

Ionization and Fragmentation of Humic Substances in Electrospray Ionization Fourier Transform-Ion Cyclotron Resonance Mass Spectrometry

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Electrospray ionization (ESI) was combined with ultra-high-resolution Fourier transform-ion cyclotron resonance mass spectrometry (FTICR MS) to characterize complex humic and fulvic acid mixtures. Lower than expected molecular weight distributions previously observed for humics when analyzed by ESI-MS have fueled speculation about a bias in favor of low molecular weight. Multiply charged ions, ionization suppression, and sample fragmentation have all been suggested as sources of this low molecular weight bias. In this work, resolution of the individual components of humic mixtures within a 1 mass-to-charge unit window was accomplished by FTICR MS at 9.4 T. At mass resolving powers between 60 000 (high mass) and 120 000 (low mass), it was possible to determine that virtually all ions present in spectra of Suwannee River fulvic and humic acid are singly charged, thus eliminating inadequate accounting for multiply charged ions as a primary source of any low molecular weight bias. The high-resolution mass spectra also revealed the presence of molecular families containing ions that differ from each other in degree of saturation, functional group substitution (primarily CH vs N and CH₄ vs O), and number of CH₂ groups. Ionization suppression and ion fragmentation were addressed for humic and fulvic acid mixtures and well-characterized poly(ethylene glycol) (PEG) mixtures with average molecular weights of 8000 and 10 000. Although these high molecular weight PEG mixtures fragment extensively under traditional positive-ion mode ESI conditions, similar fragmentation could not be confirmed for humic and fulvic acid mixtures.

Humic substances are generally considered to be the remnants and degradation products of biomolecules in soil and aquatic systems. They occur as extremely complex mixtures of large, charged organic molecules, and their characterization remains one

of the most demanding challenges in modern analytical chemistry. These ubiquitous substances influence drinking water quality and ecosystem health by affecting organic pollutant bioavailability,^{1–3} metal complexation,^{4,5} and nutrient characteristics.^{6–8} Although various liquid-phase separation techniques have been applied to the humic characterization problem, including reversed-phase liquid chromatography,^{9,10} capillary electrophoresis,¹¹ capillary isoelectric focusing,¹² size exclusion chromatography (SEC),^{13–15} field-flow fractionation,¹⁶ and ultrafiltration,^{17–19} none has provided a definitive characterization of humic substances.

Gas-phase mass spectral analysis, on the other hand, has shown great promise. The methods used initially to volatilize humic substances, such as pyrolysis and chemical derivitization,^{20,21} resulted in the loss of some compositional and structural information, however. With the advance of softer ionization

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methods (electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI)), that problem was largely alleviated, although fragmentation remains a problem with laser desorption techniques including MALDI.^{10,22} ESI is therefore emerging as the ionization method of choice for mass analysis of humic substances.^{23–29} Complications of ESI for the analysis of humic materials include formation of multiply charged ions, aggregates, sodiation and protonation of the same ions, suppression of some classes of analytes (e.g., in positive-ion ESI, the most basic ions will protonate preferentially), and small fragment losses. Large-scale fragmentation, on the other hand, is generally considered unlikely for the relatively gentle electrospray process.^{10,23–26,28} There are, however, suggestions in the literature that this assumption may not be true for humic materials. Several investigators have published ESI-mass spectra of humic and fulvic acids, as well as spectra of low and high molecular weight (low- and high-MW, respectively) fractions of humic materials, that appear highly similar.^{15,26,28} In instances in which actual differences in molecular weights have been determined for humic size fractions²⁶ or between humic and fulvic acids,¹⁵ the differences appear small (on the order of a few hundred daltons).

In general, ESI mass spectra of humic materials, no matter how they are isolated or fractionated, are dominated by ions in the low-MW region ($m/z < 2000$), suggesting a bias toward low-MW. Leenheer et al.³⁰ observed a low-MW bias for poly(acrylic acid) with ESI-quadrupole and ESI-ion trap mass spectrometry, which they attributed primarily to inadequate accounting of multiply charged ions. However, this observation cannot necessarily be extrapolated to humics. In fact, there are indications in the literature that the m/z values of humics correspond directly to true mass, which would indicate a charge state of 1. In SEC, for instance, it has been shown that ESI-MS does produce significantly higher m/z spectra for earlier eluting SEC features²⁸ although the masses determined by ESI-MS and calibration of the SEC column do not necessarily agree.¹⁵ The latter phenomenon can be explained by the difficulty of finding calibration standards that mimic the size-to-mass relationship of humic substances.³¹

Plancque and co-workers²⁷ using quadrupole time-of-flight (Q-TOF) mass spectrometry determined that most ions were either singly or doubly charged and that singly charged ions were more abundant. MS/MS data show that humic ions tend to lose m/z 44 or 18^{27,30} fragments, consistent with the expected neutral losses

of CO₂ and H₂O from singly charged ions (doubly charged ions, for instance, would lose m/z 22 and 9 fragments, respectively). It should be noted, however, that neither Plancque et al.²⁷ nor Leenheer et al.³⁰ observed individual humic ions, as pointed out by McIntyre and Jardine,³² who provided a high-resolution spectrum of certain humics, revealing several ions within a unit m/z window. Solouki et al.²³ concluded that ions in positive-ion mode ESI Fourier transform-ion cyclotron resonance (FTICR) mass spectra of two different fulvic acids were singly charged based on H/D exchange, although individual fulvic ions were again not fully resolved. Kujawinski et al.²⁹ is to our knowledge the only group that has published fully resolved spectra of humics (Mount Rainier Humic Acid and Suwannee River Dissolved Organic Matter) to date. The spectra published were in positive-ion mode and suggested that only a few humic acid ions were multiply charged. Kujawinski et al.²⁹ suggested, however, that negative-ion mode spectra were more likely to contain multiply charged ions.

Thus, before the apparent low-MW bias could be further investigated, it was necessary to clearly determine the charge state of the humic and fulvic acid ions in both ionization modes. To that end, ultrahigh-resolution spectra were used in which all ions were individually resolved. From the spectra, it is possible to measure the exact mass spacing between molecular ions (all carbons are ¹²C) and heavy isotope ($M + 1$) ions (one carbon is ¹³C) and thus determine the charge state.

Besides charge state, the effect of instrument settings on overall molecular weight distribution should also be given some consideration. In FTICR MS, two instrument parameters affect the overall molecular weight distribution of humic ions predictably and significantly—transient length^{10,29} and octopole frequency. Shorter transients produce stronger humic signals centered at slightly higher m/z . A good discussion of the phenomenon and some of the processes involved is given in a recent paper by Kujawinski et al.²⁹ Unfortunately, transients of at least 1 M-word are necessary for full resolution of humic samples. Therefore, it is currently not possible to investigate charge state, and thus the true molecular weight, of humic ions in the earliest stage of time domain data acquisition. However, transient length is relevant only for FTICR MS and thus cannot account for low-MW distributions of humics observed with other types of mass spectrometers (see, for example, refs 15, 28, and 30). Lower rf frequencies on the octopole ion guides also lead to signals centered at slightly higher m/z . This latter phenomenon occurs for all mixtures. However, decreasing octopole frequency merely shifts the overall ion abundance closer toward the upper m/z limit of the mass spectrometer without increasing charge state. Therefore, for singly charged ions, decreasing the octopole frequency cannot shift the average measured molecular weight past the upper m/z limit of the mass spectrometer ($m/z \sim 3000$ for the instrument used here). Finally, since transient length and octopole settings affect humic and fulvic acids equally, they are unlikely to account for the lack of distinction between them.

The observations cited above suggest that a careful investigation of the ionization and fragmentation of humic ions in ESI-MS is warranted. From a review of the literature, it is clear that both

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humic and fulvic acids contain a readily ionizable low-MW (below ~2000) fraction that is easily observed with ESI-MS.^{15,24,28,29} An average molecular weight somewhere below ~2000 corresponds well with average molecular weights measured for fulvic acids with various other techniques such as vapor-phase osmometry (VPO), freezing point depression, small-angle X-ray scattering, SEC, and ultrafiltration.^{13,24,33} Based on such techniques, the average molecular weight of humic acids is significantly larger (by a factor of ~5), but still below ~10 000 Da.³³

That only humic ions below ~2000 observed with ESI-MS could be explained through the following scenarios. First, it is possible that no high-MW humic acids (above ~2000) exist and that non-mass-spectral methods all give wrong, although similar, results. It should be noted however that in MALDI-TOF mass spectroscopy of humic acids with molecular weights above 2000 were detected even at threshold laser desorbing intensity, which suggests that such high-MW humic acids should exist.²² Second, it is possible that low-MW humic acids ionize more readily than heavier acids in both positive and negative electrospray ionization modes. Such preferential ionization could be based on the nature and accessibility of functional groups in the low-MW versus the high-MW fraction and on charge density since the higher molecular weight ions would have to accept multiple charges in order to fall within the m/z region of the spectrometer. For a thorough description of the principles of ESI (including those responsible for ESI selectivity), the reader is referred to a recent review article by Cole.³⁴ The important issue here is that an ion of 10 000 MW would have to accept at least four charges in order to be detected with the 9.4-T FTICR mass spectrometer used here (to fall within the most efficient m/z range in excess of 10 charges would be necessary). It is possible that the ability of high-MW humic acids to attract additional like charges is significantly lower than the ability of the lower molecular weight acids to accept one charge. Thus, it is conceivable that most of the available charge is used up by the low-MW ions that are observed in the spectrum. The high-MW ions may remain uncharged (and thus not enter the mass spectrometer) or insufficiently charged (and thus get removed in the transfer multipoles). The final scenario is that humic substances dissociate in the ESI source. The consensus assumption is that humic molecules cannot break up to a significant degree in the ESI source^{10,23–26,28} based on the known soft ionization characteristics of ESI. Conversely, the fact that some dissociation may occur is sometimes used to support the hypothesis that apparently high-MW humic acids are merely noncovalent aggregates of smaller humic acids (see, for example, Kujawinski et al.²⁹).

The purpose of the research presented here is to test whether charge competition plays a role in the apparent low-MW distribution of humic acids (i.e., test scenario two) and to investigate the validity of the assumption that ESI cannot cause complete fragmentation of covalently bound high-MW mixtures. To determine whether ionization suppression of high-MW humics based on competition for charge plays a role, high-MW humic acid molecules were isolated by tangential flow ultrafiltration (TFF). Previous experiments with 1000 or 3000 molecular weight cutoff (MWCO) membranes produced typical mass spectra dominated

by ions below 2000 Da.^{15,28} TFF membranes have molecular weight cutoff characteristics that are very susceptible to changing operating conditions and that can vary largely depending on the type of analytes.^{18,19} The TFF experiments carried out as a part of this work were therefore carefully designed to maximize the amount of low-MW material removed from the sample in order to investigate the mass spectral response of the high-MW material.

To test whether the assumption that covalently bound high-MW mixtures do not completely dissociate in the ESI source is well founded, high-MW polymer standards (poly(ethylene glycol) (PEG), with nominal average molecular weights of 8000 and 10 000, were analyzed under various conditions. As for humics, previously published ESI mass spectra of high-MW PEG show very little variation over a large molecular weight range.^{35,36} Furthermore, again similar to humics, reports disagree as to whether PEG break up in the ESI source. O'Connor and McLafferty³⁷ published mass spectra of highly charged, intact PEG up to 20 000 Da, whereas Xu et al.³⁶ provided convincing evidence that high-MW PEG (starting at 20 000) break up in the ESI source. The two groups used very different electrospray conditions, which we suspected was the reason for the difference in their results. Here it was tested whether it is possible for a high-MW mixture of covalently bound molecules (PEG 10 000) to completely dissociate in the ESI source, whether this dissociation is a function of electrospray solvent composition, and, if so, whether electrospray solvent composition has any effect on humic mass spectra.

EXPERIMENTAL SECTION

Suwannee River Humic Samples. Suwannee River humic and fulvic acid standards were acquired from the International Humic Substances Society and were stored frozen in a dark box until used. The samples were dissolved immediately before introduction into the ESI source and were never allowed to touch the lids of their vials, to minimize contamination from plasticizers and glues.

Electrospray solvent composition for each sample is listed in Table 1. The solvents were Milli-Q water, methanol (MeOH; Fisher, Purge and Trap-Grade), acetic acid (HAc; Fisher, Trace-Metal Grade), 2-propanol (ISP; Fisher, Optima Grade), and ammonium hydroxide (Fisher, TraceMetal Grade). To prevent contamination, solvents were stored in the bottles supplied by the manufacturer or in acid- and base-washed vials with removable Teflon caps.

Tangential Flow Ultrafiltration of Suwannee River Humic Acid. TFF was carried out with a 10 000 Da molecular weight cutoff regenerated cellulose membrane (Millipore Prep/Scale-TFF PLGC 10k, 2.5-ft² cartridge). Initially, 255 mL of a 24 ppm Suwannee River humic acid (SRHA) sample were prepared. Of this sample (SRHA-bulk), 240 mL was diluted to 2 L and filtered through the membrane. The sample was rediluted to the full volume of the Teflon bottle (2 L) approximately every 5 min. After each collection of ~2 L of retentate, the sample was recirculated at high flow rate and zero back pressure for 10–20 min to dislodge any high-MW material that might have collected on the mem-

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Table 1. Sample Identification and Instrumental Parameter Settings

sample ID	pre-treatment	spray solvent	mode ^a	flow (μL/min)	cap. heat ^b (A)	tube lens (V)	needle voltage (V)	acc time (s) ^c	exc voltage ^d (Vp-p)	exc range (m/z) ^e	oct freq (MHz) ^f	co-adds	data points ^g	figure ^h
SRFA	none	67% water, 33% MeOH	+	0.6	4.4	370	2000	9	100	225–2500	1.5	250	1M	1
SRFA	none	65% water, 33% ISP, 2% NH ₄ OH	-	1.0	5.4	-350	-2000	15	130	225–2500	1.5	177	1M	2
SRHA	none	50% water, 50% MeOH, 0.75mM NaOH	-	~1.5	3.1	-370	-2000	15	130	225–3000	1.5	70	1M	3
SRHA-Bulk,	none	65% water, 33% MeOH, 2% HAc	+	0.5	3.8	370	1800	15	110	225–2500	1.2	100	400	4a
SRHA-ret1	TFF	65% water, 33% MeOH, 2% HAc	+	0.5	3.8	370	1800	15	110	225–2500	1.2	100	400K	4b
SRHA-ret2	TFF and NaCl	65% water, 33% MeOH, 2% HAc	+	0.5	3.8	370	1800	15	110	225–2500	1.2	100	400K	4c
PEG 8000-10KRet	TFF	66% water, 33% MeOH, 1% HAc	+	1.0	3.7	350	1800	5	110	225–2500	1.8	25	2M	5a
PEG 10 000-10KRet	TFF	48% water, 50% MeOH, 2% HAc	+	0.5	3.4	350	1500	3	90	225–4000	1.2	20	1M	5b
PEG 10 000	none	65% water, 33% MeOH, 2% AcOH	+	0.95	5.4	350	2000	5	0.65	3000	1.5, 1.2, 1.0	25	1M, 1M, 1M	6a, 6b, 6c
PEG 10 000	none	49% water, 49% MeOH, 2% HAc	+	0.5	3.1	370	1800	5	130	225–3000	1.5	50	1M	7a
		50% water, 50% MeOH, 1 mM NaOH	+	0.5	3.1	370	1800	5	130	225–3000	1.5	50	1M	7b
		50% water, 50% MeOH, 0.1 mM NaTriflate	+	0.5	3.1	370	1800	5	130	225–3000	1.5	50	1M	7c
		50% water, 50% MeOH, 1 mM NaCl	+	0.5	3.1	370	1800	5	130	225–3000	1.5	50	1M	7d
		49% water, 49% MeOH,	+	0.5	3.1	370	1800	5	130	225–3000	1.5	50	1M	7e
SRHA	none	50% water, 50% MeOH, 0.75mM NaOH	+	0.5	3.1	370	1800	20	130	225–3000	1.5	100	1M	8a
SRFA	none	50% water, 50% MeOH, 12.4mM NaOH	+	0.5	3.1	370	1800	10	130	225–3000	1.5	200	1M	8b

^a Electrospray ionization mode. ^b Capillary heater current. ^c External ion accumulation time. ^d Excitation voltage. ^e Mass excitation range (m/z). ^f Octopole ion guide frequency. ^g Data points processed. ^h Figure where spectrum can be found.

brane. After the fractionation experiment was complete, the sample was recirculated in this manner for 25 min. Eventually 11.76 L of Milli-Q water had been added to the sample. The final volume of the retentate (fraction retained by the membrane, SRHA-ret1) was 265 mL (110% of the original volume). The membrane was cleaned with 0.12 N HCl, 0.1 N NaOH, and Milli-Q water after each use.

To check the effects of ionic strength on the efficacy of the membrane separation, a second retentate from this same sample was isolated as described above; however, the initial ionic strength was increased. This experiment included the addition of 0.1149 g of NaCl to 165 mL of SRHA-ret1, resulting in a solution that was initially 0.0119 M in NaCl. This sample was then filtered through the same TFF membrane. Eventually, 6.84 L of Milli-Q water had been added to the sample. The final volume of the salt-modified sample, designated SRHA-ret2, was 175 mL (110% of the original volume of SRHA-ret1).

All samples were stored after TFF isolation in either Teflon bottles or acid- and base-washed glass vials capped with removable

Teflon septa. Samples were stored in the refrigerator until analyzed. A 5-mL aliquot of each of the three samples (SRHA-bulk, SRHA-ret1, SRHA-ret2) was lyophilized and stored in Falcon tubes in the freezer. All three lyophilized samples were redissolved in 500 μL of electrospray solvent (Table 1) the day before ESI FTICR MS analysis.

Poly(ethylene glycol) Standards. Separate PEG standards of 8000 and 10 000 nominal average molecular weight were obtained from Aldrich and dissolved in Milli-Q water. Two liters of 5 ppm solutions of the PEG-8000 and PEG-10 000 mixtures were additionally purified by fractionation through the TFF membrane described above.

To determine the effect of electrospray conditions on PEG 10K spectra, two sets of experiments were undertaken. First, separate solutions of ~0.02 g of PEG dissolved in 50 mL of Milli-Q water, 50 mL of MeOH, 2 mL of HAc, 100 μL of 1 N NaOH, and 0.0058 g of NaCl and 0.01 g of lithium trifluoromethanesulfonate (LiTriflate, Aldrich 99.995%) or 0.2 g of sodium trifluoromethane-

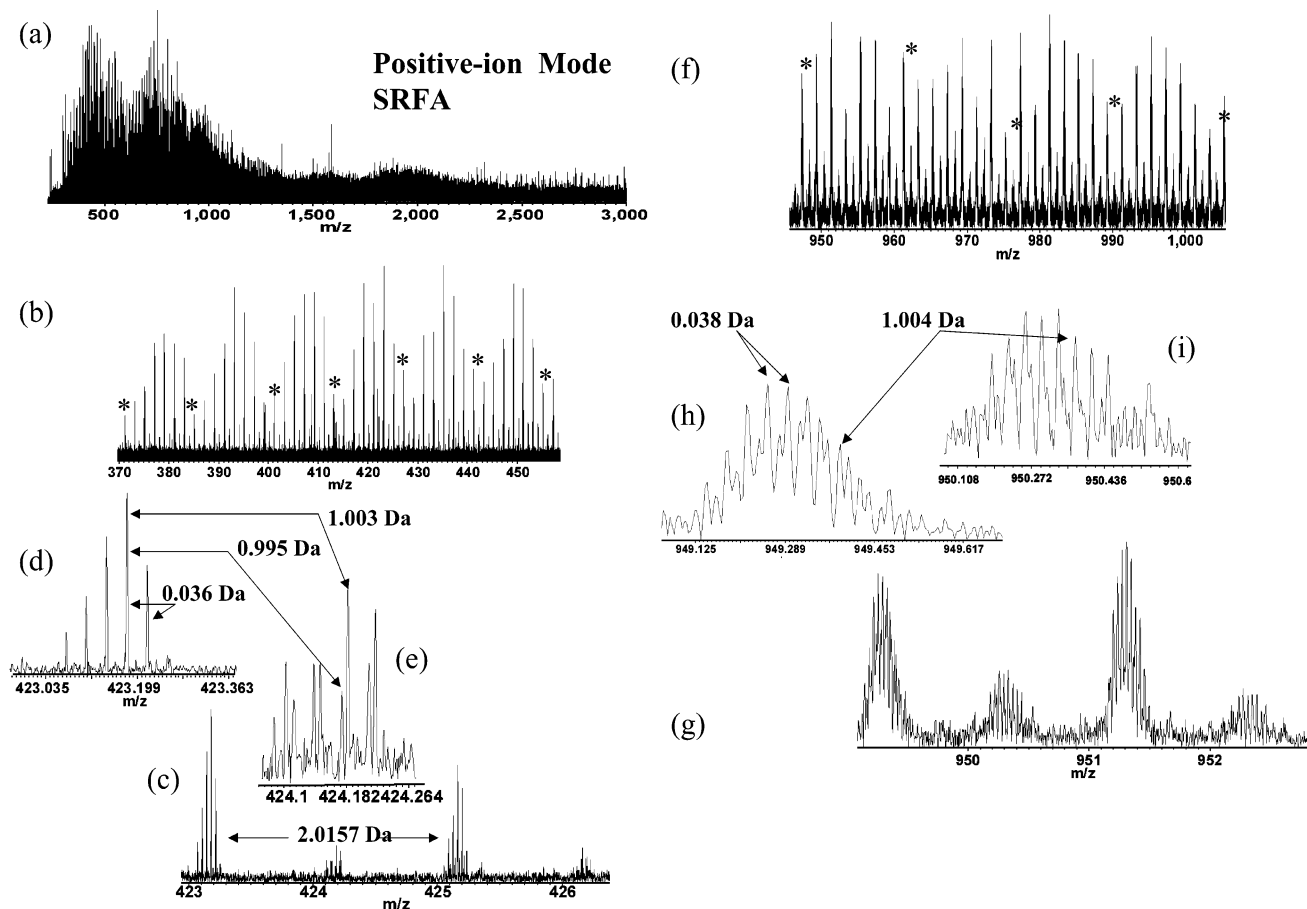


Figure 1. Positive-ion mode ESI FTICR mass spectrum of Suwannee River fulvic acid (SRFA; 5.7 mg/mL): (a) entire spectrum; (b–i) expanded regions of the spectrum. Asterisks indicate peaks separated by 14 Da.

sulfonate (NaTriflate, Aldrich, 98%) were analyzed under identical instrument conditions (Table 1). In the second set of experiments, one bulk solution of 0.02 g of PEG in 100 mL of Milli-Q water was used throughout. Then, 0.5 mL of the sample was mixed with 0.5 mL of MeOH, 20 μ L of HAc, 20 μ L of NH_4OH , or 1.5 μ L of 1 N NaOH before introduction into the ESI source. Again, identical instrument settings were used for all three samples.

Electrospray Ionization Fourier Transform-Ion Cyclotron Resonance Mass Spectrometry. ESI FTICR mass spectra were acquired with a home-built 9.4-T mass spectrometer located at the National High Magnetic Field Laboratory (NHMFL).^{38,39} Briefly, sample is pumped at microliters per minute (Table 1) through a fused-silica transfer line, a metal union supplied with the desired needle voltage, and a fused-silica capillary tip. Ions generated in the ESI source travel through a tube lens and skimmer (set to 13 V positive-ion mode and –13 V negative-ion mode). One quadrupole and three rf-only octopole ion guides follow (octopole frequencies are detailed in Table 1). Ion accumulation takes place in the second octopole, after which ions are pulsed out of that octopole, through the third, and into an open cylindrical 4-in.-diameter Penning trap ICR cell.

Instrument operating parameters (see Table 1) were optimized for each sample except when it was necessary to compare different

samples under the same conditions. It is important to note that changing instrument conditions does not affect any of the parameters of interest, i.e., charge state, mass spacing patterns, and PEG dissociation (for example, compare Figures 1, 2, and 8b, Figures 3 and 8a, and Figures 5b, 6, and 7a). It should also be noted that mass spacing patterns and m/z distribution observed in the literature are in relatively good agreement, regardless of instrument settings or type of mass spectrometer.^{10,23,24,26,28–30} The statement that instruments settings do not affect PEG dissociation is based on a careful study of changes to PEG 10K spectra as one instrument setting is changed at a time (the settings used are listed in the Results and Discussion section).

All time domain data were baseline-zeroed, zero-filled once, Hamming-apodized, subjected to fast Fourier transformation and magnitude calculation, and frequency-to-mass converted by the usual quadrupolar electric field approximation.⁴⁰

RESULTS AND DISCUSSION

Charge States of Humics. For a 9.4-T FTICR mass spectrometer employing quadrupolar excitation, the theoretical upper mass-to-charge ratio limit is nearly 120 000.⁴¹ As explained elsewhere,⁴¹ there are various theoretical upper limits to m/z inherent to the Penning trap. In addition, the observable m/z range is limited by the external multipoles in which ions are accumulated

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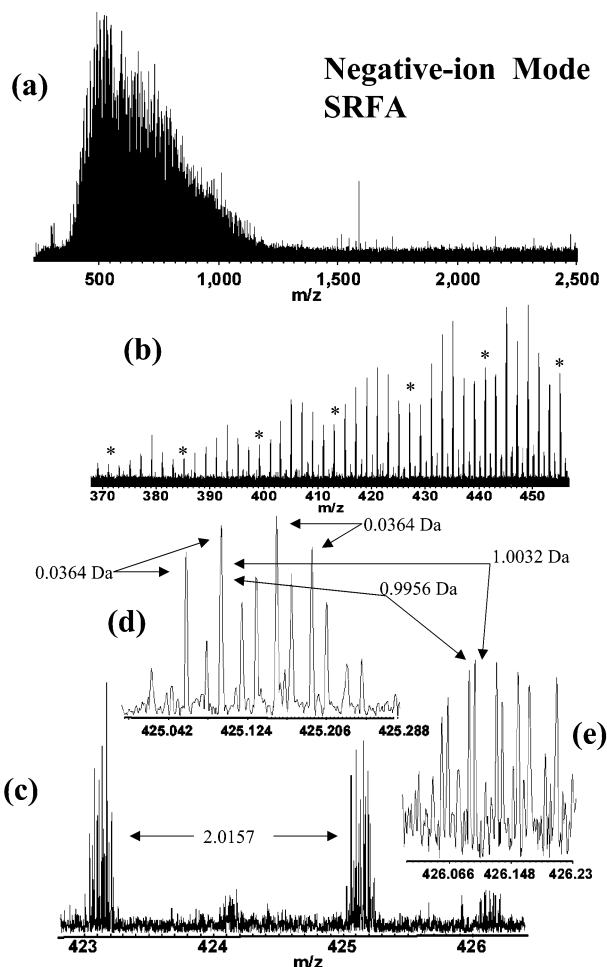


Figure 2. Negative-ion mode ESI FTICR mass spectrum of SRFA (5.5 mg/mL): (a) entire spectrum; (b–e) expanded regions of the spectrum. Asterisks indicate peaks separated by 14 Da.

Table 2. Measured Recurring Mass Differences between Ions in the Suwannee River Fulvic Acid Mass Spectrum (Figure 1).

origin	mass differences		SD ^a ($\times 10^4$)	<i>n</i> ^b
	theoretical	measured		
^{12}C vs ^{13}C	1.0034	1.0034	5.5	156
CH vs N	0.9953	0.9952	8.9	141
CH_4 vs O	0.0364	0.0364	1.3	131
H_2 vs double bond/ ring	2.0157	2.0157	2.5	125
longer CH_2 backbone	14.0156	14.0164	2.3	146

^a SD, standard deviation. ^b *n*, number of mass differences calculated.

and transmitted to the ICR cell and by the frequency ranges for the transmitter and receiver amplifiers. Under standard operating conditions for the 9.4-T instrument used here, we have been able to detect ions up to m/z 6000 and routinely to m/z 3000.

However, because electrospray ionization can produce multiple charge states for ions of a given mass, the charge states of humic ions must be determined before accurate mass assignments can be made. Fortunately, charge-state determination is possible with sufficient mass resolving power, because species containing one or more ^{13}C atoms ($M + 1$ ions) will appear $1/z$ above the m/z of the corresponding monoisotopic (e.g., all ^{12}C) ions. Thus, a singly (or doubly or triply) charged $^{13}\text{C}^{12}\text{C}_{n-1}$ ion will be higher in m/z

by 1.0 (or 0.5 or 0.33) than the corresponding $^{12}\text{C}_n$ nuclide. To produce the ultrahigh-resolution mass spectra presented here, very concentrated samples (in excess of ~ 1000 ppm), long accumulation times, and many coadds are required. It is also essential that the samples be free of salts, mineral bases, and buffers. Internal standards can only be added in trace amounts.

Humic materials generally produce mass spectra with peaks at every nominal (nearest-integer) mass^{23,24,30} with a predominance of odd-mass peaks.^{24,30} Our data show the same trends for Suwannee River humic and fulvic acid in positive as well as negative-ion mode (Figures 1–3 and 8). However, at 120 000 resolving power in the low-MW region, it becomes apparent that what appeared to be single peaks at every mass unit are in fact collections of multiple ions (Figures 1–3 and 8). Examination of the expanded regions in Figures 1–3 reveals that each primary odd-mass ion has a corresponding lower abundance ion, 1.0034 (SD = 0.000 55, n = 156) and 0.9952 (SD = 0.000 89, n = 141) higher in m/z . The even-mass peaks, 1.0034 higher in m/z , are clearly $M + 1$ ions (i.e., $^{13}\text{C}^{12}\text{C}_{n-1}$) ($13.0034 \text{ Da} - 12.0000 \text{ Da} = 1.0034 \text{ Da}$).

Admittedly, relative ion abundance is at best a qualitative measure for ESI FTICR of complex, heterogeneous mixtures because the overall abundance distribution is affected by the selectivity of the ESI source, the transfer characteristics of the multipole ion guides (e.g., Figure 6), the length of the transient, the excitation profile (i.e., chirp vs SWIFT), the signal-to-noise ratios of the very low-abundance ions, the number of data points per peak, and the differences in relative ion population from scan to scan. However, the observation that relative $M + 1$ ion abundances (assigned as such based on mass spacings) increase with overall molecular weight (Figure 1f vs b) does at least qualitatively reinforce the assignment. More importantly it helps identify higher molecular weight heavy isotope ions ($M + 1$) despite the loss in resolving power at higher m/z .

In Table 2 the average mass spacings between M and $M + 1$ ions for more than 100 ions is listed. The measured spacings correspond very well with the expected mass difference between ^{12}C and ^{13}C for singly charged ions. Based on these measurements, it is obvious that the vast majority of humic ions is singly charged. In rare instances, “bumps” can be observed along the baseline approximately halfway between the M and the $M + 1$ peaks (e.g., Figure 3c). These “bumps” are generally of very low abundance to the extent that individual peaks cannot be discerned. However, the presence of these rare “bumps” suggests that a small fraction of humic ions may in fact be doubly charged. Ions with three or more charges are not observed. Based on the very low abundance of doubly charged $M + 1$ ions and on their rare occurrence, it can be concluded that multiply charged ions do not make a significant contribution to the overall measured molecular weight of humic substances.

Although the ions at 0.9952 Da higher mass might at first glance be assigned to ^{15}N versus ^{14}N , the exact mass difference ($15.0001 \text{ Da} - 14.0031 \text{ Da} = 0.9970 \text{ Da}$) significantly exceeds the mass measurement precision. Thus, a more likely assignment is the substitution of CH by N ($14.0031 \text{ Da} - 13.0078 \text{ Da} = 0.9953 \text{ Da}$).

Patterns in Mass Spacings. The mass spacings of 1.0034 and 0.9952 Da are not the only recurring patterns in humic and

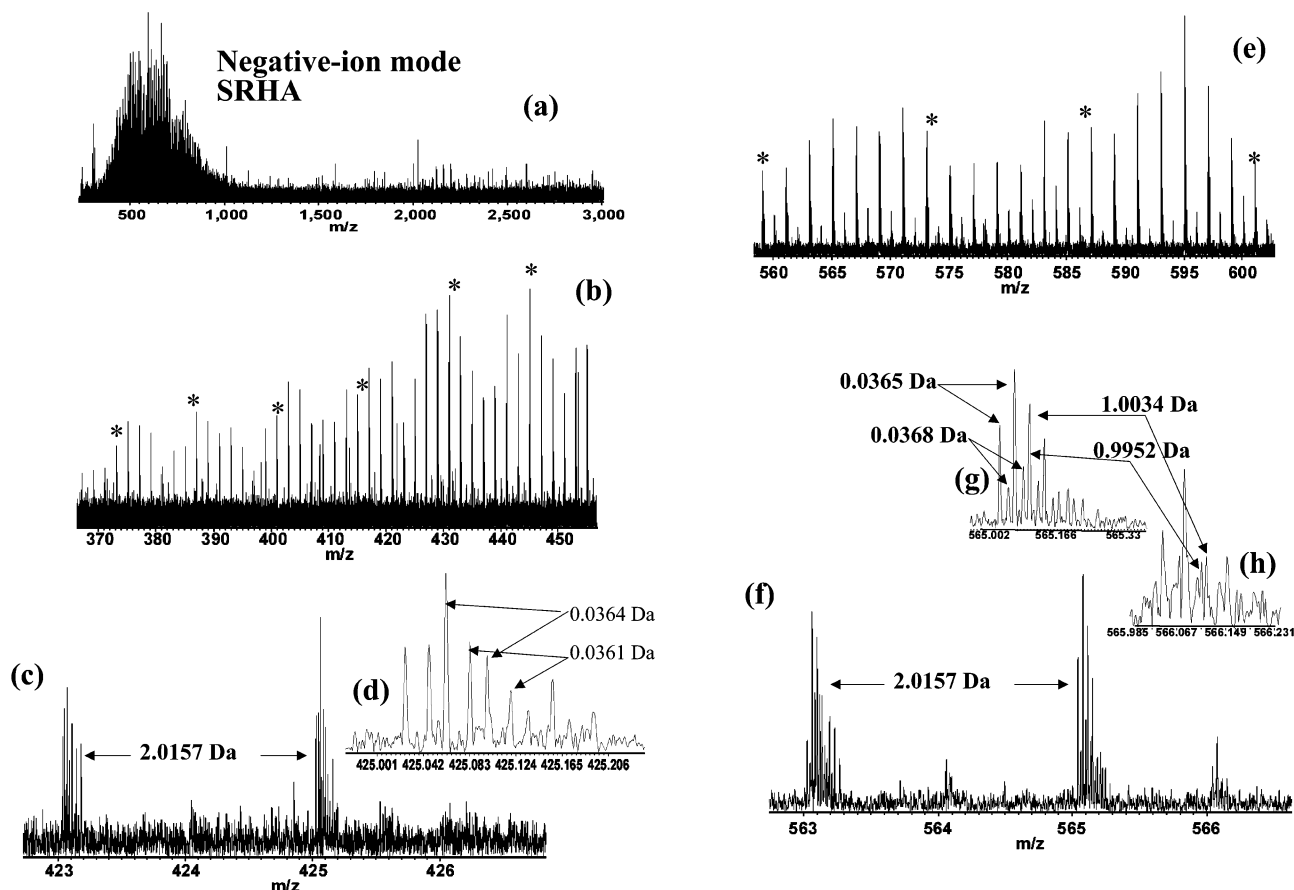


Figure 3. Negative-ion mode ESI FTICR mass spectrum of Suwannee River humic acid (SRHA; 2.8 mg/mL): (a) entire spectrum; (b–h) expanded regions of spectrum. SRHA does not have ions of enough abundance in the range between m/z 371 and 458 to distinguish individual heavy isotope ions; therefore, the mass range between m/z 559 and 602 is also included in the figure (the same region is used for Figure 8). It should be noted that the bump at $m/z \sim 425.5$ in (c) is an example one of the very rare instances in which humic ions appear doubly charged (on the other hand, the bump could arise from electronic noise as well). Asterisks indicate peaks separated by 14 Da.

fulvic acid spectra. The even spacing of prominent peaks at every other mass (~ 2 Da spacing) has been observed previously,^{24,30} as has the spacing of ~ 14 Da.^{26,27,29} Because we are able to resolve individual ions within a unit mass envelope, we are able to measure accurate mass spacings between ions (see Table 2). Although the data in Table 2 are based on the positive-ion mode spectrum of Suwannee River fulvic acid (SRFA; Figure 1), the same patterns are present in negative-ion mode mass spectra of SRFA and SRHA (Figures 2 and 3), as well as in positive-ion mode spectra of SRHA (Figure 8a). Finally, it should be noted that these patterns exist irrespective of instrument settings (compare, for example, Figures 1, 2, and 8b or Figures 3 and 8a).

The ~ 14 -Da mass spacing is generally attributed to humic ions that differ in number of CH_2 groups,^{26,27,29} as confirmed by the average experimental spacing of 14.0164 Da (SD = 0.000 23, $n = 146$); the true mass of CH_2 is 14.0156 Da. The experimental ~ 2 -Da spacing (actually, 2.0157 Da, SD = 0.000 25, $n = 125$) is most likely due to humic ions that differ from each other in degree of saturation, since each double bond (or ring) requires the loss of two hydrogen atoms ($m = 2.0157$ Da). Finally, we observed ions spaced 0.0364 Da (SD = 0.000 13, $n = 131$) apart within a unit mass interval, most likely arising from the mass difference between CH_4 and O (0.0364 Da). The CH_4 versus O mass difference could arise from species differing by ethyl versus aldehyde substituents (C_2H_5 vs CHO) or from any of several other

possibilities (e.g., CH_3 and an extra alkyl CH_2 and one less ring or double bond vs another molecule with CH_2OH instead).

Observation of a single mass spectrum at high resolution thus provides information as to the composition of humic molecules. Apparently, many of these molecules exist as families that differ in degree of saturation and functional group substitution (CH vs N and CH_4 vs O). Molecules within these families vary from one another in number of CH_2 groups. This type of information is a valuable antecedent for compositional analysis by MS/MS experiments, which is currently under way.

Besides providing some insight into the molecular composition of humic substances, the regular patterns in ion spacing allow extrapolation from the low-MW region where mass resolving power is best to the high-MW region where resolving power is sometimes insufficient for unique elemental composition assignment. Figure 1g–i demonstrates that mass spacing patterns extend to high-MW. The mass resolving power of the spectrum in Figure 1g–i ($\sim 60\,000$) is insufficient to determine unequivocally molecular ion (M) and heavy isotope ion ($M + 1$) spacings of 1.0034 Da. However, the continuation of the same patterns observed at low masses make up for the loss of resolving power. For example, it is likely that these ions are still singly charged, even though the average spacing between M and $M + 1$ ions in the region highlighted is now 1.0021 Da (SD = 0.000 77, $n = 9$) instead of 1.0034 Da.

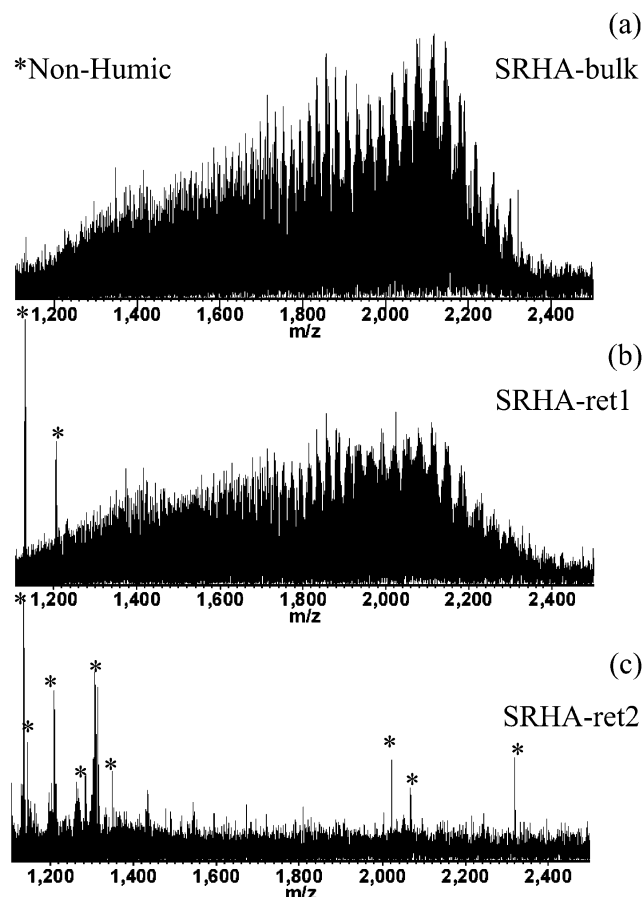


Figure 4. Positive-ion mode ESI FTICR mass spectrum of SRHA mixture: (a) unfractionated (240 mg/L); (b) after retention on 10-kDa membrane; (c) after addition of NaCl to Ret1 and subsequent retention on 10-kDa membrane. Asterisks indicate nonhumic peaks.

High Molecular Weight Isolation by Tangential Flow Ultrafiltration. The spectra in Figures 1–3 indicate not only that humic ions are singly charged but that positive- and negative-ion mode mass spectra of humic and fulvic acids consist of the same ions (Figures 1, 2, and 3b, Figures 3e–h and 8a). The fact that the ions appear at identical m/z positions in both ionization modes might seem surprising but can be understood by considering the ion types formed in each mode. The mass difference between an ion in positive-ion mode ($M + H^+$) and that same ion in negative-ion mode ($M - H^+$) is the mass of two protons, which is virtually identical to the spacing between ions that differ in one degree of saturation. The regular pattern in these spectra of ions differing in degree of saturation (H_2) has already been noted (Table 2, row 4). Thus, a molecule one degree lower in saturation falls at the same m/z value in the positive-ion mode spectrum as its more saturated counterpart does in the negative-ion mode spectrum.

The more surprising conclusion from Figures 2 and 3 is that Suwannee River humic and fulvic acids appear to be composed of many common ions. Some overlap is certainly to be expected, since the distinction between humic and fulvic acids is somewhat arbitrary, but most ions in the humic and fulvic acid spectra appear at identical m/z values over the entire mass ranges observed here. Is the ESI source somehow selective for the ions that are observed? We have already noted that electrospray ionization may

favor lower mass humics that need only a single charge to be detectable over higher mass species who need several charges.

From a bulk, unfractionated, humic or fulvic sample, it is difficult to determine whether the predominance of low-MW ions observed in these spectra is due to intact, low-MW molecules or fragment ions from larger molecules. It is also impossible to address the possibility of selective ionization suppression of larger, intact molecules. One obvious approach to addressing these issues is to isolate high-MW humic molecules before introduction into the ESI source, thus reducing competition for the limited charge available. Figure 4 contains positive-ion ESI FTICR mass spectra of a bulk Suwannee River humic acid sample (SRHA-bulk), the material retained by a 10-kDa TFF membrane in distilled water (SRHA-ret1), and a subsample of the retentate material reisolated at a higher ionic strength with the same membrane (SRHA-ret2). The 10-kDa membrane was chosen so that retention of material below 2000 Da would be minimized. To prevent aggregation of humics (which would make them appear larger), the concentration for all samples was kept below 24 ppm. Concentration factors in excess of 40 were used since Guo and co-workers¹⁸ have identified the main problem with TFF membranes to be retention of low-MW material not breakthrough of high-MW molecules.

Unfortunately, optimum conditions for the TFF separation are not ideal for ICR analysis. The samples are too dilute even after lyophilization to generate high-resolution mass spectra. In fact, the 1 M-word transient had to be cut short (see Table 1) in order to get good signal magnitude for the humic ions. Truncating the transient (i.e., Fourier transforming only the first 400K data words) not only shifts the molecular weight distribution to higher m/z but also significantly enhances humic signal magnitude. For a plausible explanation, the reader is referred to a recent article by Kujawinski and co-workers.²⁹ At 400K transient length, the humic acid signal occurs in the somewhat higher m/z range between 1000 and 2500 Da (Figure 4). Cutting the transient short also significantly decreases mass resolving power; therefore, analysis is limited to qualitative comparisons between the bulk and retentate spectra. From many previous mass spectra of humic acids in both positive- and negative-ion mode, we know that the bulk sample consists almost exclusively of singly charged ions (e.g., Figures 3 and 8a). Therefore, if competition for charge plays a major role, the removal of the low-MW, readily ionizable (as is evidenced by their abundance in humic spectra) humic molecules would allow larger molecules to acquire sufficient charge to be detectable, leading to a visible change in the overall mass spectral appearance between the bulk and retentate samples since the multiply charged, high-MW molecules are not likely to have exactly the same m/z as the low-MW material they replace. The high-MW species should also be multiply charged and therefore produce more closely spaced peaks (compare, for example, Figure 7a and b). Conversely, a significant decrease in signal magnitude is not expected since the high-MW signal should be replacing the lost low-MW signal and because multiply charged ions produce stronger ICR signals.

Panels a and b of Figure 4 compare the bulk signal with the retentate collected in distilled water. The patterns are almost identical although the relative magnitude of the overall signal of the retentate is somewhat lower (conversely, the relative abundance

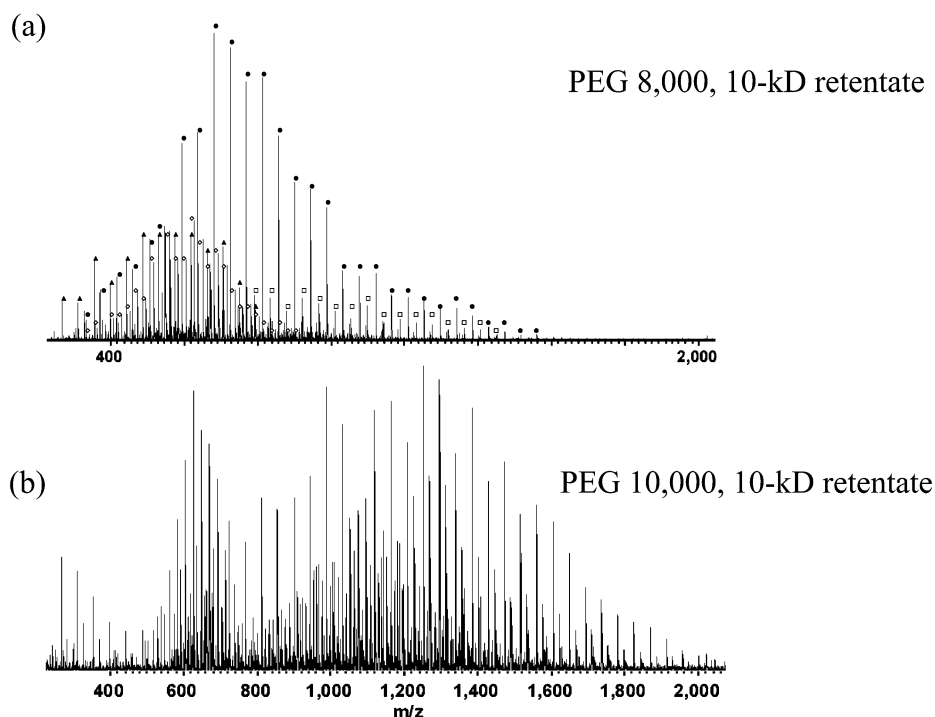


Figure 5. Positive-ion mode ESI FTICR mass spectra of PEG standards: (a) 10-kDa retentate of PEG 8000 (●, protonated, singly charged PEG; □, sodiated, singly charged PEG; ▲ = protonated, singly charged PEG minus H₂O; ◆, protonated, doubly charged PEG). (b) 10-kDa retentate of PEG 10 000. Note that instrument settings are different for the two spectra (Table 1) yet both spectra consist exclusively of low-MW PEG oligomers.

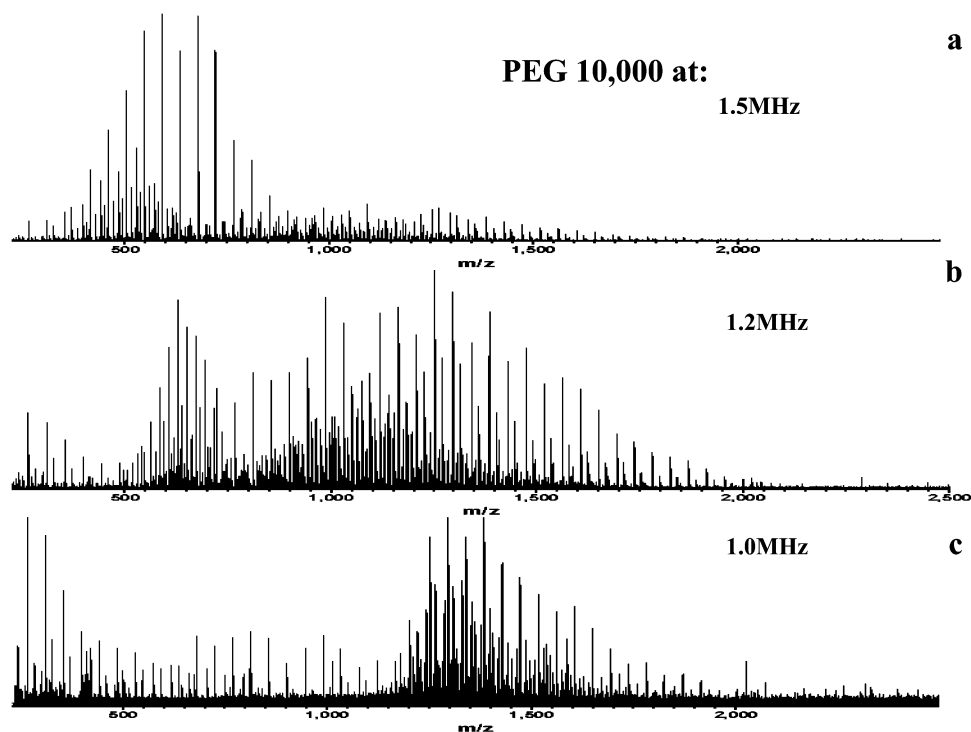
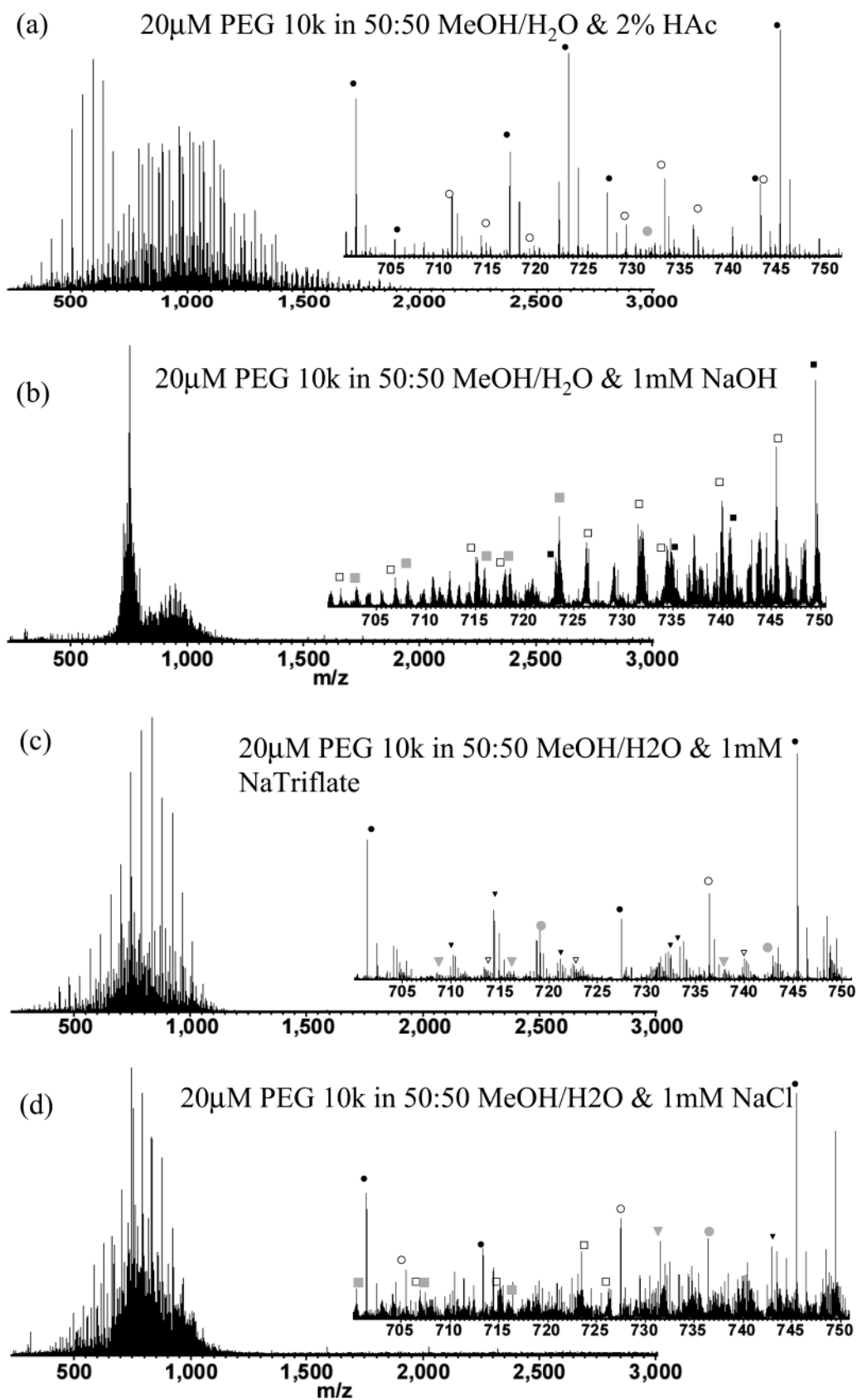


Figure 6. Positive-ion mode ESI FTICR mass spectra of PEG 10K standard at (a) 1.5-, (b) 1.2-, and (c) 1.0-MHz octopole ion guide frequency.

of nonhumic interferents is significantly higher). The most likely explanation for this trend is that the majority of the low-MW material was retained by the membrane probably because of electrostatic interactions between the membrane and the analytes. This conclusion is further confirmed by the observation that all of the yellow color of the humic acids had remained in the retentate fraction. Figure 4c shows the result of refractionating

SRHA-ret1 at initially higher ionic strength. Here no humic signal remains in the 400K transient and most of the yellow color has moved into the permeate fraction. The lack of signal in Figure 4c indicates that low-MW humic ions have been removed but not replaced with a large abundance of highly charged, high-MW humic acids. It should be noted that some humic acids apparently remained in the retentate, since their signal is detectable when



(e) 10 μ M PEG 10k in 49:49 MeOH/H₂O & 2% NH₄OH

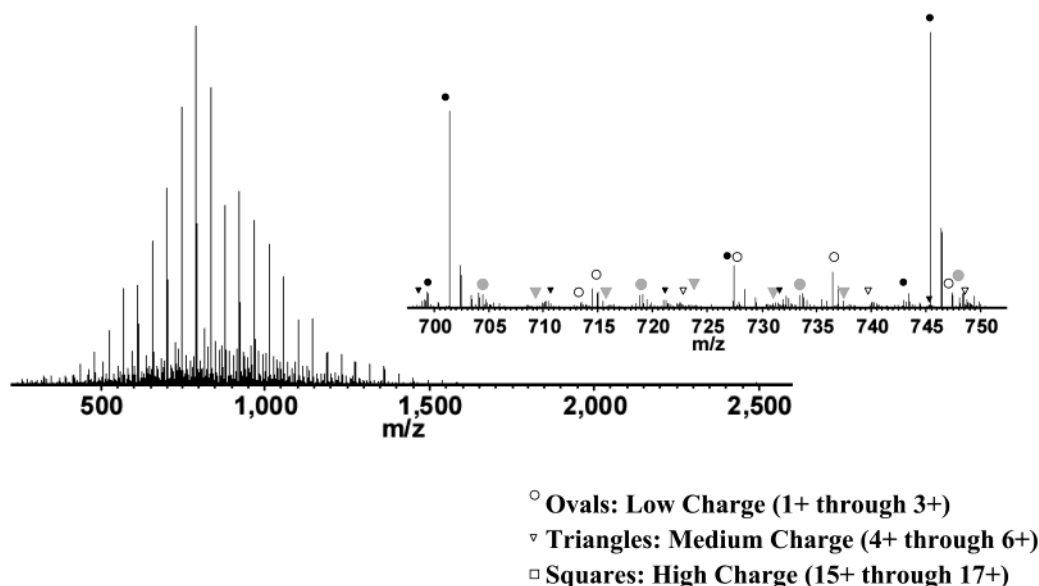


Figure 7. Effect of electrospray solvent on PEG 10 000 (~ 0.2 mg/mL). (a) Electrospray solvent contains 2% HAc (pH ~ 4); (b) solvent contains 1 mM NaOH (pH ~ 7 –8); (c) solvent contains 0.1 mM NaTriflate (pH ~ 5); (d) solvent contains 1 mM NaCl (pH ~ 6); (e) solvent contains 2% NH₄OH (pH ~ 10) (and concentration of PEG is about half that of the other samples). \bullet , singly charged (1+), \circ , doubly charged (2+), gray circle, triply charged (3+), \blacktriangledown (4+), ∇ (5+), inverted gray triangle (6+), \blacksquare (15+), \square (16+), and gray box (17+).

the transient is reduced to 64K (data not shown). This remaining signal does not contain any ions above m/z 2000, however.

Previously published findings suggest that most humic acids fall below 10 000 Da and that SRHA has molecular weights between 5000 and 10 000.³³ TFF results presented here agree that humic acids probably do not contain much material larger than 10 000. However, whereas previous results suggest that most humic acids should fall between 5000 and 10 000 Da, ESI-FTICR MS provides no evidence of any ions in that mass region. Thus far, high-charge states and charge competition have been eliminated as likely causes for this discrepancy. Although it is theoretically possible that SRHA contains a large number of high-MW molecules that do not ionize in the ESI source even in the absence of competition, dissociation of humic molecules or aggregates must be considered one of the most plausible scenarios at this point. Therefore, it is important to clearly determine whether such dissociation can occur only for noncovalent aggregates (as is generally assumed) or if it is possible for covalently bound molecules to dissociate as well.

Fragmentation of PEG Mixtures. To test whether fragmentation of solution-stable molecules in high-MW mixtures can occur, ESI spectra of PEG mixtures of 8000 and 10 000 nominal molecular weight were obtained under conditions similar to those used for humic and fulvic acids. PEG was chosen because it has been reported that high-MW PEG, like humics, show little distinction between size fractions,^{35,36} and literature reports disagree on multiple charging versus dissociation in the source as the cause.^{35,37} Also, PEG mixtures are available in many different, relatively narrow molecular weight ranges. They are well characterized, and their molecular weights are known with good certainty. In MALDI FTICR MS experiments, for instance, the mass ranges of sodiated PEG standards correspond reasonably

well with the average molecular weight indicated by the manufacturer.⁴² Finally, PEG mixtures are easily characterized because individual molecules differ from each other only in total number of C₂H₄O units.

Our initial data (i.e., data taken in acidic spray conditions for positive-ion mode and basic spray conditions for negative-ion mode) show that individual molecules in both PEG 8000 and PEG 10 000 fragment when conventional ESI spray solvents are used. Figure 5 contains spectra of PEG 8000 and 10 000 after further purification with the same 10-kDa TFF membrane used for humic and fulvic acids. Both spectra are dominated by singly charged ions. This is an unexpected similarity between PEG and humics; it should be noted that other high-MW molecules, such as proteins, are routinely observed at very high charge states.^{43–45} The highest charge state observed for PEG 8000 and 10 000 is four. Ions present are low-MW PEG oligomers. For the 10-kDa retentate of PEG 10 000 (Figure 5b), 94% of the ions observed fall below 3000 Da, and 64% fall below 2000 Da. The data in Table 3 demonstrate that accounting for charge on individual ions significantly increases the calculated molecular weights, yet these molecular weights still lie far below the true molecular weights of the polymers in solution. Similar results were obtained in negative-ion mode (data not shown).

Complete dissociation of PEG 10 000 in ESI with traditional spray solvents is reproducible regardless of the amount of HAc in the spray solvent (i.e., 0.1, 1, or 2%), further TFF purification

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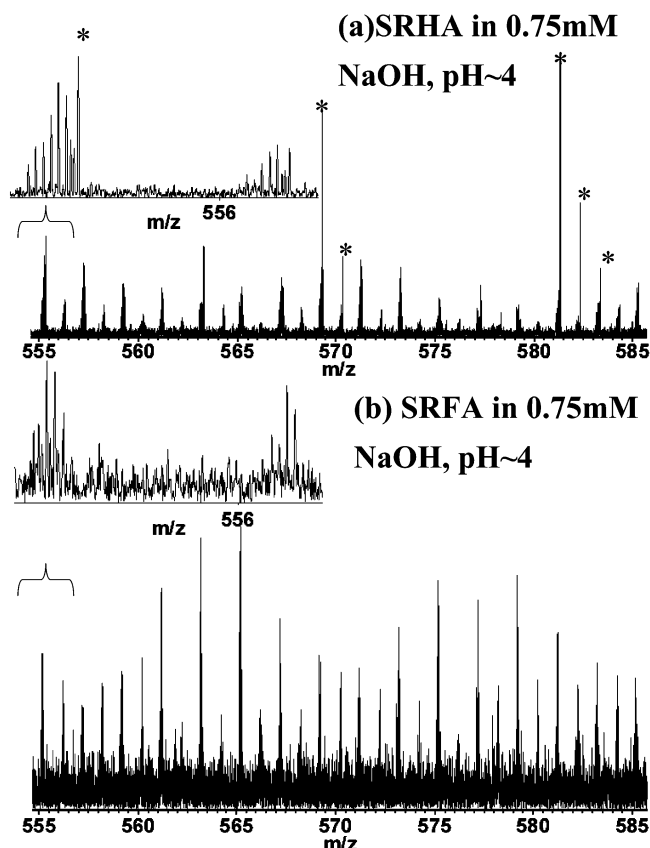


Figure 8. Selected segments of the ESI FTICR mass spectrum of SRHA and SRFA after addition of NaOH (a) SRHA, and (b) SRFA. Asterisks indicate nonhumic peaks (electronic noise or base–solvent adducts).

Table 3. Effects of Ion Charge on Average Molecular Weights of PEG 10 000 10K-Retentate (Figure 5b)

	average molecular weights		
	MW _{av} ^a	M _n ^{b,c}	M _w ^{c,d}
raw average	1695		
assuming $z = 1$ for all ions		1072	1178
accounting for charge		1541	1784

^a Peak height-weighted sum of all masses (after adjusting for charge) divided by total number of peaks. ^b M_n , number-average molecular weight, assuming all ions are singly charged. ^c See ref 44 for details regarding M_n and M_w calculations. ^d M_w , weight-average molecular weight, assuming all ions are singly charged.

of the manufacturer-provided PEG, amount of time the PEG sample spends in solution before introduction into the source (i.e., minutes to days), PEG concentration (i.e., 5.09, 789, or 5092 ppm), and instrument settings (i.e., accumulation time (3 s, 10 s), capillary heater current (0.63, 3.35, 6.31 A), needle voltage (1000 (no signal), 1500, 1800, and 3000 V), octopole frequencies (1.8, 1.5, 1.2, and 1.0 MHz (Figure 6)), tube lens voltage (350, 200 V), excitation voltage (50, 100, 130, 200 V), relaxation time (0.01 s, 10 s (very little signal))). Octopole frequency is the only instrumental parameter that significantly and predictably affects molecular weight distribution; however, even at the lowest octopole frequencies, the ion masses are below the neutral molecular weights (Figure 6). We therefore conclude that significant

fragmentation of PEG molecules occurs under conventional ESI conditions.

Positive-ion mode ESI spectra of intact high-MW PEGs published by O'Connor and McLafferty³⁷ were based on a spray solvent composed of 50% methanol, 50% water and millimolar concentrations of NaOH. We were able to reproduce those results (Figure 7b) with the same solvent mixture. The mass spectra in Figure 7 include a scale-expanded mass segment (700–750 Da) arbitrarily chosen to demonstrate the presence of highly charged ions. The PEG spectrum obtained with the more conventional acidic positive-ion mode ESI solvent (Figure 7a) is provided for comparison. It should be noted that no low-MW ions are observed in Figure 7b, suggesting that contamination or selective, high-MW ionization suppression is not responsible for the molecular weight distributions observed in Figures 5 and 6. The addition of NaCl (Figure 7c), NaTriflate (Figure 7d), and LiTriflate (spectrum not shown) to the methanol/water solvent also result in spectra containing high-MW, multiply charged ions, although some fragmentation does occur when those additives are used. A second set of experiments with the same bulk PEG solution in different spray solvents again showed no high-MW ions in HAc (or in NH₄OH) and no low-MW ions in NaOH. Interestingly, the addition of ammonium hydroxide rather than sodium hydroxide neither suppresses fragmentation nor generates highly charged ions (Figure 7e). It therefore appears that fragmentation of PEG molecules occurs in the ESI source with conventional acidic spray solvents, but addition of sodiated bases suppresses this fragmentation and produces large, multiply charged ions.

Effect of Electrospray Solvent Conditions on Humic Samples. Given that electrospray solvent conditions strongly affect the overall molecular weight distribution of high-MW PEG, it was interesting to see whether a similar change would occur for humics. Previous variations of electrospray conditions have been performed by Brown and Rice²⁴ and Kujawinski et al.²⁹ Brown and Rice were not able to fully resolve individual fulvic acid ions but did note that the addition of salt and NaOH to the spray solvent leads to the degradation of the humic signal.²⁴ Kujawinski and co-workers examined the effect of methanol concentration (75 vs 50%) but found no significant changes in the spectra.²⁹ To test how much NaOH could be added to the humic sample, SRFA was prepared at different concentrations of NaOH. It was determined that millimolar NaOH (as used for PEG) could be added without destroying the humic signal, although ion current was very low and signal-to-noise ratio significantly degraded. Furthermore, the addition of NaOH introduces large chemical noise peaks (most likely solvent–base adducts) as had already been pointed out by Brown and Rice.²⁴ Figure 8b shows the positive-ion mode mass spectrum of SRFA after addition of 0.75 mM NaOH. The patterns previously observed for humic ions are still clearly visible in the expanded mass region of Figure 8b, and it is obvious that the ions are still singly charged. More interestingly, SRHA (Figure 8a) produced the same result.

The 0.75 mM NaOH spray solution was still slightly acidic (pH ~4). Because fragmentation of PEG was completely suppressed and multiply charged ions formed only in nonacidic spray solution, both SRFA and SRHA were taken to pH ~9 by repeated 1-μL additions of a concentrated NaOH solution (data not shown). Unfortunately, the humic acid signal was completely obscured by

the chemical noise peaks under these spray conditions. The fulvic acid signal could still be made out amidst tall noise peaks showing that SRFA is singly charged even under basic, sodiated spray conditions. It should be noted, however, that SRFA appears in ESI-MS at approximately the average molecular weight indicated by most other techniques (below 2000); a large increase in charge state was therefore expected mainly for the humic acid sample. Addition of 10^{-8} M NaCl or 0.07% NH_4OH to SRHA did not appear to increase the charge state of ions observed in this sample (data not shown). The data presented here show that the addition of low concentrations of sodiated bases does not alter the charge state of humic materials. Therefore, if dissociation of humic substances occurs in the ESI source, it most likely occurs through a very different mechanism from that observed for PEG.

CONCLUSIONS

This work demonstrates that resolution of individual ions within a humic mass spectrum is possible by ultrahigh-resolution FTICR mass spectrometry. The dominant charge state of ions in ESI FTICR mass spectra of Suwannee River humic and fulvic acid mixtures is 1. Patterns in the spectra suggest that these humics are made up of molecular families that differ from each other in degree of saturation and functional group substitution (most likely CH vs N and O vs CH_4), and individual members of each family differ in number of CH_2 groups.

Investigation of the source of the apparent low-MW bias in ESI mass spectra of humics indicates that inadequate accounting for high charge states can be ruled out. Ionization suppression based on molecular weight is possible, but is difficult to determine unequivocally because of the limited mass range of the spectrometer and the shortcomings of size-based separations. No direct evidence was found for fragmentation of humics in the source despite surprising similarities (little or no distinction between size fraction, preference for $z = 1$ charge state) in ESI response of PEGs (which do break up) and humics under standard electrospray conditions. We did, however, show that it is possible for complex mixtures of covalently bound high-MW molecules to

completely dissociate in the ESI source. This observation is important to the study of not only humic substances but other complex high-MW mixtures as well. It suggests that it cannot be assumed that covalently bound high-MW molecules will not dissociate in the source and that careful experiments to investigate possible dissociation are necessary when mixtures of unknown compounds are characterized. Based on the data presented here, three possible scenarios concerning the molecular weight distribution of humic acids remain. SRHA may be composed of low-MW molecules that appear larger, however, in other techniques for molecular weight determination such as SEC, vapor pressure osmometry, and small-angle X-ray scattering. It is also possible that SRHA consists of high-MW molecules that break up in the ESI source by mechanisms different from PEG and therefore do not show the same response to sodiated bases. Finally, it is possible that SRHA consists primarily of high-MW (5000–10 000) molecules that do not ionize well in either positive- or negative-ion mode ESI or that are incapable of acquiring sufficient charge to be detected (e.g., high-MW lignin degradation products or large lipids that have few polar/ionizable functional groups).

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