Organic Geochemistry 48 (2012) 37–46

Contents lists available at SciVerse ScienceDirect

Organic Geochemistry

journal homepage: www.elsevier.com/locate/orggeochem

Purification of dinosterol from complex mixtures of sedimentary lipids for hydrogen isotope analysis

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ARTICLE INFO

Article history:
Received 18 December 2011
Received in revised form 17 April 2012
Accepted 23 April 2012
Available online 1 May 2012

ABSTRACT

We present two new methods for purifying dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) from sediments for the purpose of hydrogen isotope analysis via gas chromatography–isotope ratio mass spectrometry (GC–IRMS). The first method uses reversed phase-high performance liquid chromatography (RP-HPLC) to purify dinosterol from structurally similar 4α-methyl sterols that co-elute on GC analysis. Dinosterol purified from sedimentary sterol/alcohol fractions using this RP-HPLC method demonstrated an average yield of 80%. A very large isotope effect was observed during RP-HPLC purification, with a 560‰ range in δD value between the first and last 5% of a cholesterol standard, which is four times that during normal phase-HPLC (NP-HPLC) purification. However, we show that dinosterol recombined from 3–4 min of eluent during RP-HPLC purification yields highly reproducible and unbiased isotope values. Due to a larger isotope effect and lower sterol recovery during RP-HPLC, NP-HPLC purification is recommended for samples that do not contain 4α-methyl sterols that co-elute with dinosterol during GC. However, for samples that contain a variety of 4α-methyl sterols, RP-HPLC is more likely to yield baseline resolution of dinosterol. In the second method presented, RP-HPLC purification is preceded by NP-HPLC purification. Using this two step procedure, baseline resolution between dinosterol and all other compounds present was achieved for all samples with an average yield of 60% and, in many cases, dinosterol was purified from all other sedimentary lipids. For samples that contain a variety of 4α-methyl sterols and sitostanol concentration > 2× that of dinosterol, the two step purification method is recommended, as neither NP-HPLC or RP-HPLC alone is likely to yield baseline resolution of dinosterol.

1. Introduction

Compound-specific stable hydrogen isotope (δD) analysis of lipids from aquatic sediments is an increasingly important tool for reconstructing environmental change (e.g. Anderson et al., 2001; Englebrecht and Sachs, 2005; Schefuß et al., 2005; Hou et al., 2007; Sachs et al., 2009; Mügler et al., 2010; Tierney et al., 2010; Smittenberg et al., 2011). Source-specific biomarkers make the best targets for δD analysis for paleoclimate applications since large variations in the isotopic composition of individual biomarkers have been observed in different organisms (Sessions et al., 1999; Smith and Freeman, 2006; Zhang and Sachs, 2007). Certain sterols are particularly good targets as they tend to be source-specific, are commonly found in high concentration in a variety of aquatic environments and are well preserved in the sediment record.

However, isotope analysis of sterols for paleoclimate applications is rarely performed due to difficulty in their purification from environmental samples. Accurate and precise isotope measurements via gas chromatography–isotope ratio mass spectrometry (GC–IRMS) require baseline resolution between a target analyte and adjacent compounds. In addition, a target analyte should be well separated from preceding compounds in order to minimize memory effects in GC–IRMS (Wang and Sessions, 2008). Unfortunately, many sterols have similar molecular weight, polarity and volatility, so tend to elute close together, typically forming a broad sterol hump during GC. An ideal biomarker purification method for δD analysis is one that reproducibly achieves GC baseline resolution and is high yielding, efficient, applicable to a wide variety of sedimentary environments and does not produce isotopic bias. However, few methods have been published that allow purification of source-specific sterols with these qualities.

Dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol) would presents a particularly valuable target for paleoclimate reconstruction if it could be adequately purified from sediments for isotope analysis. It is produced almost exclusively by dinoflagellates and is found in a variety of environmental settings, from tropical lakes to the Arctic Ocean (Robinson et al., 1985; Ohkouchi et al., 1997; Sauer et al., 2001; Belicka et al., 2004; Xu and Jaffé, 2008). However, it notoriously co-elutes with other sterols during GC, particularly with structurally similar 4α-methyl sterols (Fig. A1), a variety

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0146-6380/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.orggeochem.2012.04.006

of which are produced by dinoflagellates and other algae (Robinson et al., 1984; Hallegraeff et al., 1991; Mansour et al., 1999; Leblond and Chapman, 2002). Thus, before dinosterol can be widely used for biomarker δD analysis, a procedure is needed that allows its purification from a variety of sediments with complex lipid composition.

Reports of sterol purification procedures use multiple chromatographic separation techniques, including various combinations of thin layer (TLC) and column chromatography with a variety of phases, $AgNO_3$ impregnated Si gel chromatography, normal phase-high performance liquid chromatography (NP-HPLC), and reversed phase-high performance liquid chromatography (RP-HPLC) (Hansbury and Scallen, 1980; Teshima and Patterson, 1981; Dini et al., 1982; Volkman et al., 1984; Ponomarenko et al., 2001; Sauer et al., 2001). More recently, Smittenberg and Sachs (2007) outlined a procedure for purification of dinosterol from sedimentary lipids, targeting purification from plant-derived pentacyclic triterpenoid alcohols, using KOH-impregnated Si gel column chromatography followed by NP-HPLC.

However, these methods for sterol purification were not ideal for our purpose in several ways. First, we are not aware of a study that achieves purification of dinosterol from other notoriously co-eluting 4α-methyl sterols. Because a suite of structurally similar 4α-methyl sterols is produced by dinoflagellates and other algae, a method is needed that purifies dinosterol from other 4α-methyl sterols in order for dinosterol to be routinely used for paleoclimate δD applications. In addition, most studies on sterol purification report either low yield or do not report yield. A high yield is important for isotope analysis as biomarker concentration can vary dramatically with depth in sediment and a relatively large amount of material ($\geqslant$150 ng per injection) is needed for isotope measurements via GC–IRMS. Finally, in the majority of studies, the purpose of sterol purification was compound identification and/or quantification and not isotope analysis. Thus, preservation of the analyte's isotope composition was not targeted.

Here we present two novel methods for purifying dinosterol from sediments with diverse lipid composition for hydrogen isotope analysis for paleoclimate applications. Following purification of the sterol/alcohol fraction, the first uses RP-HPLC with an Agilent ZORBAX Eclipse XDB C_{18} column to purify dinosterol from other 4α-methyl sterols. This was sufficient to achieve baseline separation of dinosterol during GC analysis for a number of sediment samples. However, some 4α-desmethyl sterols were included in the dinosterol fraction in the procedure and, for samples with a high concentration of 24-ethyl-5α-cholestan-3β-ol (sitostanol), co-elution of dinosterol and sitostanol occurred. Because of this, a second method was designed in which the sterol/alcohol fraction was subjected to NP-HPLC with a Prevail Cyano column followed by RP-HPLC on the C_{18} column. This method resulted in baseline resolution between dinosterol and all other compounds present for all of the samples investigated and in many cases allowed isolation of dinosterol from all other lipids. D/H fractionation and sterol yield with these HPLC methods were characterized and the reproducibility of dinosterol δD values was examined. Finally, two GC columns (a non-polar Agilent DB-5ms and a mid-polarity Varian VF-17ms FactorFour column) were tested to optimize the separation of dinosterol from nearby eluting compounds during GC–IRMS.

2. Experimental

2.1. Lipid extraction

Samples were taken from various depths in a 3.5 m sediment core from El Junco Lake, a freshwater lake in the highlands of San Cristobal in the Galápagos Islands. The sediment was freeze dried prior to isolation of the total lipid extracts (TLEs) using an accelerated solvent extractor (Dionex ASE 200) with three cycles of dichloromethane (DCM) and MeOH (9:1) at 100 °C and 1500 psi. TLEs were collected in 60 ml ASE vials and dried under N_2 using a Turbo-vap system (Caliper, Hopkinton, MA, USA). Heneicosanol (n-C_{21}) was added to the freeze-dried sediment as a recovery standard prior to extraction.

2.2. Purification methods – column chromatography

Neutral lipids were separated from acids and polar lipids using aminopropyl SPE cartridges (Burdick & Jackson; 500 mg/4 ml) with DCM and isopropyl alcohol (IPA) (3:1), followed by 4% acetic acid (HOAc) in diethyl ether (Et_2O). The neutral fraction was applied to a column of 5% deactivated Si gel and separated into hydrocarbon, wax ester, sterol/alcohol, and more polar fractions with hexane, 10% ethyl acetate (EtOAc) in hexane, DCM and MeOH, respectively.

2.3. Purification methods – NP-HPLC and RP-HPLC

Dinosterol was purified from the sterol/alcohol fraction via NP- and RP-HPLC. An Agilent 1100 HPLC instrument was used with an integrated autoinjector, quaternary pump and fraction collector. It was coupled to an Agilent 1100 LC/MSD SL mass spectrometer with a multimode source operated in positive atmospheric pressure chemical ionization (APCI+) mode. A HPLC pump (Waters 510) delivered additional solvent (iso-octane for NP-HPLC and MeOH for RP-HPLC) to the mass spectrometer for optimal ionization efficiency. A post-column flow splitter (QuickSplit Analytical Adjustable Flow Splitter, Analytical Science Instruments) sent ca. 2% of the sample to the mass spectrometer and ca. 98% to the fraction collector.

For a detailed account of the NP-HPLC method see Smittenberg and Sachs (2007). Briefly, dinosterol was eluted from a Prevail Cyano column (250 mm × 4.6 mm × 5 μm) with a mobile phase of 15% DCM in hexane at 1.5 ml/min. After a 27 min isocratic period, the mobile phase was gradually adjusted to 35% DCM in hexane over 27–33 min. It was then held at 35% DCM between 33 and 38 min before being flushed with 70% DCM in hexane for 10 min. Finally, the column was reconditioned with 15% DCM in hexane at 2 ml/min for 14 min and then at 1.5 ml/min for 8 additional min. Samples were dissolved in 15% DCM in hexane and 100 μl were injected.

Sterol/alcohol fractions were purified via RP-HPLC using an Agilent ZORBAX Eclipse XDB C_{18} column (250 mm × 4.6 mm × 5 μm) with a mobile phase of 5% H_2O in MeOH at 1.5 ml/min. After a 60 min isocratic mobile phase period the mobile phase was ramped to 50% hexane in EtOAc. The column was then flushed with 50% hexane in EtOAc at 2 ml/min for 10 min. Following this column rinse, the mobile phase was ramped to 5% H_2O in MeOH and the column reconditioned with 5% H_2O in MeOH at 2 ml/min for 10 min. MeOH was used as the make-up solvent and was introduced to the mass spectrometer at 0.3 ml/min. Samples were dissolved in DCM/MeOH (2:1) and 25 μl were injected.

Mass spectra were acquired in selected ion monitoring (SIM)/full scan mode. For a description of the APCI-MS conditions see Smittenberg and Sachs (2007). Dinosterol (MW 428) was identified via the m/z 411 signal resulting from loss of H_2O and addition of a hydrogen (i.e. $[M - 18 + H]^+$). Selected ions for several sterols (m/z 399, 409, 411, 413) were monitored 50% of the time while a full scan (m/z 200–800) was performed for the remaining time. Fractions were collected in 1 min intervals and 3–4 were typically combined to bracket the m/z 411 peak. The fractions immediately before and after the combined dinosterol fraction were analyzed using GC–MS to ensure that no loss of peak front or tail occurred.

Combined HPLC fractions (both RP and NP) were dried under a stream of N_2 in preparation for analysis using GC–flame ionization detection (GC–FID).

2.4. Dinosterol assignment/quantification

GC–MS was used for sterol assignment and quantification prior to HPLC purification. Dinosterol in the sterol/alcohol fractions was quantified via GC–MS in SIM mode using dinosterol and heneicosanol calibration standards. Dinosterol calibration standards were prepared by purifying sediment samples via HPLC, sequentially diluting the stock solution and quantifying via GC–FID. A series of six dinosterol and three heneicosanol quantification standards were prepared that spanned a concentration range of 1–100 ng/μl and 30–300 ng/μl, respectively. After every 10–20 samples, the standards were analyzed and calibration curves calculated to quantify the concentration of dinosterol in each sample.

The HPLC fraction before and after the fractions combined for isotope analysis was also analyzed via GC–MS in SIM mode. Using this procedure, sub-ng quantities of dinosterol could be quantified. This high sensitivity was critical in assessing whether any dinosterol had failed to be recombined following HPLC purification.

Samples were derivatized via silylation for identification purposes and via acetylation for quantification and isotope analysis. Silylated samples were derivatized with 10 μl pyridine and 20 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60 °C for 1 h. Acetylated samples were derivatized with 20 μl pyridine and 20 μl acetic anhydride (Ac_2O) at 70 °C for 30 min. A known amount of 5α-cholestane was added as a quantitation standard prior to injection.

GC–MS acquisition was performed with an Agilent 6890N GC instrument coupled to an Agilent 5975 Mass Selective Detector with either a non-polar Agilent DB-5ms column (60 m × 0.32 mm × 0.25 μm) or a mid-polarity Varian VF-17ms FactorFour column (60 m × 0.32 mm × 0.25 μm). Samples were injected using a split/splitless inlet in splitless mode 300 °C. The GC oven program was: 60–150 °C at 15 °C/min, then to 320 °C (held 28 min) at 6 °C/min. The flow of He carrier gas was 1.5 ml/min. For compound assignment a full scan (*m/z* 50–700) was performed, while for quantification, selected ions were measured.

After HPLC purification, the dinosterol samples were analyzed using GC–FID to calculate recovery during HPLC purification and assess the purity prior to isotope analysis. Following HPLC purification, the samples were acetylated, dried under a stream of N_2, and dissolved in toluene. A known amount of 5α-cholestane was added to the samples as a quantification standard. GC–FID was performed with an Agilent 6890N GC–FID instrument with an Agilent DB-5ms column (60 m × 0.32 mm × 0.25 μm). Samples were injected into a PTV inlet at 70 °C that was ramped to 450 °C over 5 min. The oven program was: 60 °C (3 min) to 220 °C at 20 °C/min, then to 325 °C at 2 °C/min under a constant flow of He at 2.5 ml/min.

2.5. Dinosterol δD measurements

Isotope measurements were performed with a Thermo Trace GC Ultra with either an Agilent DB-5ms column (60 m × 0.32 mm × 0.25 μm) or a Varian VF-17ms FactorFour column (60 m × 0.32 mm × 0.25 μm) and a pyrolysis reactor. The GC instrument was coupled to a Finnigan Delta V Plus IRMS instrument. Acetylated samples were dissolved in toluene and injected into a split/splitless inlet in splitless mode at 310 °C. The oven temperature program was: 100 °C to 220 °C at 20 °C/min, then to 325 °C (held 17 min) at 2 °C /min. The He carrier gas was held at 2.6 ml/min. The δD values were calculated with Isodat software relative to VSMOW using a co-injection standard mixture containing *n*-C_{32}, *n*-C_{40}, and *n*-C_{44} alkanes of known δD values (from A. Schimmelmann, Indiana University, Bloomington, IN, USA). The dinosterol δD values were corrected for the addition of three Hs (of known δD value) during acetylation. Triplicate analyses were typically performed on each sample and mean values and standard deviations reported. Instrument performance was evaluated and a H_3^+ factor calculated on a daily basis using H_2 reference gas and a standard of *n*-alkanes of known δD values.

2.6. D/H fractionation during RP-HPLC purification

Six standards, each containing 100 μg cholesterol (+99%, Sigma Aldrich) were prepared. Three were subjected to RP-HPLC purification using the method described here. Fractions were collected in 1 min intervals and each fraction containing at least 1 μg cholesterol was independently acetylated, quantified and its δD value measured. The waste eluent (all eluent not collected in fractions) was also quantified for cholesterol. The remaining three cholesterol standards were acetylated, quantified and analyzed for δD values without undergoing RP-HPLC purification.

2.7. Sterol recovery during HPLC purification

Cholesterol recovery in the NP-HPLC and RP-HPLC methods was investigated to quantify the loss of analyte and identify the source of analyte loss. Cholesterol (100–400 μg) was run through the HPLC system in four configurations: NP-HPLC without column, NP-HPLC with column, RP-HPLC without column and RP-HPLC with column. In each configuration, three replicates of three different quantities of cholesterol were run through the system. In each run the eluent was collected for 15 min, bracketing the cholesterol peak on the MS instrument. For the NP-HPLC and RP-HPLC runs with the columns in place, eluent was collected for an additional 15 min after the primary collection period in a separate vial. This second collection period included a column rinse with 70% DCM (100% EtOAc) in the NP-HPLC (RP-HPLC) runs. Recovery was calculated by quantifying cholesterol in samples purified via HPLC alongside a series of non-HPLC-purified cholesterol calibration standards using GC–FID.

2.8. Reproducibility of dinosterol δD values purified via HPLC

The reproducibility of dinosterol δD values was evaluated following NP- and RP-HPLC purification in order to assess the viability of the HPLC methods for isotope analysis applications. The El Junco Lake sediment samples were extracted and purified via aminopropyl and silica gel chromatography and the sterol/alcohol fractions divided between one to four aliquots. Each aliquot was independently purified for dinosterol and its δD value measured. Sample A and Sample B were divided into four aliquots; dinosterol was purified via RP-HPLC in two aliquots and via NP-HPLC in the remaining two aliquots. Sample C was divided into three aliquots; dinosterol was purified via RP-HPLC in one aliquot and via NP-HPLC in the remaining two aliquots. Samples D-H were divided into two aliquots and dinosterol was purified via RP-HPLC in both aliquots.

3. Results and discussion

3.1. Purification of dinosterol from sedimentary sterols

Smittenberg and Sachs (2007) published a method for purifying dinosterol from sediments via NP-HPLC, targeting the purification of dinosterol from pentacyclic triterpenoid alcohols. In NP-HPLC, a sample is applied to a polar stationary phase and eluted with non-polar solvents. With this system, the stereochemistry around the polar head group is the predominant factor controlling the

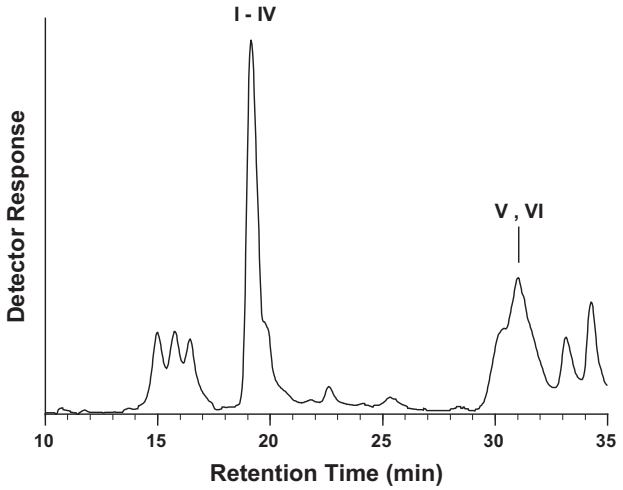

Fig. 1. Partial NP-HPLC-MS chromatogram of a representative sterol/alcohol fraction from El Junco Lake sediment (24–25 cm depth; SIM mode *m/z* 399, 409, 411 and 413). Compounds I–IV are 4α-methyl sterols while compounds V and VI are 4α-desmethyl sterols (see Table 1 for names and Fig. A1 for structures).

retention time of sterols because it plays a large role in the partitioning of the analyte between the polar stationary phase and the non-polar mobile phase. NP-HPLC therefore provides an ideal method for separating 4α-methyl sterols from 4-desmethyl sterols (lacking a Me at C-4; Fig. 1). However, although the method efficiently allows purification of dinosterol from 4-desmethyl sterols and triterpenoid alcohols (Smittenberg and Sachs, 2007), it does not allow purification of dinosterol from other 4α-methyl sterols, several of which co-elute with dinosterol during GC analysis (Table 1). Co-elution of dinosterol with other 4α-methyl sterols is an issue for many types of sediment due to the fact that a variety of 4α-methyl sterols, in addition to dinosterol, are typically produced by dinoflagellates and other algae.

The 4α-methyl sterols were separated from one another in our RP-HPLC method (Fig. 2A). In RP-HPLC a non-polar stationary phase and a polar mobile phase are used. In this chromatographic system the non-polar moiety of an analyte plays a dominant role in determining retention time; 4α-methyl sterols often differ by the location of a methyl in the side chain or the presence of a double bond and can thus be separated via RP-HPLC. Dinosterol eluted at ca. 43 min and was well separated from the other 4α-methyl sterols. For reference, the retention times of the common sterols cholesterol, stigmasterol, β-sitosterol and sitostanol, are shown in Fig. 2B, and the retention time of the pentacyclic triterpenol, taraxerol, is shown in Fig. 2C. In El Junco Lake sediments, the sterols that typically eluted in the same fractions as dinosterol during RP-HPLC were the 4α-desmethyl sterols, 23,24-dimethyl-5α-cholestan-3β-ol and 24-ethyl-5α-cholestan-3β-ol (sitostanol).

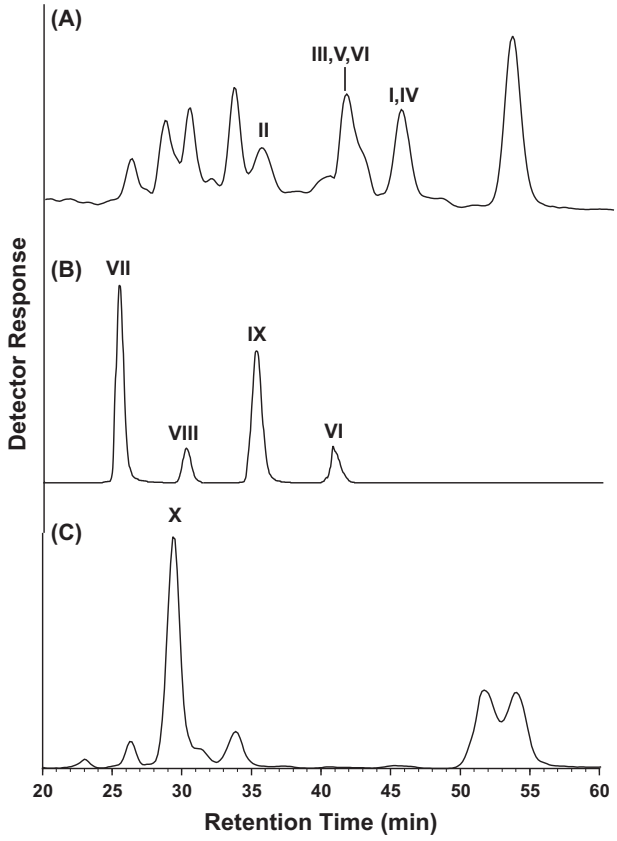

Fig. 2. Partial RP-HPLC-MS chromatograms of: (A) a representative sterol/alcohol fraction from El Junco Lake sediment at 24–25 cm depth (see Table 1 for sterol names and Fig. A1 for structures; SIM mode *m/z* 399, 409, 411, and 413), B) a sterol standard containing cholesterol (VII), stigmasterol (VIII), β-sitosterol (IX) and sitostanol (VI) (scan mode *m/z* 350–450) and C) a *Rhizophora* leaf extract, with the pentacyclic triterpenoid alcohol, taraxerol, labeled X (scan mode *m/z* 200–800).

3.2. Optimizing separation of dinosterol during GC analysis: DB-5ms vs. VF-17ms GC columns

Accurate and precise isotope measurements of dinosterol via GC–IRMS critically depend upon baseline separation from adjacent

Table 1
Comparison of GC–IRMS retention data of sterols on DB-5ms and VF-17ms capillary columns.

	Compound [a]	MW[b]	DB-5ms		VF-17ms	
			RRT[c]	Resolution[d]	RRT[c]	Resolution[d]
Sterols in NP-HPLC purified dinosterol samples						
4α,24-dimethyl-5α-cholestan-3β-ol	I	458	1.12	2.6	1.09	3.5
4α,23,24-trimethylcholest-5,22-dien-3β-ol (dehydrodinosterol)	II	468	1.13	**0.4**	1.11	1.7
4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol)	III	470	1.13	–	1.10	–
4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol	IV	470	1.13	**0.7**	1.11	**1.1**
Sterols in RP-HPLC purified dinosterol samples						
4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol)	III	470	1.13	–	1.11	-
23,24-dimethyl-5α-cholestan-3β-ol	V	458	1.14	**1.2**	1.12	2.1
24-ethyl-5α-cholestan-3β-ol (sitostanol)	VI	458	1.14	2.0	1.13	4.0

[a] Refer to Fig. A1 for structures.
[b] Molecular weight as acetate derivative.
[c] Retention time relative to cholesteryl acetate.
[d] Chromatographic resolution between associated compound and dinosterol, defined as $2(t_2 - t_1)/(w_1 + w_2)$, where t_n is the retention time and w_n is the peak width at baseline of compound n. Numbers in bold indicate resolution below baseline resolution ($R < 1.5$).

peaks. In order to optimize the separation of dinosterol from nearby eluting compounds during GC analysis, two columns were tested: a non-polar Agilent DB-5ms column (60 m × 0.32 mm × 0.25 μm) and a mid-polarity Varian VF-17ms FactorFour column (60 m × 0.32 mm × 0.25 μm). Dinosterol was purified from the sediment sterol/alcohol fractions via RP- and NP-HPLC methods and analyzed using GC–IRMS with each of these GC columns at quantities sufficient for isotope analyses (150 ng). The resolution between dinosterol and nearby eluting sterols was calculated and the results are shown in Table 1. Baseline resolution is typically defined as a resolution greater than or equal to 1.5 and higher resolution implies better separation.

In samples purified via NP-HPLC and analyzed with a DB-5ms column, dinosterol co-eluted with 4α,23,24-trimethylcholest-5,22-dien-3β-ol (dehydrodinosterol) and 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol (Table 1). When these samples were analyzed with the VF-17ms column, the resolution between dinosterol and the above compounds increased, but baseline resolution between dinosterol and 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol was not attained even for samples with a low concentration of 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol.

RP-HPLC purified samples demonstrated greater separation between dinosterol and nearby eluting compounds than NP-HPLC purified samples with both DB-5ms and VF-17ms GC columns. As with the NP-HPLC purified samples, significant improvement in chromatographic separation of dinosterol from nearby eluting compounds was obtained with the VF-17ms column vs. the DB-5ms column; the resolution between dinosterol and the closest eluting compound (23,24-dimethyl-5α-cholestan-3β-ol) was almost 2x greater with the VF-17ms GC column than the DB-5ms GC column. The preferential retention of 4α-desmethyl sterols over 4α-methyl sterols was enhanced with the VF-17ms column vs. the DB-5ms column, as might be expected due to the greater polarity of 4α-desmethyl sterols vs. 4α-methyl sterols and the greater polarity of the VF-17ms column as vs. the DB-5ms column. Only in RP-HPLC purified samples analyzed on a VF-17ms column was baseline resolution between dinosterol and all th other compounds achieved. Improved separation of dinosterol from nearby sterols after RP-HPLC purification as compared to NP-HPLC purification can be seen in Fig. 3.

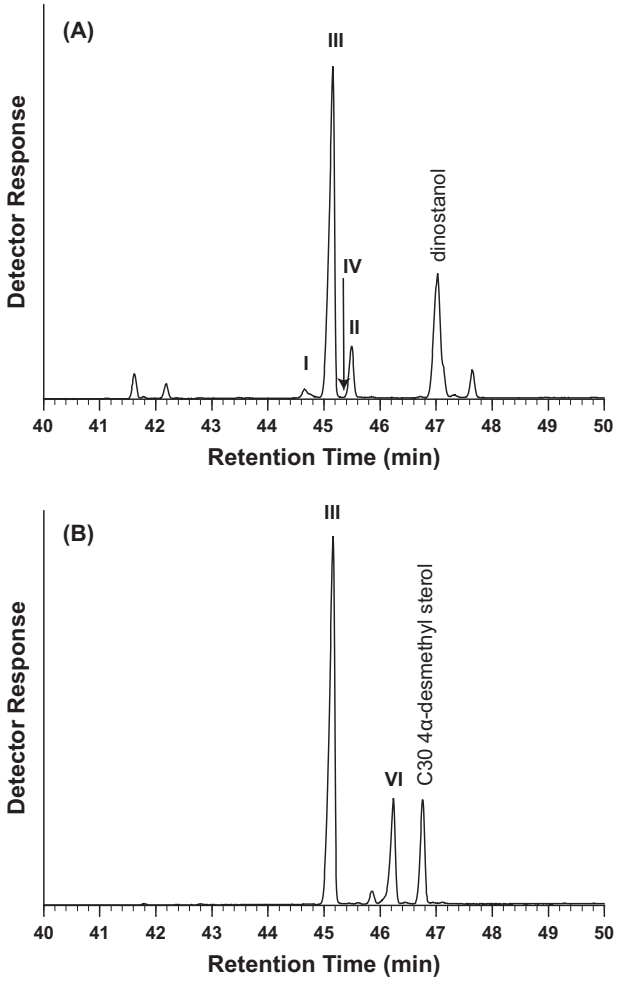

Fig. 3. Partial GC–MS TIC (*m/z* 200–800) of an acetylated dinosterol sample from El Junco Lake sediment (124–124.5 cm depth) with a VF-17ms GC column following (A) NP-HPLC purification and (B) RP-HPLC purification. See Table 1 for names and Fig. A1 for structures of sterols I–VI.

3.3. Two step HPLC purification: NP-HPLC followed by RP-HPLC

In RP-HPLC purified samples, baseline resolution between dinosterol and all the other compounds was achieved with a VF-17ms GC column for a variety of sediments in El Junco Lake (see Zhang et al. (2011) for a description of lipid distributions in the sediment). Baseline resolution of dinosterol was also achieved when this method was applied to freshwater lake sediments and a peat bog on Washington Island in the Republic of Kiribati, central tropical North Pacific, and marine meromictic lake sediments from the lagoon on Clipperton Atoll, eastern tropical North Pacific. However, there were some samples from each of the above sites in which baseline resolution of dinosterol was not achieved. Because the amount of analyte required to obtain an isotope measurement overloads the GC column, peak width broadens as analyte concentration increases. The resolution between dinosterol and 24-ethyl-5α-cholestan-3β-ol (sitostanol) therefore declined as the relative concentration of sitostanol increased and for samples in which the sitostanol concentration was >2× that of dinosterol, baseline resolution was not attained. In order to attain baseline resolution for such samples, a two step HPLC purification procedure was performed in which NP-HPLC was followed by RP-HPLC purification. During the NP-HPLC step, the 4α-desmethyl sterols were separated from the 4α-methyl sterols, while in the RP-HPLC step the remaining 4α-methyl sterols were separated from each other. This two step procedure resulted in baseline resolution between dinosterol and all other compounds for all of the 30 samples tested and in many cases gave isolation of dinosterol from all other alcohols and sterols (Fig. 4).

3.4. D/H fractionation during RP-HPLC purification

It is well known that hydrogen isotope fractionation occurs during HPLC (Hollandt et al., 1982; Smittenberg and Sachs, 2007; Schwab and Sachs, 2009; Kim et al., 2010). A more compact electron distribution in C–D bonds than C–H bonds leads to a decrease in the ability of a deuterated analyte's carbon–hydrogen bonds to donate electron density via an inductive effect, resulting in a decrease in attractive forces between the bonds and non-polar phase (Bates et al. 1987; Wade, 1999; Turowski et al., 2003). In NP-HPLC, a decrease in attractive interaction between the deuterated analyte's carbon–hydrogen bonds and the low polarity mobile phase causes increased retention of the deuterated compound (Iyer et al., 2004; Smittenberg and Sachs, 2007). This phenomenon has also been observed for tritium-labeled eicosanoids and steroids (Hollandt et al., 1982; Do et al., 1994).

In RP-HPLC, the opposite phenomenon typically occurs – decreased attraction between a deuterated analyte's carbon–hydrogen bonds and, in this case, the non-polar stationary phase

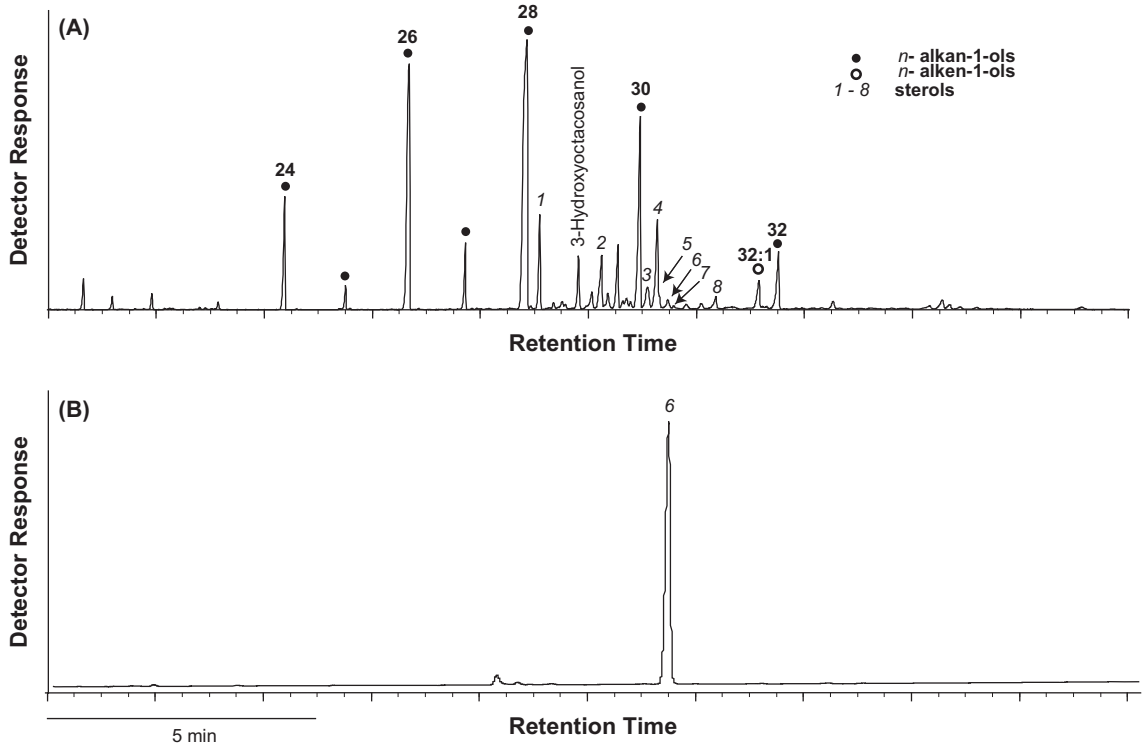

Fig. 4. GC signal of (A) a silylated sterol/alcohol fraction from El Junco Lake surface sediment, (B) an acetylated dinosterol sample following two step HPLC purification (NP-HPLC followed by RP-HPLC). Sterols: *1*, 5α-cholestan-3β-ol, *2*, 24-methyl-5α-cholestan-3β-ol; *3*, 24-ethylcholest-5-en-3β-ol; *4*, 24-ethyl-5α-cholestan-3β-ol (sitostanol); *5*, 4 α,24-dimethyl-5α-cholestan-3β-ol; *6*, 4 α,23,24-trimethyl-5α-cholest-22E-en-3β-ol (dinosterol); *7*, 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol; *8*, 4 α,23,24-trimethyl-5α-cholestan-3β-ol (dinostanol).

causes decreased retention of the deuterated compound. Thus in RP-HPLC, deuterated compounds typically elute before non-deuterated compounds (Baweja, 1986; Turowski et al., 2003).

Smittenberg and Sachs (2007) quantified the hydrogen isotope fractionation during NP-HPLC purification of a sterol (same NP-HPLC method as here). In their experiment, a cholesterol standard with a δD value of −212‰ was subjected to NP-HPLC purification using a Cyano column (250 mm × 4.6 mm × 5 μm) and a mobile phase of DCM and hexane. The eluent was collected in four 1 min fractions. A difference in δD value of 140‰ was observed between the first 6% and last 4% of the cholesterol peak.

Hydrogen isotope fractionation of a sterol during RP-HPLC purification with a C_{18} column (250 mm × 4.6 mm × 5 μm) and a mobile phase of 5% H_2O in MeOH, was quantified in this study using a similar experimental design to that of Smittenberg and Sachs (2007). A difference in δD value of 560‰ was observed between the first and last 5% of the cholesterol peak during RP-HPLC, which is 4× greater than the isotope effect observed during NP-HPLC purification (Fig. 5A). The consequence of this extremely large isotope effect is that a small loss of the front or tail of an eluting compound would result in a large bias in the isotope value. A simple mass balance calculation implies that a 1% loss in the peak front or a 2% loss in the peak tail would cause a −4‰ and +4‰ offset, respectively, in the δD value of the collected analyte, similar to the analytical error in the isotope measurements.

Secondary kinetic isotope effects have been found to vary with mobile phase composition and with different stationary phases owing to a change in the affinity of the analyte carbon–hydrogen bonds for one phase over the other (Turowski et al., 2003; Iyer et al., 2004). We postulate that the isotope effect may be greater in the RP-HPLC method here because of a larger binding energy difference between the analyte carbon–hydrogen bonds and the stationary vs. mobile phase in the RP-HPLC procedure. The NP-HPLC procedure utilizes a mid-polarity Cyano column and a low-polarity mobile phase (15% DCM in hexane) and the analyte carbon–hydrogen bonds are expected to have significant attractive interaction with both the mobile phase and the stationary phase. In contrast, the RP-HPLC procedure utilizes a non-polar C_{18} column with a highly polar mobile phase (5% H_2O in MeOH) and in this system, the analyte carbon–hydrogen bonds have a far greater attraction to the stationary phase than to the mobile phase. Due to the higher relative affinity of these bonds for the stationary phase, the attractive interaction between the carbon–hydrogen bonds and the stationary phase plays a larger role in determining the retention of the analyte. The slightly greater attraction of the non-deuterated bonds than the deuterated bonds for the C_{18} phase thus results in a large difference in retention between deuterated and non-deuterated compounds and a large isotope effect during the RP-HPLC method. A similar mechanism has been used to explain an observed increase in the isotope effect of benzene with a decrease in the MeOH/H_2O ratio of the mobile phase in RP-HPLC (Turowski et al., 2003).

The large isotope effect in RP-HPLC underscores the need for quantitative recombination of fractions containing the target analyte if an accurate isotope measurement is to be obtained. In order to avoid biasing a target analyte's isotope value through the RP-HPLC procedure, care must be taken to ensure that all fractions containing the target analyte are recombined.

3.5. Sterol recovery during HPLC purification

High-yield biomarker purification methods are important for isotope analysis as biomarker concentrations can vary widely in sediments and a relatively large amount of material (>150 ng per injection) is needed for isotope measurement via GC–IRMS. Dinosterol yield was evaluated during the NP-HPLC, RP-HPLC and two

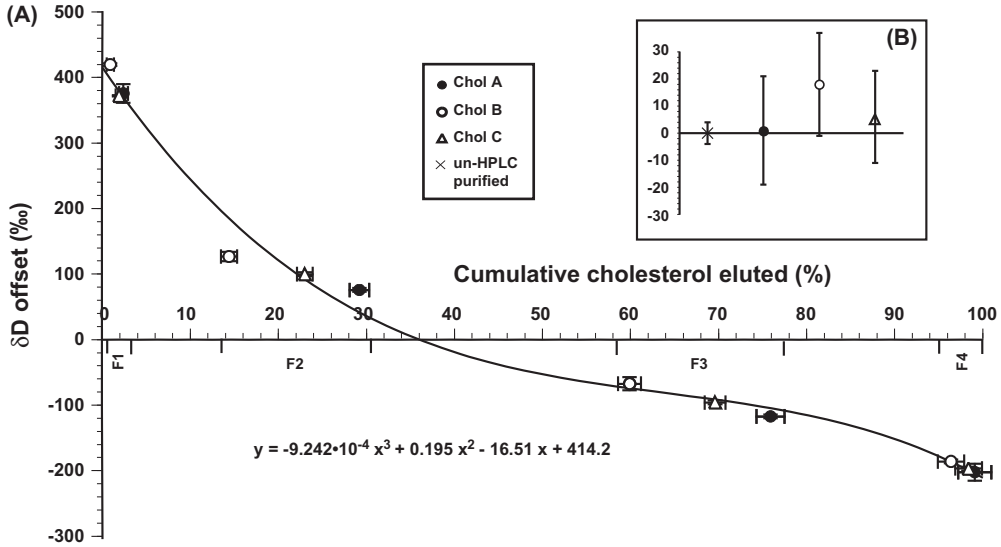

Fig. 5. (A) D/H fractionation across an eluting cholesterol peak in three cholesterol standards (Chol A, B, C) purified via RP-HPLC. *Y*-values are the δD value of cholesterol in each fraction relative to the average δD value of the non-HPLC purified cholesterol standards. *X*-values are the cumulative % of cholesterol eluted (where the midpoint is taken for each fraction). Error bars in the *y*-direction are the standard deviations of δD values from triplicate isotope analyses and error bars in the *x*-direction are standard deviations of the % cholesterol eluted arising from quantitation errors. F1, F2, F3 and F4 refer to HPLC fractions 1–4. The yield of cholesterol in Chol A, B, and C was 68%, 84% and 86%, respectively. (B) The inset shows the δD values of the theoretically combined standards (using an isotope mass balance calculation relative to the average δD value of the non-HPLC purified standards. Error bars for the theoretically combined standards (Chol A, B, C) are the standard deviations of δD values from propagating the *x*- and *y*-errors in (A). Error bars for the non-HPLC purified standards are the standard deviation of δD values from triplicate isotope analyses.

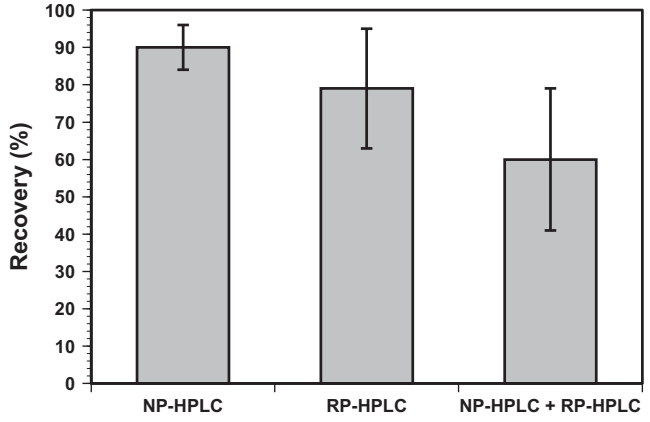

Fig. 6. Recovery of dinosterol during HPLC purification of sterol/alcohol fractions from El Junco Lake sediment using the NP-HPLC, RP-HPLC and two step HPLC purification methods; 1–16 μg of dinosterol was injected for each sample. Error bars represent standard deviations.

step HPLC purification of sterol/alcohol fractions from El Junco Lake sediment samples containing 1–16 μg of dinosterol. The average yield of dinosterol in these experiments was lower for RP-HPLC than NP-HPLC. The average yield was 90% during NP-HPLC, 80% during RP-HPLC and 60% for the sequential application of NP- followed by RP-HPLC (Fig. 6). The variability in yield was greater for RP-HPLC than for NP-HPLC and yields as low as 50% occurred during RP-HPLC.

3.6. Accuracy and reproducibility of sterol δD values purified via HPLC

Isotopic bias of sterols during RP- and NP-HPLC purification was tested in two ways. In the first, the δD value of the cholesterol that would have resulted from recombination of cholesterol fractions from the RP-HPLC fractionation experiment was estimated using the weighted sum of the δD values of each fraction. The results are shown in Fig. 5B. The theoretical δD values of the combined cholesterol fractions were within one standard deviation of the δD values of the non-HPLC purified standards, while the yield ranged from to 68% to 86%. No relationship between δD offset and yield was observed.

The estimates of the theoretical cholesterol δD values included several sources of error that would not be present for samples that were actually recombined. For instance, because a triplicate measurement of a δD value could only be obtained for fractions in which $\geqslant 1$ μg of cholesterol existed, a small amount of the cholesterol peak front and/or tail were likely missed in this experiment and the theoretical δD values would reflect any such bias. In addition, the theoretical cholesterol δD values are highly sensitive to errors in the estimated mass of cholesterol in each fraction. However, despite these additional sources of error, the results of this experiment suggest that the relatively large and variable loss of analyte that occurred during RP-HPLC did not result in isotopic bias.

In a second experiment, the reproducibility of dinosterol δD values purified via HPLC was investigated. Dinosterol was purified via NP-HPLC or RP-HPLC and analyzed via GC–IRMS with the VF-17ms GC column. Samples that had a low concentration of 4α-methyl-24-ethyl-5α-cholest-22E-en-3β-ol were selectively chosen for NP-HPLC purification in order to avoid the co-elution issues observed in the NP-HPLC purified samples. The results from this experiment are shown in Table 2. Dinosterol δD values for duplicate RP-HPLC purified samples differed by an average of 3‰, while those for duplicate NP-HPLC purified samples differed by an average of 2‰. In NP- vs. RP-HPLC purification of the same samples, the average dinosterol δD value of the NP-HPLC purified samples differed from the average of the RP-HPLC purified samples by 3–6‰. In comparison, triplicate injections with GC–IRMS gave an average standard deviation of 5‰ for the NP-HPLC purified samples and 4‰ for the RP-HPLC purified samples. Because the difference in δD value between replicate samples was on average lower than or equal to the standard deviation associated with the isotope measurement of a single sample, any bias in the isotope value caused by HPLC purification was overwhelmed by the measurement of that value.

Table 2
Reproducibility of dinosterol δD values in NP-HPLC and RP-HPLC purified samples.

RP-HPLC purified sample[a]	Dinosterol δD (‰)	SD[b]	δD Diff. between duplicates (‰)	NP-HPLC purified sample[a]	Dinosterol δD (‰)	SD[b]	δD Diff. between duplicates (‰)
A-1	−249	3	4	A-3	−249	6	2
A-2	−253	4		A-4	−247	6	
B-1	−247	7	1	B-3	−253	6	0
B-2	−248	3		B-4	−253	4	
C-1	−239	3		C-3	−245	1	3
				C-4	−242	5	
D-1	−252	4	1				
D-2	−251	3					
E-1	−244	1	3				
E-2	−241	4					
F-1	−237	5	4				
F-2	−233	2					
G-1	−257	1	5				
G-2	−252	8					
Avg.		4	3	Avg.		5	2

[a] A–G denotes different samples. Each sample was split into two to four aliquots ("1" through "4") and each independently purified via RP-HPLC or NP-HPLC.
[b] Standard deviation from triplicate δD measurements of each aliquot.

The results indicate that, regardless of the amount or source of analyte loss in the RP-HPLC method, such loss did not result in an isotopic bias. Although large D/H fractionation occurs during NP-HPLC and particularly during RP-HPLC, when all fractions containing the target sterol were recombined and baseline resolution between the target sterol and all other compounds was achieved, unbiased and reproducible δD values were obtained.

4. Conclusions

RP-HPLC purification of dinosterol from sterol/alcohol fractions of lake sediments separated it from structurally similar 4α-methyl sterols that co-eluted with dinosterol during GC. Subsequent isotope analysis with a mid-polarity VF-17ms GC column yielded superior chromatographic separation of dinosterol vs. analysis with a non-polar DB-5ms GC column. The average yield of dinosterol was 80% during RP-HPLC as compared with 90% during NP-HPLC. A large isotope effect was observed during RP-HPLC, with a 560‰ range in δD value between the first and last 5% of an eluting cholesterol standard. This is ca. 4x the isotope effect observed during the NP-HPLC method. Due to a large potential for isotopic bias, care must be taken to recombine all dinosterol-containing fractions. However, we have shown that, by combining 3–4 min of RP-HPLC eluent (bracketing the retention time of dinosterol), the isotope value of dinosterol is unbiased and highly reproducible.

Based on comparison between the purification methods here, we recommend the following procedure when testing a new set of sedimentary samples for dinosterol purification. In samples with

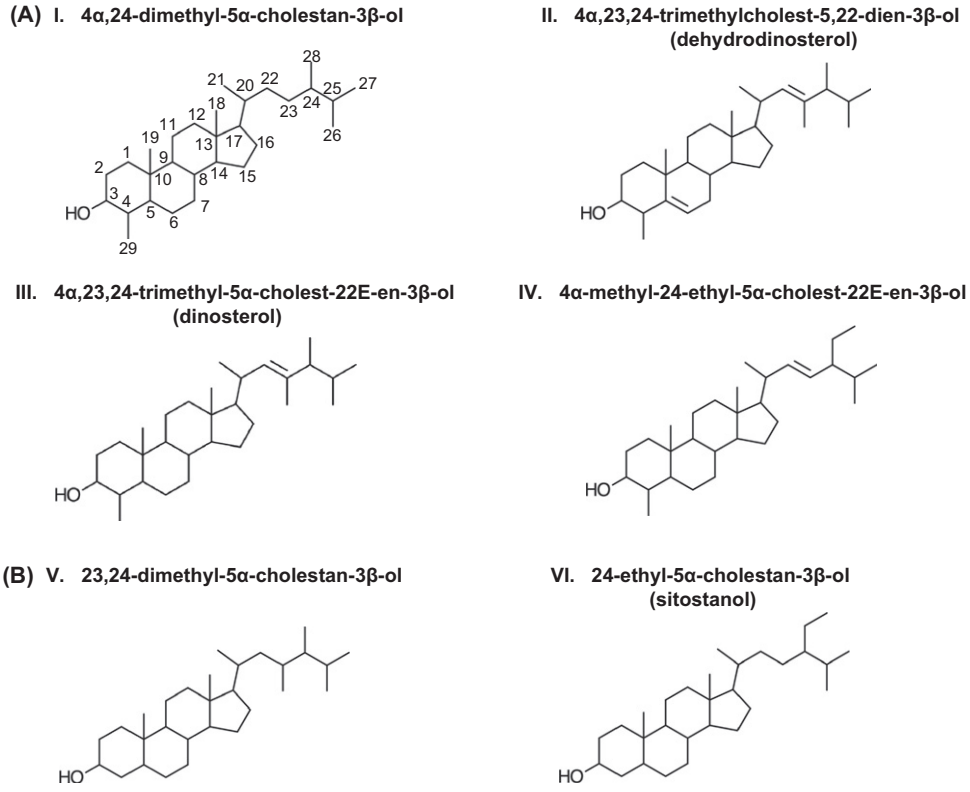

Fig. A1. Structures of the (A) 4α-methyl sterols and (B) 4α-desmethyl sterols that closely elute with dinosterol during GC.

few 4α-methyl sterols aside from dinosterol, NP-HPLC purification is a prudent first choice as baseline resolution may be achieved and higher recovery and lower potential for isotopic bias is associated with the NP-HPLC than the RP-HPLC method. In addition, we found that the alcohols and sterols dissolved readily in 100 μl hexane and DCM, which are compatible with NP-HPLC, than in 25 μl DCM and MeOH, as used for injection during RP-HPLC. However, most sedimentary samples contain significant abundances of a variety of 4α-methyl sterols due to the fact that dinoflagellates tend to produce a broad array of these compounds. Even low concentrations of certain 4α-methyl sterols co-elute with dinosterol. Thus, in samples that contain a variety of 4α-methyl sterols but a concentration of 24-ethyl-5α-cholestan-3β-ol (sitostanol) < 2× that of dinosterol, RP-HPLC is more likely to yield baseline resolution of dinosterol than NP-HPLC. An additional benefit to RP-HPLC over NP-HPLC is that the conditioning time necessary for the non-polar column in RP-HPLC is significantly lower than that for the polar column in NP-HPLC. Finally, in samples that contain a variety of 4α-methyl sterols and sitostanol concentration > 2× that of dinosterol, NP-HPLC followed by RP-HPLC is recommended, as neither NP-HPLC or RP-HPLC alone is likely to yield baseline resolution of dinosterol. This two step procedure resulted in baseline resolution between dinosterol and all other compounds for all samples tested, with recovery averaging 60%.

Improvements to the RP-HPLC method may be possible. In particular, decreasing the polarity difference between the mobile phase and stationary phase (e.g. by substituting acetonitrile (MeCN) for MeOH in the mobile phase) would likely result in higher sterol yield and reduced D/H fractionation across an eluting sterol peak, though purification of dinosterol from other 4α-methyl sterols and a reasonable retention time of dinosterol must be preserved.

Acknowledgements

This material is based upon work supported by the National Science Foundation under Grants ESH-0639640, EAR-0745982 and EAR-0823503, the American Chemical Society through Petroleum Research Fund Grant 46937-AC2, the National Science Foundation Graduate Research Fellowship Program, and the Department of Energy Global Change Education Program. The authors would like to thank O. Kawka, J. Gregersen, S. Wakeham, J. Volkman and K. Thomas for support and insight, as well as R. Smittenberg and an anonymous reviewer for thoughtful reviews.

Appendix A

See Fig. A1.

Appendix B. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.orggeochem.2012.04.006.

Associate Editor—**S. Schouten**

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