Isotope effects associated with the preparation and methylation of fatty acids by boron trifluoride in methanol for compound-specific stable hydrogen isotope analysis via gas chromatography/thermal conversion/isotope ratio mass spectrometry

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RATIONALE: Compound-specific stable hydrogen isotope analysis of fatty acids is being used increasingly as a means of deriving information from a diverse range of materials of archaeological, geological and environmental interest. Preparative steps required prior to isotope ratio mass spectrometry (IRM) analysis have the potential to alter determined δD values and hence must be accounted for if accurate δD values for target compounds are to be obtained.

METHODS: Myristic, palmitic, stearic, arachidic and behenic saturated fatty acids were derivatised to their respective fatty acid methyl esters (FAMEs). Extraction of saturated fatty acids and acyl lipids from samples, subsequent hydrolysis, then separation on a solid-phase extraction cartridge, was found to alter the determined δD values by less than one standard deviation.

RESULTS: Derivatisation was found to alter the hydrogen isotopic composition of FAMEs although this effect was reproducible and can be accounted for. The difference between the mean corrected and mean bulk δD values was always less than 6.7‰. Extraction of saturated fatty acids and acyl lipids from samples, subsequent hydrolysis, then separation on a solid-phase extraction cartridge, was found to alter the determined δD values by less than one standard deviation.

CONCLUSIONS: Overall, it has been shown that for natural abundance hydrogen isotope determinations, the isolation and derivatisation of extracted fatty acids alters the determined δD values only by a numerical increment comparable with the experimental error. This supports the use of the described analytical protocol as an effective means of determining fatty acid δD values by GC/TC/IRMS. Copyright © 2012 John Wiley & Sons, Ltd.

Gas chromatography/thermal conversion/isotope ratio mass spectrometry (GC/TC/IRMS) is emerging as a routine technique for the determination of the compound-specific stable hydrogen isotopic composition of fatty acids from a variety of materials (e.g.1–6). However, prior to the determination of hydrogen isotopic compositions by GC/TC/IRMS, the fatty acids of interest must be extracted from the sample, isolated and derivatised. The purpose of this study is to address concerns regarding the preservation of hydrogen isotope ratios in saturated fatty acids during sample preparation and derivatisation.

Hydrogen atoms, as part of organic molecules, can be exchangeable (e.g.7), with hydrogen atoms associated with hydroxyl and carboxyl groups being particularly so. These extremely exchangeable hydrogen atoms rapidly and continuously exchange with ambient hydrogen atoms, thereby affecting the δD value of the molecule as a whole and complicating the determination of stable hydrogen isotopic ratios. To avoid this problem, the exchangeable hydrogen can be removed8] or equilibrated with hydrogen of known isotopic composition.9]

Where stable isotope analyses use a compound-specific approach, polar compound classes, such as fatty acids, are typically converted into less polar derivatives to make them more amenable to GC analysis.10] Conveniently, common derivatisation methods, such as methylation11] or trimethylsilylation12] of fatty acids, also remove any exchangeable hydrogen atoms. However, a correction must be made to the measured δD values of derivatised compounds to account for the introduction of any hydrogen added during derivatisation, and any fractionation that may occur during derivatisation.

Fractionation will only occur during a process that is not quantitative, therefore, in order to prevent fractionation, derivatisation reactions are chosen so that they are quantitative with respect to the sample molecule. Boron trifluoride in methanol11] is a common methylating reagent for fatty acids and as such will be used for this study to enable future integration with carbon isotope analysis. The conversion rates for this reaction are reported to be around 99%.11,13] However, there is still scope for a kinetic isotope effect with respect to the added methyl hydrogen, derived from methanol in
many-fold excess over the fatty acid, although only a secondary kinetic isotope effect (with respect to hydrogen) is possible during this esterification, as the bond formed during the reaction is between the carboxyl carbon and the incoming methoxide oxygen. For deuterium, the primary kinetic isotope effects are of the order of 1.4–1.8 and secondary isotope effects are around 1.05–1.1.[14]

Published studies using GC/TC/IRMS for the compound-specific stable hydrogen isotope analysis of fatty acids have used several different methods for derivatisation (e.g. trimethylsilylation using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)).[15] Methylation via 2% sulphuric acid in methanol;[5] methylation via 14% w/v boron trifluoride in methanol;[16] however, little discussion about correction factors vis a vis fatty acids has been presented. In two further studies, where methylation was achieved using acetyl chloride in methanol[2] and 2% hydrochloric acid in anhydrous methanol,[16] correction for the derivatisation is achieved using parallel methylation of phthalic and succinic acid standards, of known isotopic composition, respectively. Further work on the compound-specific hydrogen isotope analysis of fatty acids cites one of the above studies (e.g.[5,17,18]).

The use of low molecular weight diazides to determine a correction factor for methylation, as used in the studies of Sessions et al.[2] and Huang et al.[16] mentioned above, is employed to increase the ratio of added methyl hydrogen to analyte hydrogen in the diester product. This reduces the error in the determination of the δD value of the methyl hydrogen. However, any fractionation occurring during derivatisation will be undetectable as the δD value of the added methyl hydrogen is unknown. To address this, Huang et al.[16] performed the methylation over a range of times and temperatures and did not observe any change in the measured δD value of the additional hydrogen greater than analytical error.

Most of the methyllating agents used for compound-specific stable isotope studies are acidic (including boron trifluoride in methanol), which presents further problems for the hydrogen isotope analysis of fatty acids. Under acidic conditions, the hydrogen α to the functional group of a fatty acid can undergo exchange with ambient hydrogen via tautomerism to the enol.[19] Obviously, any α-hydrogen exchange during methylation has to be reproducible for it to be accounted for, as must any change in δD value caused by derivatisation.

It is not only during methylation that the hydrogen isotopic composition of the fatty acids may be altered. The extraction and necessary workup of the compounds of interest also involve reactions and methods that induce fractionation or exchange of hydrogen from the analyte molecule.[20]

Typically, to analyse fatty acids absorbed in environmental matrices and artefacts, the sample is crushed before any lipids within are solvent extracted. This lipid extract is then saponified, where any acyl lipids are hydrolysed to their corresponding fatty acids, prior to being separated into acid and neutral fractions. The acid fraction, containing the fatty acids, is then derivatised and analysed by GC, GC/MS and GC/IRMS. In particular, the saponification step requires harsh conditions that involve heating the extracted lipids for 1 h at 70 °C in 0.5 M sodium hydroxide in methanol.

Although unlikely because of low chromatographic resolution, during the separation of the acid and neutral fractions by solid-phase extraction there is the possibility of chromatographic isotope fractionation.[21] Furthermore, after each step during sample preparation the lipid extract solution is typically evaporated to dryness under a gentle flow of nitrogen. This may cause slight evaporation of the analyte and possibly fractionation; fatty acid methyl esters (FAMEs) are particularly susceptible to this because of their volatility.

Therefore, the aims of this study were to: (i) to determine a reproducible correction factor for the methylation of saturated fatty acids by boron trifluoride in methanol and (ii) to establish if extraction and sample preparation (other than derivatisation) affects the δD values of fatty acids, and to correct for this or develop alternative procedures if required.

**EXPERIMENTAL**

**Correcting for changes in the hydrogen isotope composition caused by derivatisation**

Sodium arachidate (95 mg) and sodium behenate (95 mg) were synthesised from their respective fatty acids (0.11 g; Sigma-Aldrich Ltd., Poole, UK) in methanol (10 mL) by the addition of sodium methoxide solution (0.5 M in MeOH; 1 equivalent; Sigma-Aldrich) before evaporation of the solvent. The δD values, as determined by elemental analysis (hereafter referred to as bulk δD values), of these sodium salts, along with those for sodium myristate, sodium palmitate and sodium stearate (all Sigma-Aldrich), were obtained by total conversion into hydrogen at 1080 °C in a quartz reactor lined with a glassy carbon film, filled to a height of 180 mm with glassy carbon chips. Hydrogen was separated from other gaseous products on a GC column packed with a 5 Å molecular sieve at a temperature of 50 °C. The mass spectrometer used was a Geo 20–20 (Europa Scientific Ltd., Crewe, UK) with a Faraday cup collector array to monitor m/z 2 and 3. Determined δD values were referenced to IA-R002 (Iso-Analytical mineral oil, ISO-Analytical Ltd., Crewe, UK; traceable to NBS22 mineral oil). IA-R002 and IAEA-CH-7 (International Atomic Energy Agency, Vienna, Austria) were run as quality control check samples during batch analysis of the sodium salts. The bulk δD values were determined by Iso-Analytical Ltd.

Sodium myristate (n = 11; 0.011–2.995 mg), sodium palmitate (n = 6; 0.082–0.695 mg), sodium stearate (n = 5; 0.224–1 mg), sodium arachidate (n = 6; 0.011–0.191 mg) and sodium behenate (n = 6; 0.041–0.090 mg) were dissolved in MeOH (~2 mL) and acidified with HCl (3 M; ~10 μL) to pH 3, before extraction into cyclohexane (3 × 2 mL). Water (1 mL) was added after acidification to increase the separation of the organic and aqueous layers during extraction. The extracts were evaporated under a gentle stream of nitrogen before derivatisation to the corresponding FAME by heating (70 °C; 1 h) with boron trifluoride in methanol (100 μL; 14% w/v; Sigma-Aldrich; Batch #083K5300). After heating, the reaction was cooled, quenched with dichloromethane (DCM)-extracted double-distilled water (2 mL) and the FAMEs extracted into hexane (3 × 2 mL). Hexane was then removed from the FAME solutions under a gentle stream of nitrogen and the residues redissolved in hexane immediately prior to analysis by GC/TC/IRMS.
Correcting for changes in δD values during extraction and workup

From a standard solution of fatty acids (caprylic acid, 14 μL; palmitic acid, 16.5 mg; stearic acid, 18.6 mg; behenic acid, 16.2 mg; lignoceric acid, 11.3 mg; all Sigma-Aldrich), in 2:1 v/v DCM/MeOH (25 mL) six 3 mL aliquots were taken, and the remaining standard solution was stored. An aliquot (1 mL) was removed, evaporated under nitrogen and methylated, as above, and this raw fatty acid fraction was analysed by GC/TC/IRMS. The remaining 2 mL of each aliquot was evaporated to dryness under nitrogen and, to mimic the extraction of lipids from a sample, was extracted into DCM/MeOH (2:1 v/v; 10 mL; 15 min sonication). The extraction of lipids from a sample was extracted into DCM/MeOH (25 mL) six 3 mL aliquots were taken, and the remaining standard solution was stored. An aliquot (1 mL) was removed, evaporated under nitrogen and methylated, and analysed by GC/TC/IRMS. The remaining four-fifths of the lipid extract was hydrolysed with 0.5 M NaOH in 10:1 v/v MeOH/water solution (2 mL; 70 °C, 1 h), cooled and then acidified to pH 3 with 3 M HCl. Cyclohexane was used to extract the saponified lipids (3 × 3 mL) and these extracts were then combined and the excess solvent evaporated under nitrogen. A further one-fifth of this saponified lipid was removed, methylated and analysed as above.

Finally, the remaining four-fifths of the saponified lipid was separated into acid and neutral fractions using aminopropyl solid-phase extraction columns (6 mL glass reservoir; 500 mg sorbent mass; Biotage Isolute; Biotage, Uppsala, Sweden) that had first been rinsed with hexane (6 mL). The neutral fraction was eluted first with chloroform/isopropanol (2:1 v/v; 6 mL) and the acid fraction was then eluted with 2% acetic acid in diethyl ether (6 mL). Solvent was removed from these hydrolysed, separated fractions under nitrogen, prior to methylation and analysis of the acid fraction as before. For this experiment, the nitrogen flow used for evaporation was more severe than would normally be used.

GC/TC/IRMS

Stable hydrogen isotope ratios were determined by GC/TC/ IRMS performed using an Agilent 6890 gas chromatograph (Agilent Technologies UK Ltd., Wokingham, UK) coupled to a ThermoQuest Finnigan DeltaPlus XL (Thermo Fisher Scientific, Bremen, Germany) isotope ratio mass spectrometer, via a thermal conversion reactor (300 × 0.5 mm i.d.; Al2O3 1450 °C) and ThermoQuest Finnigan GC Combustion III interface.

Samples were introduced into the gas chromatograph via an Agilent PTV injector (splitless mode; 50–300 °C; purge time 1 min) and later an Agilent split/splitless injector (splitless mode; 300 °C; purge time 2 min). A fused-silica capillary column (30 m × 0.25 mm i.d.) with a 100% dimethylpolysiloxane stationary phase (Zebron ZB-1; 0.25 μm film thickness; Phenomenex Inc., Torrance, CA, USA) was used; the column flow was constant at 0.9 mL min⁻¹. The temperature programme used comprised an initial isothermal of 2 min at 50 °C followed by a ramp of 10 °C min⁻² to 300 °C and a final isothermal at this temperature for 10 min. The interface was set to backflush mode for the first 500 s of each run.

The mass spectrometer ion source pressure was 2.2 × 10⁻⁶ mbar and the electron ionization potential was 3 kV. Faraday cups were used for the detection of ions of m/z 2 (H₂) and m/z 3 (HD⁻) with ion centre performed using the HD⁻ ions. A retardation lens removed scattered (or low-energy) 4He⁺ ions. To correct for HD⁻ ions a calibration was performed daily with the Thermo ISODAT NT 2.0 application (Thermo Fisher Scientific) using pulses of laboratory tank H₂ gas over a range of 2000–10 000 mV for the m/z 2 ion beam. The HD⁻ factor was typically around 3.5 ppm V⁻¹ and had a rate of change of less than 0.1 ppm V⁻¹ day⁻¹.

To condition the reactor, two early eluting compounds (n-pentadecane and ethyl capricate; both Sigma-Aldrich) were co-injected with every run, to give an m/z 2 response of ~8000 mV while a standard (ethyl arachidate; Sigma-Aldrich) was also co-injected with sample runs. New reactors or reactors requiring significant conditioning were conditioned as follows: using a 10:1 split, with the interface split open, 1 mL of hexane was injected three times into the system (after[22]). As the front of the hexane peak was detected, the interface was switched to backflush mode and left for 5 min before the next injection.

Results were acquired and presented, in per mil, as δD values, using ISODAT NT 2.0. Data were initially calibrated to six laboratory tank H₂ peaks introduced directly into the ion source (three at the beginning and three at the end of each run), before being normalised to the V-SMOW-SLAP line using the equation of a line from a plot of measured against known δD values for a standard suite of 15 n-alkanes (C₁₅–C₃₀) mixture B, Arndt Schimmelmann, University of Indiana, Bloomington, IN, USA) injected after every two sample runs. The instrument root mean square (RMS) error was typically less than 5 %, defined as the RMS error in the measured δD values of the same n-alkane standard.[1]

As determined using a series of caprylic, palmitic, stearic, behenic and lignoceric fatty acid methyl esters repeatedly analysed by GC/TC/IRMS over a range of concentrations, the δD values produced by this instrument were approximately linear within the m/z 2 range 1000–6500 mV, with the flattest part of the curve appearing over the range 3500–5500 mV. Above 6500 mV the measured δD values became more positive at a rate of ~2.5 % V⁻¹. Furthermore, for a response of less than 2000 mV the standard deviation of repeat measurements became significantly larger than the instrument RMS error. Therefore, the samples were analysed at concentrations such that the response for compounds of interest was kept within the range 2000–6500 mV, with an optimum response of 3500–6000 mV; equivalent to ~400 ng per component.

Individual fatty acids were analysed as FAMEs and their δD values (presented as per mil) calculated by correcting for additional methyl hydrogen and any fractionation induced during methylation, as discussed in the Results and Discussion below. All samples were analysed at least in triplicate (typically in quadruplicate) and any outliers (as determined using Grubbs’ test) or data from analytical runs where the adjacent standard showed instrument RMS error greater than 8 % were discarded.
RESULTS AND DISCUSSION

Correcting for changes in the hydrogen isotope composition caused by derivatisation

Ideally, to correct for changes in δD values caused by the methylation of fatty acids to the corresponding methyl esters, using boron trifluoride in methanol, fatty acids of known isotopic composition would be methylated using methanol of known isotopic composition and the δD value of the resulting methyl ester determined. For fatty acids of natural hydrogen isotopic abundance the delta notation can be used and if no isotope effects, fractionation or competing reactions are observed, the following mass balance approximation is valid:

\[ n_{\text{FAME}}\delta D_{\text{FAME}} = n_{\text{FA}}\delta D_{\text{FA}} + n_{\text{Me}}\delta D_{\text{Me}} \]  

(1)

where \( \delta D_{\text{FAME}} \) is the δD value of the product FAME, \( \delta D_{\text{FA}} \) is the δD value of the initial fatty acid, and \( \delta D_{\text{Me}} \) is the δD value of the additional methyl hydrogens (derived from the methanol), all presented in per mil, and \( n \) is the number of non-exchangeable hydrogen atoms in each molecule or moiety. Thus, \( n \) for a fatty acid, \( n_{\text{FA}} \), is one less than the total number of hydrogen atoms in the molecule, to account for the extremely exchangeable carboxyl hydrogen. \( n_{\text{Me}} = 3 \), so:

\[ n_{\text{FAME}}\delta D_{\text{FAME}} = n_{\text{FA}}\delta D_{\text{FA}} + 3\delta D_{\text{Me}} \]  

(2)

If the above equation does not balance, an isotope effect, fractionation or a competing reaction must be occurring and a correction factor, \( K \), should be introduced to the equation, representing the mean change in the δD value of all non-exchangeable hydrogen in the resultant FAME caused during derivatisation. Thus, giving:

\[ n_{\text{FAME}}\delta D_{\text{FAME}} = n_{\text{FA}}\delta D_{\text{FA}} + 3\delta D_{\text{Me}} + n_{\text{FAME}}K \]  

(3)

Because of the non-linearity of the delta scale, the above expression is only valid for δD values of natural abundance and where any fractionation is small. Ideally, the value of \( \delta D_{\text{FAME}} \) and therefore \( K \) should be reproducible thus allowing a reproducible correction for the methylation of a fatty acid using a particular batch of BF₃·MeOH.

Although an equivalent method to that above is used for determining the correction needed for methylation during compound-specific stable carbon isotope studies, it is not possible to apply the above to hydrogen isotope determinations. First, it is not possible to accurately determine the bulk δD value of the fatty acid because of the extremely exchangeable carboxyl hydrogen; although we can easily substitute the fatty acid for its sodium salt. Second, the methanol possesses an extremely exchangeable hydroxyl hydrogen, which cannot be easily substituted without affecting the reaction. Alternatively, a sample of the methanol could be taken and the hydroxyl group substituted before bulk analysis; nevertheless, it is difficult to see how this could be achieved without inducing fractionation. Therefore, the reaction was performed on the sodium salt of the fatty acid without determining \( \delta D_{\text{Me}} \). This would still enable a correction factor to be obtained but without separation of the \( \delta D_{\text{Me}} \) and \( K \) components. Thus:

\[ n_{\text{FAME}}\delta D_{\text{FAME}} = n_{\text{FA}}\delta D_{\text{FAsalt}} + [3\delta D_{\text{Me}} + n_{\text{FAME}}K] \]  

(4)

where \([3\delta D_{\text{Me}} + n_{\text{FAME}}K]\) cannot be expanded. Note that \( n_{\text{FAsalt}} \) and \( n_{\text{FA}} \) are numerically identical. Furthermore, if we define:

\[ \delta D_{\text{Me}}^{\text{Effective}} = \left( \delta D_{\text{Me}} + \frac{n_{\text{FAME}}K}{3} \right) \]  

(5)

then

\[ n_{\text{FAME}}\delta D_{\text{FAME}} = n_{\text{FAsalt}}\delta D_{\text{FAsalt}} + 3\delta D_{\text{Me}}^{\text{Effective}} \]  

(6)

\( \delta D_{\text{Me}}^{\text{Effective}} \) contains all the terms that will have an influence on the δD value of the FAME produced from a given fatty acid sodium salt and it can easily be calculated from the δD values of the product FAME and the initial fatty acid sodium salt. Moreover, a plot of \( \delta D_{\text{Me}}^{\text{Effective}} \) against the number of non-exchangeable hydrogen atoms per methyl ester, \( n_{\text{FAME}} \), will have a slope of \( K/3 \) and an intercept of \( \delta D_{\text{Me}} \) and a plot of \( \delta D_{\text{Me}}^{\text{Effective}} \) against the carbon chain length of the fatty acid, \( N_{\text{FA}} \) (\( n_{\text{FAME}} = 2N_{\text{FA}} + 2 \) for methyl esters produced from saturated fatty acids), will have a slope of \( 3K/2 \). If there is no fractionation associated with the methylation the slope of either of these plots will be zero.

Because of the acidic conditions encountered during methylation with BF₃·MeOH (BF₃ is a Lewis acid) it is likely that the substitution of the fatty acid with its sodium salt may affect the reaction. The sodium salt must be protonated before methylation can proceed, the by-product of which will be one equivalent of sodium methoxide. The formation of sodium methoxide will increase the pH of the reaction mixture, moving the equilibrium of the reaction away from formation of the methyl ester and increasing the likelihood of incomplete conversion of the fatty acid. To avoid this, determination of \( \delta D_{\text{Me}}^{\text{Effective}} \) was performed using acidified sodium salts of fatty acids: i.e. fatty acids produced by the acidification of fatty acid sodium salts of known δD value. This method introduces its own uncertainties: specifically, during acidification the α-hydrogen of the fatty acid may undergo exchange with ambient hydrogen, via acid induced tautomerism, potently affecting the δD value of the fatty acid even before methylation.

Therefore, to calculate \( \delta D_{\text{Me}}^{\text{Effective}} \), sodium myristate (C₁₄:0), sodium palmitate (C₁₆:0), sodium stearate (C₁₈:0), sodium arachidate (C₂₀:₁) and sodium behenate (C₂₂:₀) were acidified before being methylated using BF₃·MeOH. \( \delta D_{\text{Me}}^{\text{Effective}} \) was calculated using saturated fatty acids, specifically those over a range centring on palmitic and stearic acid, as their ubiquitous occurrence and lack of double bonds make these the most likely fatty acids to be found in environmental matrices and artefacts. Furthermore, the saturation of these fatty acids is also likely to improve the preservation of their hydrogen isotope ratios compared with unsaturated fatty acids. The results are summarised in Table 1.

The equation of the line on a plot of \( \delta D_{\text{Me}}^{\text{Effective}} \) against the chain length of the derivatised acidified fatty acid sodium salt is:

\[ \delta D_{\text{Me}}^{\text{Effective}} = -17.9N_{\text{FA}} + 74 \quad (R^2 = 0.725) \]  

(7)

There is clearly some effect on \( \delta D_{\text{Me}}^{\text{Effective}} \) from \( K \), as the slope of the plot is not zero; in other words there is either an isotope effect or a competing process associated with this methylation process causing fractionation. An obvious cause...
of this effect would be exchange of the fatty acid 2-hydrogen under the reaction conditions of methylation. This is possible because the initial step in the mechanism for BF3·MeOH catalysed methylation of fatty acids is formation of a bond between BF3 and the carbonyl group of the fatty acid (Fig. 1). Once formed the intermediate can proceed via two routes (Figs. 1(a) or 1(b)), with both resulting in the eventual formation of the methyl ester (in one case with further exchange via the formation of a ketene (K) intermediate), or tautomise, resulting in 2-hydrogen exchange before methylation (Fig. 1(c)). Further tautomerism can also occur once the FAME has been produced (Fig. 1(d)). In all cases we can assume that the introduced hydrogen derives from the only extremely exchangeable hydrogen available during the reaction: the hydroxyl group of the methanol (ignoring the small amount from the fatty acid). The methyl and hydroxyl hydrogen in industrially produced methanol have the same source[24] so, barring fractionation during production, will have the same 2D value. However, after production the hydroxyl hydrogen will rapidly exchange with any ambient hydrogen. We can therefore assume that the 2D value of the hydrogen introduced to the fatty acid by 2-hydrogen exchange is 2δDMe ≤ 2δD2H ≤ −50 %, where −50 % is an estimate of the 2D value of hydrogen in local meteoric water[25,26] from which the ambient moisture within the laboratory should be predominantly derived. It is also assumed that 2-hydrogen exchange is complete, i.e. all 2-hydrogen is exchanged, for all the chain lengths studied. These assumptions enable 2-hydrogen exchange to be corrected for by adjusting Eqn. (3) as follows:

\[ n_{FAME}2\delta D_{FAME} = n_{FA}2\delta D_{FA} + 3\delta D_{Me} + n_{FAME}K \]  

(3)

\[ n_{FAME}2\delta D_{FAME} = (n_{FA} - 2)2\delta D_{FA} + 3\delta D_{Me} + n_{FAME}K + 2\delta D_{2H} \]  

(8)

\[ n_{FAME}2\delta D_{FAME} = (n_{FA} - 2)2\delta D_{FA} + 3\delta D_{Me} + 2\delta D_{2H} \]  

(9)

Assuming that 2D2H is constant across the chain lengths of the fatty acids studied (not withstanding the variation in 2D between hydrogen at odd and even carbon positions in fatty acids, e.g.[27]), the actual value of 2D2H will have no effect on the gradient of a plot of 2δDMeEffective against fatty acid chain length, as:

\[ 2\delta D_{MeEffective} = 2\delta D_{FAME} - (n_{FA} - 2)2\delta D_{FA} - \frac{2\delta D_{2H}}{3} \]  

(10)

Hence, the presence of 2-hydrogen exchange will have an effect on K, but the value of 2D2H will not. The slope of the line on a plot of 2δDMeEffective against chain length adjusted for 2-hydrogen exchange is −13.9 % ± 3.5 % (R2 = 0.754). If 2-hydrogen exchange was the only parameter contributing to K (and it affected fatty acids of varying chain length equally) the slope of this corrected line would be zero. Correcting for 2-hydrogen exchange has reduced the gradient of the slope; however, it is still not zero. Whilst not providing evidence for the presence of 2-hydrogen exchange, this suggests that 2-hydrogen exchange alone cannot account for all the observed gradient and that fractionation of either the fatty acid or methyl moieties must occur during methylation.

Under the conditions used during the methylation, methanol is in at least 600-fold excess. It would therefore be reasonable to assume that it is the incoming methyl hydrogen that is being fractionated across the range of fatty acid sodium salts tested. However, the only isotope effects possible in this reaction, with respect to methyl hydrogen, are secondary, with the methyl hydrogen being three bonds away from the new methoxyl bond being formed. Secondary isotope effects, involving an isotope three bonds from the site of reaction, do not alter reaction rates by much more than 1%[14] and therefore are very unlikely to result in a large enough fractionation to produce the observed ~130 % change in 2δDMeEffective between myristic and behenic acids (Table 1). The only reasonable explanation for the change in 2δDMeEffective is fractionation of the fatty acid hydrogen during methylation, where the change in the amount of hydrogen with the carbon number of each fatty acid results in the change in 2δDMeEffective.

In this experiment, typically less than 500 µg of fatty acid was methylated using 100 µL of 14% w/v boron trifluoride in methanol. This is less fatty acid than was used in early studies of the reaction (e.g. 4–16 mg lipid per 1 mL 14% BF3·MeOH[13]); 100–200 mg fatty acid per 3 mL 12.5%
BF₃·MeOH[11], which report around 99% conversion. The conversion in this experiment, by comparison of GC peak area with an n-tetracontane internal standard added just prior to methylation, appeared to be essentially quantitative, being always in excess of 95%. However, the fractionation observed can only be explained by some process in this procedure not being entirely quantitative. Contrary to intuition the observed fractionation suggests that the heavier hydrogen isotopomers of the fatty acyl are more favourably converted into the methyl ester than the lighter ones.

One possible source of fractionation is during the acidification and subsequent extraction of the initial sodium salts. Further investigation, by comparison with an n-tetracontane internal standard added before acidification, revealed that the conversion rates from the sodium salt into the methyl ester, via acidification, were typically only 70%. Fatty acid salts have been observed to display a range of unusual properties during acidification, forming micelles, crystals and aggregates at different pH values or concentrations; because of this fatty acid salts do not become fully acidified until pH values much lower than the pKa of the respective fatty acid.[28] For this study the salts were acidified to pH 3; however, this was determined using indicator paper, and combined with a preoccupation with preventing acid-induced α-hydrogen exchange, may have meant that the sodium salts were not fully acidified, preventing complete extraction into non-polar solvents. It is suspected that this is the major cause of the observed fractionation. A correction factor based on these results is not invalid for the intended application, because all the samples to be studied would be saponified and then acidified, using the same method as above, prior to methylation. Extracting the saponified fatty acids into a slightly more polar solvent could possibly be used to improve recovery rates in future studies.

Adapting Eqn. (6) for a fatty acid and substituting δD<sub>Methyl</sub> for the equation of the line of a plot of δD<sub>Methyl</sub> against fatty acid chain length (Eqn. (7)) gives us an expression to correct for the methylation of a saturated straight chain fatty acid:

Figure 1. Mechanism for methylation of fatty acids by boron trifluoride in methanol. Adapted from March[29] and discussion with Guy Lloyd-Jones.
\[
\delta D_{FA} = \frac{n_{FAME}\delta D_{FAME}}{n_{FA}} - 3\frac{\delta D_{MeEffective}}{n_{FA}} \tag{11}
\]

\[
\delta D_{FA} = \frac{(2N_{FA} + 2)\delta D_{FAME}}{2N_{FA} - 1} + \frac{3(17.9N_{FA} - 74)}{2N_{FA} - 1} \tag{12}
\]

This correction is only valid for the batch of boron trifluoride in methanol used during this study. There is no evidence that the relationship between \(\delta D_{MeEffective}\) and fatty acid chain length is linear across large variations in chain length and therefore this correction factor should also not be used for fatty acids outside the C14 to C22 range.

To test this correction for methylation, it was applied to methylated acidified fatty acid salts of unknown \(\delta D\) value, before comparison with subsequently determined bulk \(\delta D\) values of the fatty acid salt. This was performed in triplicate on the sodium salts of myristic, palmitic and stearic acids. The results are detailed in Table 2.

For all three fatty acids, the correction factor alters the mean uncorrected \(\delta D\) values by 5.1 \% or less; indeed, perhaps its greatest effect is to increase the reported error by about a factor of two. The mean corrected compound-specific \(\delta D\) values differ from the mean bulk \(\delta D\) values by \(-6.5\%\), \(-1.7\%\) and \(+6.7\%\) for myristic, palmitic and stearic acids, respectively. A paired t-test on the means of all three fatty acids shows the difference between the bulk and corrected \(\delta D\) values is not significant at the 95% confidence level and suggests that the correction is valid.

The greatest difference between the mean values of \(\delta D_{FAsalt}\) and \(\delta D_{FAME}\) of any particular fatty acid determined during this investigation was 10.1 \%, with the mean difference being 3.9 \% (1\% = 3.7 \%). The changes in the measured \(\delta D\) values due to the additional hydrogen introduced during methylation (using this particular batch of BF3 MeOH), and that due to the fractionation of hydrogen during methylation and workup, are of similar magnitude to the instrumental RMS error involved in the measurement. Thus, even before correction, the change in the \(\delta D\) value of a fatty acid during methylation should not present an obstacle to the interpretation of data from investigations of fatty acids with natural hydrogen isotope abundance (e.g., those of saturated fatty acids found in animals\[^{[6]}\]) and where any expected variation in the \(\delta D\) values of fatty acids from different sources is much greater than instrumental error.

**Preservation of the hydrogen isotope signal of fatty acids during extraction and workup**

In order to determine whether the extraction and preparation of a fatty acyl lipid mixture for compound-specific hydrogen isotope analysis affects the hydrogen isotope composition of components in that mixture, a standard mixture of caprylic, palmitic, stearic, behenic and lignoceric acids was extracted, saponified and separated at least six times in parallel. After each step an aliquot of the solution was taken and analysed via GC/TC/IRMS. A more severe than typical nitrogen flow was used whenever the sample solution was to be evaporated to amplify any effect produced.

The results are presented graphically in Fig. 2. For all fatty acids studied the \(\delta D\) value after full processing (labelled ‘separated’ in Fig. 2) plots within error of the \(\delta D\) value of the original fatty acid. For palmitic and stearic acids, the fatty acids of particular interest to this study, the differences between the \(\delta D\) values of the raw and fully processed fatty acids are 1.0 \% and 2.1 \%, respectively, with a minimum standard deviation per determination of 5.9 \%. It is clear that the complete process of extraction, saponification and separation has negligible effect on the hydrogen isotopic composition of fatty acids.

Interestingly, four of the five fatty acids show slight, although not significant, enrichment in deuterium after being saponified (by comparison with the unextracted fatty acids), which supports the hypothesis above that the observed fractionation during methylation could be due to the acidification and extraction of fatty acid sodium salts. For palmitic acid this enrichment in deuterium is 4.3 \% ± 8.2 \% and for stearic acid it is 3.5 \% ± 6.9 \%.

The increased error for the caprylic acid evident in Fig. 2 is associated with the method of analysis: the methyl ester of caprylic acid will elute from a gas chromatograph before ethyl caprate and \(n\)-pentadecane. As ethyl caprate and \(n\)-pentadecane were used as sacrificial standards to

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**Table 2.** Comparison of measured \(\delta D_{FAME}\), corrected \(\delta D_{FA}\) and bulk \(\delta D_{FAsalt}\). Corrected \(\delta D_{FA}\) is calculated using Eqn. (12).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Myristic (C14:0)</th>
<th>Palmitic (C16:0)</th>
<th>Stearic (C18:0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\delta D_{FAME})</td>
<td>-221.9</td>
<td>-218.1</td>
<td>-244.0</td>
</tr>
<tr>
<td>(\pm 1\sigma/%)</td>
<td>± 6.2</td>
<td>± 4.6</td>
<td>± 3.1</td>
</tr>
<tr>
<td>(n)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Corrected (\delta D_{FA})</td>
<td>-227.0</td>
<td>-218.7</td>
<td>-243.6</td>
</tr>
<tr>
<td>(\pm 1\sigma/%)</td>
<td>± 13.0</td>
<td>± 8.3</td>
<td>± 6.0</td>
</tr>
<tr>
<td>Bulk (\delta D_{FAsalt})</td>
<td>-220.5</td>
<td>-217.0</td>
<td>-250.3</td>
</tr>
<tr>
<td>(\pm 1\sigma/%)</td>
<td>± 0.4</td>
<td>± 0.5</td>
<td>± 0.5</td>
</tr>
<tr>
<td>(n)</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

**Figure 2.** The effect of lipid extraction and workup on the hydrogen isotope composition of fatty acids. Sample names refer to the step in the analytical protocol after which \(\delta D\) values were determined, thus samples labelled ‘separated’ are fully processed. Errors are \(\pm 1\sigma\), and do not include the error in the correction for methylation.
condition the reactor during GC/TC/IRMS analysis, the δD values of methyl caprate were determined using a TC reactor that was not fully conditioned and therefore they are more dependent on the initial reactor condition, resulting in increased standard deviations.

CONCLUSIONS

A correction for the effects of derivatisation by boron trifluoride in methanol has been determined, which suggests that little if any fractionation of hydrogen is caused during methylation; this is consistent with previous studies. It appears more likely that fractionation occurs during the extraction of the acidified fatty acyl salts. The combined magnitude of the change in δD values of fatty acids as a result of hydrogen introduced during methylation and any fractionation caused by methylation and subsequent extraction, is ~ for fatty acids with δD values of around −250 ‰ methylated with the batch of BF₃·MeOH used in this study ~ comparable in magnitude to the instrumental error. It has also been shown that the method of sample extraction and preparation does not significantly affect saturated fatty acid δD values.

Thus, the methylation of fatty acids by boron trifluoride in methanol is a relatively uncomplicated way to remove exchangeable hydrogen prior to compound-specific hydrogen isotope analysis, provided that the magnitude of the change in δD value of the analyte caused by methylation is determined for each different batch of BF₃·MeOH used.

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REFERENCES


