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The effect of improved chromatography on GDGT-based palaeoproxies

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ABSTRACT

The development of methods using liquid chromatography coupled to mass spectrometry to analyze glycerol dialkyl glycerol tetraethers (GDGTs) has substantially expanded the biomarker tool box and led to the development of several new proxies. Recent studies have shown that new high performance liquid chromatography methods have substantially improved separation of GDGT isomers and detection of novel isomers. Here we present a chromatographic method based on two ultra-high performance liquid chromatography silica columns capable of separating a wide range of GDGTs with good resolution and which compares favorably with previously published methods. This method was tested on a part of the global calibration set of the TEX_{86}, a proxy for sea water temperature, and on a part of the global calibration set of the MBT_{5Me}, a proxy for air temperature, and CBT′, a proxy for soil pH. Our results show that the new high resolution chromatography method leads to a significant but small offset (<0.01 or <0.8 °C) in TEX_{86}, especially at low values, while no difference is observed for the CBT′. However, for the MBT_{5Me} a significant difference is observed (<0.01 or <3 °C), especially at low values, although this difference is smaller than the calibration error (4.8 °C).

1. Introduction

Over the past decade, research into the environmental occurrence and geochemical importance of glycerol dialkyl glycerol tetraethers (GDGTs) has expanded enormously. The development of high performance liquid chromatography (HPLC)–mass spectrometry (MS) methodology (Hopmans et al., 2000) allowed analysis of the core lipids, instead of more laborious GC–MS analysis of the released carbon chains after ether cleavage. This led to the discovery of a range of new GDGTs, including crenarchaeol (Sinninghe Damsté et al., 2002) produced by Thaumarchaeota, and GDGTs with branched carbon skeletons (brGDGTs), most likely produced by soil bacteria (Sinninghe Damsté et al., 2000). These novel GDGTs were found to be widespread in marine and terrestrial environments (Schouten et al., 2000, 2013a) and several new geochemical proxies have since been introduced based on their distributions.

Schouten et al. (2002) introduced the TEX_{86} for reconstruction of sea surface temperatures based on GDGTs produced by marine Thaumarchaeota, comprising GDGTs 1–3 (numbers indicate the number of cyclopentane moieties) and the regioisomer of crenarchaeol. Hopmans et al. (2004) defined the BIT index, quantifying the relative abundance of the branched GDGTs versus crenarchaeol, to estimate the input of terrestrial organic matter into marine sediments. The relative distribution of branched GDGTs in soils was shown by Weijers et al. (2007) to contain information on mean annual air temperature and soil pH, which led to the definition of the CBT and MBT indices, respectively. Proxies based on GDGTs are now increasingly used in palaeoclimatology, palaeoceanography and palaeolimnology to reconstruct palaeoenvironmental parameters (e.g., Pearson and Ingalls, 2013; Schouten et al., 2013a).

Currently, the most commonly used analytical methodology (Schouten et al., 2013b) is a normal phase separation on a cyano (CN) column using mixtures of hexane and isopropanol as mobile phase followed by positive ion atmospheric pressure chemical ionization (APCI)-MS detection in selected ion monitoring (SIM) mode of the protonated molecules of the various GDGTs (Schouten et al., 2007). A complication in the accurate quantification of the GDGTs used in the various proxies is the imperfect separation of the various isomers of the GDGTs, resulting in both earlier eluting (as frequently observed for the isoprenoid GDGTs) or later eluting shoulders (in case of the branched GDGTs). The standard integration protocol (Schouten et al., 2009) calls for exclusion of these shoulders during integration, however, the accuracy with which this can be achieved is dependent on the quality of the chromatography, the complexity of the GDGT distribution, and the relative abundance of the isomers, resulting in analytical uncertainties.

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Recently, de Jonge et al. (2013) identified the components responsible for the late eluting shoulder often observed on the chromatographic peaks of branched GDGTs. These were found to comprise 6-methyl brGDGT rather than the 5-methyl brGDGTs. Subsequently, de Jonge et al. (2014) showed that improved HPLC separation, allowing more precise and separate quantification of the various isomers of the branched GDGTS, greatly impacted the CBT and MBT paleoproxies. The newly defined MBT'_{5Me}, which excludes the 6-methyl brGDGT, is no longer related to soil pH and showed an improved correlation with mean annual air temperature (MAT), while the newly defined CBT′, now including the 6-methyl brGDGTs, showed a much improved pH reconstruction. This improved separation was achieved by 4 HPLC silica columns in series but resulted in a total run time of 4 h, three times as long as the commonly used method. Recently, several improvements in chromatography for GDGTs were reported. Zech et al. (2012) reported improved separation between the hexamethylated brGDGTs, while Becker et al. (2013) reported improved chromatography for isoprenoid GDGTs using two Ultra (U)HPLC BEH amide columns in tandem. In addition, Yang et al. (2015) reported improved separation of brGDGTs using two UHPLC silica columns in tandem.

Here we present a chromatographic method using two UHPLC silica columns in series, that leads to baseline separation of the various isomers of the branched GDGTs, and which is fully compatible with most standard LC systems. A total analysis time of 90 min affords analysis of all GDGTs used for calculating TEX_{86}, CBT, and MBT, as well as hydroxyl (OH-) and dihydroxyl (2-OH-) GDGTs and other more recently described GDGTs (e.g., Liu et al., 2012a, b). We compared our method with previously published ones and tested the impact of improved chromatography on GDGT-based proxies by analyzing a subset of samples used in the global TEX_{86} calibration by Kim et al. (2010) and the global CBT/MBT calibration by de Jonge et al. (2014).

2. Material and methods

2.1. Samples

A representative subset of 26 samples, with TEX_{86} values ranging from 0.36–0.71, was selected from the samples previously used for the TEX_{86} calibrations by Kim et al. (2010) for re-analysis on the UHPLC columns as described below. These samples were also re-analyzed with the same LC–MS instrument using the traditional method according to Schouten et al. (2007) to prevent instrument bias impacting on the comparison of the TEX_{86} values. For comparison of MBT and CBT indices, a selection of 36 samples, previously analyzed with four Si columns in series by de Jonge et al. (2014), was made. These samples had MBT values ranging from 0.25–0.99, and CBT values ranging from −0.05 to 2.59. In addition, two composite samples (D1 and D2) from a piston core from Drammensfjord (Norway; D2-H; 59° 40.11 N, 10° 23.76 E; water depth 113 m) were used to evaluate the effects of improved chromatography on the BIT index. One of these (D1) is identical to interlaboratory standard S1 in the 2009 TEX_{86} and BIT interlaboratory study (Schouten et al., 2009). To prevent instrument bias, these samples were reanalyzed using both the new method and according to Schouten et al. (2007) on the instrument described below.

2.2. UHPLC–MS GDGT analysis

Analysis was performed on an Agilent 1260 UHPLC coupled to a 6130 quadrupole MSD in selected ion monitoring mode. Separation was achieved on two UHPLC silica columns (BEH HILIC columns, 2.1 × 150 mm, 1.7 μm; Waters) in series, fitted with a 2.1 × 5 mm pre-column of the same material (Waters) and maintained at 30 °C. GDGTs were eluted isocratically for 25 min with 18% B, followed by a linear gradient to 35% B in 25 min, then a linear gradient to 100% B in 30 min, where A is hexane and B is hexane:isopropanol (9:1, v/v). Flow rate was 0.2 ml/min, resulting in a maximum back pressure of 230 bar for this chromatographic system. Total run time is 90 min with a 20 min re-equilibration. Source settings were identical to Schouten et al. (2007). Typical injection volume was 5 μl of a 2 mg/ml solution of polar fractions obtained after aluminium oxide chromatography (Schouten et al., 2009).

In selected cases, samples were analyzed by UHPLC–high resolution accurate mass MS (HRAM-MS) on a ThermoScientific UltiMate 3000 RS series UHPLC with thermostatted auto-injector and column compartment coupled to a ThermoScientific Q Exactive Orbitrap mass spectrometer using the same chromatographic method as described above. The positive ion APCI settings were as follows: probe heater temperature, 350 °C; sheath gas (N_2) pressure, 50 AU (arbitrary units); auxiliary gas (N_2) pressure, 5 AU; spray current, 5 μA; capillary temperature, 275 °C; S-lens, 100 V. Target lipids were analyzed with a mass range of m/z 900–1500 (resolution 70,000), followed by data dependent MS^2 (resolution 17,500), in which the five most abundant masses in the mass spectrum were fragmented (stepped normalized collision energy 15, 20, 25; isolation width 1.0 Da). The Q Exactive was calibrated within a mass accuracy range of 1 ppm using the Pierce LTQ Velos ESI Positive Ion Calibration Solution (containing a mixture of caffeine, MRFA, Ultramark 1621, and *n*-butylamine in an acetonitrile-methanol-acetic solution; Thermo Scientific).

TEX_{86} values were calculated as defined by Schouten et al. (2002) and the BIT index according to Hopmans et al. (2004). Peak areas of the 5- and 6-methyl isomers of the branched GDGTs were combined for calculation of the BIT index. MBT'_{5Me}, CBT_{5Me} and CBT′ were calculated according to de Jonge et al. (2014).

The chromatographic resolution for various critical pairs was calculated using the following equation (Snyder et al., 1997):

$$Rs = 1.18(t_2 - t_1)/(W_{0.5,1} + W_{0.5,2}) \quad (1)$$

where t_1 and t_2 are the retention times of the critical pair peaks, and $W_{0.5}$ refers to peak width at half peak height.

For all calculated indices, differences in the index values between methods were assessed with paired *t*-tests. Differences were considered significant if $P < 0.05$.

3. Results and discussion

3.1. Chromatography

The improved chromatography due to the use of two UHPLC silica columns in series is illustrated in Fig. 1 for composite sediment D2 from Drammensfjord, Norway. The resolution between several critical pairs of GDGTs is shown in Table 1. The base peak chromatogram (Fig. 1A) shows the isoprenoid GDGTs eluting from 15–30 min, the brGDGTs eluting from 40–55 min, while OH-GDGTs (Liu et al., 2012b) and di-OH-GDGTs (not visible in the chromatogram shown in Fig. 1 due to low abundance) elute between 68–73 min and 82–87 min, respectively. Analysis of this extract using the same chromatographic setup but using a high resolution/accurate mass MS revealed that other previously reported ether lipids such as so called "sparsely and overly" branched GDGTs (e.g., Liu et al., 2012a), glycerol monoalkyl glycerol tetraethers or "H-shaped' GDGTs (e.g., Schouten et al., 2008), glycerol dialkanol diethers (Knappy and Keely, 2012; Liu et al., 2012c) eluted within the analytical window shown and in the same relative retention order as previously reported. The C_{46} glycerol trialkyl glycerol tetraether internal standard (cf. Huguet et al., 2006), elutes at 30 min and is separated from

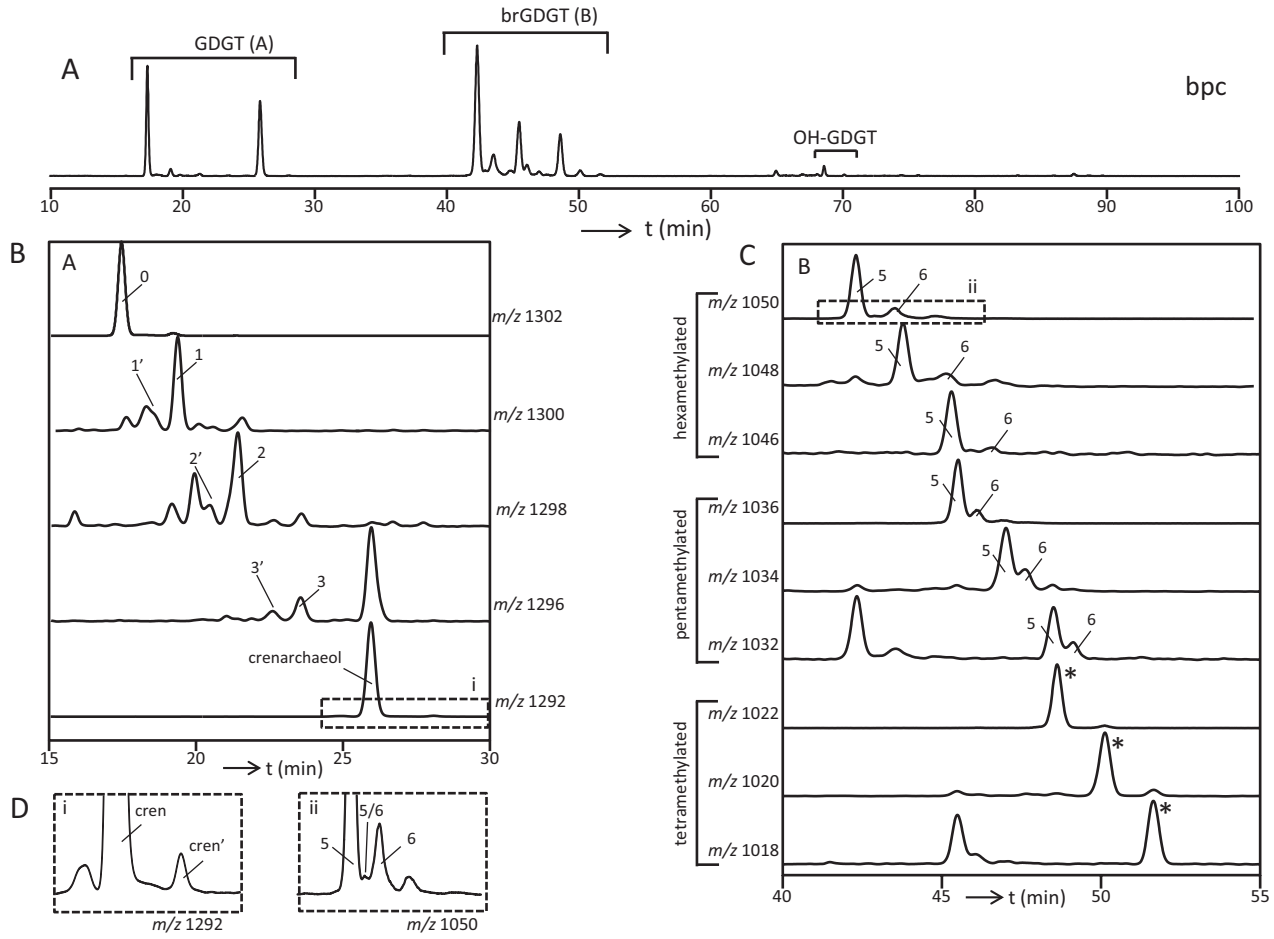

Fig. 1. LC–MS analysis of GDGTs in sediment sample D2 from Drammensfjord, Norway using two UHPLC silica column in series: (A) base peak chromatogram; (B) mass chromatograms of isoprenoid GDGTs used in TEX_{86} with number of cyclopentane rings indicated, the most abundant of the minor isomers is indicated with ′; (C) mass chromatograms of branched GDGTs showing the 5- and 6-methylated isomers for the hexa- and pentamethylated brGDGTs, while the tetramethylated brGDGTs are indicated by a *; and (D) enlargements of the area indicated by dashed boxes in (i) the mass chromatogram of *m*/*z* 1292 detailing the separation between crenarchaeol (cren) and its regioisomer (cren′) and (ii) the mass chromatogram of *m*/*z* 1050 detailing the separation between the 5- and 6-methylhexamethylated branched GDGTs (5 and 6, respectively). All mass chromatograms are at *m*/*z* values corresponding to the protonated molecules of the indicated GDGTs.

Table 1
Chromatographic resolution calculated according to Eq. 1 for critical pairs in the GDGT chromatography for different methods.

Critical pair[a]	2 × BEH HILIC Si	4 × Si	CN	2 × BEH HILIC amide[b]
GDGT-1′/GDGT-1	1.37	1.51	0.87	1.08
GDGT-2′/GDGT-2	1.46	1.71	1.14	1.16
GDGT-3′/GDGT-3	1.32	1.28	0.91	0.65
GDGT-4/crenarchaeol	1.06	nd	nd	0.83
Cren/cren′	3.71	3.85	2.51	2.04
5-HexaMe-brGDGT/ 6-hexaMe-brGDGT	1.62	1.33	–[c]	nr[d]
5-PentaMe-brGDGT/ 6-pentaMe-brGDGT	0.72	0.64	–	nr

[a] Critical pairs listed are shown in Fig. 1 and indicated by their number of rings with or without ′.
[b] Becker et al. (2013).
[c] Resolution below 0.5.
[d] Not reported.

the regioisomer of crenarchaeol (cren′) with which it typically co-elutes on the CN column.

The dominant isomers of GDGT-1, -2, and -3, used in the TEX_{86}, are now clearly separated from the previously partially co-eluting minor isomers (Fig. 1B). In fact, often multiple isomers of each GDGT are revealed, sometimes with larger apparent abundance than the isomers used in the TEX_{86}. The exact structure of these isomers is unknown, but likely are varying stereoisomers, parallel/anti-parallel conformations and/or GDGTs with double bonds (Zhu et al., 2014). Crenarchaeol (cren) and its regioisomer (cren′) are fully separated from each other (Fig. 1D). However, in many samples a third isomer of crenarchaeol eluting between crenarchaeol and its regioisomer is also observed. GDGT-4 elutes as a well-defined shoulder in front of crenarchaeol, with a resolution between peaks of 1.07 (data not shown). Comparison of the resolutions achieved with the standard CN method, the method of Becker et al. (2013) and our new method (Table 1) shows that the highest resolutions are achieved using our two UHPLC silica column method for all critical pairs of the isoprenoid GDGTs.

Separation achieved for the brGDGTs on two UHPLC silica columns (Fig. 1B) is almost identical to the improved separation on four HPLC silica columns as reported by de Jonge et al. (2014), but with further improved resolution (Table 1). The 5- and 6-methyl-hexamethylated brGDGTs are baseline separated ($Rs > 1.5$). Close examination of the chromatograms often reveals a small peak eluting between the 5- and 6-methyl-hexamethylated brGDGTs (Fig 1E). This peak represents the 5/6-methyl-hexamethylated brGDGT, recently identified by Weber et al. (2015). Baseline separation is not achieved for the pen-

tamethylated brGDGTs, although the separation is also slightly improved over the 4× HPLC silica method (Table 1). Becker et al. (2013) did not achieve baseline separation between 5- and 6-methyl-hexamethylated brGDGTs using two UHPLC BEH amide columns, although resolutions were not reported for these critical pairs. Yang et al. (2015) reported an improved separation of brGDGTs very similar to the separation presented here, also using two UHPLC silica columns in tandem but with an alternative solvent system of hexane/ethyl acetate. Unfortunately, they did not report the resolution of the various critical pairs of brGDGTs, making a quantitative comparison between the methods difficult.

Further improvements in separation can be expected by the addition of more UHPLC columns. Several GDGT peaks show hints of shoulders, and peak width varies more than expected for chemically similar compounds. However, this would result in a substantial increase in analysis time, which is undesirable for a routine method used to generation high resolution paleoclimate records.

3.2. Effect of the new separation system on GDGT proxies

In order to assess the impact of the improved separation achieved on the UHPLC columns, we reanalyzed a subset of samples, previously analyzed for the global calibration sets of Kim et al. (2010) for the TEX_{86}, and de Jonge et al. (2014) for the CBT/MBT. The samples were chosen to cover the broadest index ranges possible. We reanalyzed the selected samples for TEX_{86} on the same HPLC–MS as used for the UHPLC columns, but using the CN column to avoid differences resulting from the use of a different HPLC–MS system. All values are listed in the Supplementary Information.

3.2.1. TEX_{86}

A cross plot of the values for $TEX_{86,UHPLC}$ vs the $TEX_{86,CN}$ shows that the UHPLC method returns slightly lower TEX_{86} values ($P = 0.001$) compared to the CN column, with larger deviations for TEX_{86} values in the lower range (Fig. 2A). This is likely caused by a reduction of peak area due to removal of co-eluting peaks, which will result in lower integrated peak areas, especially in samples with an already low TEX_{86} and low relative abundances of GDGTs 1–3. However, it should be noted that the differences between the two methods is very small with an average of 0.005 TEX_{86} unit and even for samples in the lower range this typically does not exceed 0.01 unit, representing a 0.8 °C deviation, which is well within the reported calibration error of 2.5 °C reported by Kim et al. (2010) as well as interlaboratory differences which range between 1.3 to 3.0 °C (Schouten et al., 2013b).

3.2.2. MBT'_{5Me} index

The effect of the improved UHPLC separation on mean annual air temperature (MAT) reconstructions was assessed by comparing

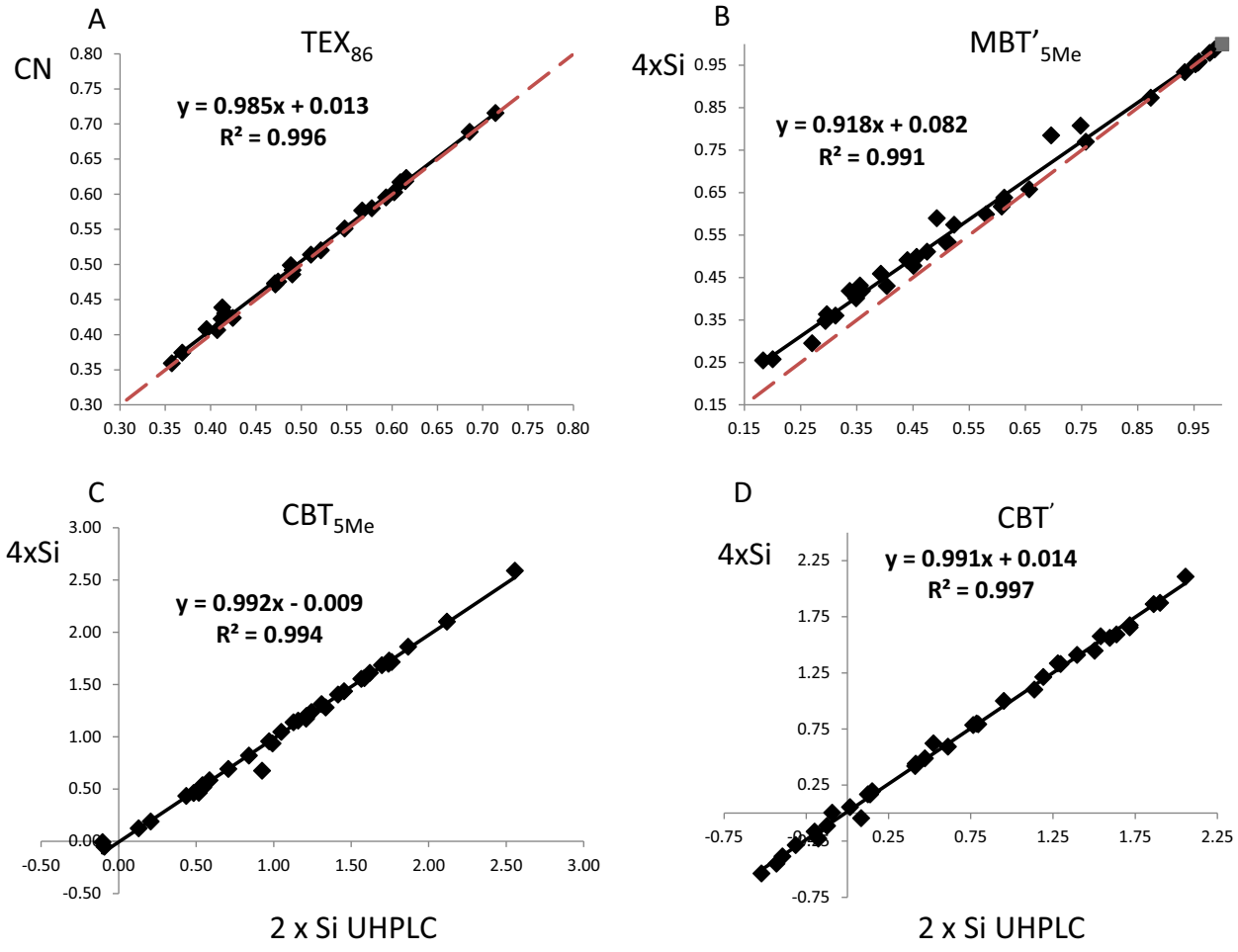

Fig. 2. Cross plots of GDGT-based proxies determined using the CN column or four Si columns vs two UHPLC silica columns: (A) $TEX_{86,CN}$ vs $TEX_{86,UHPLC}$; (B) $MBT'_{5Me,4\times Si}$ vs $MBT'_{5Me,UHPLC}$; (C) $CBT_{5Me,4\times Si}$ vs. $CBT_{5Me,UHPLC}$; $CBT'_{4\times Si}$ vs CBT'_{UHPLC}. Linear regression equations and correlation coefficients are shown in each plot; 1:1 lines (red dash) are plotted when not obscured by the data trend lines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MBT'_{5Me} values to those determined on the 4 × Si method. A comparison with the CN column method is in this case impossible as the 5- and 6-methyl-brGDGTS are not well separated using this method. The $MBT'_{5Me,UHPLC}$ is systematically lower ($P \ll 0.001$) compared to the $MBT'_{5Me,4\times Si}$ and the offset increases with lower MBT'_{5Me} values (Fig. 2B). This is most likely due to the increased sensitivity of the new separation method to detect the hexamethylated brGDGTs, and pentamethylated brGDGTs with cyclopentane moieties, which are notoriously hard to detect, especially in samples from colder regions (de Jonge et al., 2014). Improved peak shape (decreased peak width and increased peak height) due to the use of UHPLC columns will, in some cases, lead to the detection of these previously non-detectable GDGTs. As the hexa- and pentamethylated brGDGTs with cyclopentane rings are only represented in the denominator of the MBT'_{5Me} equation, this will lead to lower MBT'_{5Me} values and lower reconstructed MAT temperatures. Interestingly, de Jonge et al. (2014) shows a systematic overestimation of the MAT reconstruction vs the measured MAT in soils from colder regions where penta- and hexamethylated brGDGTs are often below the detection limit, which may partially be corrected by the improved chromatography. It should be noted that even for samples in the lower MBT'_{5Me} range, the offset does not exceed 0.01 unit, representing a ~3 °C deviation, which is still within the reported calibration error of 4.8 °C (de Jonge et al., 2014).

3.2.3. CBT_{5Me} and CBT′ indices

Comparison of the CBT_{5Me} and CBT′ values generated using the two UHPLC column versus the values obtained with four Si columns shows that $CBT_{5Me,4\times Si}$ values are slightly but significantly lower ($P = 0.04$) than the $CBT_{5Me,UHPLC}$ values with an average difference of 0.02 units. This difference is largely driven by one outlier and is reduced to 0.008 when this value is removed. Furthermore, a difference of 0.02 CBT_{5Me} unit represents a change in reconstructed pH of 0.03 which is well below the reported calibration error of 0.84 (de Jonge et al., 2014). CBT'_{UHPLC} values are not significantly different from $CBT'_{4\times Si}$ values, with an average difference of 0.008, representing 0.01 pH unit.

3.2.4. BIT index

The BIT index of the samples discussed above were all either very low (marine, <0.05) or very high (soils, >0.95) making comparisons of chromatography methods difficult. Therefore, the impact of the improved chromatography on the BIT index was assessed using composite sediment samples D1 and D2 from Drammensfjord, Norway which have intermediate BIT values. The BIT_{UHPLC} is consistently higher than the BIT_{CN} for both samples. BIT_{CN} and BIT_{UHPLC} for sample D1 were 0.59 ± 0.01 ($n = 3$) vs 0.63 ± 0.01 ($n = 5$) and 0.75 ± 0.01 ($n = 5$) vs. 0.78 ± 0.01 ($n = 5$) for sample D2. However, it should be noted that the BIT index is a qualitative measure for soil organic matter input into marine sediments and round robin studies (Schouten et al., 2009, 2013b) showed interlaboratory differences for the BIT index much larger than observed here, making the small shift in values due to improved chromatography inconsequential.

4. Conclusions

Here we have described improved chromatography for GDGTs using two UHPLC silica columns with improved resolution of all critical GDGT pairs compared to previously reported chromatographic methods. The improved chromatography has no effect on the CBT′, while the differences observed for the TEX_{86} and the CBT_{5Me} fall well within the reported error for the current global calibrations. A significant change in obtained values for the BIT index was observed, but as this index is qualitative only, the use of this index to inventory relative changes in soil organic matter input into marine sediments is not affected. Re-calibration of the MBT_{5Me} could be warranted as a significant offset is observed from values determined on four Si columns, especially for samples from cold regions. The improved resolution, improved sensitivity due to reduced peak widths and resulting enhanced peak heights (sample use is half that of the traditional method), coupled to an acceptable analysis time should allow the generation of high resolution climate records while having improved indices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.orggeochem.2015.12.006.

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