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An improved method to determine the absolute abundance of glycerol dibiphytanyl glycerol tetraether lipids

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Abstract

Isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) are specific membrane lipids derived from archaea, one of the three domains of life. These lipids can be used as biomarkers in paleo-ecological studies. GDGTs can be analyzed by high performance liquid chromatography (HPLC)/mass spectrometry (MS). To quantify GDGTs, external standard curves were run monthly on the HPLC/MS. However, external standard curves represented a snapshot and do not adjust for changes during storage, dissolution and manipulation of samples or drifts in HPLC/MS conditions. To measure absolute GDGT abundances more accurately, we tested the use of a newly synthesized C_{46} GDGT as an internal standard. The accuracy in the determination of GDGT concentration improved significantly when using the internal standard (5% standard deviation) compared to that obtained with the monthly external standard curve (43% standard deviation). Thus, the new internal standard technique will greatly improve the accuracy of GDGT abundance measurements, increase the potential of GDGTs in paleo-ecological studies.

1. Introduction

Archaea are prokaryotes which are phylogenetically distinct from bacteria and possess different membrane lipids. While bacterial membranes are generally formed by straight-chain fatty acids ester bound to glycerol, membranes of dominant archaeal groups contain lipids with isoprenoid alkyl chains bound by ether bonds (Fig. 1). Most of the cultured members of the archaeal domain producing isoprenoid glycerol dialkyl glycerol tetraethers (GDGTs) are extremophiles, but recent investigations showed that crenarchaeota, a subgroup of archaea, are ubiquitous and abundant in seawater (Hoefs et al., 1997; Massana et al., 2000; Karner et al., 2001) and lakes (Powers et al., 2004). GDGTs biosynthesized by these crenarchaeota are similar to those of their thermophilic ancestors, except for crenarchaeol, a unique GDGT containing one cyclohexane moiety in addition to the more common cyclopentane moieties (Sinninghe Damsté et al., 2002a). It is thought that the biosynthesis of

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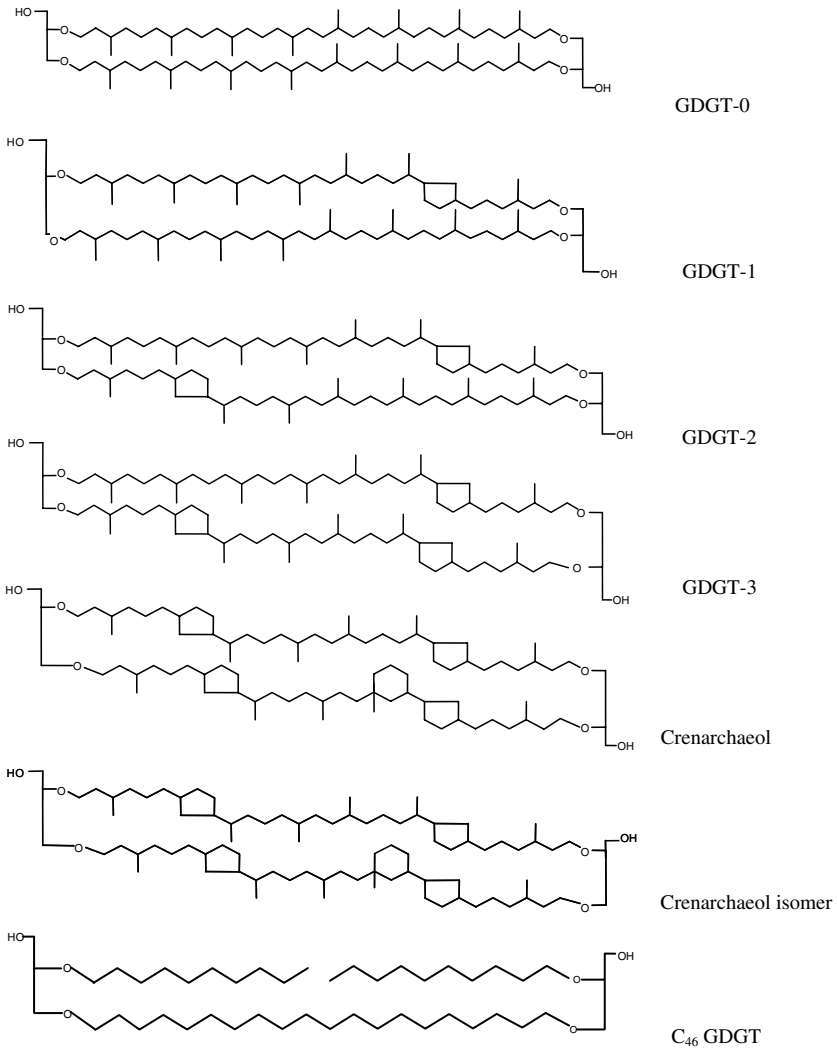

Fig. 1. Molecular structures of protonated GDGT molecules and the internal standard, C_{46} GDGT.

crenarchaeol is an adaptation to the relatively cold sea water temperatures, compared to thermophilic environments such as hot springs (Kuypers et al., 2002). For this reason, crenarchaeol (Fig. 1) is a taxonomically-specific biomarker for non-thermophilic crenarchaeota.

Crenarchaeol has been used to study the abundance of crenarchaeota in the Arabian Sea (Sinninghe Damsté et al., 2002b) and the Black Sea (Wakeham et al., 2003). Results of these studies are in general agreement with those of molecular biological studies which show that crenarchaeota occur throughout the water column (e.g. Karner et al., 2001). GDGTs are separated with high performance liquid chromatography (HPLC) and then identified by mass spectrometry (MS) (Hopmans et al., 2000). To quantify the GDGTs for the above studies, external standard curves were run, using a GDGT-0 standard (Fig. 1) (Wakeham et al., 2003). However, this technique is likely not very accurate for calculating absolute abundances as it does not account for differences in the extraction, processing, storage of the samples and drifts in MS response. Hence, the changes observed in the relative abundances in depth profiles such as those noted in the Black Sea and the Arabian Sea may be reliable, but the calculated absolute amounts might be less precise. The best way to improve the analytical accuracy in the quantification of GDGTs is to use an internal standard that can be added at the beginning of the extraction process. This internal standard should not occur naturally and needs

to be chemically similar to the GDGTs quantified. Here, we tested a C_{46} GDGT which was synthesized by Patwardhan and Thompson (1999), and compared GDGT absolute abundance measurements using this new internal standard to those calculated with the external standard curve technique for the same set of samples.

2. Experimental

As an internal standard we used a GDGT with two glycerol head groups linked by a C_{20} alkyl chain and two C_{10} alkyl chains (Fig. 1) which was synthesized by Patwardhan and Thompson (1999). A mixture of the C_{46} GDGT and crenarchaeol (1:1 w:w) was prepared and analyzed every week over a four month period to determine the relative response factor (RRF). To produce the external standard curves we used crenarchaeol as isolated previously (Sinninghe Damsté et al., 2002b) and injected amounts ranging from 10 to 200 ng. Both the internal and external standard quantification methods were tested by analyzing GDGTs in a sediment sample from the Drammensfjord (59°40′ N 10°23′ E). The sediment sample was a composite sample from a core taken in the centre of the Drammensfjord with the RV 'Pelagia' on October 29th, 1999 (Smittenberg et al., 2005). The core was freeze dried and ground to produce a homogenous sample. The freeze dried sediment sample was extracted using an Accelerated Solvent Extractor 200 (ASE 200, DIONEX) with a mixture of dichloromethane (DCM) and methanol (MeOH) (9:1 vol:vol) at 100 °C and 7.6×10^6 Pa. A solution of the C_{46} GDGT in 99% *n*-hexane: 1% isopropanol (0.01 mg/ml) was added to the total extract, with a proportion of 750 ng of the C_{46} GDGT for 1 mg of total extract. The total extract was separated using a glass pipette column filled with activated alumina by sequentially eluting with hexane/DCM (9:1 vol:vol) to obtain an apolar fraction and DCM/MeOH (1:1 vol:vol) to obtain a polar fraction. The polar fraction containing the GDGTs was filtered through a 0.45 μm pore size, 4 mm diameter PFTE filter prior to injection. The samples were analyzed by HPLC-atmospheric pressure positive ion chemical ionization mass spectrometry (APCI)-MS by applying conditions slightly modified from Hopmans et al. (2000). Analyses were performed with an HP 1100 Series HPLC/MS equipped with an auto-injector and ChemStation chromatography manager software. Separation was achieved on a Prevail Cyano column (2.1×150 mm, 3 μm; Alltech, Deerfield, Illinois, USA), maintained at 30 °C. The GDGTs were first eluted isocratically with (A) hexane and (B) propanol as follows, 99% A:1% B for 5 min, then a linear gradient to 1.8% B in 45 min. Flow rate was 0.2 ml/min. After each analysis the column was cleaned by back flushing hexane/propanol (90:10, vol:vol) at 0.2 ml/min for 10 min. Conditions for APCI/MS were as follows: nebulizer pressure 60 psi, vaporizer temperature 400 °C, drying gas (N_2) flow 6 l/min and temperature 200 °C, capillary voltage −3.5 kV, corona 5 μA (~3.2 kV). Mass spectra of the C_{46} GDGT and crenarchaeol were obtained by scanning *m/z* 800–1500. For the standard curves and GDGT quantification single ion monitoring (SIM) was used instead of mass scanning because SIM improves the signal to noise ratio and thus improves the reproducibility. SIM parameters were set to detect the protonated molecules of the five isoprenoid GDGTs (*m/z* 1302, 1300, 1298, 1296, 1292) as well as the protonated molecule of the C_{46} GDGT internal standard (*m/z* 744), with a dwell time of 237 ms per ion.

3. Results and discussion

3.1. HPLC/MS analysis of the C_{46} GDGT

Analysis of the apolar and polar fraction of the Drammensfjord sample showed that the C_{46} GDGT eluted in the same fraction as the GDGTs, i.e. the polar fraction. The LC retention time of the C_{46} GDGT varied between 22.5 and 23.3 min, eluting immediately after crenarchaeol (~21 min). The mass spectrum of the C_{46} GDGT shows a predominant protonated molecule ($[M + H]^+$, Fig. 2a). There is a minor fragment due to loss of an OH group (−18 Da) but the most abundant fragment results from the loss of one of the C_{10} chains (−140 Da) and the subsequent loss of an OH group (−158 Da). The second most abundant fragment is the loss of a C_{10} alkyl chain and glycerol group (−232 Da) (Fig. 2a). In comparison, mass spectra of isoprenoid GDGTs are dominated by the $[M + H]^+$ protonated molecule and only show minor fragments due to losses of 18 and 74 Da, which correspond to the loss of an OH and a glycerol group, respectively (Hopmans et al., 2000; Fig. 2b). The difference in fragmentation pattern is due to the fact that the C_{46} GDGT is not a macrocyclic compound in contrast to the isoprenoid GDGTs.

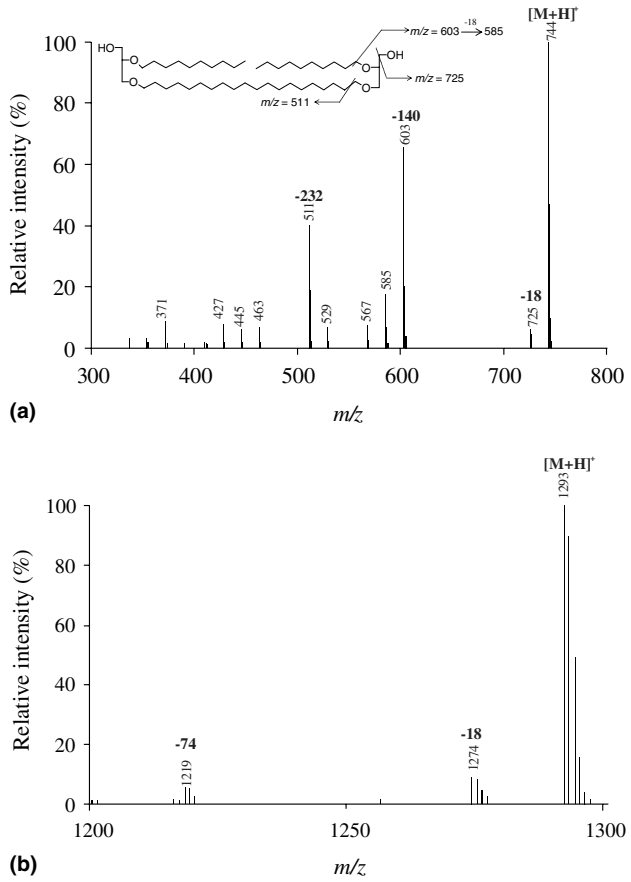

Fig. 2. APCI mass spectrum of: (a) C_{46} GDGT (m/z 743) and (b) crenarchaeol (m/z 1292). Mass spectra have been corrected for background.

3.2. Relative response factor

The response factor of both the C_{46} GDGT and crenarchaeol on the HPLC/MS was determined by SIM of both compounds at a range of concentrations (Fig. 3). The response factor of crenarchaeol is much higher than that of the C_{46} GDGT, i.e. 44.8 arbitrary units per ng injected GDGT versus 6.9 arbitrary units per ng injected GDGT respectively. This is likely due to the enhanced fragmentation of the C_{46} GDGT compared to crenarchaeol, which results in a relatively lower abundance of the protonated molecule (Fig. 2). Depending on source conditions and calibration of the MS, differences may occur in the relative fragmentation pattern of the C_{46} GDGT over time. This would affect the response factor as the protonated molecule then represents a changing percentage of the total ion signal over time. To assess this, the difference in response factor between the C_{46} GDGT and crenarchaeol was measured over time. For this, a standard mixture of C_{46} GDGT and crenarchaeol (1:1 w:w) was analyzed once every week, over a four month period to determine the relative response factor (RRF). The RRF was calculated as follows:

$$\mathrm{RRF} = \frac{(\text{Area crenarchaeol})}{(\text{Area } C_{46})} \times \frac{(\text{Weight crenarchaeol})}{(\text{Weight } C_{46})} \quad (1)$$

The RRF changed frequently, with values ranging between 2.9 and 4.8 (Fig. 4). During this study,the HPLC/MS conditions regularly changed due to power cuts and re-calibration of the MS (black arrows, Fig. 4). The most marked changes in RRF occurred after the first power cut (Fig. 4a) when the ratio changed from 4.2 to 4.8 and after MS re-calibrations when RRF values dropped from 4.7 to 3.8 and 3.8 to 2.9, respectively (Fig. 4b and d). Primary reasons for these large changes are changing conditions in the APCI source and loss of mass accuracy

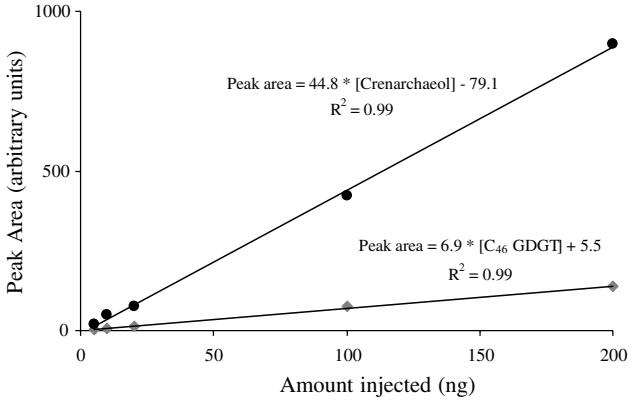

Fig. 3. Standard curves of amount injected versus peak area for crenarchaeol and C_{46} GDGT.

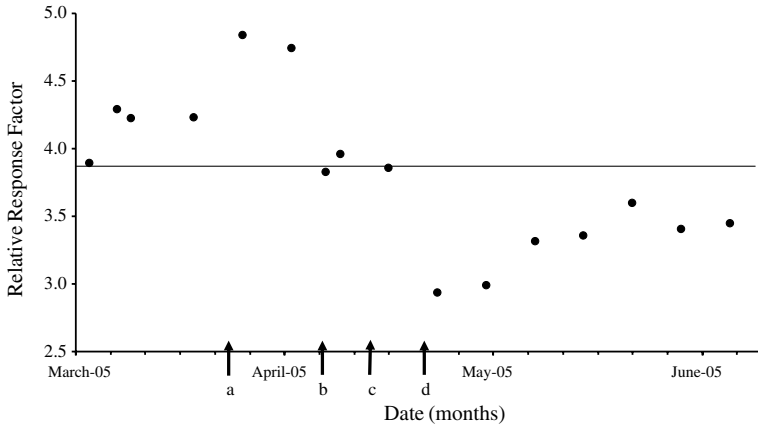

Fig. 4. Variation of the relative response factor over time. The average value is indicated by the black line. Black arrows signal changes in the HPLC/MS conditions: (a) power cut, (b) re-tuning, (c) change of parts and air-conditioning restored and (d) MS re-tuning and a power cut.

due to drifting of the mass axis. The first will change the relative degree of in-source fragmentation of the internal standard and the relative degree of ionization. The latter factor will induce changes in the RRF due to the large mass difference between the internal standard and the GDGTs. This shows that the RRF needs to be determined regularly and in particular after HPLC/MS conditions have changed.

3.3. Quantification of GDGTs using internal and external standards

Over the same period of time that the RRF stability was evaluated, we also measured GDGT abundances in a Drammensfjord sediment sample using both the internal and the external standard. These analyses were performed once every two weeks, directly after determining the RRF value. The external standard curves were determined on a monthly basis and after major alterations in the MS conditions (e.g. MS re-calibration). When calculating the absolute GDGT abundances obtained using either the internal or external standard, similar average GDGT concentrations are obtained for both methods (Fig. 5). However, the relative standard deviation is much higher when using the external standard curves (43%) than with the C_{46} GDGT internal standard (5%). This is likely because the internal standard undergoes the same work up as that of the sedimentary GDGTs. In addition, the internal standard is measured at the same time as the sample, thus reducing errors due to drifts in HPLC/MS conditions that cannot be accurately measured with an external standard. Hence, for GDGT absolute abundance measure-

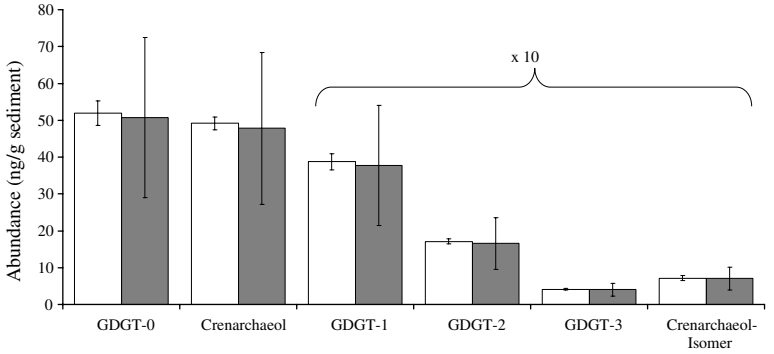

Fig. 5. Abundance of GDGT molecules in Drammensfjord sediments, calculated with internal standard (open bars) and external standard (grey bars). Relative standard deviations are shown by the vertical bars. The abundance of GDGT-1, GDGT-2, GDGT-3 and the crenarchaeol isomer are multiplied by 10.

ments the use of an internal standard will lead to improved accuracy of quantification.

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