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Rapid Communications in Mass Spectrometry 2020, **e8983**. https://doi.org/10.1002/rcm.8983

Determination of the δ^{2}H values of high molecular weight lipids by high temperature GC coupled to isotope ratio mass spectrometry

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Abstract

Rationale: The hydrogen isotopic composition of lipids (δ^{2}H$_{lipid}$) is widely used in food science and as a proxy for past hydrological conditions. Determining the δ^{2}H values of large, well-preserved triacylglycerides and other microbial lipids, such as glycerol dialkyl glycerol tetraether (GDGT) lipids, is thus of widespread interest but has so far not been possible due to their low volatility which prohibits analysis by traditional gas chromatography pyrolysis isotope ratio mass spectrometry (GC/P/IRMS).

Methods: We determined the δ^{2}H values of large, polar molecules and applied high temperature gas chromatography (HTGC) methods on a modified GC-/P/IRMS system. The system used a high temperature 7 m GC column, and a glass Y-splitter for low thermal mass. Methods were validated using authentic standards of large, functionalised molecules (triacylglycerides, TG), purified standards of GDGTs, and compared to δ^{2}H values determined by elemental analyser pyrolysis isotope ratio mass spectrometry (HTEA/IRMS); and subsequently applied to the analysis of GDGTs in a sample from a methane seep and a Welsh peat.

Results: δ^{2}H values of TGs agreed within error between HTGC/P/IRMS and HTEA/IRMS , with HTGC/P/IRMS showing larger errors. Archaeal lipid GDGTs with up to three cyclisations could be analysed: δ^{2}H values were not significantly different between methods with standard deviations of 5 to 6 ‰. When environmental samples were analysed, δ^{2}H values of isoGDGTs were 50 ‰ more negative than those of terrestrial brGDGTs.

Conclusions: Our results indicate that the high temperature GC/P/IRMS (HTGC/P/IRMS) method developed here is appropriate to determine the δ^{2}H values of TGs, GDGTs with up to two cyclisations, and potentially other high molecular weight compounds. The methodology will widen the current analytical window for biomarker and food light stable isotope analyses. Moreover, our initial measurements suggest that bacterial and archaeal GDGT δ^{2}H values can record environmental and ecological conditions.

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Introduction

The stable hydrogen isotopic composition (δ^{2}H values) of water varies systematically across the globe [1–3]. The δ^{2}H values of biological molecules, in turn, are dependent on the δ^{2}H of the H_2O available to the producing organism (source water), overprinted by biochemical processes. The δ^{2}H values of bulk organic matter and individual compounds are used across a range of disciplines, e.g., in ecology and biology to trace animal migration patterns and food webs [4,5], in forensic science to identify geographical origins of victims or suspects [6], and in food science to determine the provenance of products such as honey [7], milk [8], and meat [9]. The determination of δ^{2}H values has also resulted in substantial discoveries in archaeology, such as the earliest horse milking [10], or manuring practices [11], and has improved our understanding of past environments and precipitation regimes [12–14].

The δ^{2}H values of individual lipid biomarkers are particularly useful in paleoenvironmental studies. In particular, the correlation of lipid δ^{2}H with source water δ^{2}H has been widely documented [12,15,16], such that leaf waxes are now widely used to reconstruct past hydrological conditions [12,16–18]. Long-chain n-alkanes and other alkanes are often used in this endeavour because they are–due to their relatively high pKa (~ 50)–less susceptible to hydrogen exchange than functionalized compound classes commonly found in soils and sediments. However, a wide range of sedimentary lipids have been analysed for their stable hydrogen isotopic composition, including *n*-alkanes, fatty acids, alkenones, and, to a lesser extent, sterols and hopanols [19–23].

The routine and rapid compound-specific δ^{2}H value determination of biomarkers (as opposed to labour intensive approaches requiring compound isolation and purification) requires the application of gas chromatography, coupled to an on-line reactor containing active graphite, converting individual organic compounds into graphite, CO and H_2 [21,24–27]. The produced gas is introduced into an isotope ratio mass spectrometer monitoring *m*/*z* 2

(^{1}H-^{1}H) and 3 (^{1}H-^{2}H). This setup requires analytes to be GC-amenable [28], limiting analyses to compounds of a molecular weight and polarity low enough to elute at a typical maximum capillary column operating temperature of 320 °C. Therefore, only very few larger compounds (eluting later than a C_{36} *n*-alkane on an apolar stationary phase) have had their δ^{2}H values successfully determined. Existing measurements were achieved by implementing long isothermal holds at 320 °C but only with highly purified and ^{2}H-labelled compounds [29], due to the low GC resolution and δ^{2}H precision associated with this methodology.

However, the δ^{2}H values of large and/or polar compounds can be of significant interest. For example, the origin of vegetable oils and milk products can be constrained [30–32] with greater specificity when isotopic fingerprinting is based on individual fatty acids instead of bulk organics [33,34]. Moreover, determining the δ^{2}H values of intact triacylglycerides (TG, Suppl. Fig. 1A), instead of hydrolysed and derivatised fatty acids, could have many benefits such as eliminating derivatisation biases and increased specificity. TG are routinely characterised in food forensics by high temperature gas chromatography (HTGC[35–37]), but their ^{2}H signatures are yet to be exploited. Another potential application arises from very long-chain *n*-alkanes that are major constituents of crude oil; their δ^{2}H values could be used to assess source rock potential[17,18,38,39], or for correlating different oils and source rocks [38,40].

A third suite of applications centres on glycerol dialkyl glycerol tetraether lipids (GDGTs, Suppl. Fig. 1BC), derived from both Archaea and Bacteria and of wide interest in geochemistry. These membrane lipids are frequently used in proxies for paleotemperature and other environmental variables [41]. In many sedimentary archives, GDGTs are of mixed origins (e.g. [42,43]), and their δ^{2}H values could thus be used to distinguish terrigenous from in situ-produced GDGTs, for example in marine sediments. This would substantially improve the application of these GDGT-based proxies. Moreover, in single-source environments, the hydrogen isotopic composition of GDGTs could serve as a paleohydrological proxy, enabling reconstruction of salinity, elevation, or precipitation. More recently, it has been shown that δ^{2}H values of bacterial lipids document the metabolic state of the source organisms, potentially representing another application in biogeochemical investigations [44], and this method will allow to extend such investigations to archaea.

In order to determine the stable isotopic composition of some of these large molecules, they are often subjected to chemical degradation, and only fragments (mostly aliphatic moieties) that are more GC-amendable than the parent molecule are analysed by GC/IRMS. For TGs, this involves acid methanolysis [45]. For GDGTs, this involves ether cleavage, followed by reduction [46–51], often including laborious preparative HPLC steps for cleaning and preconcentration [52]. Aside from being labour intensive, such procedures under acidic conditions could result in hydrogen exchange.

However, recently, HTGC methods for more direct analysis of these compounds have been developed; identification and quantification of GDGTs has been achieved employing HTGC coupled to time-of-flight mass spectrometry (HTGC/TOFMS) and flame ionisation detection (HTGC/FID [53,54]). Here, we develop these methods further and demonstrate δ^{2}H analysis of polar and high molecular weight compounds by HTGC coupled to pyrolysis isotope ratio mass spectrometry (HTGC/P/IRMS). We compare the values of purchased, authentic standards (TGs), and purified standards (GDGTs) determined by elemental analyser pyrolysis isotope ratio mass spectrometry (HTEA/IRMS) with the values determined by HTGC/P/IRMS. We then report the δ^{2}H values of GDGTs in a number of environmental samples.

Experimental

Standards and environmental samples

Triacylglyceride [TG; trimyristin (TG 42:0), tripalmitin (TG 48:0), and tristearin (TG 54:0)] and *n*-alkane standards were purchased from Sigma Aldrich (Gillingham, UK). isoGDGT-2 and isoGDGT-3 standards were purified from biomass of *Sulfolobus solfataricus* (DSM 1616), which was grown in two batches (2 L each) of modified Allen medium [55] using water with a δ^2H value of -55.0 ± 0.2 ‰. Each batch was inoculated with 20 mL of a late log-phase culture, incubated aerobically at 76 °C with agitation at 200 RPM, and harvested in mid-log phase at an optical density of 0.442 (600 nm). Cells were collected by centrifugation at 4 °C, frozen in liquid nitrogen, and freeze-dried. 0.5 g of the freeze-dried cell pellet was subjected to acid hydrolysis in 5 mL of 1.5 N methanolic HCl (10 % H_2O made from 37% HCl) for 3 hours at 70°C, and lipids were extracted by ultrasonication in dichloromethane:methanol (1:1; *v/v*) as previously described [56]. The total lipid extract (TLE) was dried under a stream of N_2, dissolved in 1 mL of *n*-hexane: isopropanol (97:3; *v/v*), and filtered through a 0.45 µm PTFE filter.

To produce purified standards for both HTEA/IRMS, and for GC/P/IRMS, individual isoprenoidal GDGTs containing 2 and 3 cyclopentyl moieties (isoGDGT-2 and isoGDGT-3) were isolated by preparative normal phase (NP) high-performance liquid chromatography (HPLC). To this end, aliquots (25 µL) of the filtered TLE were injected onto an Agilent 1100 HPLC system fitted with an Econosphere NH_2 column (250 × 10 mm, 10 µm; Grace/Alltech). GDGTs were eluted isocratically with a solvent mixture of 1.35 % isopropanol (IPA) in *n*-hexane at a flow rate of 1 mL min^{-1} for 45 min, and the column was cleaned with 16 % IPA for 12 min and re-equilibrated to initial conditions for 13 min after every run. GDGTs were recovered by time-based fraction collection, according to the elution times determined by atmospheric pressure chemical ionisation-mass spectrometry (APCIMS) using an Agilent 1100 MSD quadrupole mass spectrometer (Agilent Technologies, Cheadle, UK) [57]. The collected fractions were analysed by flow injection analysis-APCIMS on the same instrument, and subsequently pooled by compound. The purity of each isolated GDGT was >97 % as assessed by NP and reverse phase HPLC/APCIMS analysis of the combined fractions [58], scanning the range *m/z* of 350–1350.

Environmental samples analysed by GC/P/IRMS included a sediment sample from a marine methane seep, and a sample from a Welsh peat [53]. In order to improve gas chromatographic performance, GDGTs were purified prior to HTGC/P/IRMS. The Welsh peat extract was passed over a column containing 130-270 mesh silica (pore size 60 Å 288608, Sigma Aldrich, Gillingham, UK) conditioned in methanol, using two column volumes of each hexane, ethylacetate/hexane 1:9 (*v/v*), 25:75, 50:50, pure ethylacetate, and methanol. Concentrations of GDGTs in the fractions were confirmed by adding triglyceride quantification standards and analysis by HTGC/FID [53]. All fractions containing GDGTs (Suppl. Fig. 2) were combined to avoid any isotope fractionation which may have occurred during column chromatography.

^{2}H analysis by HTEA/IRMS

The ^{2}H/^{1}H ratios of the triacylglycerides (TGs) and C_{50} and C_{60} *n*-alkanes were analysed viaHTEA/IRMS at Elementar UK Ltd (EUK; Stockport, UK) and University of Colorado (CUB; Boulder, USA). CUB also analysed GDGTs. CUB performed HTEA/IRMS on a Flash HT Plus elemental analyser at 1450 °C with zero blank autosampler coupled to a Delta V Plus IRMS via ConFlo-IV Interface (both Thermo Fisher Scientific, Waltham, MA, USA). At EUK, HTEA/IRMS measurements were performed using a GeovisION, which comprised a vario PYRO cube elemental analyser coupled to an isoprime visION IRMS (both EUK). Both laboratories measured samples using glassy carbon reactors in oxygen-free environments, and performed multipoint calibrations using reference materials provided by Arndt Schimmelmann (Indiana University, Bloomington, IN, USA) to normalise the measured δ^2H values against the international reference Vienna Standard Mean Ocean Water (VSMOW). CUB calibrated using 5α-androstane #3 (-293.2 ± 1.0 ‰), eicosanoic acid methyl ester #Z1 / USGS 70 (-183.9 ± 1.4 ‰), and eicosanoic acid methyl ester #Z2 / USGS 71 (-4.9 ± 1.0 ‰), and EUK calibrated using tetracosane #1: -53.0 ± 1.6 ‰, pentacosane #4: -263.6 ± 2.2 ‰ and heptacosane #3: -172.80 ± 1.6 ‰, and a standard provided by the International Atomic Energy

Agency, Vienna (IAEA CH-7: -100.2 ± 1.0 ‰). Across both labs, the standard deviation (SD) of triplicate sample analyses was typically < ±0.75 ‰.

Because the oxygen-bound H atoms of the GDGTs' hydroxyl moieties are easily exchanged, the ^{2}H content at these positions may have been altered during solvent extraction/evaporation. We therefore vapour-equilibrated the dried GDGT fractions with local deionised water (-121.8 ± 1.3 ‰) before analysis (24 h at 25 °C). GDGT fractions were then dissolved in ethyl acetate at ~10 µg µL^{-1} and 10 µL aliquots were pipetted into combusted (450 °C, 10 h) silver capsules (4x6 mm), which were pre-loaded with small discs (d = 4 mm) of combusted glass fibre filters (Whatman GF/F) as a solvent adsorbent. The solvent was then completely evaporated in a closed chamber continuously purged with N_2 (30 min at ~30 mL min^{-1}). Analysis by HTEA/IRMS was then conducted as described above.

To test for the efficiency of the vapour equilibration, a synthetic diglycerol-trialkyl-tetraether (C_{46}-GTGT; Patwardhan and Thompson, 1999) was exposed to vapour of both ^{2}H-enriched water (7 atom % ^{2}H) and local deionised water (24 h at 25 °C). Exposure to ^{2}H-enriched water vapour increased the ^{2}H content of the molecule by 0.1 atom % (from 0.014 to 0.113 atom % relative to total H), corresponding to a ^{2}H content of ~5 atom % at the OH positions after exposure (assuming all exchange is localised to the hydroxyl moieties). Exposure to natural water vapor, however, did not lead to a change in δ^2H within analytical precision of the measurement. The induced ^{2}H content at the OH positions decreased again to a ^{2}H content of ~2 atom % at the OH-positions after a 12 h exposure to ambient lab air. Together this indicates that OH-bound H of diglycerol tetraethers is readily exchanged with ambient water vapor, and any ^{2}H enrichment resulting from the evaporation of OH-containing solvents (e.g. methanol) was likely diminished either by spontaneous re-equilibration with ambient air, or by the latest through 24 h exposure to natural water vapor in a desiccator as described above.

δ^2H value determination by high-temperature HTGC/P/IRMS

Before analysis by HTGC/P/IRMS, fractions containing GDGTs and the sample from the Black Sea methane seep were dissolved in 50 µL pyridine and derivatised to trimethylsilylethers with 50 µL 99% N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA), 1% trimethylchlorosilane (TMCS), for one hour at 70 °C. The δ^{2}H value of the TMS moieties used to derivatise the hydroxyl-groups (δ^2H_{TMS}) was determined by derivatisation of sodium palmitate of a known δ^{2}H (δ^2H_P, -239.10 ‰), and analysis by GC/IRMS to yield the values of derivatised palmitate δ^2H_{TMSP}, as -82.35 ‰ according to Eqn. 1. The use of δ-values in this specific case is possible and recommended (natural abundance ranges), when larger differences are present, D/H ratios must be used.

$$\delta^2 H_{TMS} = \frac{\delta^2 H_{TMSP} \cdot 40 - \delta^2 H_P \cdot 31}{9}$$

(Eqn 1)

Values of derivatised GDGTs δ^2H_{meas} were corrected by mass balance to give δ^2H_{GDGT} with n representing the number of non-exchangeable hydrogens of the compounds and k the number of TMS groups added (1 for archaeol, 2 for GDGTs and hydroxyarchaeol; Eqn. 2).

$$\delta^2 H_{GDGT} = \frac{\delta^2 H_{meas}(n + \mathrm{k} \cdot 9)}{n} - \frac{\mathrm{k} \cdot 9 \cdot \delta^2 H_{TMS}}{n}$$

(Eqn 2)

This was combined into Eqn. 3.

$$\delta^2 H_{GDGT} = \frac{\delta^2 H_{meas}(n + \mathrm{k} \cdot 9)}{n} - \frac{\mathrm{k} \cdot 40 \cdot \delta^2 H_{TMSP}}{n} + \frac{\mathrm{k} \cdot 31 \cdot \delta^2 H_P}{n}$$

(Eqn 3)

Errors of δ^2H_{meas} were determined according to error propagation laws:

$$\sigma^2_{\delta^2 H_{GDGT}} = \sigma^2_{\delta^2 H_{meas}} \cdot \left(\frac{n + \mathrm{k} \cdot 9}{n}\right)^2 + \sigma^2_{\delta^2 H_{TMSP}} \cdot \left(\frac{\mathrm{k} \cdot 40}{n}\right)^2 + \sigma^2_{\delta^2 H_P} \cdot \left(\frac{\mathrm{k} \cdot 31}{n}\right)^2$$

(Eqn 4)

Samples were screened by HTGC/FID as described by Lengger et al. [53] before they were analysed by an Elementar isoprime visION HTGC/P/IRMS (Elementar UK Ltd., Cheadle, UK). The instrument comprised an Agilent 7890B GC fitted with an on-column injector, linked to a GC5 interface (maintained at 380 °C) and a hollow ceramic reactor, in which a stripped transfer line (Zebron Z-Guard high temperature guard column, 0.25 mm ID, Phenomenex Ltd., Aschaffenburg, Germany) was inserted carrying analytes from the GC, enabling pyrolysis at 1450 °C. A PTV injector was not available on this instrument, but was observed to inhibit elution of GDGTs in separate investigations (data not shown). Ferrules used to connect the ceramic furnace and GC-column, as well as the sample line He used as an additional carrier in the HTGC/P/IRMS system, were 100% graphite. Ion beams at *m/z* 2 and 3 were monitored via an isoprime visION mass spectrometer. The H_3^+ factor was determined daily or at least every 4 runs. Compounds were injected in ethylacetate (1 µL) and separated on a Zebron ZB-5HT analytical column (7 m × 0.25 mm × 0.25 µm, Phenomenex Ltd., Aschaffenburg, Germany) with high-temperature resistant polyimide coating, which was fitted to a transfer line (Zebron Z-Guard Hi-Temp guard column 0.25 mm ID, Phenomenex Ltd., Aschaffenburg, Germany) that was inserted directly into the reactor (with the reactor-facing side thermally stripped of polyimide coating),and an exhaust to allow diversion of the solvent peak to waste via a glass Y-splitter, in which columns were fixed with high temperature resin (Phenomenex Ltd., Aschaffenburg, Germany). He was used as a carrier gas at a flow rate of 2.2 mL min^{-1} , and the oven was programmed as follows: 1 min hold at 70 °C, increase by 10 °C min^{-1} to 350 °C, followed by an increase at 3 °C min^{-1} to 400 °C (10 min hold). Results were calibrated using a mixture of *n*-alkanes (B3, A. Schimmelmann, Indiana University, Bloomington, IN, USA) according to Sessions et al. [21,60], which was injected at least every four analyses (RMS detailed in Tab. S1), and analysed using a He flow of 1 mL min^{-1}, with a different temperature program (injection at 50 °C held for 1 min followed by an increase of 10°C min^{-1} to 300 °C and a 10 min hold). Resultant calibrated δ^{2}H values were calculated based on the derived linear regression. Root mean standard errors of normalised values of the *n*-alkane mixture were typically between 4 and 6 ‰, and never exceeded 10 ‰. Data was processed using ionOS stable isotope data processing software (Elementar UK Ltd., UK), using an automated multi-point linearisation based on the certified values of the 15 individual *n*-alkanes comprising the B3 standard.

The fractionation factor $\varepsilon_{H2O/GDGT}$ was determined from the δ^2H_{H2O} and the δ^2H_{GDGT} (Eqn. 5).

$$\varepsilon_{GDGT/H2O} = \left(\frac{\delta_{GDGT} + 1}{\delta_{H2O} + 1} - 1\right)$$

(Eqn. 5)

Results and discussion

Chromatographic method

The modifications of the GC/P/IRMS instrumentation enabled operating temperatures of up to 400 °C. Utilisation of a 7-m column and on-column injection (as previously discussed [53]) enabled elution of isoGDGTs up to GDGT-3, as well as acceptable values for the B3 standard. The HTGC/P/IRMS setup required a polyimide-coated column rather than the metal column commonly employed in HTGC-methodologies, as this allowed flow diversion via a glass Y-splitter in which the column was secured using high temperature resin (no other modifications to the standard Elementar flow diversion system were made). The glass Y-splitter ensured minimal thermal mass. The small ID of the ceramic reactor and insertion of the transfer line close to the pyrolysis site, and of contact with any metal surfaces (glass Y-splitter instead of metal valve, silicon transfer to pyrolysis site in ceramic reactor), have likely contributed to avoid the peak broadening and fronting often observed in GC/P/IRMS. Furthermore, the pneumatically operated heart-cut valve enabling diversion of the solvent away from the furnace reactor was moved to a location outside of the GC-oven in order to avoid potential leaks associated with the high temperatures. Extended (> 10 min) high temperature (> 400 °C) isothermals such as used successfully with metal columns to analyse isoGDGTs by HTGC/FID and HTGC-TOFMS [53], could not be employed to elute isoGDGTs in analogous HTGC/P/IRMS analyses due to the comparatively lower stability of the polyimide-coated columns at these temperatures.

The unusual HTGC configuration, with a short 7 m column, high flow, and on-column injector, was tested by analysing a mixture of 15 *n*-alkanes: the so-called Indiana B-standard mix routinely used for standardisation of GC/P/IRMS results. Baseline separation of individual *n*-alkane peaks and acceptable root mean square errors were achieved with this method (Fig. 1A): this standard was subsequently used for quality control and isotope calibration. Root mean square error (RMSE) and linearisation equations for all analyses of the standards are given in Supplementary Fig. 3 and Table 1, with linearisation applied to the samples based on the most contemporary analysis of the standard. RMSE for all accepted analyses were always below 10 ‰: whenever 10 was exceeded, inlet maintenance or column changes were performed. An *n*-alkane standard containing higher molecular weight compounds (up to C_{60}, Fig. 1B), a mixture of triacylglycerides (Fig. 1C), a seep sample containing GDGT-0, -1. -2, and -3, and the two GDGT standards (GDGT-2 and -3) (Fig. 1D) were analysed and chromatograms were similar to previous results employing HTGC/FID and a 7 m column [53]. The brGDGTs eluted earlier than isoGDGTs (cf. [53]).

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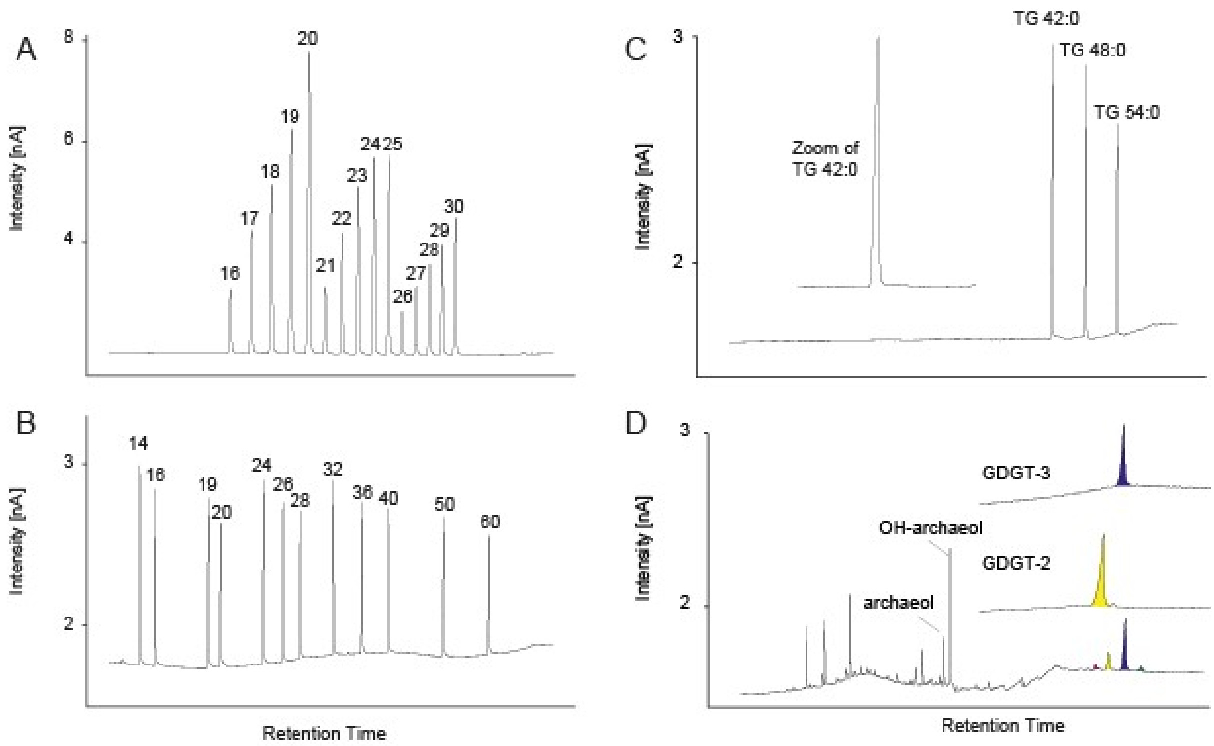

Figure 1. GC/P/IRMS chromatograms under HT conditions; different temperature ramps were applied to the different mixtures. Shown is A, a mixture of *n*-alkanes up to *n*-C_{30} with known δ^{2}H values (Indiana B3-standard); B, a mixture of long-chain n-alkanes up to *n*-C_{60}; C, triacylglycerides; and D, a sample from a Black Sea methane seep with GDGT-2 and GDGT-3 standards shown as inserts; note that the small second peak in GDGT-2 was a contaminant introduced during analysis that did not affect the measurement

Accuracy and precision of δ^2H values of high molecular weight compounds

Triacylglyceride (TG) reference compounds and purified GDGT standards were used to test the methodology for accuracy by determining the δ^2H values of these compounds by HTGC/IRMS at GC temperatures of up to 400 °C as well as by EA-analysis. The prepared isoGDGT-2 and isoGDGT-3 standards were analysed by one laboratory (CU Boulder), while the purchased standards were examined by HTEA/IRMS in two different laboratories (CU Boulder and Elementar UK Ltd). The average δ^2H values determined for the TGs were within 5 ‰ for all analyses (Tab. 1, Fig. 2). HTGC-analysed samples generally yielded δ^2H values between the values determined by the EA analyses. Standard deviations were smaller for the EA methods (< 2 ‰) than for the HTGC method (9-18 ‰, which represents 2-3× the typical precision of δ^2H value determinations by GC/P/IRMS [61], and is thus a larger error than expected). Often, precision of GC/P/IRMS measurements is determined using the same concentration, while here, injection concentrations varied. This likely contributed to the high standard deviation, and we investigate this further below. It is expected that further application of this technique – and routine analysis of TGs, as compounds of particular interest to the food industry – will lead to improvements in analytical precision as methods are improved by optimising solvents, injection temperatures, and concentrations. The δ^2H values determined for the high molecular weight *n*-alkanes with 50 and 60 carbon atoms (Table 1) were more variable among all methods and laboratories. This was surprising, and possibly a result of insufficient mixing of these large waxy compounds before distribution to other laboratories.

The δ^2H values of purified GDGTs obtained by HTEA/IRMS and HTGC/P/IRMS (Tab. 1) were not significantly different for GDGT-2 at a high confidence level (Welsh's t-test, df = 2, t = 1.32, p = 0.32). However, for GDGT-3, which eluted later, the δ^2H value derived by HTGC/IRMS was 9 ‰ higher than the value determined by HTEA/IRMS (df = 2, t = 3.32, p = 0.080). A high baseline could be a possible cause for this discrepancy. However, ionOS software applies an automated correction. Both GDGTs eluted on an isothermal baseline when samples were injected (Fig. 1D). Another cause could be fractionation due to chromatographic separation, adsorption to cold spots, or thermal decomposition. Another possibility is minor contamination of GDGT-3, resulting in a flawed HTEA/IRMS measurement but not affecting HTGC/P/IRMS measurements; however, this would be surprising as GDGT-2 and GDGT-3 were isolated from the same organism and the HTEA/IRMS results match expectations of similar δ^{2}H values. The standard deviation of 5 – 6 ‰ achieved for purified GDGTs using the HTGC/P/IRMS system is similar to the precision of lower molecular weight compounds on a conventional GC/P/IRMS instrument [61].

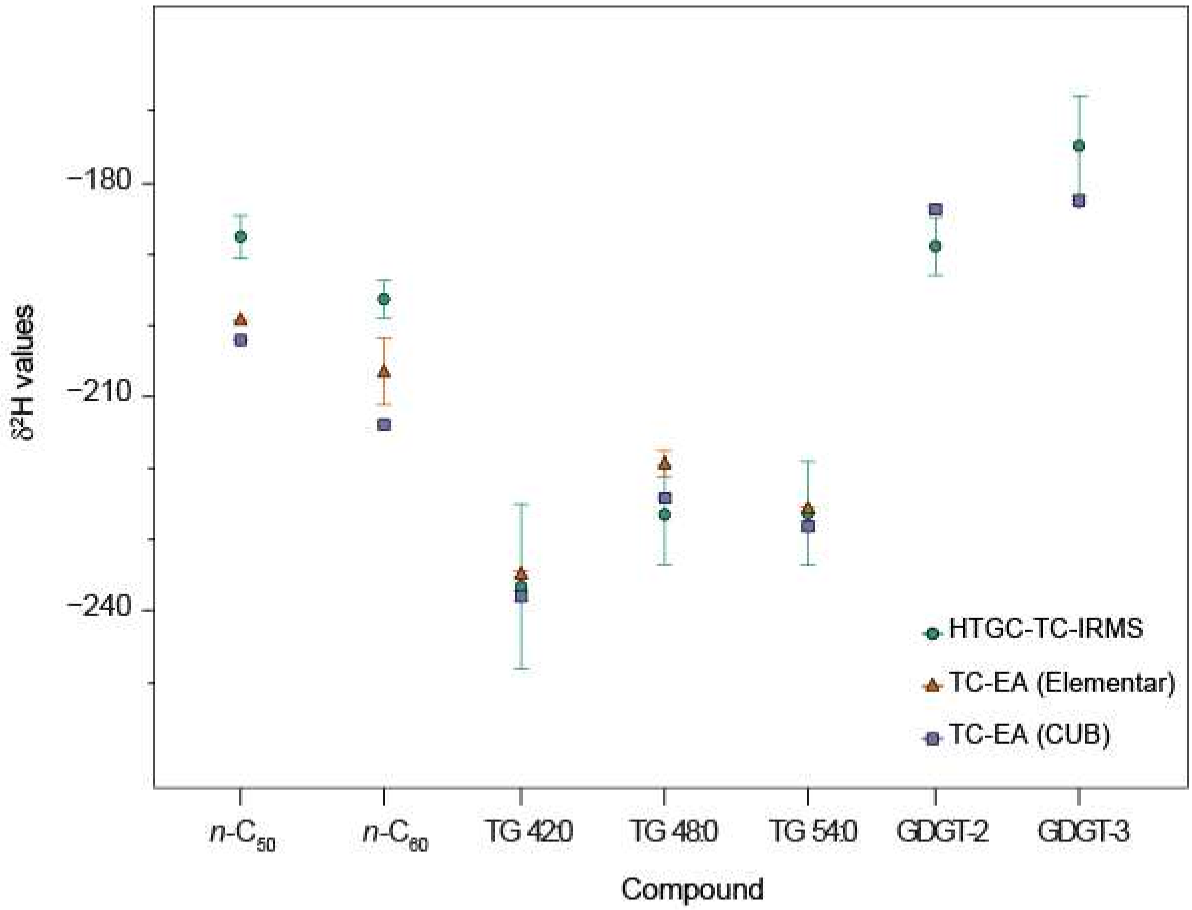

Figure 2. δ^{2}H values of purchased triacylglyceride standards and isolated GDGTs determined by HTEA/IRMS compared with values determined by HTGC/P/IRMS; values and standard errors are given in Table 1.

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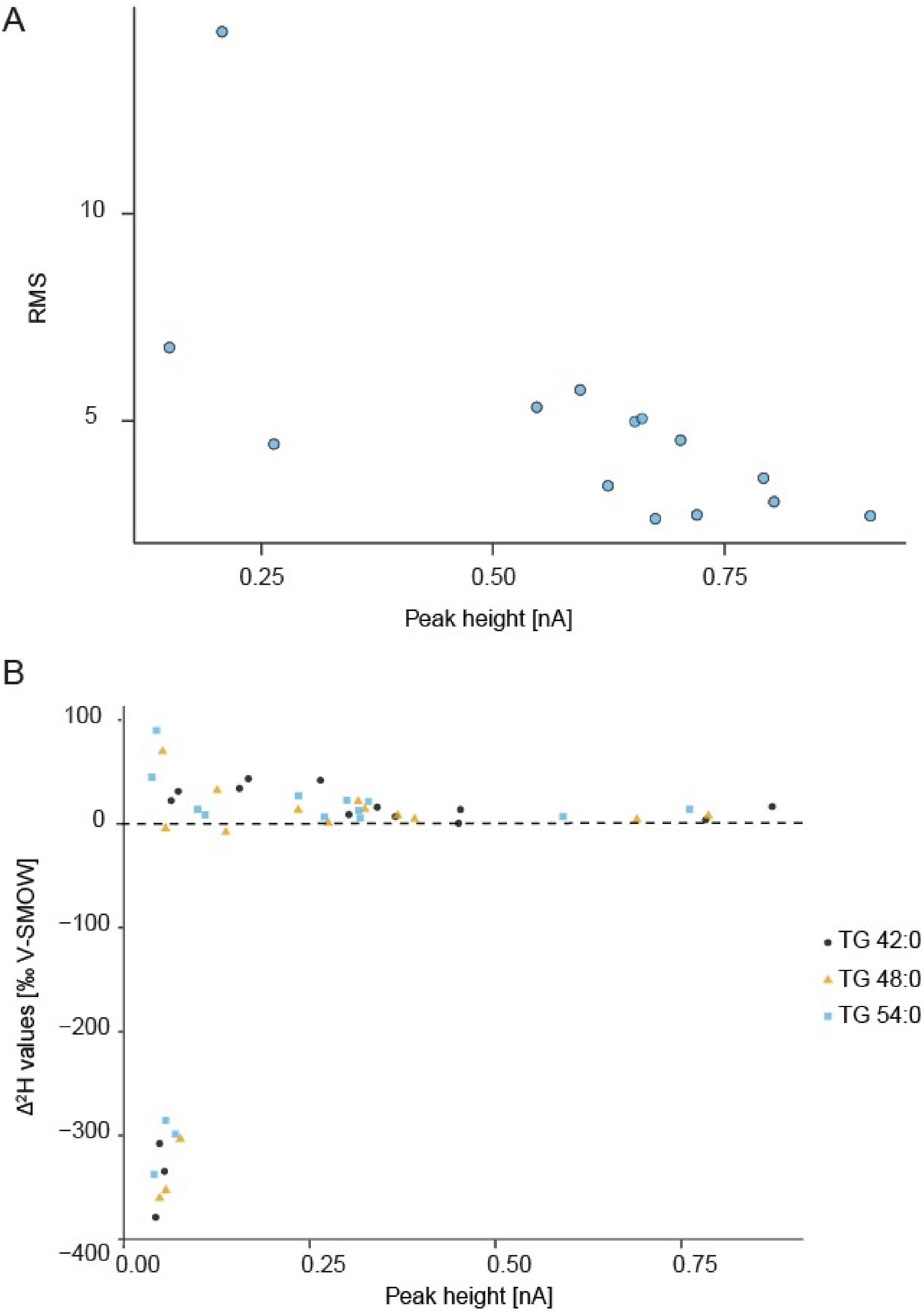
Table 1. δ^{2}H values determined by HTEA/IRMS and HTGC/P/IRMS.

	HTEA/IRMS (Elementar)			**HTEA/IRMS (CU Boulder)**			**HTGC/IRMS**		
	Mean [‰ V-SMOW]	**St. dev.**	**N**	**Mean** [‰ V-SMOW]	**St. dev.**	**N**	**Mean** [‰ V-SMOW]	**St. dev.**	**N**
GDGT-2	-	-	-	-181.6	0.4	3	-186	4	3
GDGT-3	-	-	-	-182.6	0.2	3	-173	7	3
C_{42}-TG 42:0	-235.0	0.5	4	-238.0	0.7	3	-232	9	9
C_{48}-TG 48:0	-219	2	3	-224.1	0.3	3	-223	18	7
C_{54}-TG 54:0	-225.9	0.4	4	-228.2	0.7	3	-223	12	7
***n*-C_{60} alk**	-206	5	3	-214.0	0. 4	3	-196	3	3
***n*-C_{50} alk**	-199.29	0.02	4	-202.05	0.06	3	-188	3	3

Response vs accuracy

Whilst GDGTs are ubiquitous, they are typically only present at ppm to ppb concentrations in environmental samples such as sediments and soils. In addition, many high molecular weight compounds are not very soluble in solvents suitable for GC/IRMS, and on-column injection only allows small amounts of sample to be used. Therefore, only small amounts of GDGT (ng) were injected for each HTGC/P/IRMS analysis. To assess accuracy in relationship to signal intensity, different concentrations of the TG standard were tested and compared to peak heights (Fig. 3). This yielded a response of 0.07 – 0.08 nA per ng H per compound for *m/z* 2 (equivalent to 70-80 mV on an IRMS with a 10^9 Ohm resistor on the operational amplifier for the *m*/*z* 2 faraday cup). Below ~0.25 nA peak height, values begin to deviate substantially (by ~20 ‰) from the values measured by HTEA/IRMS , with differences of up to 400 ‰ when peak heights were around 0.1 nA. We thus excluded peak heights < 0.25 nA, corresponding to less than 3.5 ng H injected on column, from any further analysis. Typical H amounts required to achieve 3-5 ‰ precision were ~10 ng, translating to *m/z* 2 peak heights of 0.7 – 0.8 nA.

Lengger, S.K., Weber, Y., Taylor, K.W.R., Kopf, S.H., Berstan, R., Bull, I.D., Mayser, J.-P., Leavitt, W.D., Blewett, J., Pearson, A., Pancost, R.D., n.d. Determination of the δ²H values of high molecular weight lipids by high temperature GC coupled to isotope ratio mass spectrometry.
Rapid Communications in Mass Spectrometry 2020, **e8983**. https://doi.org/10.1002/rcm.8983



Lengger, S.K., Weber, Y., Taylor, K.W.R., Kopf, S.H., Berstan, R., Bull, I.D., Mayser, J.-P., Leavitt, W.D., Blewett, J., Pearson, A., Pancost, R.D., n.d. Determination of the δ^{2}H values of high molecular weight lipids by high temperature GC coupled to isotope ratio mass spectrometry.
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Figure 3. Measured δ^{2}H values compared with peak heights. A, RMSE of the B3 mixture compared with peak heights of the minimum peak height in the mixture. B, Difference between δ^{2}H values of TGs determined by HTGC/P/IRMS from values determined by HTEA/IRMS, plotted vs peak height

GDGTs in environmental samples and $\varepsilon_{H2O/isoGDGT}$

A sample from a Mediterranean cold seep [53] was analysed, and δ^{2}H values for archaeol, hydroxyarchaeol, GDGT-1, and GDGT-2 were determined to be -245 ± 7, -253 ± 13, - 216 ± 15, and -225 ± 14 ‰, respectively (n=3; Fig. 1D, Fig. 4). These values show a limited range, as expected for ether lipids derived from a common archaeal source, and are similar to published δ^{2}H values of the biphytanes of GDGTs in *Sulfolobus sp.* determined after ether cleavage (-229 to -257 ‰ [46]). However, the values are not identical: the diphytanyl glycerol diether lipids archaeol and hydroxyarchaeol were ^{2}H-depleted relative to GDGTs. Though the difference between the di- and tetraethers is small, and similar to what is commonly observed between different fatty acids from the same organism [62], it could potentially reflect different archaeal origins, given that ANME-2 group Archaea appear to preferentially produce GDGTs in cold seep settings (e.g., 63). This would be particularly true if the differing source Archaea exhibit different metabolisms (see below). A sample from a Mediterranean cold seep [53] was analysed, and δ^{2}H values for archaeol, hydroxyarchaeol, GDGT-1, and GDGT-2 were determined to be -245 ± 7, -253 ± 13, - 216 ± 15, and -225 ± 14, respectively (n=3; Fig. 1D, Fig. 4). These values show a limited range, as expected for ether lipids derived from a common archaeal source, and are similar to published δ^{2}H values of the biphytanes of GDGTs in *Sulfolobus sp.* determined after ether cleavage (-229 to -257 ‰) [46]. However, the values are not identical, with the diphytanyl glycerol diether lipids archaeol and hydroxyarchaeol being ^{2}H-depleted relative to GDGTs. The difference is small, and similar to what is commonly observed between different fatty acids from the same organism [62],however, it could potentially reflect different archaeal origins, given that ANME-2 group Archaea appear to preferentially produce GDGTs in cold seep settings (e.g. [63]). This would be particularly true if the differing source Archaea exhibit different metabolisms (see below).

The $\varepsilon_{H2O/GDGT}$ for the *Sulfolobus* cultures used to purify the standards was determined as -134 ‰ and was not as large as previously reported $\varepsilon_{H2O/GDGT}$ (-213‰ to -161‰[46]). The application of this fractionation factor to the environmental iso-GDGTs would nonetheless result in an unrealistic δ^{2}H value for the seawater of -93 ‰, suggesting that metabolism, salinity, temperature, and other factors contribute strongly to the extent of fractionation.

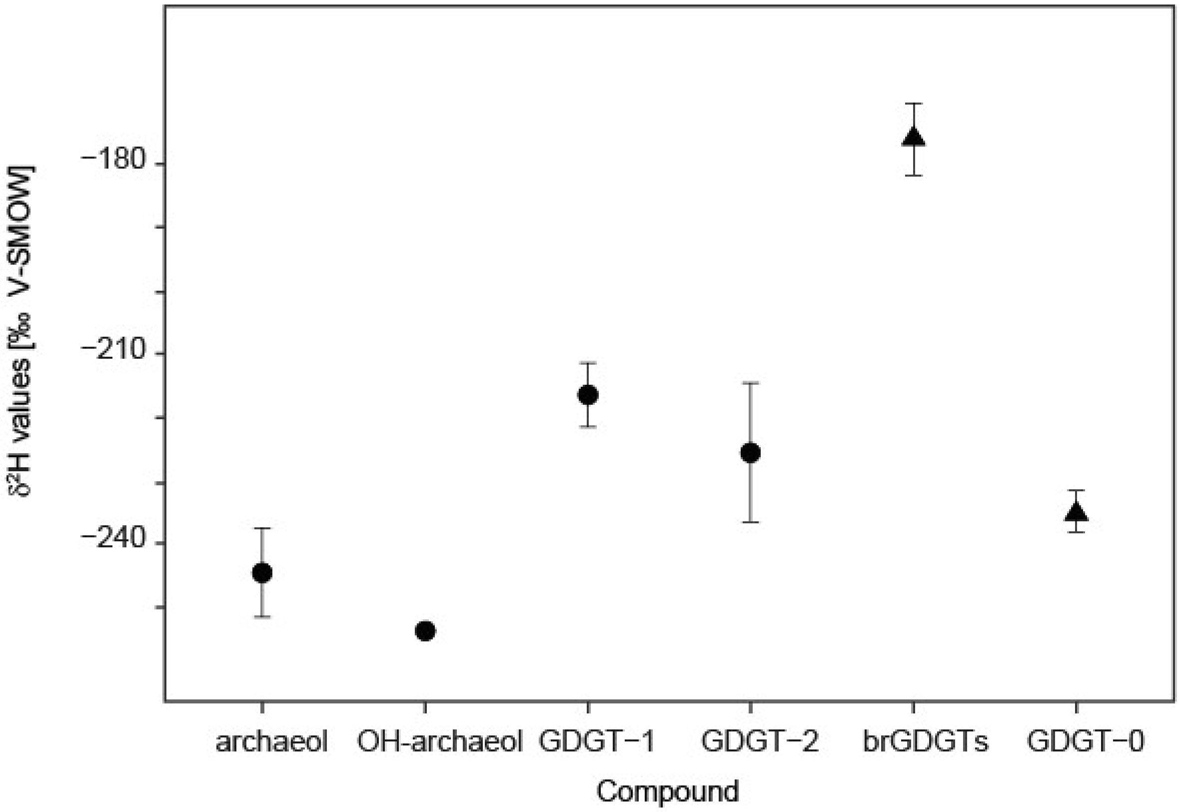

Figure 4. δ^{2}H values of ether lipids determined from environmental samples. brGDGTs and GDGT-0 were extracted from a peat (triangles) and all other compounds derived from a methane seep (circles). Error bars represent standard deviations.

Values of δ^{2}H of GDGT-0 from the peat (Suppl. Fig. 4) were similar to the isoGDGTs in the seep sample (-235 ± 3 ‰, n = 2), whereas values for brGDGTs (integrated as one peak) were relatively enriched in ^{2}H (-176 ± 6 ‰, n = 6). It is possible that the ^{2}H-enrichment of brGDGTs relative to co-occurring isoGDGTs could be due to fractionation associated with the biosynthetic pathways for isoprenoidal (isoGDGTs) vs. *n*-acyl lipids (brGDGTs), in which isoprenoidal lipids (which undergo successive hydrogenation) exhibit more ^{2}H-depleted signatures [21,64]. However, recently, it has also been shown that the energy and metabolism pathways of source organisms are highly correlated with δ^{2}H values of their lipids[44,65,66]; it is also thought that NADPH/NADH ratios and transhydrogenases play an important role, particularly in anaerobic organisms [67–70]. In general, heterotrophic bacteria consuming TCA-cycle intermediates exhibit δ^{2}H values similar to or more positive than the source water, heterotrophs assimilating carbohydrates are depleted relative to source water, and photoautotrophic and chemoautotrophic bacteria show the greatest ^{2}H-depletion [44]. While archaeal metabolisms were not examined in this work in detail, some of our results are consistent with the idea that chemoautotrophic archaea are the presumed producers of isoGDGTs in both settings, and heterotrophic bacteria are thought to be the producers of brGDGTs [71].

The differences between the peat and seep samples for isoGDGTs are unexpected: As the δ^{2}H of the peat water is likely around -52 ‰ [1] – a ^{2}H content that is depleted compared to seawater – we expected isoGDGTs from peat to also be depleted in ^{2}H relative to GDGTs from marine environments. However, isoGDGTs from peat are up to 10 to 20 ‰ more ^{2}H-enriched from peat, invoking a difference in metabolic state between the anaerobic methanogens in peat, and the anaerobic methane oxidising communities in the seep. It could also indicate synthrophy, which has been shown to affect ^{2}H values of lipids [68]. These findings speak to the potential of isoGDGT δ^{2}H analyses in probing microbial ecology and metabolic state, while brGDGTs, which are presumably of heterotrophic bacterial origin in peat settings, could prove useful as proxies for source water δ^{2}H and hydrology.

The novel HTGC/P/IRMS method enables the determination of the δ^{2}H values of compounds with a high molecular weight, including TG and GDGTs, hereby extending the range of analytes for δ^{2}H value determination. Accuracy and precision are as small as 3 ‰ in some cases and comparable to HTEA/IRMS. Our initial measurements suggest that bacterial and archaeal GDGT δ^{2}H values are likely related to both environmental parameters, and the metabolic and ecological function of the source organisms. Future applications include but are not limited to food forensics, archaeology, oil-source rock correlations, microbial ecology and paleoclimate.

Acknowledgements

We would like to thank the Editor, three anonymous reviewers, and A. Sessions for their helpful comments. The authors would like to thank Paul Sutton, Alison Kuhl, Hanna Gruszczynska, Ed Aldred, Xiahong Feng, Alec Cobban, Wolfram Meier-Augenstein, and Michiel Kienhuis for support with measurements, advice, and discussions of techniques. SKL was funded by a Rubicon Grant 825.14.014 from the Netherlands Organisation for Scientific Research (NWO, to SKL). RDP acknowledges support from ERC (Advanced Grant T-GRES, to RDP). AP and YW acknowledge support from the Swiss National Science Foundation (P2BSP2_168716), and from the Gordon and Betty Moore Foundation and US National Science Foundation (to AP). SHK acknowledges support from the US National Science Foundation. The authors thank the Natural Environment Research Council, UK, for partial funding of the mass spectrometry facilities at Bristol (contract no. R8/H10/63). WDL acknowledges support from the American Chemical Society (PRF 57209-DNI2).

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