP-HPLC, however, the polarity of the polar head group is much more important because it plays a large role in the partitioning of the analyte between the non-polar mobile phase and the polar stationary phase. NP-HPLC therefore allows a better and more predictable separation of compound classes with functional groups of varying polarity, including that imparted by stereochemical protection of alcohol groups.

Smittenberg et al. [8] reported NP-HPLC separation and collection of various alcohol fractions, including 4-methyl sterols and desmethyl sterols using a semi-preparative NH_2 column and a gradient of 1–1.8% isopropanol (IPA) in hexane. This method was developed and optimized for the separation of glycerol-dialkyl-glycerol-tetraethers (GDGT's), however, and sterol and other alcohol fractions were not well separated. The small percentage of IPA at the start of the gradient and its rise to only 1.8% leaves little room for adjustments in the mobile phase to improve separations at the front end of the chromatogram. As a strong polar solvent, even minor changes in the percentage of IPA resulting from pump noise can cause large shifts in retention time. For these reasons dichloromethane was chosen to replace IPA.

An isocratic program of 15% DCM in hexane proved to be optimal for the separation of PTAs and the 4-methyl sterols (Fig. 2). Long chain alcohols started to elute before the PTAs with the longest homologues (e.g. C_{34}) eluting well before the PTAs and the shorter homologues (e.g. C_{20}), partially overlapping with the PTAs. As expected, the less polar hydrocarbons and ketones showed short retention times. Desmethyl sterols did not elute with the isocratic program of 15% DCM, but did elute readily with 30–35% DCM (Fig. 2). Carotenoids such as isorenieratene and okenone, which are derived from green and purple photosynthetic sulfur bacteria and indicative of photic zone euxinia [29,30], are abundant in the Palau lake sediments, and could be detected both with a diode array detector, as well as by APCI–MS. Although they eluted during the 'rinse' part of the HPLC gradient (Fig. 2) and were not separated from one another with our method, we expect only small adjustments to our gradient would be required to separate them [31].

After combining various collected fractions from the HPLC that contained the same compounds according to the HPLC–MS spectra (e.g. fractions 13–15 for 4-methyl sterols (Fig. 2)), GC-FID and GC–MS analyses revealed fractions containing just one or two compound classes. Fractions with solely 4-methyl sterols could be collected in a reproducible way, and dinosterol (analyzed either as acetyl-ester or silyl-ether) typically showed baseline separation from other 4-methyl sterols (Fig. 3). In more than 50 samples this method was employed to collect between

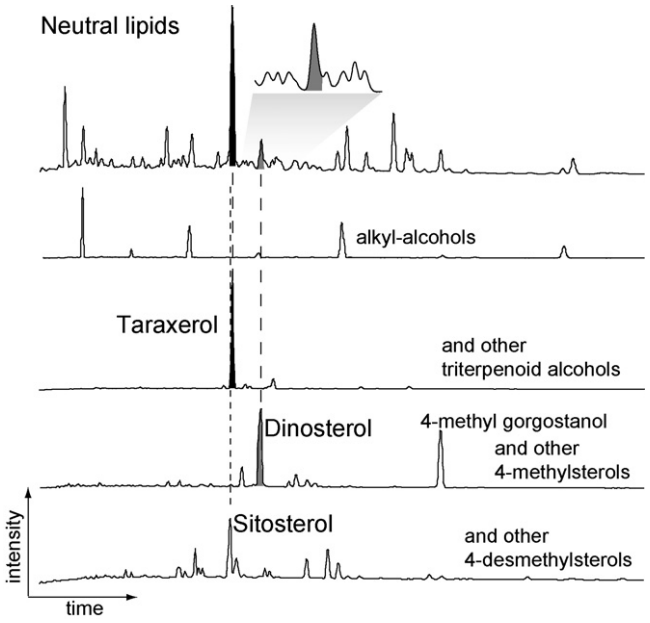

Fig. 3. Partial gas chromatograms of a neutral lipid fraction (upper) of a sediment from a saline lake in Palau, as well as chromatograms of fractions collected by semi-preparative HPLC containing various alcohol classes, including a fraction with 4-methyl sterols. Taraxerol and dinosterol are indicated with black and grey shading. Sitosterol exhibits a retention time very close to that of taraxerol. The retention time of dinosterol is very close to that of a number of pentacyclic triterpenoid alcohols (magnified).

4 and 20 μg of dinosterol for hydrogen isotopic analysis by GC–IRMS from 0.5 to 2 g of dry sediment.

4. Conclusions

Semi-preparative HPLC was applied to isolate various classes of lipid biomarkers from total lipid extracts. 4-Methyl sterols were successfully separated from notoriously co-eluting plant-derived pentacyclic triterpenoid alcohols and alkyl alcohols using a gradient of dichloromethane in hexane and an analytical Cyano column. A substantial isotope effect occurred across the chromatographic peak of a cholesterol standard, demonstrating the need to collect at least 95% of a peak for subsequent hydrogen isotope analysis. Deuterium-depleted molecules eluted early and enriched molecules eluted late. The NP-HPLC–MS method presented reduces the time and effort required to purify lipids for isotopic analysis by GC–IRMS, allowing a higher throughput of samples required to obtain detailed paleo-environmental records based on biomarker isotopic composition.

Acknowledgements

Funding was provided to J.P.S. by the US National Science Foundation grant #NSF-ESH-0639640 and by the Comer Science and Education Foundation. The Republic of Palau is thanked for allowing us to sample their saline lakes.

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