Methyl sulfates as methoxy isotopic reference materials for $\delta^{13}C$ and $\delta^{2}H$ measurements

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Abstract

RATIONALE: Stable hydrogen and carbon isotope ratios of methoxy groups (OCH_3) of plant organic matter have many potential applications in biogeochemical, atmospheric and food research. So far, most of the analyses of plant methoxy groups by isotope ratio mass spectrometry have employed liquid iodomethane (CH_3I) as the reference material to normalise stable isotope measurements of these moieties to isotope–δ scales. However, comparisons of measurements of stable hydrogen and carbon isotopes of plant methoxy groups are still hindered by the lack of suitable reference materials.

METHODS:We have investigated two methyl sulfate salts (HUBG1 and HUBG2), which exclusively contain carbon and hydrogen from one methoxy group, for their suitability as

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/rcm.8355

methoxy reference materials. Firstl, the stable hydrogen and carbon isotope values of the bulk compounds were calibrated against international reference substances by high temperature conversion- and elemental analyser isotope ratio mass spectrometry (HTC- and EA-IRMS). In a second step these values were compared with values obtained by measurements using gas chromatography isotope ratio mass spectrometry (GC-IRMS) where prior to analysis the methoxy groups were converted to gaseous iodomethane.

RESULTS: The ^{2}H- and ^{13}C isotopic abundances of HUBG1 measured by HTC- and EA-IRMS and expressed as δ-values on the usual international scales are -144.5±1.2 mUr (n=30) and -50.31±0.16 mUr (n=14), respectively. For HUBG2 we obtained -102.0±1.3 mUr (n=32) and +1.60±0.12 mUr (n=16). Furthermore, the values obtained by GC/IRMS were in good agreement with the HTC- and EA-IRMS values

CONCLUSIONS: We suggest that both methyl sulfates are suitable reference materials for normalization of isotope measurements of carbon of plant methoxy groups to isotope–δ scales and for inter-laboratory calibration. For stable hydrogen isotope measurements we suggest that next to HUBG1 and HUBG2 additional reference materials are required to cover the full range of plant methoxy groups reported so far.

Introduction

The biospheric C_1 methyl (CH_3) pool of plant origin, including mainly lignin and pectin methoxy groups (OCH_3), comprises *ca* 2.5% of the total amount of carbon in plant biomass [1]. It has been shown that plant methoxy groups have both distinct stable hydrogen ($\delta^{2}H$ values) and carbon ($\delta^{13}C$ values) isotopic signatures [2]–[7]. Relative to the bulk biomass of plants, a ^{13}C depletion of up to 50 mUr was observed for methoxy groups, the largest carbon isotope fractionation ever observed in the plant kingdom to date [8]. (Note that we follow the suggestion by Brand and Coplen [9] and express isotope δ-values in milli-Urey [mUr] (after H.C. Urey [10]) instead of in per mil [‰]; 1 mUr = 1 ‰). On the other hand, a striking ^{2}H depletion of plant methoxy groups relative to source water has been observed with a uniform apparent isotopic fractionation (~ -150 to -210 mUr), noted over a range of $\delta^{2}H$ values for meteoric water [6],[7],[11]–[17]. These particular $\delta^{2}H$ and $\delta^{13}C$ isotopic signatures have considerable potential for use as tools for investigations in biogeochemical, atmospheric and food research.

For example, the information gained from the isotope signatures of plant methoxy groups can be applied for climate reconstructions of various climate archives such as tree rings, sediments and peat cores on time scales from the Anthropocene until the Eocene [18]–[23]. Furthermore they are useful in food science for studies on the authenticity of vanillin [17],[24]–[26], in atmospheric sciences to investigate the origin and fate of C_1 volatile organic compounds such as methanol, chloromethane and bromomethane in the atmosphere [3],[6],[8],[27],[28] and in biochemical investigations to better understand bio-methylation processes [29],[30].

Stable carbon and hydrogen isotope ratiosof methoxy groups from various plant origins have been determined by site-specific natural isotope fractionation−nuclear magnetic resonance (SNIF-NMR) and isotope ratio mass spectrometry (IRMS). The principle of measurement by IRMS is the generation of gaseous iodomethane (CH_3I) via the reaction of methoxy groups with hydriodic acid (HI). This reaction was first used by Zeisel in 1885 to quantitatively determine the methoxy content of wood [31] and it is commonly referred to as the 'Zeisel method'. Later this method was applied by Galimov *et al* and Krüger *et al* for measurements of the $\delta^{13}C$ values of biogenic methoxy groups [24],[32],[33]. In the last two decades the method has been employed for the precise and rapid $\delta^{2}H$ and $\delta^{13}C$ analysis of methoxy groups by gas chromatography isotope ratio mass spectrometry (GC/IRMS) [8],[34]–[36].

The reported $\delta^{2}H$ and $\delta^{13}C$ values of plant-related methoxy groups range from -149 mUr [25] to -405 mUr [22] and from -7.1 mUr [25] to -77.2 mUr [8], respectively. In addition the $\delta^{2}H$ and $\delta^{13}C$ values of C_1-groups from extraterrestrial matter such as meteorites (carbonaceous chondrites) have been shown to be in the range of +1054 ± 626 mUr and +43.2 ± 38.8 mUr, respectively [37].

Generally, stable isotope abundance analyses require reference materials to normalise measured δ-values on the respective δ-scale. When selecting reference materials for IRMS analyses the following criteria have to be considered [38]–[41]: isotopic homogeneity (to the smallest amount to be analysed), (ii) unchanging stable isotopic composition over time, (iii) chemical similarity to the samples to ensure that errors during preparation will tend to cancel out (principle of identical treatment [39]), (iv) easy preparation, storage and handling, (v) a single chemical compound (preferably), (vi) non-hygroscopic (especially important when measuring hydrogen and oxygen isotopes), (vii) comprising only non-exchangeable hydrogen (unless the non-exchangeable $\delta^{2}H$ value is known), (viii) should not constitute a health risk

when applied for measurements.

Up until now most of the results reported in the literature from analyses of methoxy groups by IRMS were obtained using liquid CH_3I with a known isotope ratio as the reference material. Iodomethane is a liquid at room temperature with a boiling point of 42°C and high vapour pressure of 441 hPa (20°C). It is toxic (LD_{50}, rat, oral: 76mg/kg) and probably also carcinogenic. Moreover, when used as the reference material for the analysis of methoxy groups CH_3I is not treated in an identical manner to the analyte, as it is usually not heated at 130°C with HI. Furthermore, the narrow range of values of both δ^2H and $\delta^{13}C$ for industrial CH_3I do not span the full range of δ^2H and $\delta^{13}C$ values of methoxy groups found in the environment. This is a substantial drawback when utilising CH_3I as the reference standard, as the isotopic composition of samples should be spanned by standards to account for scale contraction during analysis. Finally, since δ-values are measured more precisely when the differences between the sample and standard are small a wide range of standards is highly desirable [38]. To date there is a lack of available isotope reference materials suitable for stable isotope analysis for plant methoxy groups that fulfill the requirements listed above.

There is an urgent need to establish a suite of solid reference materials for the δ^2H and $\delta^{13}C$ analysis of methoxy groups that ideally cover the whole measurement range of δ^2H and $\delta^{13}C$ values reported for the environment so that they can be applied easily on a long-term basis and be readily available for inter-laboratory comparison studies.

We investigated two salts of methylsulfuric acid (that exclusively contain carbon and hydrogen from one methoxy group; for detailed chemical structures please see Figure 1) for their suitability as stable isotope reference material for the δ^2H and $\delta^{13}C$ analysis of methoxy groups. We first performed δ^2H and $\delta^{13}C$ measurements of the two compounds by high temperature conversion (δ^2H values) and elemental analyser ($\delta^{13}C$ values) stable isotope ratio measurements (HTC-/EA-IRMS) and calibrated them against international reference substances. Next we determined the δ^2H and $\delta^{13}C$ values of the methoxy salts by transforming the salts to CH_3I using the Zeisel method and analysing them using continuous flow (CF)-IRMS. Then, the data from both methods were compared. Furthermore, we tested if there was hydrogen isotope exchange between the methyl groups of the methyl sulfate salts and the water in humid air during storage. Finally, we discuss whether both compounds are suitable reference materials for the determination of the δ^2H and $\delta^{13}C$ values of samples containing methoxy groups.

Material and Methods

Chemicals

Hydriodic acid (57 wt.% aqueous solution) was obtained from Acros (Thermo Fisher Scientific, Geel, Belgium), methyl iodide (99.5%) was purchased from Sigma-Aldrich (Seelze, Germany). Methyl sulfate sodium salt (CAS: 512-42-5) was obtained from Aldrich (Seelze, Germany, Lot: #MKBT4673V) and potassium methyl sulfate (99%, CAS: 562-54-9) was purchased from Acros (Thermo Fisher Scientific, Geel, Belgium; Lot: A0378638).

Hydrogen and carbon isotopic analyses at the Max Planck Institute for Biogeochemistry

The two methyl sulfate salts were first analysed in the stable isotope laboratory at the Max Planck Institute for Biogeochemistry (BGC-IsoLab). The hydrogen isotopic signatures of the salts were determined by High-Temperature Conversion-Isotope Ratio Mass Spectrometry (HTC-IRMS). We refer the reader to Gehre *et al.* [42] regarding method details. In short, samples and standards were weighed into Ag-capsules and placed in a Costech (Cernusco sul Naviglio, Italy) Zero-Blank 50-position autosampler. The conversion to H_2 gas was achieved with a high-temperature furnace (Hekatech, Wegberg, Germany) held at 1430 °C that was equipped with a glassy carbon tube inside a silicon carbide tube (tube in tube design) with a reverse feed He carrier gas flow rate of ~ 80 mL/min. Gehre *et al* [43],[44] and Nair *et al* [45] have shown that the HTC reactor should be filled with chromium when measuring the δ^2H signature of heteroatom (N, S, F, Cl, Br, I) containing compounds. Chromium traps the heteroatoms and thus facilitates a quantitative conversion of analyte to H_2 gas, and subsequent transport to the isotope ratio mass spectrometer [43]. Consequently, the analyses of the methyl sulfate salts and associated standards were performed using a glassy carbon tube filled with special chromium and glassy carbon chips. The HTC-furnace was coupled to a Delta^{plus} XL isotope ratio mass spectrometer via a ConFlo III open-split interface (both Thermo Fisher Scientific, Bremen, Germany).

The carbon isotopic composition of the methyl sulfates were measured on a Thermo Fisher Delta^{plus} isotope ratio mass spectrometer coupled to a Carlo Erba 1100 CE Elemental Analyser (Thermo Fisher Scientific, Rodano, Italy) via a ConFlo III open-split interface. The measurement procedure has been described previously [46][39][47]. In brief, samples were

introduced into the EA via an autosampler. The samples were combusted at 1020 °C in the oxidation oven, and then reduced at 650 °C. Water was removed with a Nafion^{TM} and magnesium perchlorate trap before the sample gas was introduced into the gas chromatograph held at 80 °C, containing a HaysepQ filled 2m packed column (IVA-Analysetechnik, Meerbusch, Germany). The He-carrier gas flow rate was 110 mL/min.

Generation of iodomethane from methyl sulfate salts

CF-IRMS measurements were conducted at Heidelberg University, Institute of Earth Sciences, Heidelberg, Germany (HU). The δ^2H and δ^{13}C values of the sodium and potassium methyl sulfate salts were measured as methyl iodide (CH_3I), released upon treatment of the samples with hydroiodic acid (HI) using the method described by Greule *et al* [34],[35]. Hydriodic acid (HI) (0.25 mL) was added to the salt samples (hydrogen analysis: 5 mg; carbon analysis: 2 mg) in a crimp glass vial (1.5-ml; IVA Analysentechnik). The vials were sealed with crimp caps containing PTFE lined butyl rubber septa (thickness 0.9 mm; part # IVA70311101, IVA Analysentechnik) and incubated for 30 min at 130 °C. After heating, the samples were allowed to equilibrate at room temperature (22 ± 0.5 °C) for at least 30 min before an aliquot of the headspace (10 – 90 µL) was directly injected into the respective GC/IRMS system as described below using a gastight syringe (100 µL, SGE Analytical Science, Melbourne, Australia).

CH_3I standard preparation

For measurements made where only CH_3I standard was added, crimp vials (1.5-ml; IVA Analysentechnik) were first sealed and then 1-2 µL of liquid CH_3I standard was injected into each vial through the septum using a 10µL GC autosampler syringe (Hamilton Company, Reno, NV, USA; part #80300). The vials were placed into an A200S autosampler (CTC Analytics, Zwingen, Switzerland) (22 ± 0.5 °C, air conditioned room) and headspace gas (10-90µL) injected into the GC/IRMS system using a gastight syringe following the equilibration period.

Hydrogen stable isotope analysis using GC/HTC-IRMS

The δ^2H values of the CH_3I were measured using an HP 6890N gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with an A200S autosampler, coupled to a Delta^{PLUS}XL isotope ratio mass spectrometer via a thermo conversion reactor [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., reactor temperature 1450 °C] and a GC Combustion III Interface (ThermoQuest Finnigan, Bremen, Germany).

The gas chromatograph was fitted with a Zebron ZB-5MS capillary column (Phenomenex, Torrance, CA, USA) (30m x 0.25mm i.d., d_f 1µm) and the following conditions were employed: split injection (4:1), initial oven temperature at 30 °C for 3.8 min, ramp at 30 °C/min to 100 °C. Helium 5.0 was used as the carrier gas at a constant flow rate of 0.6 mL/min.

A tank of high purity hydrogen gas 6.0 (Alphagaz^{TM} 2 H_2, Air Liquide, Düsseldorf, Germany) was used as the working reference gas. The H_3^+ factor was 2.27 ppm/nA.

All δ^2H values were normalised by a two-point linear calibration [48] using the δ^2H values of two CH_3I working standards relative to VSMOW. The δ^2H values of the CH_3I working standards were calibrated against international reference substances (VSMOW2 [δ^2H_{VSMOW} = 0.0 ± 0.3 mUr] and SLAP2 [δ^2H_{VSMOW} = -427.5 ± 0.3 mUr]) by HTC-IRMS at the BGC-IsoLab. The calibrated δ^2H values in mUr vs VSMOW for the two CH_3I working standards were −173.0 ± 1.5 mUr (n=9, 1σ) and −66.2 mUr ± 1.2 mUr (n=8, 1σ).

Carbon stable isotope analysis using GC/C-IRMS

The δ^{13}C values of CH_3I were measured using the same analytical system as described for hydrogen measurements but with the difference that the gas chromatograph and the mass spectrometer were coupled by an oxidation reactor [ceramic tube (Al_2O_3), length 320 mm, 0.5 mm i.d., with Cu/Ni/Pt wires inside (activated by oxygen), reactor temperature 960°C] and different GC conditions were employed: split injection (10:1), initial oven temperature at 40 °C for 3.8 min, ramp at 30 °C/min to 100 °C. Helium was used as carrier gas at a constant flow rate of 1.8 mL/min.

A tank of high purity carbon dioxide 4.8 (Kohlendioxid ISO-TOP, Air Liquide) was used as the working reference gas.

All the $\delta^{13}C$ values were normalised by two-point linear calibration using the same CH_3I working standards described above, relative to VPDB. The $\delta^{13}C$ values of the CH_3I working standards were calibrated against international reference standards (NBS 19 [$\delta^{13}C_{VPDB}$ = 1.95 ± 0.05 mUr] and NBS 22 [$\delta^{13}C_{VPDB}$ = -30.03 ± 0.04 mUr]) by EA-IRMS at BGC-IsoLab. The calibrated $\delta^{13}C$ values in mUr vs VPDB for the two CH_3I working standards were -70.04 ± 0.13 mUr (n=4, 1σ) and -60.72 mUr ± 0.05 mUr (n=7, 1σ).

Hydrogen isotope exchange experiments

To test if there was hydrogen exchange between the two methyl sulfate salts and water in humid air during storage, both salts were stored over a vessel containing water of known δ^2H value in a desiccator under vacuum for three weeks (100 % humidity). The exact δ^2H value of the water (vapour) did not need to be known (exactly) because the aim of this experiment was to see if the post-experiment δ^2H values of either compound or even both had changed significantly. Both sulfate salts dissolved shortly after the desiccator was evacuated due to hygroscopicity. Therefore, after the desiccator was vented the vessel containing water was exchanged for a vessel containing drying agent (silica gel) to allow both salts to re-crystallize. Subsequently both salts were analyzed at HU (GC/TC-IRMS) and the δ^2H values before and after the storage experiment were compared.

Results and discussion

The stable hydrogen and carbon isotope ratios of sodium methyl sulfate (HUBG1) and potassium methyl sulfate (HUBG2) were measured using two different analytical techniques (HTC- and EA-IRMS and CF-IRMS). While the bulk material of the methyl sulfates was pyrolysed (δ^2H values) and oxidised ($\delta^{13}C$ values) during HTC- and EA-IRMS analysis, CH_3I (δ^2H and $\delta^{13}C$ values) was analysed using CF-IRMS following its formation by reaction of the methyl sulfates with HI. The results, which are shown in Table 1, are compared and discussed in detail below.

Stable hydrogen isotope results

The δ^2H_{VSMOW} values for hydrogen of HUBG1 and for HUBG2 measured by HTC-IRMS are -144.5 ± 1.2 mUr (n=30) and -102.0 ± 1.3 mUr (n=32), respectively. The errors shown represent the combined uncertainties which were calculated by error propagation containing contributions from the precision of replicate measurements, the bias of experimental processes, the uncertainty of δ-values in reference materials used to fix and normalize the δ – scale and the algorithms applied to correct and normalize the data [38]. The complete propagated uncertainty of the methyl sulfates $U_{HUBG1/2}$ is calculated as:

$$U_{HUBG1/2} = \sqrt{u_{CRM}^2 + u_{HS}^2 + u_{HUBG1/2}^2}$$

where u_{CRM} indicates the uncertainty of the in-house standards analysed against primary reference standards, u_{HS} indicates the measurement uncertainty of the in-house standards, and $u_{HUBG1/2}$ indicates the measurement uncertainty of the methyl sulfates HUBG1 and HUBG2. All uncertainties are multiplied by the Student's factor (t) at a 90 % confidence limit to account for the limited number of analyses.

The methyl sulfate values measured at BGC-IsoLab were normalised to the SMOW/SLAP isotopic δ scale using a two point calibration based on two in-house reference standards, Pet-J1 (polyethylene powder; -78.2 ± 0.4 mUr) and Jena-Pristane (Pristane; -362.6 ± 0.5 mUr). Both standards are calibrated against VSMOW2 (0 ± 0.3 mUr) and SLAP2 (-427.5 ± 0.3 mUr). IAEA-CH7 (-100.3 ± 2 mUr) was interspersed throughout the samples and used as a quality control.

For comparison, the δ^2H values of HUBG1 and HUBG2 analyzed by GC/TC-IRMS (measured as CH_3I generated by the 'Zeisel-method') are -143.2 ± 3.3 mUr and -102.5 ± 3.2 mUr. These values are in very good agreement with the values obtained by HTC-IRMS. This excellent data agreement might be explained by the fact that the CH_3I working standards used to normalise the results of the GC/TC-IRMS measurements at the laboratory of Heidelberg University were previously calibrated at BGC-IsoLab (see Material and Method section), the same laboratory where the HTC-IRMS measurements of HUBG1 and HUBG2 in this study were conducted. Moreover, the two CH_3I working standards applied to correct the δ^2H values for scale-compression for two-point normalisation cover a range of -66.2 to -173.0 mUr and thus include the δ^2H values of HUBG1 and HUBG2 (-143.2 mUr and -102.5 mUr).

The above provided errors for GC/TC-IRMS measurements represent the combined uncertainties which were calculated by error propagation including the same contributions as mentioned for the EA-IRMS measurements. These are somewhat higher than the HTC- and EA-IRMS measurements (3.3 versus 1.2 mUr, and 3.2 versus 1.3 mUr, respectively, for HUBG1 and HUBG2). However, the precision of the replicate measurements ('external precision' including chemical replication uncertainty) of δ^2H values is 1.5 and 1.0, respectivel,y for HUBG1 and HUBG2. This is in the same range as previously reported for substances such as vanillin, pectin and wood [34].

Exclusion of hydrogen exchange between methoxy groups and ambient water during storage

The 'hydrogen-exchange-experiment' (for detailed description refer to Material and Methods section) and GC/TC-IRMS measurements for both HUBG1 and HUBG2 before and after the experiment were all performed at HU. After a time period of three weeks where both sulfate salts were stored over water of known isotopic signature (δ^2H_{VSMOW} = -59.5 mUr) the stable hydrogen isotope ratios did not change (Table 2). This unambiguously indicates that there was no hydrogen exchange between ambient water and the methyl group of the sulfates during the storage experiment. This is in agreement with previous considerations and measurements that esterified methyl groups are considered to be chemically stable in that the hydrogen atoms of the methoxy moiety do not exchange with those of plant water during ongoing metabolic reactions in the plant [11][34]. This experiment shows that the methyl sulfate salts can be reliably utilised as reference materials for normalization of stable hydrogen isotope data of organic matter containing methoxy groups if the 'Zeisel method' is employed for formation of CH_3I. Nevertheless, if the δ^2H values of methyl sulfate salts are measured by HTC-EA-IRMS it is mandatory to carefully remove any adsorbed water prior to analysis.

Stable carbon isotope results

The $\delta^{13}C_{VPDB\text{-}LSVEC}$ values for HUBG1 and HUBG2 measured by EA-IRMS are -50.31 ± 0.16 mUr (n=14) and +1.60 ± 0.12 mUr (n=16), respectively. The errors shown were calculated in the same way as those provided for the hydrogen isotopic ratios. They represent the combined uncertainties containing the same contributions as mentioned for the hydrogen

measurements above. The methyl sulfates measured at the BGC-IsoLab by EA-IRMS were calibrated to the VPDB scale using the reference material IAEA-603 (+2.46 ± 0.01 mUr) and the in-house standard as the second scale anchor (Acetanilide; -30.06 ± 0.1 mUr), which was calibrated against the two international secondary standards NBS 22 (-30.03 ± 0.04 mUr) and LSVEC (-46.6 mUr). As of June 2018 IUPAC no longer recommends the use of LSVEC as a scaling standard for the VPDB scale (Press release; http://iupac.org/standard-atomic-weights-of-14-chemical-elements-revised). LSVEC has previously been shown to be an inadequate $\delta^{13}C$ isotopic standard [49],[50]. Over time, LSVEC carbon atoms exchange with atmospheric CO_2 carbon atoms. This causes a gradual contamination and ^{13}C-isotopic fractionation of LSVEC. The BGC-Isolab is currently involved in an effort to produce a replacement standard for LSVEC. The envisaged replacement is USGS44, a Merck high purity $CaCO_3$ with a preliminary value of -42.15 ± 0.05 mUr, available at https://isotopes.usgs.gov. It is likely that USGS44 will replace LSVEC in the future. Therefore, BGC-IsoLab also analysed HUBG1 and 2 using USGS44 as a second scale anchor. The reader should note that the provided USGS44 value is a preliminary value and may change slightly upon its final publication. However, if necessary, users of HUBG 1 and 2 can easily recalculate the values of HUBG1 and 2. The preliminary $\delta^{13}C$ values of HUBG1 and HUBG2, calibrated against IAEA-603 and scaled to USGS44 (-42.15 ± 0.05 mUr) are -50.17 ± 0.08 mUr and +1.60 ± 0.05 mUr, respectively (cf. Table 1).

Comparable with the measurement of the $\delta^{2}H$ values, the $\delta^{13}C$ values of HUBG1 and HUBG2 were also measured by GC/TC-IRMS after conversion to CH_3I by the 'Zeisel-method'. The $\delta^{13}C$ values are -47.73 ± 0.16 mUr for HUBG1 and +4.42 ± 0.19 mUr for HUBG2. These data show an offset of 2.58 mUr and 2.82 mUr, respectively, from the $\delta^{13}C$ values measured at BGC-IsoLab. This offset can be explained by the fact that the results of GC/TC-IRMS measurements at laboratory of Heidelberg were normalised using the same two CH_3I working standards which were used for normalisation of the stable hydrogen measurements. However, the range of $\delta^{13}C$ values of the CH_3I working standards (-70.04 mUr and -60.72 mUr) is narrow and unfortunately does not span the isotope range of the methyl sulfates (-47.73 mUr and +4.42 mUr) and thus these standards are not ideal to correct for scale-compression. This probably caused the differences in the $\delta^{13}C$ values measured by the two laboratories. However, we note that in each instance the offsets in the $\delta^{13}C$ values for HUBG1 and 2 are more positive by nearly the same value. This may indicate that when liquid CH_3I is applied as a reference material (1-2µL of liquid CH_3I is injected into a 1.5-mL vial

and a fraction of headspace gas injected into the GC/IRMS system; for details refer to the method section) to normalize samples prepared by the "Zeisel-Method" other factors causing ^{13}C fractionation might be involved. Thus, again this would highlight that it is important to follow the principle of identical treatment of reference materials and samples [39]. The adoption of HUBG1 and 2 as reference standards would facilitate following the IT Principle when analysing methoxy groups.

The above provided errors for GC/TC-IRMS measurements represent the combined uncertainties which were calculated by error propagation including the same contributions as mentioned for the EA-IRMS measurements. These are in the same range as the EA-IRMS measurements (0.16 versus 0.16 and 0.19 versus 0.12 for HUBG1 and HUBG2, respectively).

Conclusion

There is a lack of suitable reference materials to reliably normalise stable carbon and hydrogen isotope measurements of plant methoxy groups. In order to rectify this situation we conducted investigations with two methyl sulfate salts to determine if they could be adopted for this purpose. Both salts were calibrated against international stable isotope reference materials by HTC- and EA-IRMS to be δ^2H_{VSMOW} = -144.5 ± 1.2 mUr and $\delta^{13}C_{VPDB}$ = -50.31 ± 0.16 mUr for HUBG1 and δ^2H_{VSMOW} = -102.0 ± 1.3 mUr and $\delta^{13}C_{VPDB}$ = +1.60 ± 0.12 mUr for HUBG2, respectively. Quantities of each salt ranging between 0.52 and 0.65 mg for $\delta^{13}C$ values and between 6.91 and 7.10 mg for δ^2H values were used for single EA-IRMS measurements.

Using a different analytical approach where the methyl groups of the methyl sulfate salts were converted to CH_3I by reaction with HI and subsequent analysis of the generated CH_3I using GC/IRMS we obtained δ^2H_{VSMOW} values of -143.2 ± 3.3 mUr (HUBG1) and -102.5 ± 3.2 mUr (HUBG2) using sample amounts of ~5mg of each salt. These results are in excellent agreement with the values measured by HTC-IRMS. The precision ('external precision' including chemical replication uncertainty) for δ^2H values of replicate measurements (n=14) obtained by GC/IRMS is ±1.5 mUr and ±1.0 mUr for HUBG1 and HUBG2, respectively.

To measure the stable carbon isotope ratios of methoxy groups using GC/IRMS, approximately 2mg of each salt is required. The results show an offset of about 2 to 3 mUr

from the EA-IRMS measurements which might be explained by the lack of available suitable reference materials required to correct for scale compression for GC/IRMS analysis. The precision of the replicate measurements of $\delta^{13}C$ values obtained by GC/IRMS is ±0.21 mUr, and ±0.40 mUr, for HUBG1 and HUBG2, respectively.

The results regarding stable carbon isotope analysis might have a substantial impact on the $\delta^{13}C$ values of plant methoxy groups that have been previously reported [7],[12],[14],[19],[25],[26],[35],[51]. Most of the $\delta^{13}C$ values of wood methoxy groups that have been measured in the past (~ranging from -20 to -30 mUr) were normalised with CH_3I reference materials with very negative $\delta^{13}C$ values (~ -60 to -70 mUr) may need to be corrected by 2 to 3 mUr towards more negative values. For example, these corrections would bring the $\delta^{13}C$ values of wood methoxy groups presented in the studies of Gori *et al* [12] and Mischel *et al* [14] closer to the respective $\delta^{13}C$ values of plant cellulose and whole wood.

An additional experiment clearly showed that the hydrogen atoms of the methyl sulfate salts do not exchange with hydrogen from atmospheric water vapour. Thus the storage and preparation of HUBG 1 and 2 standards are much easier than the storage and preparation of CH_3I, which evaporates easily, and is unstable, releasing I_2 when in contact with air. Furthermore, both sodium- and potassium methyl sulfate are much less harmful than CH_3I, which is thought to cause cancer (Sigma Aldrich SDS sheet 2018). This is another important and practical reason to find a replacement for CH_3I. Furthermore, using methyl sulfates for generation of CH_3I is advantageous because these standards allow the user to follow the IT Principle.

The $\delta^{13}C$ values determined for HUBG1 and HUBG2 span a relatively wide range of $\delta^{13}C$ values (-50 to +2 mUr) thus covering most of the natural $\delta^{13}C$ values of terrestrial plant methoxy groups that have been reported so far. The respective $\delta^{2}H$ values of HUBG1 and HUBG2 cover a narrow range on the δ-scale for $\delta^{2}H$ value measurements of methoxy groups that lie in the range of ~ -100 to -150 mUr. Therefore, in future work we aim to prepare several alternative wood reference materials from subtropical and temperate regions that ideally span a range of $\delta^{2}H_{VSMOW}$ values from around -200 to -350 mUr.

Following the recommendations for selecting reference materials for IRMS analysis as outlined in the introduction we recommend that the two methyl sulfate salts HUBG1 and HUBG2 should be adopted as suitable reliable reference materials to normalise the $\delta^{13}C$ values of methoxy groups. For stable hydrogen isotope measurements of plant methoxy

groups we suggest that next to HUBG1 and HUBG2 additional reference materials are required to cover the full range of δ^2H values of plant methoxy groups reported so far. Moreover, the two investigated methyl sulfates might also serve as reference materials for bulk measurements of the δ^{13}C and δ^2H values of organic matter.

Acknowledgments

This study was supported by the German Science Foundation DFG (KE 884/6-3, KE 884/8-2)

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Table 1. Stable hydrogen and carbon isotope values of HUBG1 and HUBG2 analysed by HTC- and EA-IRMS (MPI-BGC) and CF-IRMS (HU-BGC).

HUBG1				**HUBG2**			
MPI-BGC[1]		*HU-BGC*[2]		*MPI-BGC*[1]		*HU-BGC*[2]	
δ^2H_{VSMOW}	n	δ^2H_{VSMOW}	n	δ^2H_{VSMOW}	n	δ^2H_{VSMOW}	n
-144.5 ± 1.2 mUr	30	-143.2 ± 3.3 mUr	14	-102.0 ± 1.3mUr	32	-102.5 ± 3.2 mUr	14
$\delta^{13}C_{VPDB}$	n	$\delta^{13}C_{VPDB}$	n	$\delta^{13}C_{VPDB}$	n	$\delta^{13}C_{VPDB}$	n
-50.31 ± 0.16 mUr	14	-47.73 ± 0.16 mUr	35	+1.60 ± 0.12 mUr	16	+4.42 ± 0.19 mUr	35
-50.17 ± 0.08 mUr[3]	*14*	---	---	*+1.60 ± 0.05 mUr*[3]	*16*	---	---

[1] *IsoLab at the Max Planck Institute for Biogeochemistry, Jena, Germany*

[2] *Heidelberg University, Institute of Earth Sciences,*

[3] *values calibrated against IAEA-603 and scaled to USGS44*

Table 2. Results of the 'hydrogen-exchange-experiment' measured by GC-HTC-IRMS (HU).

	HUBG1	HUBG2
δ^2H_{VSMOW}	-142.4 ± 1.4 mUr	-100.1± 1.5 mUr
n	15	15
	↓ ↓ ↓ ↓ ↓	↓ ↓ ↓ ↓ ↓
storage in desiccator for 3 weeks (under vacuum, over water*) initial δ^2H value of the water = -59.5 mUr		
	↓ ↓ ↓ ↓ ↓	↓ ↓ ↓ ↓ ↓
δ^2H_{VSMOW}	-142.9 ± 0.9 mUr	-100.8 ± 1.4 mUr
n	15	15

***** *water with a ^2H signature significantly different from that of both HUBG1 and HUBG2*

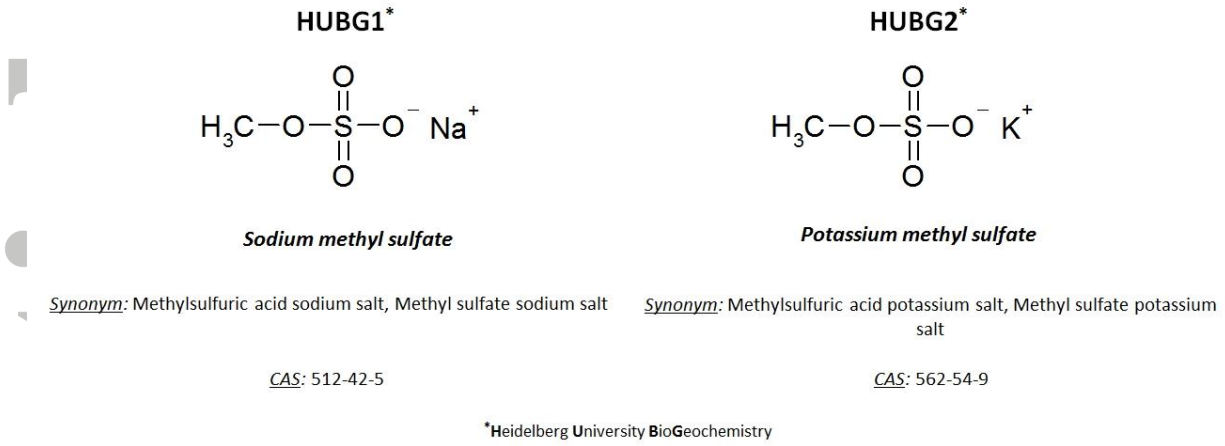

Figure 1: Information on the investigated methyl sulfates.