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High-Resolution Fourier Transform Ion Cyclotron Resonance Mass Spectrometry of Humic and Fulvic Acids: Improvements and Comparisons

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Full structural characterization of complex mixtures such as humic acid extracts has been elusive because of insufficient compound resolution with conventional techniques. Using electrospray ionization coupled with Fourier transform ion cyclotron resonance mass spectrometry, we were able to resolve individual compounds within humic and fulvic acid mixtures (mass resolving power ~80 000 at 300 *m*/*z*). We examined two samples in detail: (1) dissolved organic matter (primarily fulvic acids) from Suwannee River, GA, and (2) a humic acid extract from a degraded wood collected on Mt. Rainier, WA. Sample conditions (such as solvent, pH, and concentration) and instrument parameters (such as source voltages, trapping potentials, and excitation parameters) were optimized to yield the highest mass resolving power with the least mass discrimination in positive ion mode. High resolving power was achieved with low ion densities combined with coadding numerous scans. The increased resolution allowed molecular-level comparisons of the two samples which in turn could be used to estimate the relative similarity of individual compound distribution as well as an indication of the dominant diagenetic processes in the two source environments.

Humic and fulvic acids are important components of soil refractory organic matter and play critical roles in sediment processes such as metal reduction and pollutant transport. Structural characterization of humic and fulvic acids has been difficult due to their complexity and to the fact that conventional structural methodologies cannot provide detailed molecular-level information. The mixtures are composed of molecules that are macromolecular (>500 Da), hydrophilic, and highly polar. Nuclear magnetic resonance (NMR), an often-used noninvasive method, provides bulk functional group information such as the relative contributions of carboxylic acids, aromatic functionalities, and aliphatic carbon chains. NMR results can be used to generate estimates of the general character of the humic or fulvic acid, but taken alone, they are not adequate to identify particular compounds within a complex mixture.[1] Gas chromatography/mass spectrometry (GC/MS) has been used extensively to examine pyrolytic or chemolytic fragments of humic materials.[2] Attempts to characterize the molecular weight distribution of these mixtures have relied on size exclusion techniques such as gel chromatography and have suggested that humic acids are large, covalently linked macromolecules (1−100 kDa).[3] However, recent studies have begun to shift this premise to a concept of noncovalently linked aggregates of biologically derived molecules such as fatty acids, alcohols, and sugars.[4,5] Thus, both the molecular-level composition and the molecular weight distributions of complex organic mixtures are poorly understood and merit detailed study.

Electrospray ionization mass spectrometry (ESI MS) has been used successfully to examine macromolecular polar compounds such as proteins.[6] With electrospray ionization, polar macromolecules are multiply charged prior to acceleration into mass spectrometers. The mass spectrometers used for ion detection have included standard ion trap quadrupole-based instruments,[7] time-of-flight mass analyzers,[8,9] and ion cyclotron resonance cells.[10,11] Although recent reports have shown that humic substances can be ionized readily by ESI, resultant mass spectral data

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are very complex with multiple peaks at every m/z.[10−14] Brown and Rice conducted the most extensive analysis to date of the use of ESI Fourier transform ion cyclotron resonance mass spectrometry (FTICR MS) in the characterization of humic materials, specifically fulvic acids.[10] While their data represented the highest resolution of the individual components of fulvic acid extracts at the time, the mass resolving power of their analyses was insufficient to resolve individual peaks at high m/z as well as determine accurate mass and exact molecular formulas at low m/z.

The mass resolving power of FTICR mass spectrometry depends on many factors, most notably the magnetic field and ion density within the ICR cell. Higher magnetic field allows inherently better mass resolving power than lower magnetic fields.[15] However, at a particular magnetic field, optimization of the ion density within the ICR cell is critical to achieving high mass resolving power. High ion densities in the cell lead to deleterious ion−ion interactions which then result in space charge effects and signal damping.[16,17] When coherent phase locking of ions with similar cyclotron frequencies (peak coalescence) occurs, ions behave as a single population with an intermediate (and unstable) cyclotron frequency. Thus, at high ion number, resolving power is poor. To achieve high mass resolving power, it is important to keep ion densities in the cell to a minimum. However, for complex mixture analysis, it is equally critical to keep the concentration of individual components above the detection limit. Therefore, a delicate balance must be maintained between these two opposing factors—the total number of ions must be low enough to avoid space charge effects but high enough to allow detection of individual compounds.

In this paper, we describe the results of ESI FTICR MS analyses of two humic/fulvic acid samples. Using lower ion densities than previous studies, we tested the ability of numerous coadded scans to increase the resolution and mass resolving power of our instrument. In addition, we tested other instrument and solution parameters to optimize the mass resolution of individual components. The parameters we used enabled us to achieve mass resolving powers significantly higher than any other broadband FTICR study to date on these complex samples.[10−13]

EXPERIMENTAL SECTION

Sample Preparation. Two samples were the focus of the presented study. Dissolved organic matter (DOM) from the Suwannee River, GA, was collected via reverse osmosis and ultrafiltration by Dr. E. M. Purdue (Georgia Institute of Technology, Atlanta, GA). The sample was desalted using an H^+-saturated cation-exchange resin and lyophilized. A stock solution of Suwannee River DOM (SRDOM) was prepared by dissolving 10 mg in 2 mL of ultrapure water (Resi-Analyzed, J.T. Baker). Humic acids from a degraded wood sample (collected from Mt. Rainier, WA[18]) were extracted using 0.5 M NaOH. A stock solution of Mt. Rainier humic acid (MRHA) was prepared by dissolving 10 mg in 2 mL of pH 8 NH_4OH. Working solutions were diluted from the stock solutions with ultrapure water and methanol such that the concentration of organic matter was 1.25 mg mL^{-1} and the ratio of water to methanol was either 25:75 or 50:50.

Instrumental Parameters. Most MS analyses were performed using a commercial 7-T ion cyclotron resonance mass spectrometer (Apex IIe, Bruker, Billerica, MA) with an electrospray ionization source and a homemade heated capillary inlet.[19] Samples were infused into a tapered 50-μm-i.d. micro-ESI needle at 300 nL min^{-1}.[20] The temperature of the heated capillary inlet was varied from 75 to 95 °C, depending on water content. The capillary entrance voltage was varied from 2200 to 2700 V, and the capillary exit and skimmer voltages were maintained at 390 and 2 V, respectively. Samples were accumulated in the external ESI hexapole for 0.8 s and then transferred to the ICR cell where they were trapped using Sidekick (1.5 V trapping). The experimental FTICR time domain data (bandwidth 1.28 MHz, size 1 MWord) were acquired using either the commercially available Bruker data station or a MIDAS data station.[21] The averaged time domain ICR signal (14 000−22 000 scans) was subjected to baseline correction followed by Hanning apodization and one zero-fill before Fourier transformation and magnitude calculation.

Mt. Rainier humic acid was also analyzed using a home-built 9.4-T ESI FTICR MS at the National High Magnetic Field Laboratory.[22] A solution of 1.25 mg mL^{-1} in 75:25 MeOH−H_2O was infused into a micro-ESI needle at 400 nL min^{-1}. The capillary needle voltage was set at 1800 V. Ions were accumulated in the external octopole (frequency set at 1.65 MHz) for 10 s before transfer to the ion cyclotron cell. Data were collected for 1.6 s (2 MWord) by the MIDAS data station. The averaged time domain ICR signal was composed of 150 coadded scans and was analyzed in the same manner as the 7-T data.

Kendrick Mass Analysis. We used the Kendrick mass analysis approach[23] to test the degree to which our analyses were complementary to the data obtained by NMR. Briefly, all observed

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masses are normalized to the nominal mass of a functional group, in this case, CH_2.

$$\text{Kendrick mass} = (\text{observed mass}) \times (\text{nominal mass of } CH_2)/\text{exact mass of } CH_2$$

$$\text{KMD} = (\text{Kendrick nominal mass} - \text{Kendrick mass}) \times 1000$$

The Kendrick mass defect (KMD) is then calculated and all data are sorted according to KMD. Compounds with identical KMD values represent members of a CH_2 homologous series and are easily discerned from the plot as a straight line parallel to the *x*-axis.

RESULTS AND DISCUSSION

Improvements over Previous Studies. The most important improvement in the FTICR mass spectra of humic substances over previously published studies is the mass resolving power. Instead of broad humps at each nominal mass, we were able to resolve individual components at each nominal mass in both the MRHA and SRDOM samples. The most important modification to previous methods was the low ion concentration with significantly higher number of scans prior to Fourier transformation. By lowering the total number of ions in the cell, we were able to ameliorate some of the space charge effects that lowered the resolution of prior investigations. Previous studies were able to circumvent some space charge effects by analyzing only narrow mass ranges using SWIFT ion isolation and then summing the narrow-band spectra to produce a full spectrum.[12] However, accumulating a full broadband spectrum may give relative ion intensities that are more reflective of the original sample. Quantitative assessment of this issue is beyond the scope of this report but clearly merits attention in future studies.

Minimization of Space Charge Effects. Space charge effects were evaluated by comparing MRHA spectra as a function of processed data size (Figure 1). The FID for this sample is reasonably well-behaved with a strong signal at the beginning that tapers off exponentially with time. However, the fraction of the FID that is processed has large ramifications on the spectrum observed. When one-eighth (64 kWord) of the transient is processed by fast Fourier transform (FFT), a large broad hump of peaks is observed between 400 and 1200 *m*/*z*. Although the peaks are unresolved within this data size, they indicate the presence of material in this size range in the sample. As more data are processed (256 kWord), the broad hump of peaks decreases in magnitude and disappears completely when 1 MWord data are processed.

The eventual loss of the peaks above 800 *m*/*z* is most likely due to space charge effects within the ICR cell that dampen the signal of high molecular weight ions. If the signals for these high-*m*/*z* (low-frequency) components do not last for the entire scan time, noise will be averaged into the Fourier transform and thus decrease the magnitude of the signal at high *m*/*z*. Hanning apodization further exacerbates the observed damping effect by removing the initial portion of the signal prior to Fourier transformation.

The predicted ion−neutral collision rates for the low-*m*/*z* versus high-*m*/*z* species do not change significantly enough

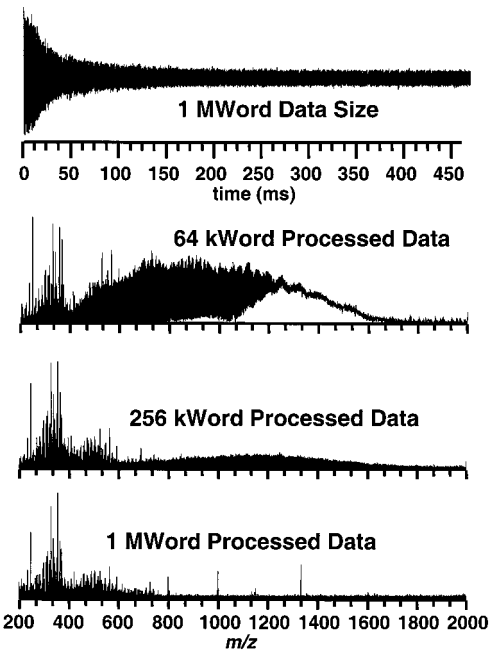

Figure 1. Unprocessed and processed data for Mt. Rainier humic acid. The top panel shows the FID time transient for 1 MWord data collected (average of 18 000 scans). The next three panels show the Fourier transformed data as a function of the amount of data processed.

(<12%) to account for this phenomenon. In fact, the species at lower *m*/*z* have a higher collision rate predicting the opposite behavior. Most importantly, ion−ion interactions between components of the complex mixture may result in short-lived ion signals for closely spaced frequencies.[17] This effect would be most pronounced for signals that occur at high *m*/*z* because (1) lower frequencies are more closely spaced and thus more difficult to fully resolve and (2) there is an increasing number of components with increasing nominal mass resulting in higher ion densities at these lower frequencies. Two conditions can be used to ameliorate the effect of deleterious ion−ion interactions: (1) decreased ion density either through lower ion number or larger cell volume and (2) higher magnetic field. For our purposes, we lowered the ion number and used extensive signal averaging to improve the signal-to-noise ratio and achieve high resolving powers.

Extensive signal averaging works only if the ion population is relatively consistent between scans. If the ion population is constantly changing, the averaged frequencies are not stable from scan to scan and peaks can drift in the frequency domain. This effect was examined in our data for the Mt. Rainier HA sample (Table 1) by comparing two spectra, containing 120 and 22 700 scans, respectively. Only 128 kWord of data were acquired for the 120-scan spectrum, so we compared it to 128 kWord of the 22 700-scan spectrum. In the selected mass region shown in Table 1, the peak locations were identical within the error of the instrument at 128 kWord, implying that the average ion population did not change between analyses. However, the decrease in resolving power with increased scans shows that some peak broadening has occurred as a result of shifting ion densities (and thus shifting frequencies). This effect is offset by the improvement in resolving power due to the increased number of scans and concomitant data transient length (Table 1).

Table 1. Peak Locations and Mass Resolving Power for a Selected Region of Two MRHA ESI FTICR Mass Spectra (601−605 amu)[a]

120 scans, 28 KWord		22 700 scans, 128 KWord		22 700 scans, 1 MWord	
peak location	resolving power	peak location	resolving power	peak location	resolving power
				601.112	28 600
601.155	7100	601.148	4300	601.148	28 600
				601.185	10 000
				602.115	21 400
nd[b]		602.166	5300	602.152	34 200
				603.089	28 500
603.162	4300	603.163	3600	603.125	28 500
				603.161	28 500
				604.131	21 300
nd		604.152	2400	604.164	28 400
				604.178	8100
				605.103	12 200
605.156	4300	605.152	4300	605.139	28 400
				605.175	6 800

[a] The first data set (columns 1 and 2) represents a spectrum with 120 coadded scans (128 KWord data collected). The second data set (columns 3 and 4) represents a spectrum with 22 700 coadded scans (128 KWord processed of 1 MWord data collected). The third data set (columns 5 and 6) represents the same spectrum as columns 3 and 4 (all 1 MWord data processed). [b] nd, not detected.

Effect of Magnetic Field Strength. The effect of higher magnetic field was evaluated using the 9.4-T FTICR MS at the National High Magnetic Field Laboratory (Tallahassee, FL). This instrument has both a larger ICR cell and a higher magnetic field than the 7-T FTICR MS originally used. The spectrum generated by the 9.4-T instrument showed higher mass resolving power at 700 *m*/z after significantly fewer scans (Figure 2). This result supports the need for higher field systems since space charge effects are significantly minimized, enabling higher ion densities to coexist in the cell. The number of scans was substantially reduced in the 9.4 T (150 scans) over the 7 T (22 700 scans), which translated into an acquisition time difference of hours (1−2 h on the 9.4 T versus 18−24 h on the 7 T). Furthermore, the individual components were better resolved in the 9.4 T than in the 7 T (Figure 2 insets).

Effect of Sample Preparation. The effect of solvent was considered in this study by comparing samples diluted in 75:25 and 50:50 MeOH−H_2O (Figure 3). Significant shifts in molecular weight distribution would indicate changes in degree of aggregation or precipitation within the individual components of the humic acid extract. However, the molecular weight distribution in both spectra is similar, suggesting no changes in aggregation tendency. There is a slight shift in the 50:50 solution (Figure 3 bottom) toward lower *m*/*z*. It is not clear, however, whether this shift is real, whether it is solvent-induced, or both. In addition, the space charge effects discussed earlier exacerbate the interpretation of data in the high-*m*/*z* range (>1000 *m*/*z*). However, it is encouraging to note no major differences between spectra acquired with two different solvent systems.

Other Effects. Other solvent and instrument conditions important in generating high-resolution mass spectra included salt concentrations and capillary end voltages. High salt concentrations resulted in an unstable spray (excessive "spitting"). The stock MRHA solution was diluted with water instead of pH 8 NH_4OH to avoid increasing the salt concentration past the limit needed for stable spray formation. The other parameter important in the study of humic and fulvic acid mixtures is the voltage at the end of the capillary. For routine analyses in our laboratory, the capillary end voltage ranges from 70 to 120 V. In the case of the natural organic matter analyzed here, however, the capillary end voltage needed to be significantly increased in order to detect compounds within the range analyzed. These higher voltages may have broken weak noncovalent interactions between individual compounds. This phenomenon is consistent with other authors' hypotheses[4] that humic and fulvic acids are aggregates, rather than large covalently linked macromolecules (>10 kDa). However, recent

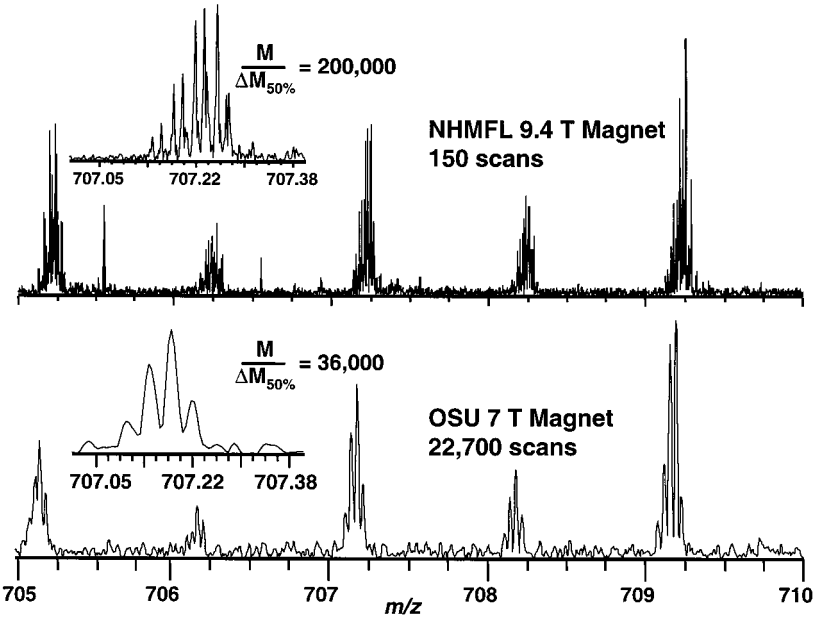

Figure 2. Effect of magnetic field on Mt. Rainier humic acid MS spectra. The top panel shows the MRHA spectrum acquired with a 9.4-T magnet (150 scans), and the bottom panel shows the MRHA spectrum acquired with a 7-T magnet (22 700 scans). The insets for both panels highlight the difference in compound resolution between the two magnets.

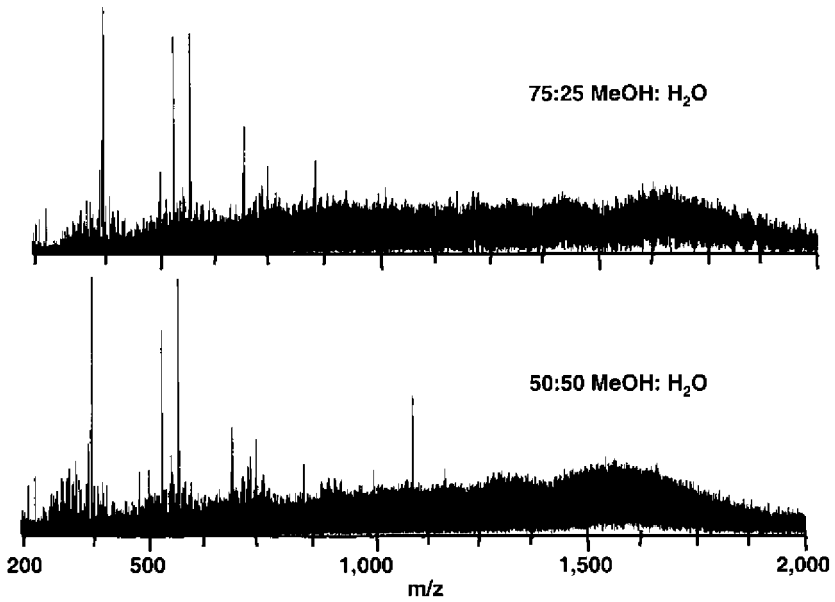

Figure 3. Effect of solvent conditions on Mt. Rainier humic acid MS spectra. Both spectra were acquired on a 7-T FTICR MS and represent the average of 14 000 scans. Both spectra were acquired with a 1.25 mg mL^{-1} solution of MRHA in either 75:25 (top) or 50:50 MeOH−H_2O (bottom).

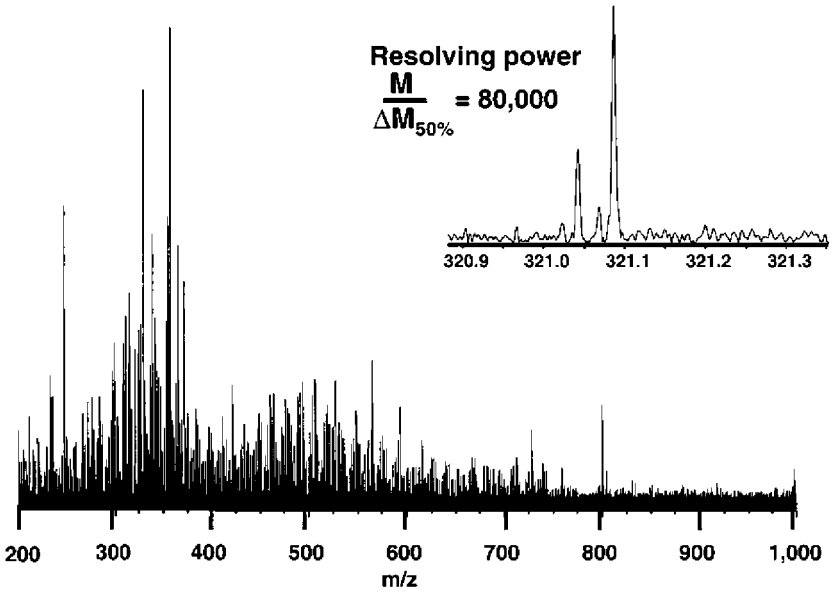

Figure 4. Positive ion mass spectrum of Mt. Rainier humic acid. The spectrum was acquired with a 1.25 mg mL^{-1} solution of MRHA in 75:25 MeOH−H_2O and represents the average of 22 700 scans on a 7-T FTICR MS. The inset shows the expanded region around 321 *m*/*z* and highlights the resolving power and resolution capabilities of this method (80 000 at 321 *m*/*z*).

studies have suggested that the appearance of low molecular weight peaks may be due to fragmentation of higher molecular weight species.[7] Resolution of this controversy is beyond the scope of this study. Clearly, experiments with model compounds are necessary to ascertain the presence or absence of fragmentation.

MRHA and SRDOM Spectra. The FTICR mass spectra for both MRHA (Figure 4) and SRDOM (Figure 5) nicely illustrate the complexity of humic and fulvic acid samples. There is a cluster of peaks at every nominal mass between 400 and 1200 m/z (MRHA) and between 300 and 900 m/z (SRDOM). The mass resolving power ranges from 80 000 at low m/z to 40 000 at higher m/z. This resolving power is high enough to separate 4−8 peaks per nominal mass (insets in Figures 4 and 5). Since these spectra were acquired in positive ion mode, the compounds observed are either protonated or complexed with a sodium ion. Few peaks are observed at half the nominal mass and the isotope peaks are one nominal mass higher than the original compound, suggesting these compounds are primarily singly charged species. Blank spectra were significantly different from sample spectra with a few sharp peaks scattered within the mass range, primarily due to electronic noise in the laboratory.

The molecular weight distributions of MRHA and SRDOM are quite different (Figures 4 and 5). The difference in these distributions will be due to a combination of two factors: different sources (lignin degradation products for MRHA and peat swamp drainage for Suwannee River DOM) and different diagenetic processes within the source environment (brown rot fungi-mediated degradation for MRHA and a variety of microbial

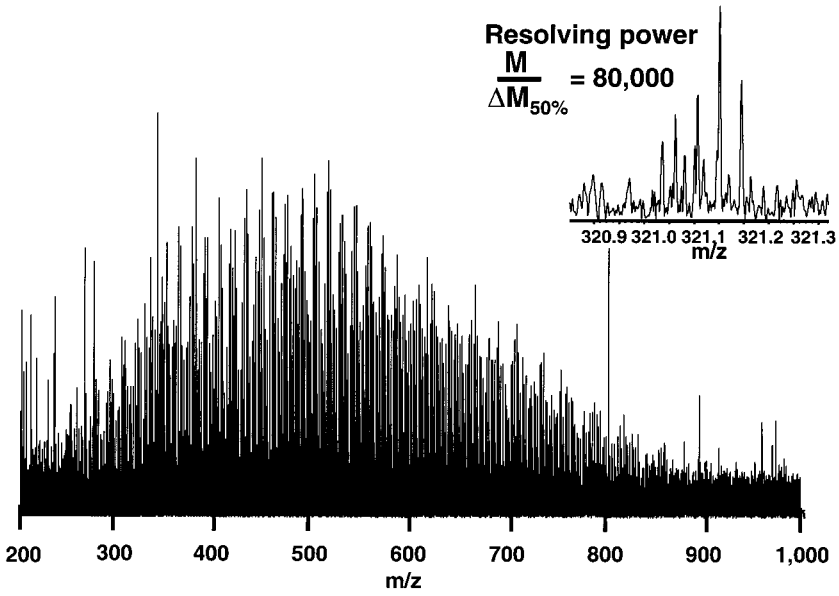

Figure 5. Positive ion mass spectrum of Suwannee River dissolved organic matter. The spectrum was acquired with a 1.25 mg mL^{-1} solution of SRDOM in 75:25 MeOH−H_2O and represents the average of 20 000 scans using a 7-T FTICR MS. The inset shows the expanded region around 321 *m*/*z* (same as in Figure 4).

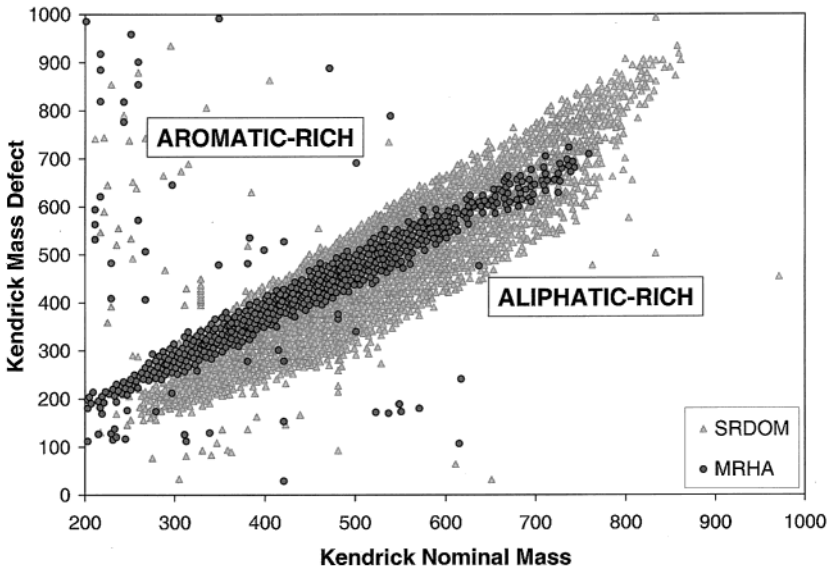

Figure 6. Bulk Kendrick mass plot for Mt. Rainier humic acid and Suwannee River DOM. The Kendrick mass defect was plotted against the Kendrick nominal mass for all odd *m*/*z* compounds within Mt. Rainier humic acid (black circles) and Suwannee River DOM (gray triangles).

alterations for SRDOM). The compound distributions within each sample can be used to elucidate common components within the two mixtures. The Mt. Rainier humic acids are assumed to be derived primarily from lignin.[18] Thus, we can make a further assumption that the peaks in the Suwannee River sample occurring at the same exact molecular mass may also be due to lignin. While this comparison is by no means conclusive at this stage, the ability to conduct molecular-level comparisons is unprecedented in this field. Furthermore, sequential assignment of natural organic matter components using complementary techniques will allow molecular-level examinations of diagenetic processes within different source environments.

Kendrick Mass Analysis. The Kendrick mass defect plot (Figure 6) can give a great deal of qualitative information about our samples. When the Kendrick mass defect of odd m/z compounds within MRHA (551 compounds) and within SRDOM (2123 compounds) were plotted against their m/z, the differences in the bulk properties of the two samples were clear. Compounds within MRHA were concentrated in a narrow band with relatively high Kendrick mass defect. Some compounds within SRDOM overlapped completely with MRHA compounds, suggesting similar if not identical chemical composition. However, there was a significant fraction of SRDOM compounds that had lower Kendrick mass defects than the compounds within MRHA, consistent with a larger aliphatic contribution.

The degree of oxidation and unsaturation increases with increasing KMD while saturation and reduction increases with decreasing KMD. We split the data into two groups, odd and even m/z. In ESI MS, odd m/z corresponds to an even mass and thus generally, an even number of nitrogen atoms (0, 2, 4, etc.). Due

to the low N content of humic acids, it is most likely that odd m/z compounds contain primarily C, H, and O. From the data plot of odd m/z (Figure 6), we can surmise that the high molecular weight components of our samples are either more aromatic or more oxygenated (or a combination of both) than their low molecular weight counterparts. From NMR, it has been shown that MRHA contains more aromatic functionalities than SRDOM.[8] Visual inspection of these data suggests that the ionization efficiencies of all compounds within MRHA and SRDOM are similar and that we are measuring a representative slice of the sample that is chemically similar to the material observed in the NMR and wet chemical studies conducted by others.

Kendrick mass analysis was also conducted to ascertain the degree to which CH_2 patterns dominated the spectrum. Many compounds fit a series of increasing CH_2 units, but the series contained few components (generally <10). Lengthy series of compounds are ideal because once the elemental composition of the lowest molecular weight compound is determined, the elemental compositions of all the compounds within the series are known. A good example of this is explained in detail for petroleum-derived compounds in Hughey et al.[23] In our data set, the short series indicate that CH_2 is not the dominant building block of these compounds. This is not surprising given the number of processes that contribute to the composition of these complex mixtures.

In the degraded wood sample (MRHA), the dominant parent compound is lignin and other structural materials.[18] Therefore, the samples observed in the ESI spectrum should contain numerous lignin oxidation products as well as biological material derived from the fungi responsible for wood degradation. The Suwannee River sample, on the other hand, is the product of drainage from the Okefenokee Swamp (GA) and should contain compounds derived from terrestrial plant degradation (e.g., degradation products of swamp grasses and lignin) as well as biological components derived from microbial communities. There is no a priori reason to expect CH_2 patterns to be the dominant building blocks of the compounds within these mixtures. Normalization with other simple functional groups (such as OCH_3 or OH) gave results identical to that for normalization with CH_2. This implies that the compounds in humic mixtures are far too complex to be separated on the basis of simple functional groups. Nonetheless, this exercise is instructive for an initial assessment of the dominant patterns within these large data sets and helps focus further structural work.

Implications for Analyses of Complex Mixtures. This study has shown that resolution of individual compounds within complex mixtures can be achieved with a 7-T ESI FTICR MS. High resolution is achieved when the appropriate balance between space charge problems (high ion number) and signal-to-noise ratio is maintained. However, there are additional concerns that need to be considered when such complex mixtures are analyzed. First, variations in ionization efficiency among the different classes of compounds within these samples can result in spectra with compound distributions that are not representative of the original sample. For example, hydrocarbons are not ionized at all during electrospray and so any such compounds would not be observed in the mass spectra. In addition, N-containing compounds are preferentially ionized in positive ion mode[24] and thus may have peak magnitudes that are not reflective of their concentration in the original sample. While hydrocarbons and N-containing compounds are minor components of humic substances,[25] we cannot ascertain the degree to which our data are affected by different ionization efficiencies. This topic is the subject of continuing work in our laboratory.

Second, the mode (positive or negative) in which the spectra are collected will undoubtedly lead to different compound distributions. Previous investigators have observed changes in compound and molecular weight distributions between the two modes.[7,9,10] Positive ion spectra were collected for this study since previous work had shown that negative ion mode may induce greater fragmentation and is more likely to contain multiply charged ions.[7,9]

CONCLUSIONS

The spectra of MRHA and SRDOM provided here represent a significant advance in the structural and chemical characterization of natural organic matter. The use of ESI coupled with FTICR MS has elucidated both compound and molecular weight distributions of components within these complex mixtures. The combination of low ion densities and numerous coadded scans has allowed high resolution and mass resolving power for peaks of <1000 amu. Some of these peaks are so well resolved that accurate mass can be determined at low m/z. The application of Kendrick mass analysis further elucidates structures within these complex mixtures by highlighting patterns based on chemical composition. This combined approach is applicable to the study of any complex mixture such as polar constituents of petroleum and bacterial lysates.

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