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An efficient approach to eliminate steryl ethers and miscellaneous esters/ketones for gas chromatographic analysis of alkenones and alkenoates

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ABSTRACT

Long-chain alkenones (LCAs) and alkenoates (LCEs) are highly valuable biomarkers for paleotemperature reconstructions. A major problem, however, for accurate quantification of these compounds using gas chromatography (GC) is co-elution with steryl ethers, wax esters, saturated ketones and other numerous mid-polarity compounds frequently encountered in marginal marine and lake sediments. Co-elution during GC separation is prevalent, particularly if the full homologous series of alkenones and alkenoates are to be analyzed. Taking advantage of the presence of two or more double bonds in LCAs and LCEs, the conventional silica gel impregnated with silver nitrate has previously been used to remove co-eluting compounds for LCAs. However, this conventional argentation chromatography is hampered by the extreme instability of silver nitrate, poor reproducibility, low recovery and short lifetime. Here we demonstrate a highly efficient flash chromatographic approach based on silver thiolate chromatographic material (AgTCM) that overcomes the shortcomings of the traditional argentation chromatography and allows repeated sample preparation (up to 62 samples in one test) with little loss in separation efficiency. AgTCM selectively extracts LCAs and LCEs and effectively eliminates co-eluting compounds including steryl ethers and wax esters for the subsequent gas chromatography (GC) analysis. This new method, therefore, allows low-cost and high-throughput sample preparation for comprehensive quantification of the full homologous series of LCAs and LCEs in marine and lake sediments.

1. Introduction

Long-chain alkenones (LCAs) and alkenoates (LCEs) are a series of C_{35} to C_{42} aliphatic unsaturated ketones produced by Isochrysidales, an order of haptophyte algae [1–3]. LCAs are important biomarkers for paleotemperature reconstructions from ocean and lake sediments [1,4–7], because their degrees of unsaturation vary linearly with temperature of the water in which the compounds are biosynthesized. LCAs are recalcitrant biomarkers that can survive millions of years of sedimentary burial while still retaining the unsaturation ratios, making them among the most ideal biomarkers for paleotemperature reconstructions in a variety of timescales [8,9]. Conventionally, paleotemperature reconstructions focus on C_{37} alkenones because of their high natural abundances and relative ease of analysis [8–12]. However, recent studies indicate that longer chain LCA homologues [13] and LCEs [14] may provide critical new paleoclimate and paleoenvironmental information in marginal ocean environments with multiple LCA producers [13,14]. A new GC separation method now permits simultaneous analysis of all homologues of C_{36} to C_{40} alkenones and alkenoates [15], overcoming the longstanding problem of LCA/LCE interference using the conventional non-polar GC columns. However, co-elution with wax esters, steryl ethers and numerous other unknown compounds still poses major difficulties for simultaneously analyzing the full suite of LCAs and LCEs. Saponification would remove wax esters together with LCEs hence cannot be used if LCEs-based indices are desired. In some cases, even C_{37} alkenones co-elute with steryl ethers or other miscellaneous compounds, causing major distortion of reconstructed sea surface temperatures if co-eluting compounds are not completely removed [15–17]. Such distortion can sometimes go undetected without careful gas chromatography-mass spectrometer (GC–MS) examination [18].

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Due to the fact that silver ion (Ag^+) can form reversible complexes with double bonds in organic compounds [19,20], silica gel impregnated with $AgNO_3$ (also called argentation chromatography) has traditionally been the method of choice for purifying lipids containing varying numbers of carbon-carbon double bonds, including LCAs [21,22]. However, Ag^+ can be readily reduced to Ag upon exposure to light (Ag metal does not have any affinity for double bonds in chromatography); hence the chromatographic procedure must be carried out in total darkness [19]. The high light sensitivity also requires that silica gel impregnated with silver nitrate be freshly prepared and stored without exposure to light [19]. These requirements create significant operational inconvenience and often lead to irreproducible retention volumes. In addition, because the electrostatic interaction between silver ions and the silanol functional group are weak, the silver ions are liable to bleed, especially in relatively polar solvents such as acetone and methanol. Ag^+ can also oxidize unsaturated compounds, causing lower compound recovery [23,24] and potentially altering LCA unsaturation ratios. Therefore, it is difficult to use silver-nitrate-based argentation chromatography for purifying large number of alkenone samples for high-resolution paleotemperature reconstructions from sediment cores.

Recently a new chromatographic medium called silver thiolate chromatographic material (AgTCM), characterized by its sulfur-containing functional group covalently bound to a silver atom, has been reported [22–26]. AgTCM eliminates the key drawbacks of traditional argentation chromatography, allowing for convenient, reproducible and consistent separation of organic compounds containing variable numbers of double bonds. For example, AgTCM has been used to successfully separate mixtures of compounds by different numbers of double bonds [23,24], and separate triglycerols with variably unsaturated fatty acids [25,26] and purify individual LCAs using high performance liquid chromatography (HPLC) [22]. Recently, Rama-Corredor et al. demonstrated a normal phase (silica gel) HPLC method to eliminate steryl ethers co-eluting with C_{37} di- and tri- unsaturated alkenones. Purified alkenones were subsequently analyzed on a nonpolar polydimethylsiloxane) GC column [18]. However, routine use of HPLC for cleaning a large number of alkenone samples, often required for paleoclimate studies, is relatively difficult in practice for most earth science laboratories and the running cost is relatively high. It is also unclear if the HPLC procedure is capable of purifying the full homologous series of C_{36} to C_{40} LCAs and LCEs from numerous types of co-eluting compounds. On the other hand, steryl ethers cannot be separated from alkenones using conventional gravity or flash silica gel column chromatography (which separates compounds based on polarity differences) routinely used for cleaning alkenones for sea surface temperature (SST) reconstructions [26].

The objectives of the present study are 1) to develop an optimal solvent scheme of using an AgTCM flash column for purification of the C_{36} to C_{40} homologous series of LCAs and LCEs from sediment samples; 2) to test reusability of the AgTCM flash columns for LCAs and LCEs, including retention of the double bond ratios; and 3) to demonstrate how the optimized AgTCM purification can rectify a highly challenging paleotemperature reconstruction from marine sediment cores that contain large amounts of steryl ethers, wax esters and other miscellaneous coeluting compounds.

2. Experiment Section

2.1. Materials

The solvents, including acetone, dichloromethane, trichloroethylene and hexane, were HPLC grade purchased from Fisher Scientific (NJ, USA). Mercaptopropyl silica gel (40–63 μm particle size, 60 Å pore size) was obtained from SiliCycle Inc. (Quebec, Canada). Silver nitrate (analytical grade) and internal standard (*n*-Hexatriacontane) were obtained from Sigma-Aldrich (MA, USA).

2.2. Preparation of AgTCM

The preparation of AgTCM ($\equiv Si{-}(CH_2)_3{-}S{-}Ag$) was performed according to the published procedure by suspending 1.6 g of 3-mercaptopropyl functionalized silica (2.26 mmol SH) in 20 ml of a water-methanol (1:1) solution containing 460 mg of $AgNO_3$ (2.71 mmol) [23,24]. The pH of the slurry was ~1–2 due to the formation of HNO_3 when silver thiolate is formed. The excess Ag^+ was monitored by taking an aliquot of the solution and precipitating AgCl with a drop of NaCl 5% solution. The mixture was stirred at room temperature for 2 h, then filtered and washed with 60 ml of deionized water and 40 ml of methanol to completely remove any excess $AgNO_3$ and HNO_3. After filtering and washing with deionized water and methanol, AgTCM (faint yellow) powder was dried at 60 °C for 16 h [23].

2.3. Origin of sediment samples

Since our central objective is to demonstrate the capability of AgTCM for eliminating a variety of interfering compounds, such as steryl ethers, wax esters, and various carbonyl-containing compounds of similar polarity, we purposefully select the following suite of different marine and lake sediments containing different types of co-eluting compounds for this study (Table S1):

1) Samples from marine sediment cores 343310 and 343300 from Disko Bugt [28], West Greenland (Fig. S1). These samples contain exceptionally large amounts of co-eluting compounds with alkenones, especially steryl ethers, wax esters and compounds of similar polarity. We first use these samples to develop an optimal solvent elution scheme to eliminate steryl ethers and wax esters. We then perform a down core study of site 343310 to compare the changes in the paleotemperature reconstructions with and without our purification procedures. The optimal solvent scheme is also applied to one sample from a marine sediment core in the northern Icelandic Shelf JR51-GC35 [27] to demonstrate the effectiveness of simultaneous purification of alkenones and alkenoates (Fig. S1) because this sample contains relatively high concentration of alkenoates;
2) A surface sediment sample from Lake Étang des Vallées. The compounds co-eluting with alkenones in this lake (and other lakes we have studied so far) differ greatly from those in marine sediments [13,29]. The co-eluents are a series of saturated alkyl ketones with variable carbonyl positions and numerous other unidentified compounds. This sample is selected to demonstrate that the same solvent scheme developed for marine sediment samples can also be readily applied to purify lacustrine alkenones that often co-elute with a different set of interfering compounds (i.e., not steryl ethers);
3) Sample from Braya Sø lake surface sediment, Southwestern Greenland [30] is used to test the retention of various unsaturation ratios (Table 2) after repeated separations using our optimized solvent elution scheme;
4) Down core samples of site 343310, cleaned by reusing the same AgTCM flash column, are selected to compare with previously published data from the same sediment core [28] and regional paleptemperature records from southwestern Greenland [30]. All separated fractions are analyzed by GC or GC–MS to test the stability of solvent retention volume and ensure that alkenones and alkenoates do not elute in non-designated fractions.

2.4. Sample preparation and extraction

Sediment samples (3–5 g) were freeze-dried, then extracted with 11 ml cells using dichloromethane: methanol (DCM:MeOH = 9:1, v/v) with an accelerated solvent extractor ASE200 (Dionex) at 120 °C and 1200 psi. The void columns were filled with fine sand (40–100 mesh, ACROS Organics™, obtained from Fisher Chemical, USA) pre-baked at 500 °C for 5 h. Activated copper was added into the 40 ml collection vials of the ASE200 during solvent extraction in order to remove sulfur. Prior to use, copper was sonicated in 0.1 N HCl for 1 min, then rinsed with deionized water and MeOH. Ocean sediments often contain various concentration of sulfur. Copper treatment is a routine procedure to remove sulfur. Removing sulfur is particularly important here because of its interference with AgTCM. Total lipid extracts were separated into hexane, DCM, and methanol fractions using silica gel flash chromatography. The DCM fraction contains LCAs and LCEs. When LCE analyses were not desired, saponification was performed to remove LCEs along with other esters (e.g, wax esters). The saponification was carried out by heating the extract at 65 °C for 3 h in 1 ml of 1 M KOH in MeOH:H_2O (95:5, v/v). After cooling to room temperature, 5% NaCl was added into the mixture, then acidified to pH 2 with 1 N HCl in H_2O, and extracted with hexane three times (1 ml *n*-hexane was used each time). Hexane extracts were combined, dried in the fume hood under nitrogen gas at room temperature, and then transferred to 2 ml vials with 250 μl inserts for GC analysis [31].

Saponification is effective in removing compounds containing ester functional groups including wax esters. However, in many samples mentioned above, there are still high concentrations of co-eluting compounds with LCAs even after saponification. In addition, the removal of LCEs by saponification also removes alkenoates as a potentially important source of paleotemperature information, since these compounds are also temperature-sensitive [14,15]. Ideally, LCAs and LCEs can be purified without saponification. In this study, we prepared both saponified and unsaponified samples from ocean and lakes (Table S1) to test our separation methods.

2.5. Flash chromatography of AgTCM

Alkenones (and alkenoates in samples that were not subjected to saponification) prepared using above steps were separated using the AgTCM columns. 1 g of AgTCM is dry packed into a glass pipette with about 0.3 cm inner diameter, and the packed column is about 5.5 cm in height. Separation was achieved by using a sequence of solvents with increasing polarities (solvent elution volumes are discussed in detail in Section 3.2). Fractions of 250 μl were collected in glass vials with inserts, and then evaporated to dryness and re-dissolved in 50 μl of hexane with *n*-Hexatriacontane as the internal standard for quantification and analyses by GC and GC–MS. After each sample separation, the AgTCM columns were eluted with 10 ml acetone to remove any residual compounds. The column was then eluted with hexane for conditioning, in preparation for next sample separation. In our test samples, no alkenones or alkenoates were found in the acetone eluents, indicating the absence of sample-to-sample carry overs.

2.6. Instrumentation

The solvent collections in this study were analyzed with an Agilent 6890$^+$ GC system equipped with a flame ionization detector (FID) and an Rtx-200 capillary column (105 m × 250 μm × 0.25 μm) at Brown University. The analytical methods were identical to those used by Zheng et al. [15]. Samples were injected onto the column using a split/splitless injector (2 μl injections, pulsed splitless mode) with the injector port temperature set to 320 °C. The carrier gas (H_2) was held at a constant flow rate of 1.0 ml min^{-1}. The following oven temperature program was used: initial isothermal at 60 °C (hold 1 min), ramp by 20 °C /min to 255 °C, ramp 3 °C /min to 315 °C (hold 20 min). Analytical precision was determined based on replicate analyses of the same Braya Sø lake LCAs as the laboratory standard. The following alkenone and alkenoates are quantified using GC-FID: $C_{37:4}$ Me, $C_{37:3a}$ Me, $C_{37:3b}$ Me, $C_{37:2}$ Me, $C_{38:4}$ Et, $C_{38:3a}$ Et, $C_{38:3b}$ Et, $C_{38:2}$ Et. The compound ratios calculated are listed in Section 2.7, and the analytical errors for the alkenone index were reported as ±1 Standard Deviation or SD (±Standard Error or SE): UK' 37, 0.004 (0.0005) [6]. Alkenones and alkenoates were identified by comparison of GC retention times of an alkenone standard mixture isolated from Lake Braya Sø, Greenland [15] and Isochrysidales cultures [32]. When confirmation of compound identification was needed, sample fractions were analyzed by GC–MS. Individual alkenones and alkenoates were identified by comparison with published mass spectra [1]. GC–MS analyses were performed using an Agilent 6890 N GC system coupled to an Agilent 5973 N quadrupole mass spectrometer (MS) using a VF-200 ms capillary column (60 m, 250 μm i.d., 0.10 μm film thickness). The GC conditions used for GC–MS analysis were the same as those used for GC-FID, except that the flow rate of helium was 1.3 ml/min. The MS was set to an ionization energy of 70 eV and a scan range of 40–600 *m/z*.

2.7. Alkenone and alkenoate unsaturation indices

The alkenone unsaturation indices and the isomeric ratio of alkenone (RIK_{37}, RIK_{38E}) were all calculated by the following equations: $\%C_{37:4} = 100 \times C_{37:4}/(\text{sum of } C_{37} \text{ LCAs})$; UK 37 = $(C_{37:2} - C_{37:4})/(C_{37:2} + C_{37:3} + C_{37:4})$; UK' 37 = $C_{37:2}/(C_{37:2} + C_{37:3})$; UK" 37= $C_{37:4}/(C_{37:3} + C_{37:4})$; UK 38 Et = $(C_{38:2}Et - C_{38:4}Et)/(C_{38:2}Et + C_{38:3}Et + C_{38:4}Et)$; UK 38Me = $(C_{38:2}Me - C_{38:4}Me)/(C_{38:2}Me + C_{38:3}Me + C_{38:4}Me)$; UK 39 = $(C_{39:2}Et - C_{39:4}Et)/(C_{39:2}Et + C_{39:3}Et + C_{39:4}Et)$; C_{38}/C_{37} = $(C_{38:2} + C_{38:3} + C_{38:4})/(C_{37:2} + C_{37:3} + C_{37:4})$; $RIK_{37} = C_{37:3a}/(C_{37:3a} + C_{37:3b})$; $RIK_{38E} = C_{38:3a}Et/(C_{38:3a}Et + C_{38:3b}Et)$, where UK stands for unsaturated ketones. Carbon number (n) and number of double bonds (m) of alkenones are designated by Cn:m, where Me denotes methyl ketones, Et denotes ethyl ketones.

3. Results and discussion

3.1. Steryl alkyl ethers

Steryl alkyl ethers are C_9 to C_{10} alkyl chain ether- bonded to the hydroxyl group of sterols (Fig. 1 and S2) [16,17,33]. These compounds have been reported to occur in marine sediments overlain by cool surface water (such as high latitude or seasonal upwelling regions) [16,17]. They are also thought to be particularly abundant in marine sites with major influence from continental inputs [17]. The specific biological source of these compounds is currently unclear, but is thought to be eukaryotic phytoplankton or their zooplankton consumers [16]. These ethers coelute with alkenones on the conventional, 100% polydimethylsiloxane nonpolar GC columns (e.g., $C_{27:1}$-$C_{11:1}$ steryl ethers coeluting with $C_{37:3}$ alkenone) and can significantly bias SST alkenone measurements [16,17]. In fact, on GC-FID equipped with the 100% polydimethylsiloxane GC column, co-elution of C_{37} alkenones with steryl ethers is so severe that it is virtually impossible to detect the co-elution without GC–MS analysis [18]. Because C_{37} alkenones are routinely analyzed using the nonpolar stationary phase on a GC-FID in most paleoclimate laboratories around the world [34–37], and GC–MS analyses are not always performed to confirm the identity and purity of these compounds, the stakes are particularly high for inad-

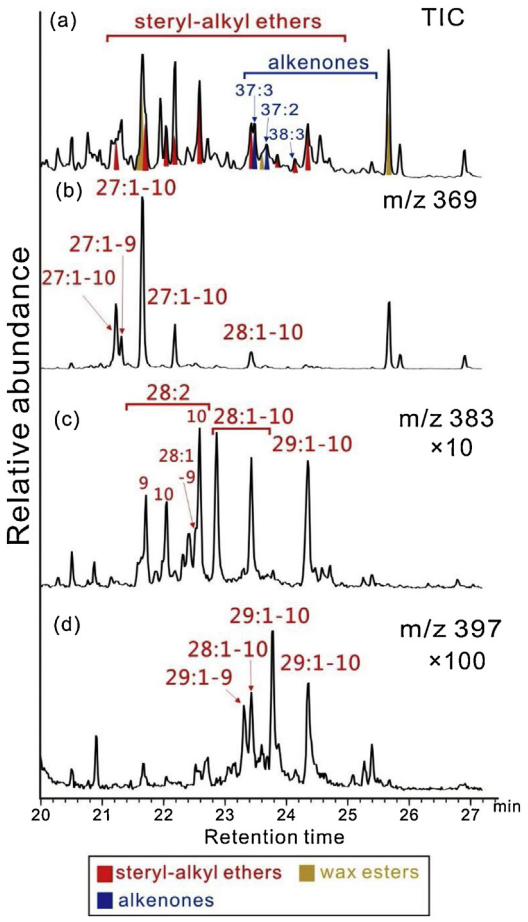

Fig. 1. Partial total ion chromatogram and mass chromatograms for *m/z* 369, *m/z* 383 and *m/z* 397 (representing C_{27}, C_{28} and C_{29} sterol moieties, respectively) of the 191 cm depth sample from marine sediment core 343300 [28], West Greenland (Fig. S1). The GC–MS analysis was performed on the DCM fraction of the total sediment extract. Large numbers of steryl ethers and wax esters are present in the sample and coelute extensively with alkenones. Carbon number (n), number of double bonds (m) of the sterol residual, and carbon number (x) of alkyl moiety of the steryl ethers are designated by n:m –x. Example mass spectra of steryl ethers, wax esters and other unknown coeluting compounds are given in Fig. S2 and A3.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

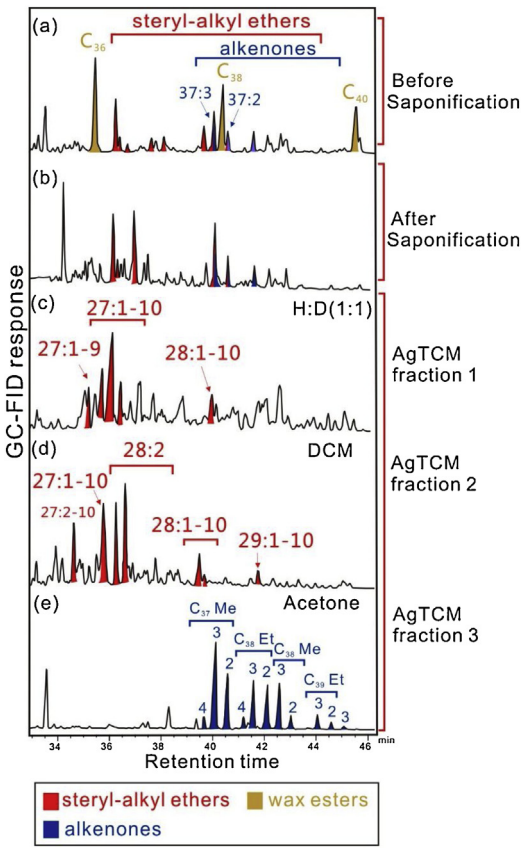

Fig. 2. Partial GC-FID chromatograms of 280 cm depth sample from site 343300 of Disko Bugt, West Greenland, showing LCAs, co-eluting compounds including steryl ethers, wax esters and other unknown compounds at various stages of sample purification. (a) before saponification: wax esters (brown) and steryl ethers (red) coelute extensively with LCAs (blue); (b) after saponification: wax esters and LCEs have been removed, but steryl ethers and other unknown compounds still coelute with LCAs; (c) and (d) coeluting compounds are collected during AgTCM treatment using hexane: DCM (1:1 v/v) and DCM, respectively, using the solvent scheme 3 (Table 1). (e) alkenone fractions eluted with acetone. For alkenones, Me and Et designate methyl and ethyl alkenones, respectively. Carbon number (n), number of double bonds (m) of sterol residual, and carbon number (x) of alkyl moiety of the steryl alkyl ethers are designated by n:m –x. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

vertently obtaining erroneous SST reconstructions in marine sites prone to steryl ether inputs.

Our recent application of a mid-polarity, trifluoropropylmethylsiloxane stationary phase for alkenone and alkenoate analyses appear to partially alleviate this problem [15,31]. Relative to alkenones, most steryl ethers elute significantly faster on the mid-polarity RTX-200 and are readily detected (Fig. 1), hence significantly reducing the likelihood of inadvertently reporting erroneous SSTs. Unlike nonpolar GC stationary phase that separates compounds primarily based on boil point differences, more polar compounds attain longer retention times on RTX-200. Relative to non-polar GC columns, the steryl ethers elute faster than alkenones on RTX-200 GC column, reflecting an overall lower polarity of the steryl ethers than alkenones.

Samples from marine sediment cores 343310 and 343300 from Disko Bugt, West Greenland [28] contain exceptionally large amounts of steryl ethers: alkenones are barely visible without purification (Fig. 1). These compounds with C_{27}, C_{28} and C_{29} sterol moieties can be readily detected using mass chromatograms of *m/z* 369, 383 and 397 (Fig. 1 and S2). The distribution of steryl ethers in these samples is characterized by the dominance of $C_{27:1}$ and $C_{28:1}$ steryl ethers and a relatively low abundance of $C_{28:2}$ and $C_{29:1}$ steryl ethers. Unlike on a nonpolar GC stationary phase where C_{37} alkenones co-elute with C_{27} steryl ethers [18], C_{37} alkenones mainly co-elute with C_{29} steryl ethers on the more polar RTX-200 column used (Fig. 1). If samples are not saponified, there are also a series of C_{37} to C_{38} wax esters co-eluting with C_{37} to C_{39} alkenones in our samples (Figs. 1 and 2a). Saponification does not remove coeluting steryl alkyl ethers, hence alkenones cannot be accurately measured on GC-FID, regardless of the type of GC columns used (Figs. 1 and 2b).

3.2. Optimization of solvent elution schemes for purification of LCAs and LCEs

To develop an optimal solvent elution scheme, we selected samples from marine sediment core 343300 [28] of West Greenland (Fig. S1) that contain large amounts of steryl ethers. We performed following tests to identify an optimal elution scheme for purification of LCAs and LCEs. We used trichloroethylene, acetone, dichloromethane, hexane and their different gradient mixture as the eluents (Table 1). In all of our tests, steryl ethers eluted much faster than alkenones and alkenoates, hence our main focus is to avoid overlaps.

Table 1
The solvent elution schemes for simultaneous purification of LCAs and LCEs by AgTCM.

Scheme No.	Hex (ml)	Hex:DCM (ml)				DCM (ml)	Acetone (ml)	Removal impurities
		7:1	5:1	2:1	1:1			
1	–	–	–	3.75	1.25	1.25	1.25	N
2	1.25	2.5	1.25	–	1.25	1	2	Y
3	–	–	–	–	2	1	1	Y
Scheme No.	TCE (ml)					DCM (ml)	Acetone (ml)	Removal impurities
4	2.5					1.25	1.25	N

Hex=Hexane; DCM = dichloromethane; TCE = trichloroethylene. The scheme 3 is proposed as the optimal scheme for separating LCAs and/or LCEs from impurities.

Table 2
The retention of the double bond ratios of various alkenones after repeatedly cycling the same sample from Lake Braya Sø through one single AgTCM pipette column three times (using the solvent scheme 3 described in Table 1).

Alkenone indices	TEST 1	TEST 2	TEST 3	1σ
UK 37	−0.52	−0.51	−0.52	0.003
UK' 37	0.12	0.12	0.11	0.004
UK" 37	0.40	0.40	0.40	0.000
UK 38 Et	−0.44	−0.43	−0.43	0.004
UK 38Me	−0.27	−0.27	−0.26	0.004
UK 39	−0.22	−0.23	−0.24	0.007
C_{38}/C_{37}	0.73	0.74	0.74	0.008
RIK_{37}	0.62	0.61	0.61	0.003
RIK_{38E}	0.31	0.31	0.30	0.003

1σ denotes standard deviation for various ratios from three replicate elutions. Please see Section 2.7 for definition of various indices.

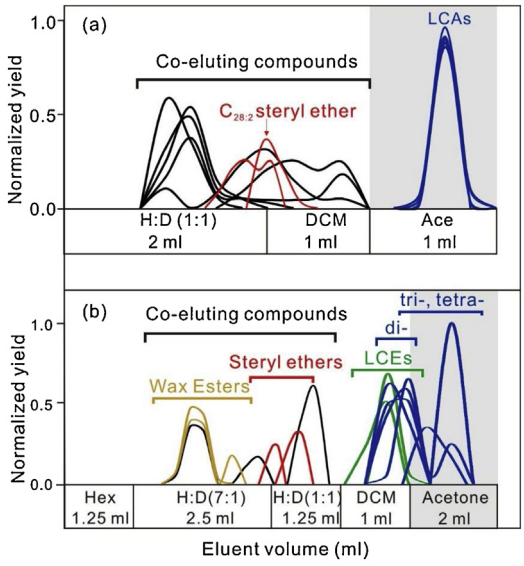

Fig. 3. Normalized yields vs eluent volume for LCAs (blue) and LCEs (green) purification using an AgTCM pipette column. The separation tests were performed on 370 cm depth (a), and 340 cm depth (b), respectively, from marine sediment core 343300, Disko Bugt, West Greenland. In solvent scheme 3 (a), alkenoates were removed by saponification prior to AgTCM column clean-up. In solvent scheme 2 (b), non-saponified DCM fraction collected on a plain silica gel column from total solvent extract was used. Scheme 2 (b) is capable to resolving LCEs and LCAs from wax esters (brown) and steryl ethers (red), although LCEs (green) elute slightly faster than LCAs (Blue). Solvent eluent volumes are noted below the graphs where Hex = hexane. H:D = hexane :dichloromethane (v/v); DCM = dichloromethane; Ace = acetone. Di-, tri-, and tetra- denote di-, tri- and tetra- unsaturated alkenones. Each line represents smoothed solvent elution volume curve of one individual compound, which is established by measuring the concentration of the compound (quantified on GC-FID with *n*-hexatriacontane as an internal standard) in consecutively collected 250 μl solvent intervals. Different compound classes are represented by different colors: blue = alkenones, green = alkenoates, red = steryl ethers, brown = wax esters; black = unknown co-eluents. Only selected major components in each compound class are plotted here for illustration purpose, as compounds of similar polarity would generally elute in the same solvent fractions. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Some LCAs and LCEs were eluted in the DCM fraction using Scheme 1, together with some co-eluting compounds (hence the initial solvent polarity was too high). Using scheme 2, wax esters and steryl ethers were successfully removed, and the LCAs and LCEs were separated from complex matrix compounds. However, LCAs and LCEs were partially eluted in two fractions (Fig. 3). LCAs and/or LCEs were fully separated from co-eluents using scheme 3. Attempts were also made to replace the hexane and DCM mixture with other pure solvents such as trichloroethylene to further simplify the solvent scheme, but the results were unsatisfactory. For example, in scheme 4 (Table 1), LCAs and the co-eluents were both eluted in the trichloroethylene fraction. Therefore, both schemes 2 and–3 are excellent candidates for separating LCAs and/or LCEs. Using Scheme 3, all LCEs and/or LCAs elute in the acetone fraction with no need to manually recombine two fractions afterwards. For most samples, scheme 3 is likely sufficient, especially for samples that have undergone saponification to remove wax esters and alkenoates. In practical applications for specific samples (which may contain variable sets of co-eluents), if scheme 3 cannot eliminate all co-eluting compounds, we recommend reducing the polarity of the initial solvents (e.g. higher hexane over DCM ratios). However, in all our testing and separation described below, we find scheme 3 is optimal for simultaneously purifying LCAs and LCEs.

3.3. *Underlying mechanism for AgTCM separation of LCAs and LCEs from co-eluents*

Our experimental results indicate that steryl ethers elute significantly faster than alkenones and alkenoates on AgTCM. This is perhaps not too surprising for steryl ethers containing one double bond [16–18] while alkenones with two or more double bonds should interact more strongly with AgTCM. It is, however, not immediately apparent why steryl ethers with two double bonds would also elute significantly faster than alkenones and alkenoates with two double bonds (Fig. 3).

We attribute the greater mobility of steryl ethers than alkenones to the following mechanisms: 1) in general, double bonds within the fused cyclic ring structures (e.g., sterols) have significantly lower interaction with AgTCM than those in alkyl chains, probably due to greater steric hindrance in the cyclic structures [23]; 2) steryl ethers, with a long saturated alkyl substitution on one side of ether bonds, are significantly less polar than alkenones and alkenoates. This is already well demonstrated based on their GC retention time on the mid-polarity RTX200 column discussed above (Fig. 1 and 2a-e). AgTCM contains ~65% of uncovered silanol (Si-OH) groups that separate compounds according to their polarity, with more polar compounds retained more strongly [23,25]. 3) The relatively lower polarity of steryl ethers compared to alkenones are also well demonstrated by their faster elution on the normal phase silica gel HPLC column [18]. AgTCM takes advantage of differences in both unsaturation and polarity to resolve alkenones from steryl ethers. In contrast, silica gel HPLC separation takes advantage of only the polarity differences [18]. In the case of using plain

silica gel, it is mandatory to employ the high pressure liquid chromatography with its small and uniform particle size and relatively sophisticated equipment in order to maximize the effect of separation based only the polarity differences (a flash column cannot accomplish sufficient separation) [18]. Overall, AgTCM is clearly superior over silica gel in terms of chromatographic selectivity for eliminating co-eluents for alkenone and alkenoate analyses. As a result, it is possible to use a small flash column, rather than an HPLC to accomplish similar objectives.

3.4. Test of separation effects on ocean and lake samples

We have also performed LCA and LCE purification on a variety of samples, such as a sample from the JR51-GC35 sediment core [15,27] that contain steryl ethers, wax esters and other unknown co-eluting compounds (Fig. S4). These interfering compounds are successfully removed after AgTCM treatment, and all LCAs and LCEs were eluted in acetone fraction (Fig. 4). The purification method developed in this study have also been successfully tested on a number of lake sediments samples, although none of the lake sediment samples contained steryl ethers [15,29]. A specific example is the purification of alkenones from surface sediment of Lake Étang des Vallées, which contain large amounts of unsaponifiable co-eluting compounds (Fig. S5), among which are various saturated ketones with variable carbonyl positions. As shown in Fig. 4, coeluting compounds are also fully removed after AgTCM flash column elution using scheme 3.

3.5. Repeatability of LCA distributions

It is important that AgTCM purification procedure does not affect various index values based on LCAs and LCEs. To test the reproducibility of the index ratios before and after AgTCM treatment, we performed repeated separation of LCAs in the surface sediment sample from Braya Sø, West Greenland using the solvent scheme 3 [30]. As shown in Table 2, distribution of alkenones remains virtually unchanged upon three times of repeated separation using the same AgTCM pipette column.

3.6. Reusability of the same AgTCM pipette column

HPLC column equipped with AgTCM produces consistent separations with little change in retention during repeated injections [22,25,38]. We test the reusability of AgTCM flash column by consecutively loading 35 samples from different depth of marine sediment core 343310 onto the same AgTCM column, and subsequently examined all fractions by GC-FID (and GC–MS when compound identification is needed). Between each reuse of the AgTCM flash column, we use 10 ml of acetone to rinse the column and then 10 ml of hexane to re-condition the column before loading the next sample. No alkenones are found in acetone eluents used to clean-up the column between samples, indicating absence of compound carry overs. In all cases, LCAs are well purified from steryl ethers and other co-eluents, and DCM fractions of AgTCM elution contained no alkenones.

The maximum number of samples that have been successfully purified using one single AgTCM column is 62 so far – it is likely many more samples can be purified before the AgTCM flash column needs to be replaced (Fig. S6). The only visual change observed during our repeated sample separation is that the top most 0.5 cm of the flash column slightly darkens over repeated usage although the impact on the separation efficiency appears to be minimal. One critical factor for a long lasting AgTCM column, however, is the absence of sulfur in the sample, as sulfur could remove silver ion bonded onto the thiolate functionalized silica gel. Therefore, samples should be pretreated with activated copper to remove sulfur prior to using AgTCM flash column.

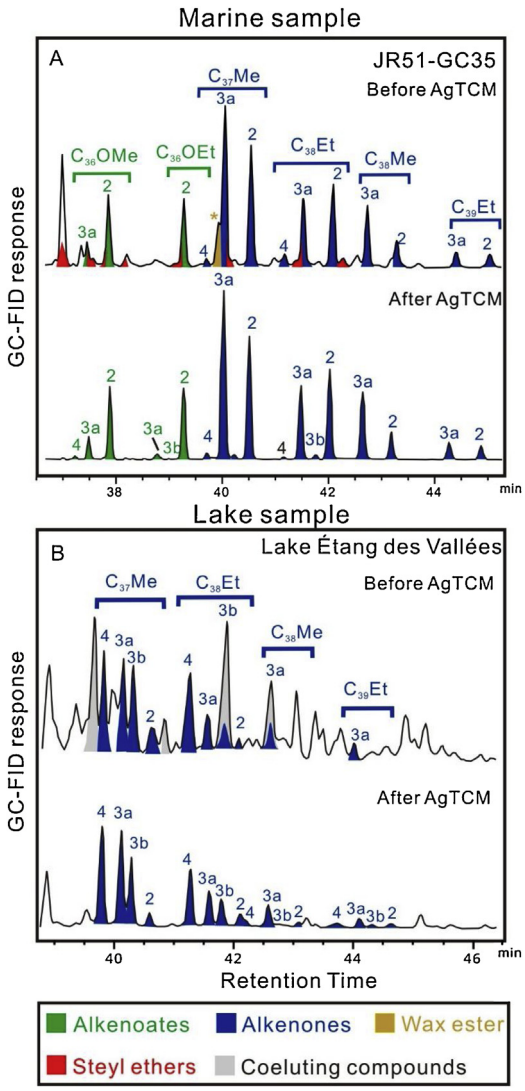

Fig. 4. Partial gas chromatograms illustrating the performance of AgTCM in cleaning up an unsaponified sample from marine sample from core JR51-GC35 (16–17 cm depth), Northern Icelandic shelf (A) [11]; and a saponified lake surface sediment sample from Lake Étang des Vallées, France (B) [29]. The solvent scheme used here is scheme 3 as illustrated in Fig. 3a. This test also indicates solvent scheme 3 allows full recovery of alkenoates as well as alkenones. OMe/OEt represent long chain alkenoates (LCEs), and Me/Et denote LCEs and LCAs with a methyl or an ethyl group, respectively, with the corresponding carbon numbers indicated by C_x. The numbers 2, 3, and 4 on top of GC peaks indicate the number of double bonds in alkenones or alkenoates. 3a and 3b denote double bond positional isomers for tri-unsaturated LCAs or LCEs [22,29]. Note that compounds coeluting with sedimentary alkenones in Lake Étang des Vallées are not steryl ethers, but are alkyl ketones and other unidentified compounds (Fig. S5).

4. Comparisons of alkenone-inferred paleotemperature reconstruction before and after AgTCM

We performed LCA cleaning on sediment core samples form site 343310 in central West Greenland [28]. GC-FID chromatograms of alkenone fractions eluted using DCM from silica gel columns contain large numbers of co-eluting compounds, including steryl ethers, wax esters and other unknown compounds in 343310 (Figs. 1 and 2). In the previous study, Moros et al. (2016) used two gas chromatographs connected in tandem, with two GC columns for identification and quantification of $C_{37:2}$, $C_{37:3}$ and $C_{37:4}$ alkenones

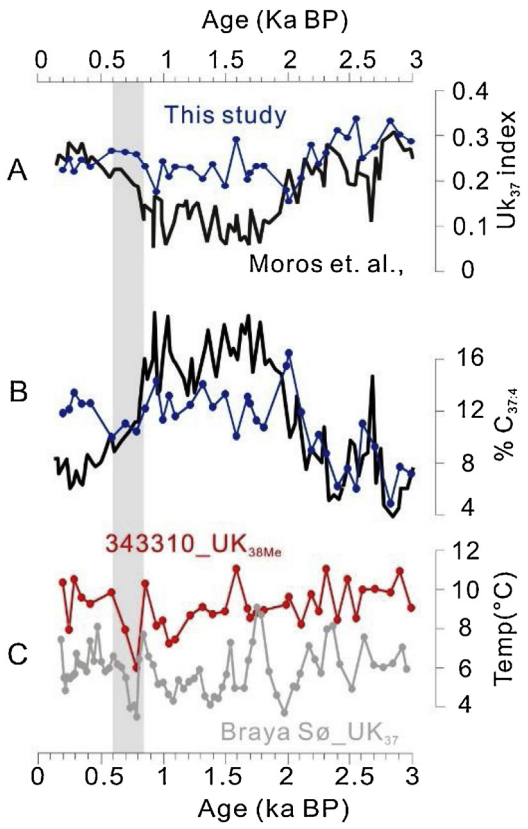

Fig. 5. Comparison of down core variations in UK 37 (A) and $\%C_{37:4}$ (B) index values for site 343310 between results from this study (blue lines) and the previously published data (black lines) for the past 3000 years [28]. In (C), sea surface temperature changes inferred from C_{38} methyl ketones (UK 38Me) (red line) are compared with a nearby alkenone-based temperature reconstruction from Lake Braya Sø (grey line), Southwestern Greenland [30]. UK 38Me index values are transferred into sea surface temperature using T= (UK 38Me+0.061)/0.034. This equation is derived by combining the relationship of UK 38Me and UK' 37 (UK 38Me= UK' 37-0.1), as estimated from downcore samples in Herbert et al. [6], and the UK' 37 temperature calibration T= (UK' 37-0.039)/0.034 in Prahl et al. [7]. The analytical error for alkenone unsaturation ratio is of 0.0005 UK' 37 units, which translate into inferred temperature uncertainties of about 0.15 °C [6]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

[28]. However, detailed methodology and gas chromatograms were not presented in the Moros et al. [28], and it is difficult to know if the separation method consistently eliminated the co-eluting compounds for C_{37} alkenones in all samples. As shown in one example from 280 cm depth of 343300 (Fig. 2), the LCA purification results using AgTCM are satisfactory.

In the ocean, the $\%C_{37:4}$ values of alkenones are thought to be sensitive to surface water salinity [12], and are used as an indicator of meltwater input in marine sediment core 343310 [28]. From 3.5 to 0.8 ka, our $\%C_{37:4}$ values show similar trend as those reported by Moros et al. [28], although our results are less extreme between 2 and 1 ka (Fig. 5). However, there are clear different trends between two $\%C_{37:4}$ records after 0.8 ka (Fig. 5). We find co-eluting compounds in this interval are among the highest for the whole sediment core, which may have resulted in analytical bias in the previous study [28]. In the study by Moros et al. [28] authors inferred a decreasing freshwater flux from 0.8 ka onwards from the declining $\%C_{37:4}$, but our data instead suggest increasing meltwater from 0.6 ka to present.

Our UK 37 variations display even greater differences from those reported by Moros et al. [28]. The unusually low index values observed between 2.6 to 1.6 ka was previously interpreted as high meltwater flux. However, if translated into temperature changes, the temperature would have dropped by as much as 6.5 °C, which is likely unrealistic. After removing co-eluted compounds, we find much smaller amplitude of change, corresponding to only 1–2 °C.

One additional concern for alkenones in 343310 is that this site is located in close proximity to land (Fig. S1), with major meltwater discharge from Greenland Ice Sheet [28]. The region is completely covered by sea ice from January to April. While UK 37 inferred temperature record after removing co-eluting compounds is less extreme, it still differs significantly from alkenone inferred temperatures obtained from adjacent southwestern Greenland lakes (Fig. S7) [30]. One possible scenario is that some of the alkenones in the sediment are produced by brackish Isochrysidales species, especially during times of high meltwater flux that reduces the salinity of the marginal sea waters, and/or during the seasonal ice melt creating reduced local salinity in the surface of the ocean. The salinity levels measured in July 2007 illustrate that the bottom water transported by West Greenland Current are higher in temperature and salinity, while the surface sea water are lower in salinity and temperature with the polar water sandwiched in between [28]. Therefore, we speculate that brackish Isochrysidales species may contribute significant amount of alkenones to the sediments at 343310.

To alleviate this problem, we analyzed unsaturation ratios of C_{38} methyl alkenones – this is possible to achieve after removing the complex co-eluting compounds using AgTCM. UK 38Me is advantageous over UK 37 in cases C_{37} alkenones are produced by a mixture of Group II (e.g., *Isochrysis galbana*) and Group III (*Emiliania huxleyi*), because the Group II species do not produce C_{38}Me alkenones [15,32]. The temperature signal from UK 38Me should thus derive from alkenones produced by *E. huxleyi* only. As shown in Fig. 5c, the UK 38Me inferred temperature changes from 343310 display remarkable similarity to those from the nearby lakes [30]. In particular, the abrupt temperature drop around the time of disappearance of the Norse population is consistent in both records. Overall, our new clean-up procedure allows effective and efficient removal of co-eluents and has potential to greatly improve the accuracy of paleoclimate reconstructions.

5. Conclusions

We demonstrate a highly efficient, low-cost and convenient liquid chromatographic method based on silver thiolate chromatographic material (AgTCM) for purifying LCAs and LCEs. This method overcomes the shortcomings of the traditional argentation chromatography (instability, inconsistency, low recovery etc). While HPLC equipped with a plain silica gel column has recently been shown to be effective in eliminating co-elution of steryl ethers with C_{37} alkenones [18], the relative high operational cost of the HPLC, along with the fact that the separation mechanism depends only on the relatively small differences in polarity between steryl ethers and alkenones, may limit the use of HPLC to special circumstances. Our new flash column chromatography based on AgTCM takes advantage of differences in both polarity and degree of double bond interactions between alkenones and steryl ethers, offer a highly efficient and low cost solution to this challenging analytical problem. The new approach thus allows conventional laboratories of paleoclimatology and paleoceasonography to conduct alkenone-based SST reconstruction in regions previously unamenable for such study due to co-elution problems. Our test of down core samples from West Greenland indicates that it is essential to fully remove steryl ethers and other complex co-eluents prior to GC analysis in order to obtain correct index values from the full homologous series of LCAs. In marginal ocean environments with potential presence of alkenones produced by brackish Isochrysidales, unsaturation ratios of C_{38} methyl ketones may provide more realistic sea surface paleotemperature reconstructions.

More specifically, AgTCM flash column chromatography offers: (1) the ability to purify LCAs and LCEs simultaneously from complex samples; (2) high level of reusability which greatly simplifies and shortens sample preparation processes, and dramatically reduces cost; (3) comprehensive analysis of all alkenone and alkenoate-based indices including C_{36}OMe, C_{38}Et, C_{38}Me and even C_{39}Et (rather than indices based on C_{37} alkenones only); (4) the potential for simplifying LCA fractions for more accurate compound-specific isotopic analysis. Overall, our new flash column clean-up procedure, when combined with the improved GC separation of Zheng et al. [15], may greatly improve the analytical accuracy and efficiency for comprehensive quantification of individual LCAs and LCEs from the ocean and lake environments.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chroma.2019.02.064.

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