RESEARCH ARTICLE

Comparison of three methods for the methylation of aliphatic and aromatic compounds

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Rationale: Methylation protocols commonly call for acidic, hot conditions that are known to promote organic 1H/2H exchange in aromatic and aliphatic C–H bonds. Here we tested two such commonly used methods and compared a third that avoids these acidic conditions, to quantify isotope effects with each method and to directly determine acid–exchange rates relevant to experimental conditions.

Methods: We compared acidic and non-acidic methylation approaches catalyzed by hydrochloric acid, acetyl chloride and EDCI (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide)/ DMAP (4-dimethylaminopyridine), respectively. These were applied to two analytes: phthalic acid (an aromatic) and octacosanoic acid (an aliphatic). We analyzed yield by gas chromatography/flame ionization (GC/FID) and hydrogen and carbon isotopic compositions by isotope ratio mass spectrometry (GC/IRMS). We quantified the 1H/2H exchange rate on dimethyl phthalate under acidic conditions with proton nuclear magnetic resonance (1H-NMR) measurements.

Results: The 1H and 13C values and yield were equivalent among the three methods for methyl octacosanoate. The two acidic methods resulted in comparable yield and isotopic composition of dimethyl phthalate; however, the non-acidic method resulted in lower 1H and 13C values perhaps due to low yields. Concerns over acid-catalyzed 1H/2H exchange are unwarranted as the effect was trivial over a 12-h reaction time.

Conclusions: We find product isolation yield and evaporation to be the main concerns in the accurate determination of isotopic composition. 1H/2H exchange reactions are too slow to cause measurable isotope fractionation over the typical duration and reaction conditions used in methylation. Thus, we are able to recommend continued use of acidic catalysts in such methylation reactions for both aliphatic and aromatic compounds.

1 INTRODUCTION

Stable hydrogen isotopic compositions (δH values) of organic compounds are widely used in organic geochemistry, biochemistry, ecology, climate, and forensics. Applications include the study of the hydrologic cycle and climate,3,4,13 biosynthetic pathways of lipids,7,8 paleoelevation,9,20 diagenesis of organic matter,11 and food authenticity and provenance.12,13 In many compounds, hydrogen positions include those in C–H bonds that are non-exchangeable after biosynthesis and preserved under low-temperature sedimentary conditions. They carry the original biosynthetically fixed H, although even these H atoms may be exchanged under elevated temperatures and over geologic time.14,15 In contrast, O–H bonds, as found in cellulose or fatty acids, contain H atoms that are readily exchangeable with ambient water in the plant.16,17 In order to recover signals relating to the original plant biosynthesis, analyses typically target only the carbon-bound hydrogens, by masking exchangeable O–H H atoms. The preparative steps for cellulose involve ‘nitrification’ (i.e. formation of nitric acid esters17), whereas derivatization of aliphatic and aromatic acids typically involves esterification with methanol. Here, we examine isotopic exchange associated with reaction conditions used for esterification prior to isotopic analysis.

1.1 Acid-catalyzed esterification

Organic compounds with carboxylic acid groups are commonly esterified with methanol prior to gas chromatography (GC) analysis,18,19 using acidic catalysts. Hydrochloric acid (HCl) and acetyl chloride
(CH₃COCl), which forms anhydrous HCl in methanol, are among the most widely used catalysts of aliphatic and aromatic acids. The carboxylic acid is initially protonated and undergoes an exchange reaction with the alcohol to give the reaction intermediate. The intermediate can then lose a proton to become an ester. The equilibrium point of this reaction is displaced so that esterification proceeds virtually to completion in excess alcohol. However, Brønsted acid catalysts can also promote H atoms bound to an aromatic ring to undergo $^1$H/$^2$H exchange via electrophilic aromatic substitution (Figure 1). Thus, during esterification, hydroxyl H atoms in methanol can exchange with aromatic H atoms in an analyte molecule and the $\delta^2$H values can equilibrate.

Understanding acid-catalyzed isotope exchange during esterification is relevant to the study of aromatic compounds, but also indirectly for the study of aliphatic compounds such as the alkanoic acids derived from plant leaf waxes and microbial lipids, because aromatic structures are commonly used as part of the isotopic determination. One method to determine the $\delta^2$H values and the carbon isotopic composition ($\delta^{13}$C values) of methyl groups added in aliphatic acid esterification steps involves the use of phthalic acid. Phthalic acid of known isotopic composition (measured independently as a sodium salt) can be methylated and measured by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) to calculate the isotopic composition of the methyl hydrogens and carbon via mass balance. Because phthalic acid has an aromatic ring, conventional acid esterification conditions should enable $^1$H/$^2$H exchange on the aromatic H atoms (Figure 1). Such exchange may result in an incorrect estimation of the overall dimethyl phthalate isotopic composition and mass balance estimation of the $\delta^2$H value of methyl hydrogens, needed when calculating the isotopic composition of the original fatty acid. However, the magnitude of such an effect is currently unknown.

### 1.2 Non-acidic coupling reagents to promote esterification

Alternatively, non-acidic approaches to methylation exist that may obviate the above concerns. In this study, we tested a non-acidic method using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) as a coupling reagent with 4-dimethylaminopyridine (DMAP) as the catalyst (Figure 2). Carbodiimides, due to their accessibility and versatility, are ranked as one of the most important classes of compounds in organic chemistry. The most widely used carbodiimide reagent, dicyclohexylcarbodiimide (DCC), makes insoluble urea byproducts requiring additional cumbersome purifications. Hence we chose EDCI, a water-soluble carbodiimide with a water-soluble urea byproduct, combined with DMAP which has been shown to increase the efficiency of esterification by 10-fold. We compared this approach with conventional acidic methylation reactions. EDCI and DMAP are not expected to lead to $^1$H/$^2$H exchange because the reaction conditions are buffered, and the merits of these reagents for organic geochemical applications are tested here.

### 1.3 Method comparison

In this study, we tested two acidic and one non-acidic method of esterification as applied to phthalic acid and octacosanoic acid to evaluate any isotopic fractionation and practical issues arising with each using GC/IRMS and GC/FID approaches. We further directly measured the $^1$H/$^2$H exchange rate in phthalic acid methylation with 5 vol% HCl using proton nuclear magnetic resonance ($^1$H-NMR). Based on the exchange rate, we estimate the proportion of aromatic H atoms substituted over 12 h of reaction time from the initial $\delta^2$H values of the reactants. The goal is to quantify the $\delta^2$H and $\delta^{13}$C perturbation possible with each esterification method and to make practical recommendations for esterification prior to GC/IRMS analyses.

### 2 EXPERIMENTAL

#### 2.1 Phthalic acid and methanol of known isotopic composition

The hydrogen isotopic composition of phthalate was measured in Na-phthalate to exclude exchangeable, carboxylic acid hydrogen atoms and the $\delta^{13}$C value was measured from the free diacid via offline combustion at Indiana University (Bloomington, IN, USA). Offline combustion consisted of phthalic acid combustion in quartz ampoules and cryogenic purification of combustion gases in a vacuum line. The aromatic hydrogen isotopic composition was $-95.5 \pm 2.2$‰ ($\sigma$, $n = 4$) and the carbon had a $\delta^{13}$C value of $-27.21 \pm 0.02$‰ ($n = 4$). The uncertainties reported represent replicate precision. Unquantified uncertainty in the absolute value may exist, and the largest source of concern would be water inclusion in the Na-phthalate crystals. In order to mitigate against the influence of water inclusions, the crystals were warmed before combustion.

Methanol (MeOH) was 99.8% anhydrous and the $\delta^2$H value of the methyl group was $-141 \pm 3$‰ ($n = 3$), calculated via mass balance between bulk methanol and hydroxyl hydrogen at Indiana University.
Excess sodium metal and a small amount of methanol were reacted in an evacuated, sealed ampoule to liberate H₂ gas from the hydroxyl hydrogen for isotopic determination. The uncertainties reported represent replicate precision. Unquantified uncertainty in the absolute value may exist; the largest source of such error would be associated with residual methanol vapor, although this is mitigated against by allowing the reaction to proceed for several days. The hydroxyl hydrogen has a δ²H value of ~27% and thus can provide the relatively ²H-enriched hydrogen source for exchange into the phthalate aromatic ring during acid-catalyzed equilibration. The other H⁺ in the 5 vol% catalyst (from HCl and water) can exchange freely with methanol’s hydroxyl hydrogen and their isotopic compositions are unknown. Nonetheless, the dominant source of exchangeable H atoms is from methanol, which is 95% of the reaction solvent. The δ¹³C value of the methanol was -46.77 ± 0.04‰, from bulk measurement. The isotopic compositions of phthalic acid and methanol were used to calculate the predicted isotope values of dimethyl phthalate via Equation 1 for hydrogen and Equation 2 for carbon.

\[
\delta²H \text{ dimethyl phthalate } H \times 10 = \delta²H \text{ phthalic acid } H \times 4 + \delta²H \text{ methyl } H \times 6
\]  

(1)

\[
\delta¹³C \text{ dimethyl phthalate } C \times 10 = \delta¹³C \text{ phthalic acid } C \times 8 + \delta¹³C \text{ methyl } C \times 2
\]  

(2)

2.2 | The acidic methods: HCl and CH₃COCl

For the HCl-catalyzed methylation, 3 mg of phthalic acid or 3 mg of C₂₉ n-alkanoic acid were methylated with 1 mL of a mixture of 5 vol% HCl and 95% MeOH in a tightly sealed 15-mL culture tube at 70 °C overnight. For the CH₃COCl-catalyzed methylation, the same procedure was followed using CH₃COCl (instead of HCl) and reacting at 50 °C overnight. Practical guidance on methylation protocols are provided in the supporting information, and are briefly summarized here.

Following overnight methylation reactions, the samples were removed from the heat, and 1 mL of Milli-Q water (Millipore Milli-Q Plus QPAK 2; Millipore, Billerica, MA, USA) was then added to produce an aqueous phase. The analyte was extracted using liquid-liquid extraction (LLE) with 1 mL of dichloromethane (DCM) for dimethyl phthalate and hexane for n-alkanoic acid methyl esters. For each extraction (repeated 3 or more times), the mixture was vigorously shaken, left to partition into two phases and the organic phase, containing dimethyl phthalate or fatty acid methyl ester (FAME), was extracted by pipette and dried by passage through a column of anhydrous Na₂SO₄. The organic fraction was further purified by passing through a silica column (5 cm × 40 mm Pasteur pipette, 5 wt% water-deactivated silica gel, 100–200 mesh) eluting with three column volumes of DCM for dimethyl phthalate and for FAMEs.

2.3 | The non-acidic method: EDCI and DMAP

A general procedure for synthesis of ester derivatives from Lehmann et al. was followed, with adjustments for scale. EDCI (22.5 mg; 8 mol ratio to phthalic acid or octacosanoic acid) and DMAP (1.1 mg; 0.5 mol ratio) and 3 mg of phthalic acid or octacosanoic acid (C₂₉ n-alkanoic acid) were added to 1 mL of isotopically known methanol and the solution was kept at room temperature overnight. The solution was diluted with 1 mL of Milli-Q water. The methylated product was extracted by the addition of 1 mL of ethyl acetate. The mixture was shaken vigorously to allow LLE to proceed, and then allowed to settle into two phases, and this process was repeated at least three times. The organic fraction, containing dimethyl phthalate or methyl octacosanoate, was dried by passage through a column of anhydrous Na₂SO₄. It was further purified by eluting the ester fraction through a silica column with three column volumes of ethyl acetate.

2.4 | GC/MS/FID analysis

The methylation products generated in this study, including dimethyl phthalate and C₂₉ FAME, were identified and quantified using gas chromatography (Agilent 6890 series) coupled with a single quadrupole mass-selective detector (Agilent 5973, using electron ionization with an ionization energy of 69.9 eV) and flame ionization detection (GC/MS/FID instrumentation supplied by Agilent, Santa Clara, CA, USA). The instrument was equipped with a Rxi-5 ms column (30 m × 0.25 mm, film thickness 0.25 μm supplied; Restek Corp., Bellefonte, PA, USA) with a 4 mL min⁻¹ constant column flow rate, split passively 4:1 (based on theoretical calculations following Poiseuille’s law) between the mass spectrometer (via a 80 cm × 0.25 mm silica capillary) and the flame ionization detector (via a 120 cm × 0.1 mm silica capillary). Helium was used as the carrier gas. The initial temperature of 50 °C was held for 3.5 min, followed by a temperature ramp of 20 °C min⁻¹ to 300 °C and maintained for 10 min. A volume of 1 μL of sample was injected in splitless mode. Absolute abundance was calculated using a calibration curve of an in-house standard (mixture of n-alkanes and n-alkanoic acids) and their peak area response on the flame ionization detector. Yields were calculated based on the chemical equation using masses of reagents, quantified product abundances and are reported as % of predicted product. All uncertainties are reported as standard deviations (s) unless stated otherwise.

2.5 | GC/IRMS analysis

Compound-specific hydrogen and carbon isotopic compositions were measured using GC/IRMS. Samples were introduced via a programmable temperature vaporizing (PTV) injector in splitless mode at 50 °C into a Trace gas chromatograph (Thermo Scientific). The gas chromatograph was equipped with a Rxi-5 ms column (30 m × 0.25 mm, film thickness 0.25 μm) and connected via an Isolink with a pyrolysis furnace at 1400 °C for hydrogen isotopic analysis, and via a combustion furnace at 1000 °C for carbon isotopic analysis, through a Conflo IV interface to a DeltaV™ isotope ratio mass spectrometer (all supplied by Thermo Scientific, Bremen, Germany). The gas chromatograph was operated with a helium flow rate of 1 mL min⁻¹ from an initial temperature of 50 °C, followed by a ramp of 20 °C min⁻¹ to 300 °C, and held for 5 min.

For hydrogen, the H²⁺ factor was measured daily to correct for the formation of H₂⁺ in the ion source, and to check for linearity, with a mean value over the course of measurements reported here of...
3.6 ± 0.35 ppm mV⁻¹ (n = 6) across a range of 1–8 V. For carbon, the
standard deviation of CO₂ pulses spanning 1–8 V was determined
daily to check for linearity and the standard deviation across that range
averaged 0.02 ‰. Gas peaks of H₂ or CO₂ as appropriate were
co-injected bracketing the analyte peaks during the GC/IRMS run.
Two of these peaks were used for standardization between sample
and standard, while the rest were treated as unknowns to assess
precision. Data were then normalized to the Vienna Standard Mean
Ocean Water (VSMOW)/Standard Light Antarctic Precipitation
(SLAP) hydrogen isotopic scale via comparison with an external
standard with 15 n-alkanes (C₁₆–C₃₀ n-alkane mixture of type A,
isotopic standard from Indiana University), with δ²H values ranging
from −46 to −227 ‰. For carbon, the isotopic results were normalized
to Vienna PeeDee Belemnite (VPDB)/lithium carbonate prepared by
H. Svec (LSVEC) by comparing with the A mix standard with δ¹³C values
ranging from −28.6 to −33.3 ‰. The standard was run daily throughout
the sequence. Measured δ²H values of the samples are normalized
using a regression between the known and measured values of the
standards. The root-mean-square error of replicate analysis of the
external standard (A mix) averaged 4.6 ‰ for hydrogen and 0.2 ‰
for carbon. The results are reported using conventional delta (δ)
notation in permil (‰).

2.6 | ¹H/²H exchange rate using ³H-NMR

We conducted a proton nuclear magnetic resonance (³H-NMR) kinetic
experiment to measure the rate of ¹H/²H exchange on dimethyl
phthalate in 5 vol% HCl (12 M) using a Varian VNMRS-600 spectrometer
(supplied by Varian, Inc., now Agilent Technologies, Palo Alto, CA, USA)
at the University of Southern California (Los Angeles, CA, USA). We
reacted 30 mg of phthalic acid in a solution of 0.60 mL of methanol-d₄
(C₂H₂O²⁻H) and 5 vol% HCl in a Wilmad J Young NMR tube (type of
NMR tube with a Teflon cap that can be attached to a vacuum line,
supplied by Wilmad-labglass, Vineland, NJ, USA) sealed with plastic
cap and paraffin, in an oil bath at 70 °C. We measured the phthalate
H peak areas daily for 10 days after the overnight esterification
reaction. The tube was placed back in the oil bath after measurement
to maintain the reaction conditions over the 10 days. The peaks were
analyzed with a relaxation delay of 10 s. A decrease in peak area
detected by ³H-NMR indicated exchange of ³H on the phthalate with
²H from the methanol-d₄ solvent.

3 | RESULTS AND DISCUSSION

3.1 | Product yield

We found that product yields varied between methods and analytes.
For dimethyl phthalate, the acidic methods resulted in >70 % product
yield, whereas the carbodiimide method resulted in yields <30 %
(Figure 3, Table S1, supporting information). For methyl octacosanoate,
all methods yielded >80 % product yield, but again yields were highest
for the acidic methods, >94 % (Table S2, supporting information).
Low product yield (observed here for the dimethyl phthalate using
the non-acidic method) is a concern given the potential for isotopic
fractionation associated with incomplete reaction or recovery. Low

![FIGURE 3 Dimethyl phthalate isolated yield and isotopic results. Carbon and hydrogen isotopic measurements are compared with known dimethyl phthalate values (i.e., target values) calculated by isotopic mass balance. Diamonds represent carbon isotope results whereas circles represent hydrogen isotopes. [Color figure can be viewed at wileyonlinelibrary.com]](image)
3.1.2 Yield for the carbodiimide method
The isolated yield of dimethyl phthalate was low (mean 22%) using the carbodiimide method and this could be due to incomplete reaction or loss during purification. We monitored the reaction with thin layer chromatography (TLC) at the beginning and end of the reaction (after 12 h) and we confirmed that the product had formed while phthalic acid had been completely consumed. Additional evidence for reaction completion was obtained by testing for the absence of phthalic anhydride, a reaction intermediate for the carbodiimide method, at the end of the experiment by GC/MS. Thus, we can rule out incomplete reaction.

Low product yield using carbodiimide reagents for esterification has been previously noted, due to the carboxylate being consumed during the formation of an N-acylurea byproduct. Addition of DMAP as a catalyst inhibits the byproduct formation and facilitates the reaction to form the ester, which has been shown to increase yield. Following this protocol, we obtained <30% expected yield of dimethyl phthalate, while obtaining >80% yield of methyl octacosanoate. The low yield of dimethyl phthalate may be explained by its greater volatility than methyl octacosanoate.

We tested yield response to various reaction conditions such as reagent concentration, temperature and duration of reaction for the carbodiimide approach, although tests of intermediates showed that reactions went to completion. In case isolation and purification steps were the issues affecting low isolation yields we tested different solvents for extraction, as well as citric acid washing and brine washing in efforts to improve the isolated product yield. Each of these efforts failed to generate yield >30% (Table 54, supporting information). Having experimented considerably with this approach, we find it is not currently a pragmatic protocol for low concentration (3 mg) dimethyl phthalate derivatization applications. In contrast, the method does have promise as a viable method for derivatization of aliphatic acids.

Nevertheless, we note additional practical challenges for the use of the carbodiimide method prior to GC analyses. We found it necessary to clean the syringe, inlet and trim the column after running the carbodiimide samples by GC/IRMS probably due to urea persisting in the mixture even after LLE. If these compounds were injected in measurable quantity, due to their high polarizability and protic nature, they could interact with the GC column. We do not, however, observe any humps or fronting and tailing of the analyte peak in the chromatogram; our analytical results for both quantification and isotopic composition therefore appear to be unaffected by any such polar contaminants. Regardless of analyte, the carbodiimide method presented challenges for subsequent GC analyses that should be addressed if this approach is to be adopted for aliphatic GC applications.

3.2 Isotopic analyses
The expected isotopic ratios of dimethyl phthalate calculated from mass balance (Equations 1 and 2) were δ13C values of −12.3‰ and δ15N values of −31.1‰. For carbon isotopes, acidic methods successfully resulted in values that are close to those expected (Figure 3). Acetyl chloride and HCl methods produced δ13C values of −31.5 ± 0.0‰ (n = 3) and −31.3 ± 0.1‰ (n = 3). The non-acidic method resulted in a more negative value of −32.7 ± 0.4‰ (n = 4).

The hydrogen isotopic composition of dimethyl phthalate differed between the acidic and non-acidic esterification methods (Figure 3). Dimethyl phthalate produced with acetyl chloride and HCl resulted in δ2H values of −105 ± 2‰ (n = 3) and −107 ± 1‰ (n = 3), respectively, thus being equal within uncertainty although +16 to +18‰ more 2H-enriched than expected. The carbodiimide method resulted in a more negative δ2H value of −137 ± 1‰ (n = 4). We calculated the initial methyl hydrogen and carbon isotopic composition of methanol by mass balance and found that none of the methods accurately assessed the (known) initial methyl δ2H and δ13C values. Acidic methods produced 25–29‰ more positive estimates for methyl δ2H values whereas the carbodiimide method produced 25‰ more negative estimates than the known value of the methanol, −141 ± 3‰, n = 3 (Table 1). This inaccuracy is greater than analytical errors, the largest of which is the calibration to the international VSMOW-SLAP isotopic scale (see section 2). This suggests an isotopic fractionation during the methylation reaction and/or purification and one that differs between the acidic and non-acidic methods.

3.2.1 Is acid-catalyzed exchange a cause of 2H-enrichment?
We directly measured 1H/2H exchange rates for dimethyl phthalate by 1H-NMR under conditions relevant to the acidic methylation method. We found the same exchange rates (slopes) within uncertainties for the hydrogens on the alpha and beta carbon positions of dimethyl phthalate (Figure 4). Using the mean slope as the exchange rate constant (4.94 × 10⁻⁴ h⁻¹), this corresponds to an exchange half-life (the time to exchange 50% of aromatic hydrogens, = (ln2) / k) of 1403 h (58.5 days) at 70°C. For a 100‰ deuterium in δ2H values between aromatic H and methanol hydroxyl H, this would induce a change in the δ2H value of the aromatic H of just 0.035‰ over a 1-h reaction, which is not detectable by GC/IRMS. For the reagents described here, with 70‰ deuterium in the δ2H values of dimethyl phthalate and methanol hydroxyl H, and a 12-h reaction time (0.4 % exchange), we would expect 0.3‰ 2H-enrichment. Even with a 12-h reaction (0.4% exchange) and a 1000‰ deuterium, such as might be encountered working on extraterrestrial samples, a shift of only 4‰ is predicted, which is still smaller than the analytical uncertainties for GC/IRMS. Although 1H/2H exchange is taking place as expected, we directly establish, for the first time, that the rates are too slow to cause any significant changes in δ-values over the reaction conditions used in the HCl method. Still, when using strong acids as catalysts in methylation, it is good practice to not extend reaction times.

3.2.2 Is evaporation a cause of 2H-fractionation?
Guarding against evaporation is always a concern for volatile analytes intended for isotopic analyses, requiring careful laboratory handling. Vapor pressure isotope effects vary between molecules, depending on intramolecular vibrations (bonding) and frequency shifts on condensation, both of which vary with temperature. We directly tested fractionation for dimethyl phthalate under worst-case scenario laboratory handling with evaporative losses to demonstrate the scale of possible errors. To mimic extreme sample preparation error, we
TABLE 1  Hydrogen and carbon isotope ratios calculated for C28 and C16 FAMEs and propagated uncertainties based on the precision and accuracy of dimethyl phthalate analyses

<table>
<thead>
<tr>
<th></th>
<th>δ²H values (%)</th>
<th>δ¹³C values (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HCl</td>
<td>CH₂COCl</td>
</tr>
<tr>
<td>Mean dimethyl phthalate</td>
<td>-107</td>
<td>-105</td>
</tr>
<tr>
<td>Uncertainties</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Precision</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ii. Accuracy</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Estimated methyl group</td>
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<td>-112</td>
</tr>
<tr>
<td>Assigned FA value</td>
<td>-100</td>
<td></td>
</tr>
<tr>
<td>Propagated δ²H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Precision</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>ii. Accuracy</td>
<td>6</td>
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</tr>
<tr>
<td>C16 FAME calculated</td>
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<td>Propagated δ¹³C</td>
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<td></td>
</tr>
<tr>
<td>i. Precision</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>ii. Accuracy</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

³Reproducibility of phthalic acid methylation and preparative chemistry.

²Difference between known and measured dimethyl phthalate values from Figure 3.

³Propagated standard deviation calculated following the approach of Polissar and D’Andrea.

3.2.3  δ²H- and δ¹³C-depletion using the carbodiimide method

The carbodiimide method resulted in more negative δ²H and δ¹³C values than expected for dimethyl phthalate compared with the known isotopic composition. We infer a kinetic isotope effect, with the preferential reaction of the lighter isotope, by the carbodiimide method. Although we have not fully resolved the cause of the isotopic offset using this method (Figures 3 and 4) we suggest that securing higher yields and determining the potential for kinetic isotope effects with the carbodiimide reaction may be relevant questions for future research if this method is to be used as preparative method prior to GC/IRMS analyses.

3.3  Implications for aromatic and aliphatic acid esterification

For methyl octacosanoate, prepared by each of three methods, the δ²H and δ¹³C values were within 3 % and 0.5 % uncertainty envelopes, respectively (Figure 5). Thus, all three methods are suitable preparative methods for GC/FID and GC/IRMS applications for long-chain fatty acids, although the acidic methods remain the recommended ones.

For phthalic acid, we found that the two acidic methods are equivalent, and δ¹³C values were as predicted (within 0.4 %, similar within uncertainties), although we found that the δ²H values were elevated by 15 % (Figure 3). Although acidic exchange can lead to ²H-enrichment, the rates established here are far too slow to explain observations. Small ²H-enrichment can be reproduced through evaporation; however, evaporation cannot fully explain the observed enrichment given the high yields obtained, given that ~50 % of the analyte would have to be lost to explain such an enrichment. Thus, the remaining offset may reflect a fractionation
associated with the methylation reaction. Nevertheless, from a practical standpoint, we emphasize that volatility and evaporation are greater concerns than acidic exchange on aromatic compounds, when pursuing phthalic acid methylation or methylation of reagents with similar or lower boiling points. We recommend that analytical conditions be carefully designed and monitored to minimize evaporative losses and further isotopic enrichment, by testing quantitative yields by GC/FID in parallel with isotopic determinations by GC/IRMS.

In contrast, the non-acidic carbodiimide method performed poorly in phthalic acid methylation experiments in terms of product yield and depletion in both $^2$H (by up to 15 %) and $^{13}$C (by up to 2 %; Figure 3). We hypothesize that the low isolated yield is related to the isotopic depletion but the yield could not be improved in our attempts shown here and thus the consequent isotope effects could not be directly tested. Since acidic exchange is not quantitatively important for these applications, we have no reason to extend testing of the non-acidic method here.

Phthalic acid methylation is commonly used to determine the isotopic composition of methyl groups in methanol used for fatty acid esterification. While the different methods induce isotopic offsets from predicted values in this study (Table 1), the error in the isotopic composition of the methanol becomes trivial for long-chain $n$-alkanoic acids due to the small proportion of methyl hydrogens and carbon. For example, a 54 % offset in the methyl $^2$H value becomes 2 % for the $^2$H value of C$_{28}$ FAME and 5 % for C$_{16}$ FAME and a 7 % offset in the methyl $^{13}$C value becomes 0.2 % and 0.3 % for the $^{13}$C values of C$_{28}$ and C$_{16}$ FAME. These accuracy and precision errors contribute to methyl group uncertainty, and to the propagated uncertainties in the calculation of analyte $^2$H values. Overall, the magnitude of methyl hydrogen uncertainty on derivatized compounds remains small relative to other sources of uncertainties in the estimation of high molecular weight aliphatic acids, e.g., C$_{28}$ $n$-alkanonic acid, a constituent of plant leaf waxes (Table 1). For short-chain lipids such as the C$_{16}$ $n$-alkanonic acid (common in microbial, plant and animal cells) the mean isotope values differ more between methods (up to 5 %) and uncertainties increase. For practical guidance, we provide detailed protocol and error propagations in the supporting information.

4 | CONCLUSIONS

Methyl esterification is a commonly used derivatization procedure for both aromatic and aliphatic compounds with carboxylic acid groups prior to GC analysis. We have studied hydrogen and carbon isotopic fractionations during methylation using two widely used acidic catalysts and one non-acidic method using a carbodiimide. We found practical advantages to the acidic methods over the carbodiimide method, notably higher isolated yields. We determined that the rates of $^1$H/$^2$H exchange ($4.94 \times 10^{-6}$ h$^{-1}$) are too slow to be of quantitative importance and thus find that the acidic conditions do not present problems for most applications, even with aromatic compounds. We thus recommend following one of the acidic protocols.

A phthalic acid standard is commonly used to determine the isotopic composition of the methanol used to derivatize both the standard and the aliphatic or aromatic acid analyte for GC/IRMS applications. Using phthalic acid and methanol standards of known values here, we establish that for both carbon and hydrogen isotopic analyses the carbodiimide method produces systematically lower values than the acidic methods. As we have shown that acidic exchange is not quantitatively significant, the greatest issues appear to be low yield (carbodiimide method) and evaporation (under poor laboratory handling scenarios). Although we observe a small systematic $^{13}$C- and $^2$H-enrichment in dimethyl phthalate from known reagents using the acidic methods, the propagated errors for long-chain $n$-alkanonic acid applications are smaller than the analytical uncertainty of $^2$H values by GC/IRMS at least for high molecular weight analytes.

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