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Naturally present fatty acids as internal calibrants for Fourier transform mass spectra of dissolved organic matter

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Abstract

The analysis of dissolved organic matter (DOM) by Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) has gained wide interest recently, driven primarily by its ultrahigh resolving power and mass accuracy. Accurate calibration of mass spectra is a key step to successfully decipher the DOM components. We propose a simple and accurate method to internally calibrate the peaks in the complex spectra without the need to add a calibrant. Mass spectra of DOM samples from the Dismal Swamp, Virginia, and the lower Chesapeake Bay display the presence of naturally occurring fatty acids which can be readily recognized and calibrated with accuracies <0.1 ppm. Once calibrated with fatty acids, approximately 80% of all peaks in the DOM mass spectra can be assigned unique molecular formulas with accuracies <0.4 ppm. Although the formula errors for the assigned molecular formulas do increase with increasing m/z, the dynamic range of the fatty acids used as calibrants is sufficient because high m/z values (>600) have formulas with an average error of <0.6 ppm. Because fatty acids are ubiquitous components of most DOM, this approach is applicable to a large variety of DOM samples.

Introduction

Dissolved organic matter (DOM) is a complex assemblage of organic molecules from natural waters, and information on its chemical composition is crucial if we are to understand its source, reactivity, and global cycling. An improved understanding of its composition is also essential to understand how pollutants react with DOM and possibly become less bioavailable due to this interaction (Traina et al. 1996; Akkanen and Kukkonen 2003; Gourlay et al. 2005). Dissolved organic carbon (DOC) is a significant component of the global carbon cycle, accounting for a pool of active carbon (680 × 10^{15} g C) that is approximately equal to that of atmospheric carbon dioxide (Hedges 1992; Eglinton and Repeta 2003). DOM has defied complete molecular level characterization by most analytical techniques, primarily because it exists as a

highly functionalized, complicated polyelectrolyte mixture. To date, <10% of DOM can be characterized as amino acids, sugars, and other chemicals using traditional chromatographic analyses (Perdue and Ritchie 2003). Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) has recently changed this analytical shortcoming by providing the first molecular-level details for DOM (Kujawinski et al. 2002, 2004; Llewelyn et al. 2002; Stenson et al. 2002, 2003; Kim et al. 2003a, 2003b, 2004, 2006; Kramer et al. 2004; Koch et al. 2005; Hockaday et al. 2006; Sleighter and Hatcher 2007). The ultrahigh resolving power (>400,000) and mass accuracy (<1 ppm) of FTICR-MS provides the ability to assign unique molecular formulas to thousands of components in a single DOM sample.

Accurately calibrating the FTICR mass spectrum is key to successful molecular formula assignments (Muddiman and Oberg 2005; Kujawinski and Behn 2006). One must meticulously calibrate the spectrum both externally and internally to achieve the mass accuracy of 1 ppm that is generally needed for unique formula assignments at masses <500 Da. Typically, external calibration is achieved by use of a synthetic standard or a manufacturer's specific tuning mix, with accuracies of 2–5 ppm. Internal calibration can be applied by use of numerous methods that basically add an internal calibrant. Many studies of DOM using the instrumentation at the National High Magnetic Field Laboratory in Tallahassee, FL, have employed a dual-spray injection technique (Hannis and Muddiman 2000) to simultaneously coinject calibrants

into the source (Kim et al. 2003a, 2004, 2006; Hockaday et al. 2006). The ions from the calibrants are accumulated with analyte ions in the hexapole, and then both calibrant and analyte ions are transferred to the ICR cell. The peaks in the resulting mass spectrum can be calibrated by reference to the exact m/z of the calibrant ions. This approach is successful if no overlap exists between calibrant and analyte peaks; however, the complicated nature of DOM spectra places constraints on this requirement. To overcome these complications, the sample is analyzed separately without calibrants, and the resulting spectrum is then internally calibrated by use of the exact m/z values of the major DOM peaks in the spectra previously obtained in the presence of calibrants. The major disadvantage of this approach is that each sample must be analyzed at least twice, doubling instrument time. Furthermore, the DOM signals can easily be overwhelmed by the added standards, especially for samples with low DOC concentrations.

Internal calibration to <1 ppm is also possible without this dual-spray procedure. Essentially, the protocol described above is used, but the sample is mixed with the internal standard before ionization and usually analyzed again separately without the standard (Kujawinski et al. 2002, 2004; Llewelyn et al. 2002; Stenson et al. 2003; Koch et al. 2005). However, this method also requires twice the instrument time for data acquisition.

In this study, we describe a new internal calibration approach for DOM that takes advantage of the fatty acids naturally present in the sample. Although the petroleum community has previously calibrated spectra with a homologous series of compounds known to be present (Schaub et al. 2005; Fu et al. 2006; Klein et al. 2006a, 2006b), this is the first time, to our knowledge, that such calibration has been accomplished with DOM samples, which are compositionally quite different from petroleum. Fatty acids, mainly those with carbon chain lengths of 14 to 32 (C_{14} to C_{32}), are ubiquitous components of DOM (Slowey et al. 1962; Mannino and Harvey 1999; Minor et al. 2001; Kaiser et al. 2003; Frazier et al. 2005; McCallister et al. 2006). Saturated fatty acids from C_{14} to C_{32} are typically derived from terrestrial vegetation, whereas mono- and polyunsaturated fatty acids, usually with carbon numbers <22, are characteristic of plankton (Mannino and Harvey 1999). McCallister et al. (2006) determined concentrations of fatty acids in DOM from the York River estuary, Virginia, to be in the range of 0.4 to 2.9 μg fatty acid/mg organic carbon.

Fatty acids are ideal for use as internal calibrants, mainly because they have high ionization efficiencies in negative ionization mode owing to their carboxyl group (Henriksen et al. 2005). In addition, because saturated fatty acids are hydrogen rich and have a high mass defect (distance displaced from the exact nominal mass), they tend to separate well from other ions typically detected in DOM mass spectra. Saturated fatty acids with mass defects in the range of 0.2 to 0.4 reflect mid-length fatty acids (C_{14}–C_{22}), and longer-chain fatty acids (C_{23}–C_{40}) appear at mass defects of 0.4 to 0.6. Accordingly, they can be readily recognized in the spectra. Furthermore, by

use of Kendrick mass defect (KMD) analysis, we can easily identify the homologous series of saturated fatty acids in our samples. KMD analysis categorizes m/z values that differ only by the exact mass of a certain functional group, such as a CH_2 group (Stenson et al. 2003; Kujawinski and Behn 2006; Sleighter and Hatcher 2007). Saturated fatty acids have a generic formula of $C_nH_{2n}COOH$, which give a KMD value of 0.9480. These criteria are used to confirm the presence of saturated fatty acids in DOM. Internal calibration of ultrahigh-resolution mass spectra can be performed accurately and with ease by use of the fatty acids that are unambiguously present in these complex DOM samples.

Materials and procedures

Sample preparation—To illustrate the use of fatty acids as natural calibrants, we chose 2 different DOM samples. The first was from the Great Dismal Swamp in Suffolk, Virginia. This site represents the swampy, highly terrestrial DOC headwaters of the Elizabeth River system. The water has an ambient pH of about 3.3–4.5 and DOC concentrations in the range of 60–140 ppm C, depending on the time of sampling and recent precipitation (Johannesson et al. 2004). We filtered Dismal Swamp water (250 mL) through a 0.1-μm polycap cartridge filter (Whatman) and acidified it to pH 2. All of the filtrate was extracted with a 47-mm solid phase C_{18} extraction disk (3M, Empore), and adsorbed organic matter was eluted with 20 mL LC-MS–grade methanol (Fisher Scientific). The second sample, collected from on board the R/V *Hugh R. Sharp*, was taken from surface water of the Chesapeake Bay mouth using Niskin bottles on a CTD rosette. With a pH of about 8.0, a salinity of 25, and DOC concentrations in the range of 1–3 ppm C, this water is typical coastal ocean water. We filtered about 2 L Chesapeake Bay water through doubly stacked 47-mm precombusted 0.7 μm glass fiber filters (Whatman). All of the filtrate was then C_{18} extracted using the same procedure described above.

To confirm that the mass spectral peaks were indeed fatty acids, each DOM sample was spiked with a fatty acid standard (Sigma-Aldrich) to a final concentration of 0.001 mg fatty acid/mL DOM solution. The fatty acid standard comprised 8 saturated fatty acids in the range of C_{15}–C_{30} (Table 1). An approximate concentration of 0.02 mg/mL of each fatty acid was prepared in 50:50 (vol/vol) methanol:acetonitrile with 0.1% ammonium hydroxide.

To show that this fatty acid standard could be used to calibrate other mixtures of compounds, we prepared a simple, easily ionized, and readily available peptide mixture (Sigma-Aldrich) to which our fatty acid standard was added. The peptide mixture consisted of numerous peptides (Table 1), each at a concentration of approximately 0.15 mg/mL in 50:50 (vol/vol) methanol:water. For mass spectral analysis, the fatty acid standard and the peptide mixture were diluted to final concentrations of 0.005 and 0.015 mg/mL, respectively.

Table 1. Composition of the fatty acid and peptide standards.

	Formula	Exact MW negative ion	Final concentration, mg/mL
Fatty acid			
n-Pentadecanoic acid	$C_{15}H_{30}O_2$	241.216755	4.96×10^{-3}
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	255.232405	4.95×10^{-3}
n-Nonadecanoic acid	$C_{19}H_{38}O_2$	297.279355	4.57×10^{-3}
n-Eicosanoic acid	$C_{20}H_{40}O_2$	311.295005	4.38×10^{-3}
n-Docosanoic acid	$C_{22}H_{44}O_2$	339.326306	4.57×10^{-3}
n-Tetracosanoic acid	$C_{24}H_{48}O_2$	367.357606	4.92×10^{-3}
n-Hexacosanoic acid	$C_{26}H_{52}O_2$	395.388906	4.54×10^{-3}
n-Triacontanoic acid	$C_{30}H_{60}O_2$	451.451506	4.79×10^{-3}
Amino acid sequence			
Glu-Asn-Gly	$C_{11}H_{16}N_4O_6$	299.099708	1.48×10^{-2}
Val-Pro-Leu	$C_{16}H_{29}N_3O_4$	326.208530	1.53×10^{-2}
Val-Thr-Cys-Gly	$C_{14}H_{26}N_4O_6S$	377.150029	1.41×10^{-2}
Met-Leu-Phe	$C_{20}H_{31}N_3O_4S$	408.196251	1.87×10^{-2}
Lys-Val-Ile-Leu-Phe	$C_{32}H_{54}N_6O_6$	617.403207	1.49×10^{-2}

The exact molecular weight of the negative ion is the exact molecular mass – 1 H. The final concentrations are those that were introduced into the mass spectrometer.

Instrumentation—Before analyzing the C_{18} extracted DOM samples, a blank of 50:50 (vol/vol) methanol:water with 0.1% ammonium hydroxide was analyzed on the FTICR-MS to ensure that no fatty acids or peptides from previous analyses would contaminate the sample spectra. This blank analysis confirmed that both standards were completely rinsed from the ion source before analyzing the DOM samples. The C_{18} extracted samples were diluted with LC-MS–grade water (Fisher Scientific) to a final sample composition of 50:50 (vol/vol) methanol:water. To increase the ionization efficiency, ammonium hydroxide was added to all samples immediately before MS analysis, bringing the pH to about 8. The samples were continuously infused into the Apollo II ESI ion source of a Bruker Daltonics 12 Tesla Apex Qe FTICR-MS, housed at the College of Sciences Major Instrumentation Cluster (COSMIC) at Old Dominion University. Samples were introduced by a syringe pump operating at a rate of 120 µL/h. All samples were analyzed in negative ion mode, and electrospray voltages were optimized for each sample. To acquire the optimal resolving power, the ion accumulation time and the number of coadded transients, collected with a 4 MWord time domain (the FID data set acquisition size), were adjusted for each sample. Ion accumulation times were in the range of 0.1–2.0 s, and the number of scans was 25–100. Each summed FID signal was zero-filled once and Sine-Bell apodized before fast Fourier transformation and magnitude calculation using the Bruker Daltonics Data Analysis software. The instrument was initially externally calibrated with PEG (polyethylene glycol).

Assessment

Fatty acid standard and peptide mixture—The fatty acid standard was first analyzed separately to optimize instru-

mental conditions (1.0-s ion accumulation and 50 coadded transients). As Fig. 1A shows, each fatty acid is numbered 1–8, and its corresponding *m/z* value is shown in the inset. The spectrum was internally calibrated using the fatty acids' known masses as the calibrant list. The calibration errors, measured as deviation from a quadratic fit of the fatty acid masses, are all <0.3 ppm. Once this fatty acid standard was optimized alone, we combined it with the peptide mixture to test the fatty acids' ability to accurately calibrate a mixture with a more diverse mixture of components. The negative ion mass spectrum for the peptide mixture to which the fatty acid standard was added is shown in Figure 1B, and the peptides are labeled a–e with their corresponding *m/z* values shown in the inset. The peak abundance relationships of fatty acids in this mixture are greatly changed with the addition of peptides, which is likely due to the charge competition between peptides and fatty acids during the electrospray process (Cech and Enke 2000, 2001). The exact reasons for this phenomenon need to be further explored, and ongoing research within our group is currently aimed at determining how ionization efficiencies affect the observed relative abundances of various DOM moieties in the mass spectra. Molecular formula assignments for the peptides were made by inserting their *m/z* values into a molecular formula calculator (Molecular Formula Calc v.1.0, ©NHMFL, 1998), which generated empirical formula matches using carbon, hydrogen, oxygen, nitrogen, sulfur, and phosphorus. Formula errors were determined by comparing the *m/z* value to the calculated exact mass of the assigned formula. Table 2 shows the mass accuracy from the internal calibration and errors in assigning molecular formula matches for the peptides. The
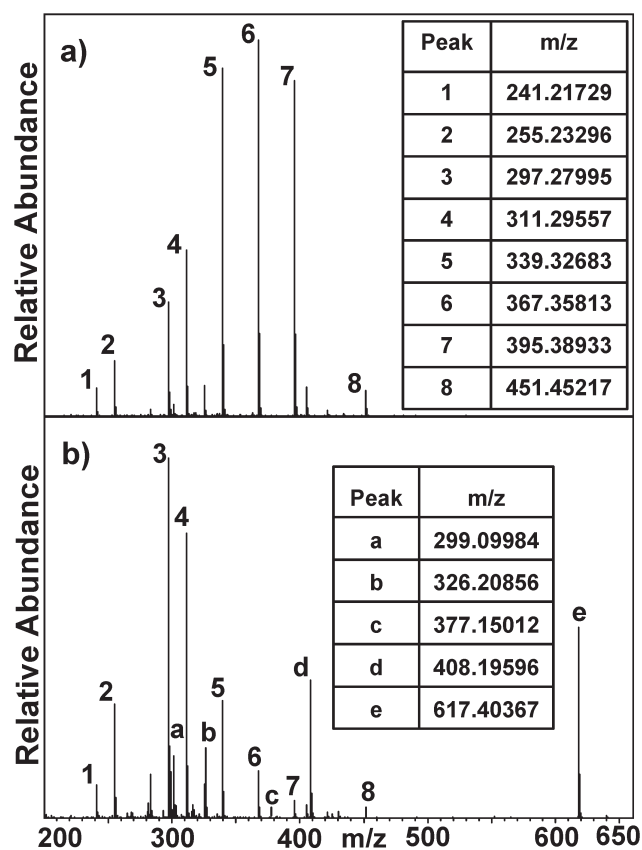
Peak	m/z
1	241.21729
2	255.23296
3	297.27995
4	311.29557
5	339.32683
6	367.35813
7	395.38933
8	451.45217

Peak	m/z
a	299.09984
b	326.20856
c	377.15012
d	408.19596
e	617.40367

Fig. 1. (A) Negative ion mass spectrum of the fatty acid standard. The exact *m/z* values of the fatty acids (peaks 1–8) are listed in the inset. (B) Negative ion mass spectrum of the fatty acid standard mixed with the peptide standard. The exact *m/z* values of the peptides (peaks a–e) are listed in the inset. Each spectrum had an optimal resolving power with an ion accumulation time of 1.0 s and 50 coadded transients.

root mean square deviation (RMSD) was calculated to measure the differences between the observed *m/z* values and the exact masses calculated from the assigned molecular formulas, by the formula shown below:

$$RMSD = [(1/n) \, \Sigma(x_{1,i} - x_{2,i})^2]^{1/2}$$

where n is the number of values in the dataset (5 in this case), x_1 values are the observed *m/z* values, and x_2 are the calculated exact masses. The RMSD for the peptide mixture calibrated by the fatty acid standard was 2.5×10^{-4}. Clearly, this peptide mixture was well calibrated by using the fatty acids, as indicated by the high mass accuracy of the internal calibration, the low errors for the molecular formula matches, and the low RMSD.

DOM samples—The Dismal Swamp DOM and the Chesapeake Bay DOM mass spectra are shown in Fig. 2A and B, respectively. It is apparent from the insets of Fig. 2 that these mass spectra contain thousands of peaks, with up to 15 peaks per nominal mass over the entire *m/z* range of 200–650. Other investigators have observed the same degree of complexity for DOM (Kujawinski et al. 2002; Kim et al. 2003a; Stenson et al. 2003). The ultrahigh resolving power (>400,000 broadband), defined as the exact *m/z* value divided by the full width at half maximum (FWHM) of that peak, is absolutely essential to resolve each peak in samples such as DOM. We determined that the peaks in the spectra were singly charged, which is consistent with previous studies (Kujawinski et al. 2002; Stenson et al. 2002; Kim et al. 2003a). The large peak at *m/z* 368.97651 is an artifact from the C_{18} extraction and is present in all of our C_{18} extracted samples.

As mentioned above, saturated fatty acids have a higher mass defect than other DOM compounds, which simplifies their identification (insets of Fig. 2). To confirm that these high mass defect peaks are in fact fatty acids, each DOM sample was spiked with the fatty acid standard (0.001 mg fatty acid/mL DOM solution) and analyzed using the same conditions as above. The signal enhancement for the previously assigned fatty acid peaks in the spiked samples, as shown in Fig. 3, verified the natural presence of the fatty acids. The cluster at lower mass defect stood out over the fatty acid peak when only the DOM sample was analyzed, but when spiked with the fatty acid standard, the high mass defect fatty acid peak dominated over the entire nominal mass. Although only 2 nominal mass regions of the Chesapeake Bay DOM

Table 2. Mass accuracy of the fatty acids used for internal calibration of the mixture (fatty acids standard with the peptide mixture) and error values for the molecular formula matches to each peptide.

Fatty acid	Formula	Mass accuracy, ppm	Molecular formula calculator	Error from molecular formula calculator, ppm
n-Pentadecanoic acid	$C_{15}H_{30}O_2$	–0.028	$C_{11}H_{15}N_4O_6$	0.44
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	0.073	$C_{16}H_{28}N_3O_4$	0.092
n-Nonadecanoic acid	$C_{19}H_{38}O_2$	–0.046	$C_{14}H_{25}N_4O_6S$	0.24
n-Eicosanoic acid	$C_{20}H_{40}O_2$	–0.15	$C_{20}H_{30}N_3O_4S$	–0.71
n-Docosanoic acid	$C_{22}H_{44}O_2$	0.13	$C_{32}H_{53}N_6O_6$	0.75
n-Tetracosanoic acid	$C_{24}H_{48}O_2$	0.16		
n-Hexacosanoic acid	$C_{26}H_{52}O_2$	–0.15		

The root mean square deviation (RMSD) calculated for the peptide mixture was 2.5×10^{-4} (see text for details of this calculation).
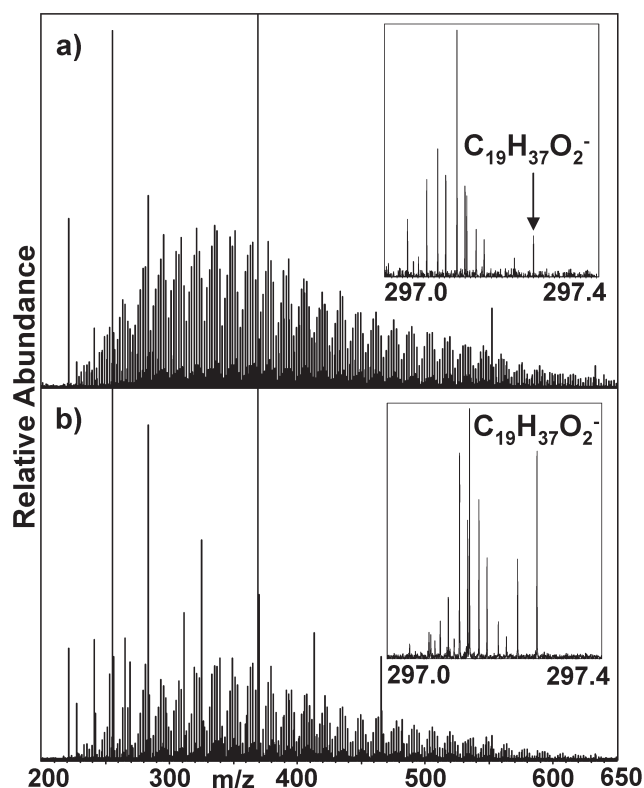
Fig. 2. (A) Negative ion mass spectrum of the C_{18} extracted Dismal Swamp water. (B) Negative ion mass spectrum of the C_{18} extracted Chesapeake Bay water. Each spectrum was optimized for the number of peaks and highest resolving power using an ion accumulation time of 1.0 s and 100 coadded transients. The insets show an expanded region of 297.0–297.4. In the inset, the peak with the highest m/z is the C_{19} fatty acid, which is well separated from the other peaks at the 297 nominal mass.
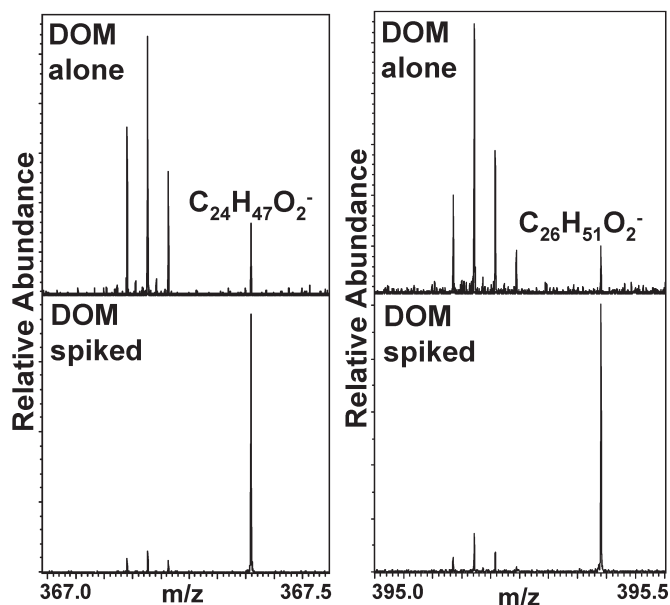

Fig. 3. Negative ion mass spectra of the C_{18} extracted Chesapeake Bay water spiked with the fatty acid standard. The top spectrum in each is the DOM sample alone and the bottom is the DOM spiked with the fatty acid standard. The peak with the highest mass defect in each set is the fatty acid, whose enhancement is apparent when spiked with the standard.

are shown as examples in Fig. 3, the enhancement for each fatty acid in both DOM samples is analogous. It should be noted that the peaks we labeled as naturally present fatty acids may not be n-fatty acids (straight chain). Because mass spectrometry does not distinguish between structural isomers, each peak likely contains numerous isomers with varying degrees of branching.

The DOM samples examined here showed fatty acid distributions from C_{14} to C_{26}, as determined by their KMD for the homologous series of saturated fatty acids (KMD 0.9480). It should be noted, however, that smaller fatty acids are likely naturally present within these samples, but the FTICR-MS typically discriminates against ions with m/z values <225 (Sleighter and Hatcher 2007). The mass spectra were internally calibrated with the naturally present fatty acids, and then all the m/z values with a signal-to-noise ratio >5 were inserted into the molecular formula calculator. Below m/z 400, only 1 formula fell within the 1.0-ppm error limit, providing an unequivocal assignment. Above m/z 400, where multiple

formulas exist for 1 m/z value, the correct formula was assigned by use of KMD analysis and the formula extension approach described by Kujawinski and Behn (2006). This procedure leads to approximately a thousand individual formulas for peaks in each sample spectrum. The mass accuracies from the internal calibration for the fatty acids were <0.1 ppm (Table 3). This value is slightly lower than that of the fatty acid standard, but we do not believe that the difference is significant since calibration values vary from sample to sample. Using this internal standard approach, the vast majority (approximately 80%) of formula assignments for peaks in DOM are within 0.4 ppm, and <5% of the formula assignments have an error >0.8 ppm (Fig. 4). These low error values allow us to confidently state that the range of fatty acids used was adequate for our formula assignments. Although the dynamic range of the calibrants (225–400 m/z) covered only about 50% of the range of DOM components (200–650 m/z), the fatty acids do accurately calibrate the DOM peaks >400 m/z. Fig. 5 shows the calculated average formula errors for each m/z range, subdivided for every 25 m/z units. Over the range of the calibration, from m/z 225 to 400, the average formula errors are <0.1 ppm. The formula errors began to slightly increase once the analyte peak was outside the calibration range. These errors increased to 0.5 ppm when the m/z exceeded 550 and then reached a maximum average error of approximately 0.6 ppm above that. An equivalent plot was also constructed by calculating the RMSD for each m/z range, rather than average formula error. As with the average formula

Table 3. Mass accuracy of the fatty acids used for internal calibration and the error values for the molecular formula matches to those fatty acids in the C_{18} extracted Dismal Swamp water and Chesapeake Bay water.

Fatty acid	Formula	Mass accuracy, ppm	Molecular formula calculator	Error from molecular formula calculator
Dismal Swamp				
Tetradecanoic acid	$C_{14}H_{28}O_2$	–0.020	$C_{14}H_{27}O_2$	–0.018
Pentadecanoic acid	$C_{15}H_{30}O_2$	0.047	$C_{15}H_{29}O_2$	0.066
Hexadecanoic acid	$C_{16}H_{32}O_2$	–0.024	$C_{16}H_{31}O_2$	–0.016
Heptadecanoic acid	$C_{17}H_{34}O_2$	0.017	$C_{17}H_{33}O_2$	0.022
Octadecanoic acid	$C_{18}H_{36}O_2$	–0.036	$C_{18}H_{35}O_2$	–0.049
Nonadecanoic acid	$C_{19}H_{38}O_2$	–0.004	$C_{19}H_{37}O_2$	–0.013
Eicosanoic acid	$C_{20}H_{40}O_2$	0.019	$C_{20}H_{39}O_2$	0.019
Chesapeake Bay				
Hexacosanoic acid	$C_{26}H_{52}O_2$	–0.095	$C_{26}H_51O_2$	0.086
Tetracosanoic acid	$C_{24}H_{48}O_2$	0.099	$C_{24}H_{47}O_2$	0.098
Docosanoic acid	$C_{22}H_{44}O_2$	0.044	$C_{22}H_{43}O_2$	0.047
Henicosanoic acid	$C_{21}H_{42}O_2$	0.003	$C_{21}H_{41}O_2$	–0.012
Eicosanoic acid	$C_{20}H_{40}O_2$	–0.064	$C_{20}H_{39}O_2$	–0.077
Nonadecanoic acid	$C_{19}H_{38}O_2$	0.040	$C_{19}H_{37}O_2$	0.054
Octadecanoic acid	$C_{18}H_{36}O_2$	–0.011	$C_{18}H_{35}O_2$	–0.014
Heptadecanoic acid	$C_{17}H_{34}O_2$	–0.007	$C_{17}H_{33}O_2$	–0.015
Hexadecanoic acid	$C_{16}H_{32}O_2$	–0.024	$C_{16}H_{31}O_2$	–0.016
Pentadecanoic acid	$C_{15}H_{30}O_2$	0.000	$C_{15}H_{29}O_2$	–0.017
Tetradecanoic acid	$C_{14}H_{28}O_2$	0.015	$C_{14}H_{27}O_2$	0.026

Fatty acids in these samples are naturally present and were identified based on their Kendrick mass defect of 0.9480.

error plot, the RMSD was nearly constant up to m/z 400, and then the RMSD values increased up to m/z 600. The range of RMSD values was 2.5×10^{-5} to 7.4×10^{-5} for m/z 225 to 400. The RMSD increases to 3.0×10^{-4} up to m/z 550 and reaches 3.9×10^{-4} above that. This information, along with the fact that <20% of the total formulas have an error >0.5 ppm, verifies that this range of fatty acids is sufficient for the internal calibration of the entire m/z range of the DOM mass spectra. Of course, the presence of fatty acids with higher masses in DOM samples would extend the range of accuracy beyond that described here.

Comments and recommendations

The ultrahigh resolving power and mass accuracy of FTICR-MS has the ability to extensively characterize DOM samples, but an accurate calibrating procedure is required to successfully obtain molecular formulas from the complex mass spectra usually obtained for these substances. The compositional differences elucidated by FTICR-MS of the 2 very different DOM samples discussed in this study will be reported elsewhere (Sleighter and Hatcher, 2008). Our method demonstrates a new protocol for internal calibration that uses the easily recognizable fatty acids naturally present in DOM samples. This procedure achieves accurate, high-quality mass spectral assignments, reduces instrument time, and maintains the ultrahigh resolving power that is required

for FTICR-MS analysis of DOM. Although other studies using a synthetic internal standard, whether mixing with the sample before ionization or using a dual-spray injection technique, accomplish the mass accuracy required for data interpretation and formula assignments, they also call for multiple analyses for each sample and are therefore much more time consuming.
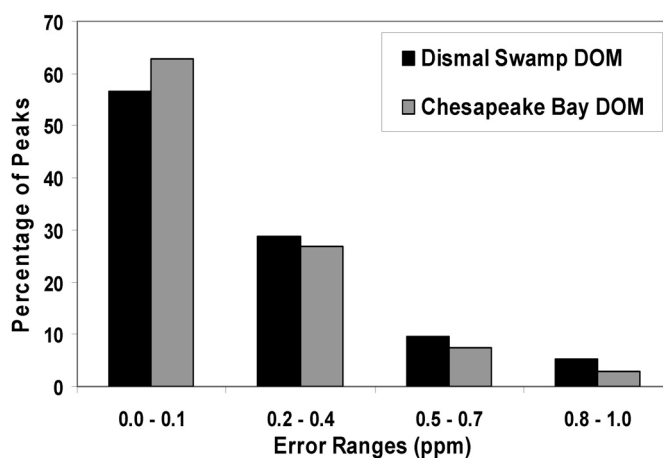

Fig. 4. The percentages of molecular formula matches that exist in each ppm error range for the C_{18} extracted Dismal Swamp water (black) and the C_{18} extracted Chesapeake Bay water (gray). The ppm error ranges are from the absolute error values determined by the molecular formula calculator.

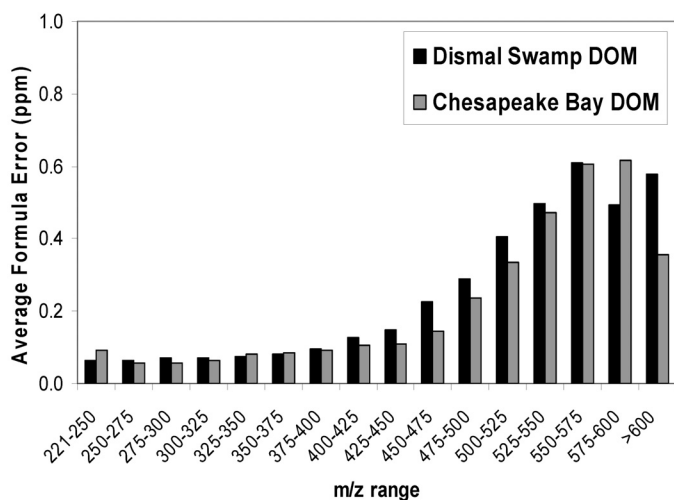**Fig. 5.** The calculated average formula error values (ppm) for each m/z range in the C_{18} extracted Dismal Swamp water (black) and the C_{18} extracted Chesapeake Bay water (gray). The average formula errors were calculated from the absolute error values (determined by the molecular formula calculator) of each molecular formula in the given m/z range.

Our method eliminates the need for multiple analyses. Also, because our calibrants are naturally present within the sample make-up, they eliminate 2 other major problems associated with a synthetic calibrant: interfering overlap between calibrants and analyte, and balancing the relative abundances of calibrants with that of each analyte. As previously mentioned, fatty acids are ubiquitous components of many DOM samples, as well as other natural organic matter (NOM) samples [i.e., humic and fulvic acids, particulate organic matter (POM), soil and sedimentary organic matter (SOM), atmospheric organic matter (AOM), etc.], and this method can most likely be applied to their FTICR-MS analyses as well.

The particularly critical aspects of this procedure are that fatty acids are present in measurable abundance in the DOM samples and they exist across a wide range of m/z values. Even when the concentrations of fatty acids are low in the natural samples, their high ionization efficiency in ESI will allow for their detection using FTICR-MS. Once the range of fatty acids present is determined for the sample, the internal calibration can be performed quickly and accurately without the concern of the impeding overlaps of calibrants with analyte peaks.

Although here we have focused on the saturated fatty acids naturally present in DOM, it should be recognized that numerous unsaturated fatty acids (mono- and polyunsaturated) as well as di-acids, COOH–(CH2)$_n$–COOH, also exist in DOM samples. Both unsaturated fatty acids and di-acids were observed in these 2 DOM samples, and these components can also be used for internal calibration. These compounds, if they exist in measurable abundance in other samples, could be used to fill in gaps where saturated fatty acids may not exist, as well as to increase the dynamic range of the overall calibration.

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