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Uncertainty in paleohydrologic reconstructions from molecular δD values

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Abstract

Compound-specific δD measurements can be used for quantitative estimation of source water δD values, a useful tracer for paleohydrologic changes. Such estimates have quantifiable levels of uncertainty that are often miscalculated, resulting in inaccurate error reporting in the scientific literature that can impact paleohydrologic interpretations. Here, we summarize the uncertainties inherent to molecular δD measurements and the quantification of source water δD values, and discuss the assumptions involved when omitting various sources of uncertainty. Using standard protocols from measurement science, we derive the equations necessary to quantify these various sources of uncertainty. We show that analytical uncertainty is usually improperly estimated and that after apparent fractionation between δD values of source water and molecule, normalization of data to the VSMOW scale introduces the largest amount of uncertainty. Lastly, to facilitate systematic error reporting we provide an Uncertainty Calculator spreadsheet to conveniently calculate uncertainty in δD measurements.

1. INTRODUCTION

The hydrogen isotopic composition (δD) of water is a sensitive tracer for phase changes and can be used to examine the modern hydrologic cycle. The δD values of organic molecules preserved in sediments are increasingly used to investigate past changes in the hydrologic cycle. Because hydrogen in the organic molecules of most photosynthetic organisms derives from source water, the δD values of these molecules reflects that of the lake, stream, soil or ocean water in which the organism grew (DeNiro and Epstein, 1981; Sauer et al., 2001; Huang et al., 2002; Sachse et al., 2004; Hou et al., 2008; Feakins and Sessions, 2010; Polissar and Freeman, 2010; Sachse et al., 2012; Kahmen et al., 2013a,b). Environmental and biosynthetic isotopic fractionations result in modification of source water hydrogen isotopic composition as hydrogen is incorporated into organic molecules (Sessions et al., 1999; Kahmen et al., 2013a,b). The cumulative isotope modification between source water and molecule is defined as the apparent isotopic fractionation (ε_{app}) and is an observed value that incorporates multiple, poorly understood fractionation steps. Thus, δD values of source water and ε_{app} are the fundamental determinants of molecular δD values.

Uncertainty in estimates of source water δD values from molecular δD measurements stems from (i) uncertainty in ε_{app} and (ii) analytical uncertainties in the determination of molecular δD values. There are differing and inconsistent practices for reporting these uncertainties in the scientific literature, and the implications of the true uncertainty of measurements used for paleohydrologic interpretations remain underappreciated. Previous authors have discussed strategies for accurate and precise measurement of compound-specific δD values (Burgoyne and Hayes, 1998; Sessions et al., 2001a,b; Bilke and Mosandl, 2002; Wang and Sessions, 2008) and a number of authors have investigated approaches for referencing stable isotope measurements (Werner and Brand, 2001; Paul et al., 2007; Zhang et al., 2012). However, there has been no systematic treatment

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of the data reduction and error analysis required for reporting and interpreting molecular isotope values. Here we (i) review the sources of uncertainty inherent to molecular hydrogen isotope measurement, (ii) derive analytical solutions to facilitate calculation and propagation of these uncertainties, (iii) make specific recommendations for reporting uncertainty of δD values for paleohydrologic reconstructions and (iv) provide an electronic worksheet to facilitate determination and systematic reporting of uncertainty (Electronic Annex). Our analysis is based upon fundamental principles of measurement and statistics but is specifically tailored for factors relevant to molecular δD measurements.

2. SOURCES OF UNCERTAINTY

2.1. Analytical uncertainty

Molecular δD values are primarily measured by coupling a gas chromatograph through a pyrolysis reactor to an isotope ratio mass spectrometer (GC–pyr–irMS) (Burgoyne and Hayes, 1998). The analyst measures the $^2H/^1H$ of an analyte peak in the chromatogram relative to the $^2H/^1H$ of a reference peak measured in the same chromatogram. The reference peak(s) is usually a pulse of laboratory H_2 (refgas) introduced at the beginning and/or end of the chromatogram. Less commonly, a reference molecule is co-injected with the sample and elutes within the sample chromatogram. In this manner, a δD value is determined for the analyte on a laboratory-specific reference scale. If needed, the δD value of the analyte molecule is then corrected for any hydrogen added to the molecule during sample processing (e.g. during replacement of exchangeable hydrogen in carboxylic acid and alcohol groups by methylation, silylation or other derivatization reactions). δD values are then transferred from the laboratory-specific scale to the internationally agreed upon Vienna Standard Mean Ocean Water (VSMOW) scale to allow comparison of δD measurements made on different materials (e.g. water) and in different laboratories. Normalization to the VSMOW scale requires δD measurement of the laboratory reference standard (reference gas or coinjected standard) relative to a molecular standard of known δD composition. The reference gas δD value on the VSMOW scale can then be used to determine the δD value of any analyte on the VSMOW scale. Results are reported in delta notation:

$$\delta = \frac{R_{sample}}{R_{VSMOW}} - 1 \quad (1)$$

where $R = {}^2H/{}^1H$, the ratio of deuterium to hydrogen. Because the range of δD values in natural materials is small ($\sim$0 to $-$0.3), they are usually reported in 10^3 units indicated by a ‰ symbol.

Fig. 1 provides a schematic diagram tracing the measured, known and calculated values that are sources of uncertainty in δD analysis. Uncertainty in the isotopic composition of biosynthetically-sourced hydrogen depends upon (in order of decreasing magnitude) (i) the uncertainty of the δD value of the reference H_2 on the VSMOW scale ($\sigma_{refgas,VSMOW}$) determined from molecular standards, ii) the measurement uncertainty of the δD value of the analyte in a chromatogram ($\sigma_{sample,refgas}$), and (iii) the uncertainty of the δD value of any hydrogen added to the molecule ($\sigma_{CH_3,VSMOW}$). Each of these sources of uncertainty is in

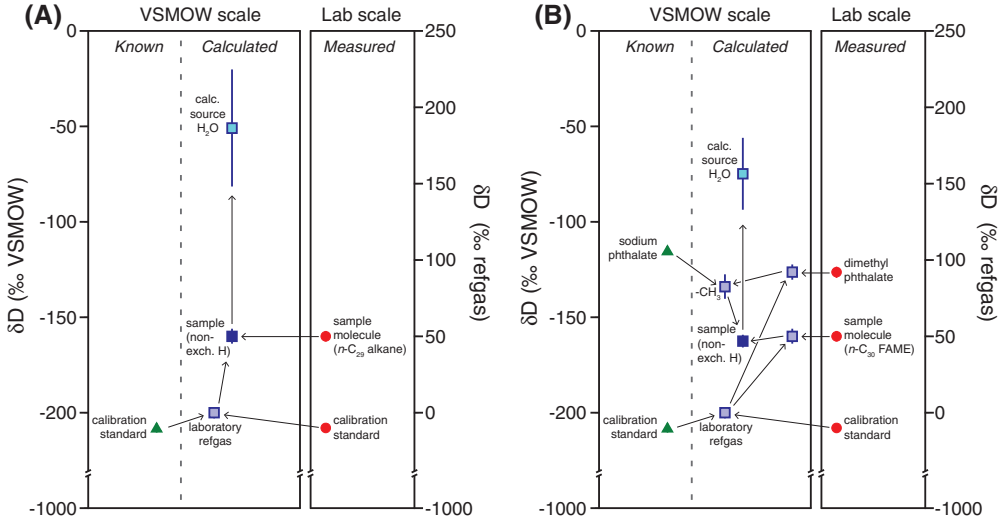

Fig. 1. The relationship between measured and calculated isotope values and laboratory and VSMOW scales for (A) a C_{29} *n*-alkane and (B) a C_{30} *n*-alkanoic acid derivatized as a methyl ester (*n*-C_{30} FAME). The δD value of the methyl group ($-CH_3$) added to the acid is determined by derivatizing sodium phthalate of known isotopic composition to form dimethyl phthalate. The calibration standard is a mixture of molecular standards whose isotopic compositions are known. Arrows indicate the variables needed to calculate a particular isotope value. The smaller uncertainty for source H_2O calculated from the *n*-C_{30} FAME relative to *n*-C_{29} alkane probably reflects the limited geographic range of the empirical calibration datasets used to determine ε_{app} for *n*-alkanoic acids (Hou et al., 2007; Chikarishi and Naraoka, 2007) compared to that for *n*-alkanes (reviewed by Sachse et al., 2012). The VSMOW and Lab isotopic scales are equivalent in the absence of the heavy isotope (i.e., at -1000‰) but scale differently depending on the absolute deuterium/hydrogen ratio of the reference gas.

turn comprised of measurement uncertainties stemming from the analyst's lab and the reported uncertainties from standard reference materials (Fig. 1 and Table 1). In all there are up to five separate, quantifiable uncertainties that contribute to the uncertainty of molecular δD value and a sixth (ε_{app}, discussed below) for estimating δD of source water (Table 1). Typically, only one of these six sources of uncertainty (internal laboratory precision) is reported in the literature, overestimating the confidence with which a δD value is known. The goal of this paper is to provide the stable isotope community with a systematic approach for incorporating all sources of error into the final reporting of a δD value.

The $\sigma_{sample,VSMOW}$ values include the precision of repeated measurements of the same material in a given laboratory ($\sigma_{sample,refgas}$) and the uncertainty in δD value of the reference hydrogen ($\sigma_{refgas,VSMOW}$). Both values are time-dependent and generally stabilize to a larger value over days to years of analysis. If the reference is laboratory hydrogen gas, $\delta D_{refgas,VSMOW}$ values should be determined by measuring molecular reference standards through the same GC–irMS interface as samples (Werner and Brand, 2001). The δD values of the molecular reference standards on the VSMOW scale ($\delta D_{std,VSMOW}$) and their uncertainties $\sigma_{std,VSMOW}$ are known, having been determined by offline methods. The $\sigma_{refgas,VSMOW}$ values include uncertainty from the measurement of the molecular reference standards through the GC–irMS ($\sigma_{std,refgas}$) and from the offline methods $\sigma_{std,VSMOW}$. Similarly, if a co-injected reference compound (refcpd) is used in place of a reference gas, then the $\sigma_{refcpd,VSMOW}$ value reflects the combined uncertainty of its δD value ($\sigma_{refcpd,VSMOW}$ determined by offline methods) and the measurement uncertainty ($\sigma_{sample,refcpd}$) of that molecule by GC–irMS.

The δD value of hydrogen added to samples during derivatization ($\delta D_{CH_3,VSMOW}$) is usually determined by derivatizing a molecule of known δD composition with a derivatizing agent (e.g., by methylating a sodium salt of a carboxylic acid that has been measured by offline methods), measuring the δD value of the resulting molecule ($\delta D_{MCH_3,VSMOW}$) by GC–irMS, and determining the $\delta D_{CH_3,VSMOW}$ value by isotopic mass balance. Uncertainty in this value ($\sigma_{CH_3,VSMOW}$) depends on the uncertainties associated with measuring the derivatized molecule by GC–irMS ($\sigma_{MCH_3,refgas}$) and the offline measurement of the non-exchangeable hydrogen on this molecule ($\sigma_{MNa,VSMOW}$). In cases where the δD_{CH_3} value can be measured directly (e.g. acetic anhydride for the derivatization of alcohol groups), the uncertainty is simply that reported from determination by offline methods.

3. ANALYTICAL SOLUTIONS FOR ERROR PROPAGATION

The following section provides an analytical solution for the δD value of the non-exchangeable hydrogen in a sample molecule and formulation of an equation to quantify its uncertainty.

3.1. Analytical solution for data reduction

The D/H ratio of a sample measured relative to laboratory reference gas (or co-injected reference molecule) is converted to the VSMOW scale using the D/H ratio of the reference gas (or molecule) relative to VSMOW.

$$\frac{R_{sample}}{R_{VSMOW}} = \frac{R_{sample}}{R_{refgas}} \frac{R_{refgas}}{R_{VSMOW}} \quad (2)$$

Table 1
Description of variables including values and uncertainties used in the example calculations (Section 4.4 and EA-1).

Variable	Value	Uncertainty	Represents the isotope composition of:	Scale
Measured				
$\delta_{sample,refgas}$	+53 to + 99	±2.3 (s_p)[a]	Derivatized sample molecule	Lab
$\delta_{std,refgas}$	−66 to +199	±0.5 to 4.0 (s)[a]	Molecular standard, needed for $\delta_{refgas,VSMOW}$ calculation	Lab
$\delta_{MCH_3,refgas}$	+92	±3.0 (s) [a]	Derivatized standard, needed for $\delta_{CH_3,VSMOW}$ calculation	Lab
Calculated				
$\delta_{sample,VSMOW}$	−161 to −125	±2.5 to 3.0 (*SEM*)	Derivatized sample molecule	VSMOW
$\delta_{wax,VSMOW}$	−162 to −125	±2.4 to 3.0 (*SEM*)	Non-exchangeable hydrogen in underivatized sample molecule	VSMOW
$\delta_{refgas,VSMOW}$	−203.7	±2.2 (s)[b]	Laboratory reference gas	VSMOW
$\delta_{CH_3,VSMOW}$	−140.8	±4.4 (*SEM*)	Hydrogen added during derivatization	VSMOW
δ_{H_20}	−61 to −18	±26.4 to 27.6 (s)	Inferred source water	VSMOW
Known				
$\delta_{std,VSMOW}$	−254 to −49	±0.3 to 2.9 (s)[a]	Reported value of molecular standard for reference gas calibration	VSMOW
$\delta_{MNa,SMOW}$	−115.0	±2.0 (s)[a]	Reported value of non-exchangeable hydrogen in standard	VSMOW
ε_{app}	−108.3	±24.9 (s)[b]	Isotopic fractionation between lipid and source water	
f_w	0.95		Mole fraction of H in the derivatized sample that is sourced from the original, underivatized sample molecule	
f_{nx}	0.4		Mole fraction of H in the derivatized standard that is sourced from the original, underivatized standard molecule	

[a] The uncertainty statistic used in error propagation is *SEM*, which is calculated from the unbiased sample standard deviation (s) or pooled standard deviation (s_p) and number of measurements (n).
[b] The uncertainty statistic used in error propagation is s, the unbiased sample standard deviation (see text for discussion).

Using Eq. (1), Eq. (2) can be expressed using δ notation:

$$\delta_{sample,VSMOW} = \delta_{sample,refgas} + \delta_{refgas,VSMOW} + (\delta_{sample,refgas}) \times (\delta_{refgas,VSMOW}) \quad (3)$$

The instrument software typically applies Eq. (3)automatically to calculate δD values on the VSMOW scale after δD values of the reference gas on the VSMOW scale ($\delta_{refgas,VSMOW}$) are supplied by the analyst. The following relationship is used to calculate laboratory reference gas δD values on the VSMOW scale ($R_{refgas,VSMOW}$):

$$\frac{R_{refgas}}{R_{VSMOW}} = \frac{R_{std}}{R_{VSMOW}} / \frac{R_{std}}{R_{refgas}} \quad (4)$$

where R_{std}/R_{VSMOW} is the known isotopic composition of the offline standard on the VSMOW scale and R_{std}/R_{refgas} is the measured isotopic composition of this standard relative to the laboratory reference gas.

This relationship expressed using delta notation is:

$$\delta_{refgas,VSMOW} = \frac{(\delta_{std,VSMOW} + 1)}{(\delta_{std,refgas} + 1)} - 1 \quad (5)$$

3.1.1. Effects of derivatization

Prior to δD measurement, molecules with exchangeable hydrogen are derivatized to remove the non-exchangeable hydrogen and to improve gas chromatography. By isotopic mass balance, the D/H of non-exchangeable hydrogen in a derivatized molecule is:

$$R_{sample} = fR_{wax} + (1 - f)R_{CH_3} \quad (6)$$

where *wax*, *sample* and CH_3 refer to the nonexchangeable hydrogen in the underivatized molecule, all hydrogen in the derivatized molecule and the derivatization hydrogen, respectively, and f is the mole fraction of hydrogen in the derivatized molecule that is from the original underivatized molecule ($f = N_{wax}/[N_{wax} + N_{CH_3}]$).[1] Using the example of a long-chain *n*-alkanoic acid (*wax*) derivatized as a wax ester (*sample*), and the mole fraction of hydrogen in the derivatized sample from the original underivatized sample molecule (f_w), Eq. (6)can be expressed using δ notation and solved for the isotopic composition of non-exchangeable hydrogen:

$$\delta_{wax,VSMOW} = \frac{1}{f_w}\delta_{sample,VSMOW} - \frac{1 - f_w}{f_w}\delta_{CH_3,VSMOW} \quad (7)$$

The δD value of the added hydrogen (δ_{CH_3}) is generally determined by derivatizing and measuring a molecule with nonexchangeable hydrogen of known δD composition. Using the example of a sodium salt of a carboxylic acid (MNa), its methyl ester derivative (MCH_3) and the mole fraction of hydrogen in the derivatized standard that is from the underivatized standard molecule (f_{nx}), Eq. (7)can be recast to solve for the derivatizing hydrogen (CH_3) as:

$$\delta_{CH_3,VSMOW} = \frac{1}{1 - f_{nx}}\delta_{MCH_3,VSMOW} - \frac{f_{nx}}{1 - f_{nx}}\delta_{MNa,VSMOW} \quad (8)$$

3.1.2. General equation for data reduction

Combining Eqs. 3, 7, and 8 yields a general analytical solution for the isotopic composition of non-exchangeable hydrogen in a molecule expressed only in terms of variables either measured in the laboratory ($\delta_{sample,refgas}$, $\delta_{MCH_3,refgas}$, $\delta_{refgas,VSMOW}$) or reported from offline analyses ($\delta_{MNa,VSMOW}$):

$$\delta_{wax} = \frac{1}{f_w}\left[\delta_{sample,refgas} + \delta_{refgas,VSMOW} + \delta_{sample,refgas}\delta_{refgas,VSMOW}\right] - \frac{1 - f_w}{f_w}\left[\frac{1}{1 - f_{nx}}(\delta_{MCH_3,refgas} + \delta_{refgas,VSMOW} + \delta_{MCH_3,refgas}\delta_{refgas,VSMOW}) - \frac{f_{nx}}{1 - f_{nx}}\delta_{MNa,VSMOW}\right] \quad (9)$$

Eq. (9)includes a value for $\delta_{refgas,VSMOW}$ calculated from laboratory measurements by Eq. (5). Table 1 describes the variables used in Eq. (9).

3.1.3. Calculating δD of source water

Values of source water δD (δ_{H_2O}) are related to δD values of non-exchangeable hydrogen in a molecule (δ_{wax}) through the apparent fractionation factor:

$$\alpha_{wax-H_2O} = \alpha_{app} = \frac{\delta_{wax} + 1}{\delta_{H_2O} + 1} \quad (10)$$

often expressed using the notation $\varepsilon = \alpha - 1$. Water δD values are calculated from molecular δD values using:

$$\delta_{H_2O} = \frac{\delta_{wax} + 1}{\varepsilon_{app} + 1} - 1 \quad (11)$$

Eqs. 5, 8, 9, and 11 are incorporated into the electronic spreadsheet (Electronic Annex 1).

3.2. Propagation of uncertainties

Eqs. 5 and 9 together provide for δD calculation of the non-exchangeable hydrogen in a sample molecule ($\delta_{wax,VSMOW}$) based upon three laboratory measurements ($\delta_{sample,refgas}$, $\delta_{MCH_3,refgas}$, $\delta_{std,refgas}$) and two values determined by offline methods elsewhere ($\delta_{MNa,VSMOW}$, $\delta_{std,VSMOW}$). Note that we use δ_{wax} to refer to the isotope composition of the non-exchangeable H in a molecule, while δ_{sample} refers to the composition of all hydrogen. For a derivatized molecule δ_{sample} includes hydrogen added from the derivatizing group. Uncertainty in $\delta_{wax,VSMOW}$ can be determined by standard methods for the propagation of errors assuming uncorrelated and normally distributed errors in the measured and 'known' values (Table 1) (Bevington and Robinson, 2003). Generally, for $x = f(a, b\ldots)$, the uncertainties can be propagated to give the uncertainty in x assuming normally distributed and uncorrelated uncertainties in a and b, (and ignoring covariance terms which are negligible here):

$$\sigma_x^2 = \left(\frac{\partial x}{\partial a}\right)^2\sigma_a^2 + \left(\frac{\partial x}{\partial b}\right)^2\sigma_b^2 + \cdots \quad (12)$$

[1] The isotope ratios (R) in Eq. (6)are an approximation of the exact form using fractional abundances: $F = {}^2H/({}^2H + {}^1H)$. This approximation introduces only minor errors unless the abundance of D becomes significant relative to H, such as for artificially enriched samples (Sessions and Hayes, 2005).

The uncertainty in the δD value of reference gas calculated from a single molecule using Eq. (5) is:

$$\sigma^2_{\delta_{refgas,VSMOW}} = \left(\frac{1}{\delta_{std,refgas}+1}\right)^2 \sigma^2_{\delta_{std,VSMOW}} + \left(\frac{(\delta_{std,VSMOW}+1)}{-(\delta_{std,refgas}+1)^2}\right)^2 \sigma^2_{\delta_{std,refgas}} \quad (13)$$

The uncertainty in the δD value of non-exchangeable hydrogen of the sample molecule (from Eq. (9)) is:

$$\sigma^2_{\delta_{wax,VSMOW}} = \left(\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{sample,refgas}}\right)^2 \sigma^2_{\delta_{sample,refgas}} + \left(\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{refgas,VSMOW}}\right)^2 \sigma^2_{\delta_{refgas,VSMOW}} + \left(\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{MCH_3,refgas}}\right)^2 \sigma^2_{\delta_{MCH_3,refgas}} + \left(\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{MNa,VSMOW}}\right)^2 \sigma^2_{\delta_{MNa,VSMOW}} \quad (14)$$

where

$$\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{sample,refgas}} = \frac{1}{f_w}(1+\delta_{refgas,VSMOW}) \quad (15)$$

$$\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{refgas,VSMOW}} = \frac{1}{f_w}(1+\delta_{sample,refgas}) - \frac{1-f_w}{f_w(1-f_{nx})}(1+\delta_{MCH_3,refgas}) \quad (16)$$

$$\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{MCH_3,refgas}} = -\frac{1-f_w}{f_w(1-f_{nx})}(1+\delta_{refgas,VSMOW}) \quad (17)$$

$$\frac{\partial\delta_{wax,VSMOW}}{\partial\delta_{MNa,VSMOW}} = -\frac{f_{nx}(1-f_w)}{f_w(1-f_{nx})} \quad (18)$$

and the uncertainty of the reference gas δD value on the VSMOW scale is given by Eq. (13). The uncertainty in the reference gas determined by molecular standards, and sample measurement uncertainty are the two largest sources of analytical error. The uncertainty due to correction for derivatization hydrogen is relatively minor (Table 2).

Finally, the uncertainty in source water δD value calculated from the molecular δD value (Eq. (11)) is:

$$\sigma^2_{\delta_{H_2O}} = \left(\frac{1}{\varepsilon_{app}+1}\right)^2 \sigma^2_{\delta_{wax,VSMOW}} + \left(\frac{(\delta_{wax,VSMOW}+1)}{-(\varepsilon_{app}+1)^2}\right)^2 \sigma^2_{\varepsilon_{app}} \quad (19)$$

The uncertainty in ε_{app} is the greatest contributor to reconstructed water δD values (Table 2). Eqs. 13, 14, and 19 are incorporated into the Uncertainty Calculator (Electronic Annex 1).

3.3. Standard deviation or standard error of the mean?

There is much confusion in the scientific literature concerning the use of standard deviation versus standard error of the mean. Uncertainty in nearly all published lipid δD measurements is quantified using the standard deviation; however, the standard error of the mean is the appropriate statistic. The standard deviation describes the variation of a set of measurements (e.g. replicates of the same sample) relative to the sample mean (Taylor, 1997):

$$s_x = \frac{\sum(x_i-\bar{x})^2}{n-1} \quad (20)$$

As the number of measurements increases, the sample standard deviation, s_x approaches the population standard deviation, σ_x. The standard deviation also describes the uncertainty of a *single* measurement (or realization) drawn from an underlying population. In contrast, the standard error of the mean (*SEM*) describes the uncertainty from using the sample mean of a set of measurements to estimate the population mean, and is therefore the appropriate statistic to quantify the uncertainty in the mean of a set of lipid δD measurements (Taylor, 1997). This value decreases toward zero as the number of sample measurements increases:

$$SEM = \frac{s_x}{\sqrt{n}} \quad (21)$$

where n is the number of measurements used to calculate the mean ($\bar{x}$). While the uncertainty of sample δD is best described by the standard error of the mean, as we will explain below, the uncertainty of both the reference gas value as determined by molecular standards and the apparent fractionation factor are best described by the standard deviation.

Table 2
The influence of various sources of uncertainty on the propagated uncertainty. The table reports the hypothetical reduction in uncertainty achieved by eliminating different sources of uncertainty. Note, due to the squared terms in the error propagation equation (Eq. (12)) error reductions do not sum linearly. Calculations are based on the example dataset (Section 4.4 and EA-1).

	$SEM_{sample,VSMOW}$ (% uncertainty reduction)	$SEM_{wax,VSMOW}$ (% uncertainty reduction)	$s_{H20,VSMOW}$ (% uncertainty reduction)
Correct propagated uncertainty	2.6 ‰	2.6 ‰	26.8 ‰
If this uncertainty were zero:			
$s_{sample,refgas}$	2.3 (−12%)	2.2 (−14%)	26.7 (−0.2%)
$s_{refgas,VSMOW}$	1.2 (−53%)	1.3 (−50%)	26.6 (−0.4%)
$s_{MCH_3,refgas}$	2.6 (0%)	2.6 (0%)	26.8 (0%)
$s_{MNa,VSMOW}$	2.6 (0%)	2.6 (0%)	26.8 (0%)
$s_{\in_a}$	2.6 (0%)	2.6 (0%)	2.9 (-89.1%)
All analytical uncertainty[a]	0.0 (−100%)	0.0 (−100%)	26.6 (−0.6%)

[a] Includes all measured and known values in Table 1 except ε_{app}.

3.4. VSMOW–VSLAP normalization

Our treatment of data reduction and analytical uncertainty ignores the normalization procedure recommended to formally realize the VSMOW scale, which is defined by the δD values of two standards: Vienna Standard Mean Ocean Water (VSMOW $\equiv 0‰$) and Vienna Standard Light Antarctic Precipitation (VSLAP $\equiv 428‰$) (Coplen, 1994). Procedures to calibrate molecular δD measurements originally included this normalization by analysis of a mixture of 15 *n*-alkanes with widely varying isotopic values (Sessions et al., 2001a,b). However, it was later recognized that memory effects within the pyrolysis reactor led to a systematic bias of the laboratory-to-VSMOW scaling factor (Wang and Sessions, 2008). These memory effects are subtle and pervasive, and currently limit the ability to accurately apply the VSLAP normalization procedure. However, the lack of VSMOW–VSLAP normalization is generally of minor importance because typical samples and standard analytes span only a limited range of δD values, and the δD value of the commonly used molecular standards for laboratory reference gas calibration are determined offline with the VSLAP normalization procedure. Future analytical efforts are required to control or eliminate this memory effect and allow VSLAP normalization (c.f. Wang et al., 2009).

4. QUANTIFYING UNCERTAINTY

4.1. Uncertainty in the δD value of laboratory reference gas

Typically, the laboratory reference gas is calibrated with a reference standard composed of a mixture of many different molecules. Theoretically, the reference gas values determined from each molecule in the mixture should be identical within measurement uncertainty. However, in practice this is not usually the case. Instead, the differences in reference gas δD values determined from the means of different reference molecules are larger than the standard error of the mean of any individual reference molecule (Fig. 2). Therefore, reference gas uncertainty calculated from any individual reference molecule (Eq. (13)) underestimates the true uncertainty in realizing the VSMOW scale. The larger than expected differences are likely due to memory effects in the pyrolysis reactor (Wang and Sessions, 2008) and other poorly constrained processes that evolve during a GC analysis. There is no *a priori* reason to assume that any individual standard molecule in a mixture better reflects the behavior of a sample molecule; therefore, all available standard molecules should be used to estimate the $\delta D_{refgas,VSMOW}$ value, and the uncertainty thereof ($\sigma_{refgas,VSMOW}$) is not properly described by the standard error of the mean.

Based upon these considerations we recommend the following for determination of the laboratory reference gas (Fig. 2):

1. Measure multiple molecular standards.
2. Define the laboratory reference gas value as the mean of the mean values determined from the different standard molecules.
3. Use the standard deviation of the means of the reference gas values determined from the different standard molecules to decide which of the following approaches to use to quantify the uncertainty in the reference gas:

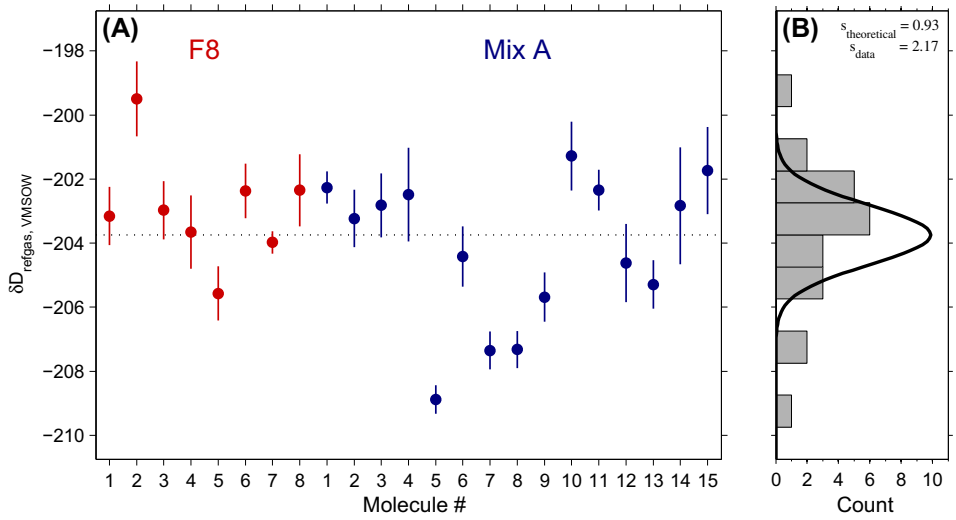

Fig. 2. Determining the reference gas δD value and its uncertainty. (A) 23 independent mean values of $\delta D_{refgas,VSMOW}$ determined using individual standard molecules in mixtures F8 and Mix A (12 injections of F8 and 7 of Mix A). The error bars show ± 1 standard error of the mean for each of the 23 molecules, calculated using Eqs. (13) and (20). The horizontal line is the mean of these values and is assigned as the value of the reference gas. (B) A histogram showing the frequency distribution of the 23 mean values in 1‰ bins centered around the assigned reference gas value. The black curve is the theoretical distribution expected if each of the 23 molecules returned identical mean values for the reference gas within the uncertainty calculated as the error bars in panel A. In the example shown here, the theoretical standard deviation of the mean values is 0.93 ‰ (black curve) while the actual standard deviation of the means is 2.17 ‰ (1σ, gray bars). In this case the standard deviation of the means (i.e., the full dispersion of mean values rather than the standard error) should be used to quantify the uncertainty in the reference gas.

Case 1. If the standard deviation of the means of the reference gas values is less than the analytical uncertainty calculated with Eq. (13) (Fig. 3A), determine the *SEM* of the means (Eq. (20)) using the standard deviation and n equal to the number of molecules in the standard mixture(s).

Case 2. If the standard deviation of the means of the reference gas values is greater than the analytical uncertainty calculated with Eq. (13) (Fig. 3B), use the standard deviation of the means as the reference gas uncertainty.

The supplemental Uncertainty Calculator (Electronic Annex 1) includes both calculations and enables the user to easily determine whether their data fall into Case 1 or Case 2.

4.2. Uncertainty of replicate measurements

The analytical uncertainty of molecular δD measurements is often reported for a sample based upon the dispersion of replicate measurements on that sample (e.g., a sample is measured three times and the standard deviation of the three values is used to quantify the sample uncertainty). However, this is approach is inaccurate for the following three reasons:

1. *When the number of replicates is small, the sample standard deviation is a biased and poor predictor of the population standard deviation.* Estimates of the standard deviation based on small values of n (e.g. $2 \leqslant n \leqslant 10$) contain large uncertainties. This effect is illustrated in Fig. 4, which shows the sampling distribution of the sample standard deviation (s) as a function of sample size (n). The standard deviation of two replicate measurements is often reported in the literature as the measurement uncertainty. However, when $n = 2$ the sample standard deviation systematically underestimates the population standard deviation and the uncertainty in the standard deviation is extremely large (the 95% confidence interval ranges from 2% to 224% of the true standard deviation, Fig. 4B). Slightly larger numbers of replicates only offer modest improvement: the 95% confidence interval for $n = 10$ ranges from 55% to 146% of the true standard deviation (Fig. 4C). Thus, for small n the standard deviation for any particular replicated sample can dramatically underestimate or overestimate the true measurement uncertainty for that sample and on average will be less than the true analytical uncertainty.

We recommend determining the analytical uncertainty from (1) the pooled long-term standard deviations of measured laboratory standards and/or (2) pooled standard deviations from replicate measurements on many samples. With either method, each standard deviation should be corrected for bias due to sample size prior to calculating the pooled standard deviation:

$$s_{unbiased} = \frac{s}{c(n)} \tag{22}$$

The sample standard deviation s is divided by a bias correction factor c that is a function of the number of replicates, n (Holtzman, 1950):

$$c(n) = \sqrt{\frac{2}{n-1}}\frac{\Gamma\left(\frac{n}{2}\right)}{\Gamma\left(\frac{n-1}{2}\right)} \tag{23}$$

where Γ is the gamma function. The bias-corrected standard deviations from either approach can be pooled by calculating a weighted mean:

$$s_p = \sqrt{\frac{\sum(n_i - 1)s_i^2}{\sum(n_i - 1)}} \tag{24}$$

Both of these approaches can increase n significantly to better estimate the population standard deviation, and thus provide a better estimate of analytical uncertainty (Fig. 4). The standard error of each sample mean due to analytical uncertainty is then calculated from the pooled standard deviation and n using Eq. (20). Fig. 5 shows this approach applied to an example dataset. Both the bias correction and pooling of standard deviations are incorporated into the Uncertainty Calculator (EA-1).

2. *The sample standard deviation often systematically underestimates the uncertainty unless replicates are measured over widely spaced time intervals.* The magnitude of

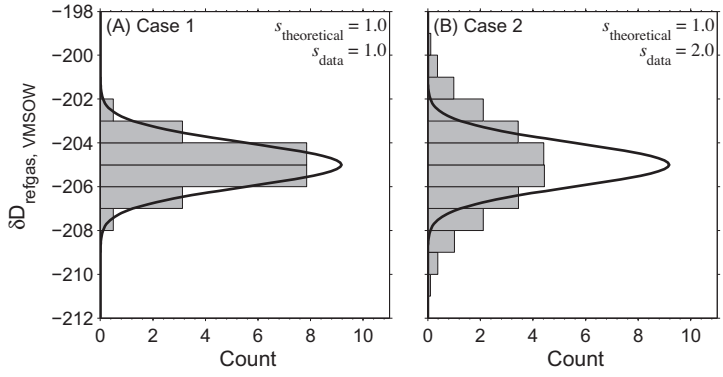

Fig. 3. Theoretical distributions for determining the uncertainty of the reference gas value from a mixture of molecular standards. (A) The standard deviation of the reference gas values determined from different standard molecules (histogram; s_{data}) is equal to the theoretical standard deviation (black curve; $s_{theoretical}$) determined using Eq. (13). The standard error of the mean should be used to quantify uncertainty in the reference gas with n equal to the number of standard molecules (in this example, $n = 23$ and $\sigma_{refgas,VSMOW} = 1.0/\sqrt{23} = 0.21‰$. (B) The standard deviation of the reference gas values is greater than the theoretical standard deviation. The standard deviation of the reference gas values determined from different standard molecules should be used to quantify uncertainty in the reference gas ($\sigma_{refgas,VSMOW} = 2.0‰$).

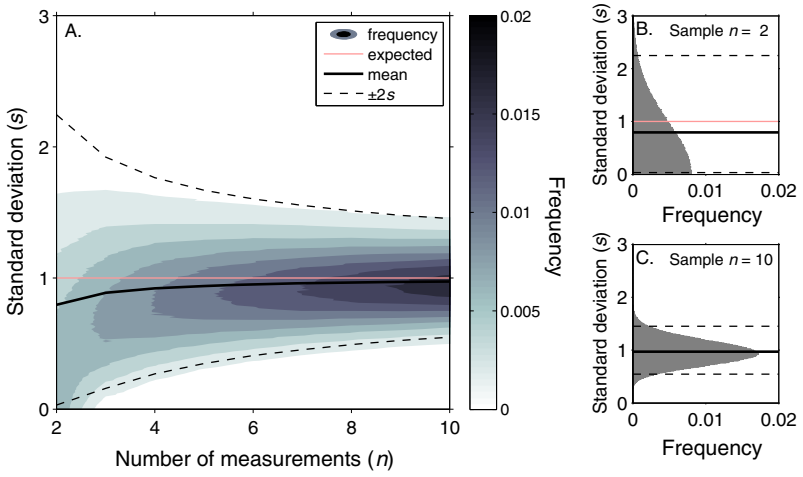

Fig. 4. (A) Relationship between sample size (n) and the distribution of sample standard deviation values (sampling distribution of the sample standard deviation) determined from 10^6 random datasets for each integer value n and a population standard deviation equal to 1.0 (thin horizontal red line in all panels). Thick solid line is the mean standard deviation and dotted lines are the 95% range. For small values of n the standard deviation is underestimated and has a large uncertainty. The underestimation (bias) should be corrected as described in the text. Distributions of standard deviations from sample replicates for (B) $n = 2$ and (C) $n = 10$ illustrate the bias and uncertainty at small sample size. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analytical uncertainty increases with time between measurements. Thus, precision improves for replicates closely spaced in time (Werner and Brand, 2001). This effect can be substantial with molecular δD measurements: back-to-back injections of the same sample may differ by less than 1‰ while injections several weeks apart may differ by 2–4‰. Therefore, a better metric for measurement uncertainty is the long-term (weeks to years) precision of replicates.

3. *The sample standard deviation ignores the uncertainty in the δD value of the reference gas hydrogen needed for normalization to the VSMOW scale ($\sigma_{refgas,VSMOW}$).* This important and quantifiable source of uncertainty is ignored in the vast majority of reported molecular δD values. The problem is easily remedied by including this uncertainty in the manner described above.

4.3. Quantitative comparisons of molecular δD values

Usually, the goal of comparing molecular δD values is to infer changes in the δD composition of source water. This

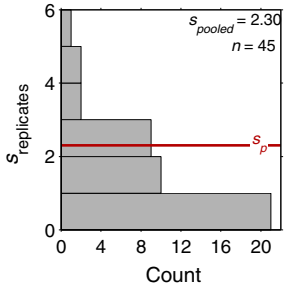

Fig. 5. Frequency distribution of standard deviation values from replicate measurements on samples of C_{30} n-alkanoic acids derivatized as a methyl esters (n-C_{30} FAMES) from the example dataset (data in EA-1). Standard deviations are corrected for bias from small n (Eqs. (22) and (23)) and are pooled by calculating a weighted mean (Eq. (24)).

requires assumptions concerning the apparent fractionation (ε_{app}) between source water and molecular δD values. At present, ε_{app} is based on finite observations of isotopic fractionation between source water and molecules, and is the end result of an unknown number of poorly understood fractionation steps, including those related to evaporative enrichment of soil water and leaf water and molecular biosynthesis. Published data reveal a large range of ε_{app} among, as well as within, plant functional types (reviewed by McInerney et al., 2011; Sachse et al., 2012). It is unclear how much of the observed variability is a result of environmental or climatic controls on ε_{app}, inherent biological variability, or data quality. Therefore, existing ε_{app} values come from empirical observations with quantifiable uncertainties that should be considered and discussed when using these empirical relationships to quantify source water δD, or relative changes therein. *Even if source water is not being explicitly quantified, interpreting differences among molecular δD values requires assumptions concerning the apparent fractionation factor and obliges the researcher to discuss its value and uncertainty.*

How uncertainty in the ε_{app} value is quantified depends upon the source of variability in the ε_{app} calibration datasets. One interpretation is that there is a very narrow 'true' range for ε_{app} and the calibration variability primarily reflects uncertainty in the data quality (e.g. source water δD value and timing of plant growth). In this interpretation, the mean value of the ε_{app} calibrations is the best estimate for the 'true' value of ε_{app} and the uncertainty is given by the standard error of the mean. A second interpretation is that there are different values for ε_{app} and the calibration variability reflects differences in ε_{app} across plant types, ecosystems and climate gradients. Following this interpretation the mean value of the ε_{app} calibrations is still the appropriate estimate for ε_{app} but the uncertainty is given by the standard deviation of the calibration data. The difference arises because in the second case there is no reason to assume that

the mean ε_{app} value provides the best estimate for a particular sample or site.

Existing ε_{app} calibration data support the second interpretation. Differences in ε_{app} are observed between plant functional types, ecosystems, photosystems, and across climate gradients. Therefore, it is likely that the variability of ε_{app} within groups (e.g. broad plant-functional types or ecosystems, Chikaraishi and Naraoka, 2007; Hou et al., 2007; Polissar and Freeman, 2010; McInerney et al., 2011; Sachse et al., 2012) reflects further differences that could be revealed with narrower groupings. Therefore the standard deviation of existing ε_{app} calibrations should be used to discuss and interpret the uncertainty in source water δD estimates.

4.4. Example calculation

The following example illustrates the approach to properly quantify uncertainty in reported molecular δD values. All calculations and results are based upon measurements of molecular standards (Mix A and F8, from Arnt Schimmelmann, Indiana Univ.), and C_{30} *n*-alkanoic acid samples derivatized as methyl esters (*n*-C_{30} FAME; data provided in EA-1). The isotopic composition of the methyl group was determined by derivatizing phthalic acid to dimethyl phthalate and comparing its δD value to that reported for sodium phthalate prepared from the same phthalic acid.

First, determine the reference gas value on the VSMOW scale from multiple GC-irMS measurements of isotope standards and their known δD values (measured offline). For both the *reported* reference standard values and the *measured* reference standard values on the laboratory scale, the Uncertainty Calculator spreadsheet takes as inputs the mean, standard deviation and number of replicate measurements (n) and calculates the unbiased standard deviation and the standard error of the mean (*SEM*). For each standard molecule, the spreadsheet then calculates the δD value and *SEM* of laboratory reference gas on the VSMOW scale. Finally, the spreadsheet calculates the mean of all *SEM* values as the "theoretical uncertainty" and the standard deviation of reference gas δD values from each peak as the "data uncertainty." The analyst compares these two values to determine whether Case 1 or Case 2 applies (see Section 4.1). In this example, the calculation results in a reference gas value of -203.7 ± 2.2 ($1s$) ‰ VSMOW (Fig. 2). The uncertainty reflects Case 2 because the reference gas values determined from each individual reference molecule are statistically different from one another (i.e., the standard deviation of their means, 2.17‰, is greater than the theoretical analytical uncertainty, 0.93‰, calculated with Eq. (13)).

Second, determine the analytical uncertainty of GC–irMS measurements by pooling the standard deviations for all the samples that have replicate measurements. For each sample, the analyst calculates the mean, standard deviation and n for the molecule(s) of interest. The spreadsheet uses these as inputs to calculate the unbiased standard deviation for each molecule and sample, and then determines the pooled analytical standard deviation from the $(n-1)$-weighted average of the unbiased standard deviations (s_p; Eq. (24)). This pooled value is used by the spreadsheet for subsequent error propagation. For the example dataset, the calculated analytical standard deviation (s_p) is 2.3 ‰, and its relationship to the distribution of unbiased standard deviations for all *n*-C_{30} FAME samples in the dataset is shown in Fig. 5.

Last, determine the δD value and uncertainty of a sample on the VSMOW scale and the δD value and uncertainty of source water inferred from the sample. The spreadsheet uses the following inputs:

1. The mean δD value for each sample, the pooled analytical standard deviation (s_p), and number of replicate measurements of each sample (n), all of which have been determined in previous steps.
2. The mean δD value and uncertainty (standard deviation) of the reference gas, determined in a previous step.
3. The mean δD value and *SEM* of the phthalic acid methyl ester (PAME). The *SEM* needs to be provided by the analyst, and can be determined exclusively from multiple measurements of the PAME, or by dividing s_p (as determined previously) by the square root of the number of PAME measurements (Eq. (21)).
4. The mean and *SEM* of sodium phthalate, determined from offline measurements and entered in the spreadsheet by the analyst.
5. The mole fraction of H in the sample molecule (f_w) and in the sodium phthalate (f_{nx}) that was not added by the derivatizing reagent, calculated by the analyst. For *n*-C_{30} FAME, $f_w = (2 \times 30 - 1)/(2 \times 30 + 2)$ and for dimethyl phthalate, $f_{nx} = (4)/(4 + 6)$.
6. The apparent fractionation (ε_{app}) and standard deviation determined from empirical studies and supplied to the spreadsheet by the analyst.

The spreadsheet calculates the *SEM* of (1) the sample molecule, *n*-C_{30} FAME, (2) the phthalic acid dimethyl ester, and (3) the reported value of sodium phthalate. The spreadsheet then calculates the δD value and *SEM* of the non-exchangeable hydrogen in the sample molecule on the VSMOW scale from Eqs. (9) and (14). After error propagation, the δD value and uncertainty of the non-exchangeable hydrogen in the *n*-C_{30} FAME (Sample 1 of Example 5 in EA-1) is -148.2 ± 3.0‰ VSMOW (±1*SEM*). After incorporating the value and uncertainty of ε_{app} (-108.3 ± 24.9‰, $\pm 1s$), the empirical relationship between the δD value of source water and C_{30} *n*-alkanoic acid, the inferred source water δD value is -44.7 ± 26.9‰ VSMOW ($\pm 1s$).

This example illustrates the relative importance of individual sources of uncertainty on the final propagated uncertainty. The reference gas uncertainty is the largest contributor to both $\sigma_{sample,VSMOW}$ and $\sigma_{wax,VSMOW}$, followed by the analytical measurement uncertainty of a sample (Table 2). Both of these sources are only minor contributors to the uncertainty in inferred source water isotopic composition ($\sigma_{H_20,VSMOW}$), which is dominated by uncertainty in ε_{app} (Table 2).

4.5. Application to molecular $\delta^{13}C$ measurements

The data reduction and error propagation strategy outlined here is directly applicable to reporting $\delta^{13}C$ values

from GC–irMS measurement. The only practical changes are that the mole fractions f_w and f_{nx} must refer to carbon rather than hydrogen atoms, and the apparent fractionation factor (ε_{app}) is not applicable. The value ε_{app} is replaced by the photosynthetic fractionation (ε_p) or some other precursor/product relationship with an empirically determined value and an uncertainty that is propagated in the same manner as that for ε_{app}.

5. CONCLUSIONS

We describe a method for error analysis and reporting for molecular δD measurement that is based upon fundamental principles of measurement science. Our analysis and discussion demonstrates that analytical uncertainty is usually improperly estimated and that the importance of uncertainty in apparent fractionation (ε_{app}) between δD values of source water and molecule (the largest single contribution to source water δD uncertainty) is underappreciated. Future studies that reduce uncertainty in ε_{app} could have a large impact on reducing overall uncertainty in molecular δD-based paleohydrologic reconstructions.

Specific recommendations for quantifying and reporting uncertainty are:

1. **Quantify uncertainty in primary measurements**. δD measurement (made relative to a laboratory reference gas or co-injected reference molecule) of two or three analytes is needed to calculate the δD value of any sample molecule on the VSMOW scale. The analytes are: i) sample molecule, ii) molecular standard(s) of known δD value (used to determine the laboratory reference gas on the VSMOW scale), and, if necessary iii) derivatized molecule of known δD value (used to determine δD of hydrogen added during derivatization). An appropriate estimate of the uncertainty in each of these measurements is required to quantify the uncertainty in the δD value of a sample molecule on the VSMOW scale.
2. **Report the uncertainty in primary measurements and calculated values**. All primary measurements should be reported along with an estimate of the uncertainty in the measurement and the basis for this estimate. Uncertainties should be propagated and reported for all calculated δ values using the methods detailed in this paper.
3. **Determine measurement uncertainty from many replicates**. Sample standard deviation determined from an insufficient number of replicate measurements (e.g., $n < 10$–15) provides a poor estimate of the population standard deviation, leading to large uncertainty of the sample standard deviation and standard error of the mean, and systematic underestimation of the measurement uncertainty. A more accurate estimate of measurement uncertainty is provided by the long-term standard deviation of laboratory standards or the pooled standard deviation of sample replicates (corrected for small n bias as described in the text) of all available samples. Standard errors of the mean for individual samples can then be calculated from these values.
4. **Explicitly address the treatment of apparent fractionation between lipid and source water**. The δD value of a biosynthesized molecule is fundamentally determined by the δD value of the hydrogen source and the isotopic fractionations that occur during the transfer of this hydrogen to the molecule. These fractionation steps and their magnitudes, variability and controls are poorly understood; furthermore, their combined impact (apparent fractionation, ε_{app}) is the largest source of uncertainty when estimating source water δD values. At present, uncertainty in ε_{app} values for broad groupings of plant functional types and photosystems is best described by the standard deviation of the calibration data not the standard error of the mean. When making paleohydrologic interpretations based on the differences in molecular (or estimated source water) δD values among different samples, it is important to acknowledge the assumptions concerning ε_{app} (e.g. if ε_{app} is assumed to remain constant through time). These assumptions should be discussed even if source water δD values are not explicitly quantified.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.gca.2013.12.021.

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