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Molecular Resolution and Fragmentation of Fulvic Acid by Electrospray Ionization/Multistage Tandem Mass Spectrometry

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Molecular weight distributions of fulvic acid from the Suwannee River, Georgia, were investigated by electrospray ionization/quadrupole mass spectrometry (ESI/ QMS), and fragmentation pathways of specific fulvic acid masses were investigated by electrospray ionization/ion trap multistage tandem mass spectrometry (ESI/MST/ MS). ESI/QMS studies of the free acid form of low molecular weight poly(carboxylic acid) standards in 75% methanol/25% water mobile phase found that negative ion detection gave the optimum generation of parent ions that can be used for molecular weight determinations. However, experiments with poly(acrylic acid) mixtures and specific high molecular weight standards found multiply charged negative ions that gave a low bias to molecular mass distributions. The number of negative charges on a molecule is dependent on the distance between charges. ESI/MST/MS of model compounds found characteristic water loss from alcohol dehydration and anhydride formation, as well as CO_2 loss from decarboxylation, and CO loss from ester structures. Application of these fragmentation pathways to specific masses of fulvic acid isolated and fragmented by ESI/MST/MS is indicative of specific structures that can serve as a basis for future structural confirmation after these hypothesized structures are synthesized.

Humic substances (environmental degradation products of plant, fungal, and bacterial biopolymers) are significant in the binding of toxic chemicals in soil and water,[1] as a source of nutrients for plant growth,[1] in the formation and maintenance of good soil structure,[1] and in their reaction with chlorine to produce chlorinated disinfection byproducts in drinking water.[2] Specific molecular structures of humic substances have not been previously determined because of the complexity of structures and molecular mixtures. An overview article[1] on humic substance

structure states, "The reality is that, despite numerous attempts to fractionate humic substances by many researchers over several years, no discrete fractions have ever been isolated, or are likely to be isolated, which could or would reasonably be considered to approach chemical purity". Gas chromatography/mass spectrometry (GC/MS) has been used to characterize humic substances by applying degradation techniques, such as chlorination, ozonation, hydrolysis, pyrolysis, oxidation, and reduction and, optionally, chemical derivatization (methylation and acetylation) to produce more volatile substructures that can be identified. Humic core structures that resist these degradation approaches have been identified; however, carboxyl and hydroxyl functional groups are usually destroyed by these degradative methods. Nondegradative methods for humic substance characterization, such as nuclear magnetic resonance (NMR) spectrometry[3] and infrared (IR) spectrometry,[4] characterize aggregate group structures of whole humic substance mixtures. This lack of molecular resolution has been a primary limitation in structural studies of humic substances.

Recent research[5−8] using electrospray ionization mass spectrometry (ESI/MS) applied to aquatic fulvic acids has generated molecular ion distributions that appear to be within the range of molecular mass distributions determined by vapor pressure osmometry, field flow fractionation, size exclusion chromatography, and equilibrium ultracentrifugation. Aquatic fulvic acids have moderate molecular weight distributions that range from 200 to 2000, facilitating specific structural analyses as compared to humic acids that have much greater molecular weights. Buffer composition, fulvic acid concentration, solvent composition, negative versus positive ion detection, pH, and ionic strength all affect fulvic acid molecular mass distributions.[5] However, ESI/MS is reported to have sufficient sensitivity to detect specific molecules at low fulvic acid concentrations where aggregate formation is not a problem,

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and multiply charged ions that complicate spectral interpretations can be minimized if the correct conditions are used.[7]

The objective of this study is to apply multistage tandem ion trap mass spectrometry coupled to ESI to the molecular resolution and fragmentation of fulvic acid isolated from the Suwannee River, Georgia. Multistage tandem mass spectrometry can selectively isolate and fragment both parent and product ions. Detailed structural elucidation by multiple, sequential stages of specific ion isolation and fragmentation with an ion trap mass spectrometer has been described as multistage or multistep tandem mass spectrometry.[9,10] Up to four stages of multistage tandem mass spectrometry have been used to sequence cyclic peptides,[10] determine the composition of complex mixtures of surfactants in crude extracts,[11] and determine specific linkage positions in glycosphingolipids and oligosaccharides.[12,13]

The experimental approach used was to optimize the experimental conditions based upon the use of various poly(carboxylic acids) that model fulvic acid properties. Monocharged parent and product ions and interpretable fragmentation pathways were the objectives of this research on model compounds. After characterization of the model compounds, fulvic acid was analyzed and the fragmentation results of parent and product ions were used for hypothetical structural analyses of certain individual fulvic acid molecules that can be used to guide future standard synthesis for ultimate structural confirmation studies.

EXPERIMENTAL SECTION

Sample and Standards. The Suwannee River was sampled at its origin at the outlet of the Okefenokee Swamp near Fargo, GA, during November 1983. Isolation and characterization of the fulvic acid fraction, which constituted 66% of the dissolved organic carbon, is described in previous reports.[14,15] Chemical standards discussed in this report were purchased from Aldrich Chemical Co, (Milwaukee, WI). with the exception of pyromellitic acid, which was purchased from Chem Service (West Chester, PA). All standards were used as acquired without additional purification except for lasalocid acid (sodium salt) and glycyrrhizic acid (monoammonium salt), which were converted to acid form with a hydrogen-form ion-exchange resin. Sample and standard solutions were analyzed unfiltered to eliminate bias from selective sorption onto filter media.

Instrumentation. *Experiments with ESI/Quadrupole MS.* Samples and standards were dissolved in UV grade 50/50 water/ methanol and initially analyzed by flow injection analysis on a Hewlett-Packard Series 1100 LC/MS with electrospray ionization. The flow rate of the 25/75 water/methanol mobile phase was 0.2

mL/min. The quadrupole mass spectrometer scanned from 100 to 1000 m/z with capillary exit at 70 V for both positive or negative polarity. For electrospray ionization, the drying gas was 350 °C at 12 L/min with 35 psig nebulizer pressure and capillary voltage of 4000 V. The scan range was often increased to 3000 to detect larger mass ions, along with ramped quadrupole voltages to increase high-mass ion transmission. Source capillary exit voltage was decreased to 50, based on results with standards, to maximize formation of molecular ions and minimize adducts (positive mode) or fragmentations (negative mode). Although the instrument can scan down to m/z 50, the possibility of interference from solvent ions precluded this.

Experiments with ESI/Ion Trap/MS and Multistage Tandem MS. Standard and fulvic acid solutions were analyzed using a Bruker Esquire ion trap mass spectrometer (Bruker Daltonics, Bellerica, MA), equipped with an orthogonal ESI interface (Hewlett-Packard Co, Palo Alto, CA). Solutions of standards and Suwannee River fulvic acid were analyzed by infusion directly into the ESI interface at 5−10 μL/min in 75% methanol/25% water. The mass range analyzed varied between m/z 100 and 2000. After initial experiments, the mass range was shortened to a maximum of m/z 800−1000 to improve dwell time and sensitivity. The source temperature was held at 350 °C. The infused solutions were nebulized by coaxial flow of nitrogen through the spray needle at 240 kPa. Desolvation was enhanced by a countercurrent flow of nitrogen gas of 12 L/min. Negative ions were generated and transferred to the ion trap by maintaining a 3−3.25-kV potential energy difference between the needle and capillary end cap. The end plate was held at 500 V relative to the capillary end cap, and the capillary exit varied from −20 to −70 V. For each scan, 50 000 ions were accumulated and every 5 scans summed. Ions were accumulated in the trap for up to 0.2 s. Helium was introduced into the ion trap at 6 × 10⁻⁶ mbar for the purpose of ion cooling within the trap and to facilitate fragmentation of ions by the application of the rf fragmentor voltages during tandem mass spectrometry (MST/MS) experiments. For the MST/MS experiments, the width of the m/z window for the isolated ion was set between 1 and 3. HPLC/MS spectra of the fulvic acid indicated that there were significant peaks at all masses at unit resolution. After isolation, the supplementary rf voltage applied to the ion trap end caps to induce CID was varied between 0.2 and 1.4 V, depending on the ion isolated and the degree of fragmentation achievable or desired. Typically, IT/MS spectra were generated such that the isolated ion was 20−60% of the largest product ion in the spectra.

Two series of MST/MS experiments were performed. The first experiments, an initial series of up to four sequential steps of isolation and fragmentation, identified the predominant fragments. The second series of experiments were performed to confirm that the fragments were the result of a fragmentation of a singly charged ion and did not result from the combination of singly and multiply charged ions at a single mass-to-charge ratio. For this second series, the range of product ions monitored was extended so that masses 2−4 times greater than the precursor ion were monitored, and the supplementary rf voltage incremented from 0.2 to 1.4 V. This was done to determine whether collisions of lower energy would result in loss of charge and the production of higher m/z ions.

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(14) Leenheer, J. A.; Noyes, T. I. *U. S. Geol. Surv. Water−Supply Pap.* **1984**, No. 2230.

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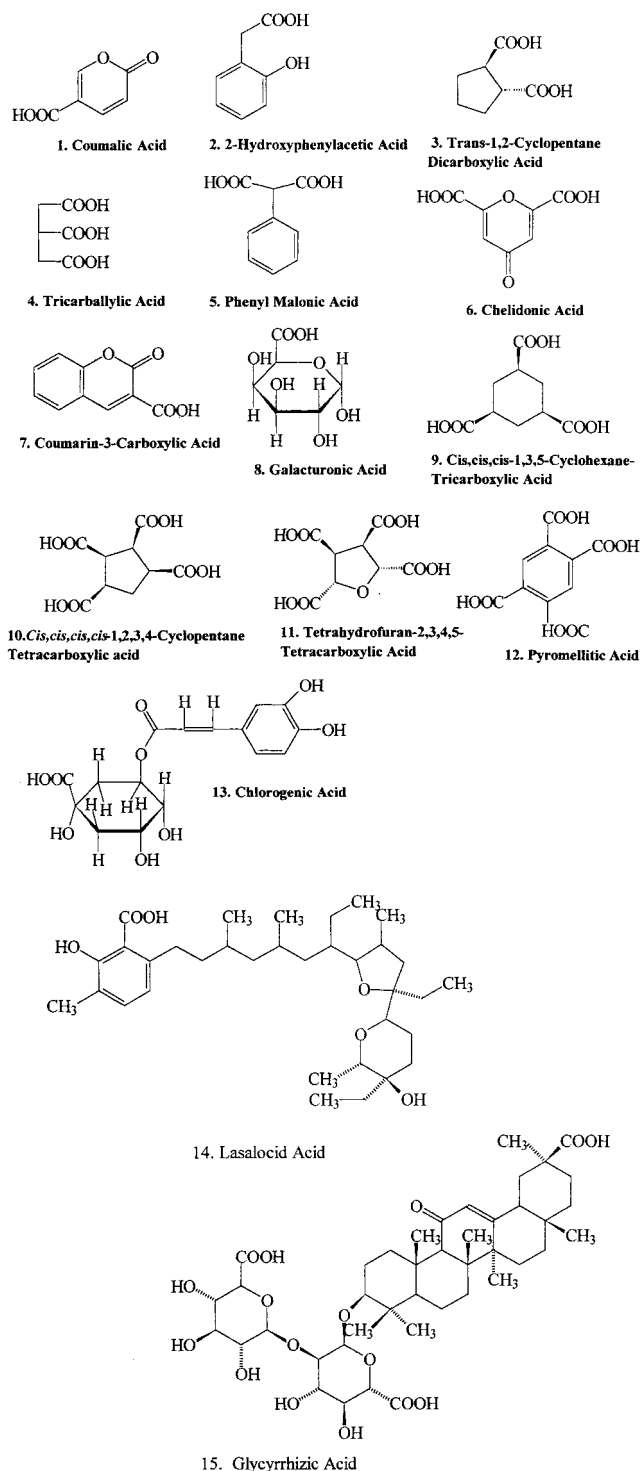
Figure 1. Structures of carboxylic acids analyzed by positive and negative electrospray.

RESULTS AND DISCUSSION

ESI/Quadrupole MS of Model Compounds. Carboxylic acids were selected (Figure 1) that had hydroxyl and/or carboxylic acid distributions in the molecule that are characteristic of the structure of humic material. Typically, positive ion spectra were less intense and had more spurious noise ions than the negative ion spectra. The major ions are tabulated in Table 1. For positive ion spectra, exclusive generation of protonated molecular ions [M + H]$^+$ was rare. Sodium adducts were common, along with a range of others. In addition to the monosodium adduct, adducts such as 2 sodium minus 1 hydrogen, or 3 sodium minus 2 hydrogen, formed. Sodium adducts, preformed in solution or during ionization, commonly occur in electrospray ionization.[16] The extent of sodium adduct formation was somewhat dependent on concentration in solution or amount injected, with more adducts formed at higher concentrations. However, increased sodium concentration created a wider range of sodium adducts, not a dominance of the monosodium adduct. Under these conditions, it would be difficult to drive ionization to one or two dominant ion forms in order to procure the simplest spectra.

Negative ion electrospray spectra were simpler and more intense. Under 70 V capillary exit voltages, many fragments were observed, for example, for *cis,cis,cis,cis*-1,2,3,4-cyclopentanetetracarboxylic acid (**10**): [M − H − H$_2$O]$^-$ (rel intensity, 100), [M − H]$^-$ (60), [M + Na − 2H]$^-$ (10), [M − H − H$_2$O − CO$_2$]$^-$ (50), M − 2H − H$_2$O − 2CO$_2$]$^-$ (15). Loss of neutral fragments (H$_2$O and CO$_2$) and generation of even-electron ions is characteristic of ESI/LC/MS fragmentation. By lowering the voltages to 50 V, however, for the most part, the dominant ion (base peak) of the spectra for these standard carboxylic acids was the deprotonated molecular ion [M − H]$^-$ with very few fragments or adducts (Table 1). Exceptions to this tended to lose a carboxylic group (**5**−**7**). A few compounds (**4**, **8**) predominantly lost water to produce [M − H − H$_2$O]$^-$. Some dimers [2M − H]$^{2-}$ of **6** and **13** and some dideprotonated [M − 2H]$^{2-}$ ions formed. From this point on, all analyses were done with capillary exit at 50 V. Lowering the capillary exit further generally caused a considerable loss in overall intensity.

Under these electrospray ionization conditions, only a few of the smaller molecules did not produce predominantly a deprotonated molecular ion, even over the range of structures analyzed. Dominant loss of carboxylic acid occurred (**5**−**7**) for those compounds that readily decarboxylate. Certain molecules lost water though processes such as alcohol loss and/or anhydride and ester formation (**4**, **8**). Aromatic alcohols (phenols) rarely lost water. Larger molecules, such as chlorgenic acid, more effectively stabilized the charge and did not fragment.

Because we planned to apply these ionization conditions to fulvic acid, which contains many different acid structures with a range of proton dissociation constants (pK_a),[17] there was concern about the effects of pK_a on the uniformity of ionization response. Two acids with very different pK_a values, tetrahydrofuran-2,3,4,5-tetracarboxylic acid (THTCA), pK_{a1} 0.95[17] and caproic (or 1-hexanoic) acid, pK_a 4.83,[18] were tested for their ionization response. Under the conditions described here (50/50 water/MeOH in samples, 25/75 water/methanol mobile phase), the ionization response for the deprotonated molecular ion [M − H]$^-$ averaged about 80% for caproic acid and 100% for THFTCA. Relative intensities of [M − H]$^-$ for replicate injections at various dilutions ($n = 12$) varied with a standard deviation of 18% for caproic acid and 6% for THTCA. Because the relative intensities of these two acids were essentially equal, differences in pK_a had only a minor effect on ionization under these conditions.

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Table 1. Results of Positive and Negative Electrospray Ionization at 50 V of Selected Carboxylic Acids

| no.	compound [CAS no.]	MW	[M + H]	[M + Na]	other [charged ion]	m/z (relative intensity)			
						[M − H]	[M − H − H$_2$O]	[M − COOH]	other [charged ion]
1.	coumalic acid (2-pyrone-5-carboxylic acid) [500-05-0]	140			179 [M + K] (95), M + 71 (100)	139 (100)			301 [2M + Na − 2H] (15), 457 [3M + Na + H$_2$O − 4H] (30), 603 [4M + 2Na − 3H] (10), 619 [4M + 2Na + H$_2$O − 5H] (15)
2.	2-hydroxyphenylacetic acid [614-75-5]	152	na[a]			151 (100)		107 (50)	325 [2M + Na − 2H] (25)
3.	trans-1,2-cyclopentanedicarboxylic acid [1461-97-8]	158		181 (70)	197 [M + K] (100)	157 (100)			
4.	tricarballylic acid [99-14-9]	176		199 (100)	391 [2M + K] (40)	175 (100)		135 (100)	293 [M + 3Na − 2H] (10)
5.	phenylmalonic acid [26613-89-0]	180		203 (100)	311 (40), 207 (40)		157 (45)		
6.	chelidonic acid [99-32-1]	184	185 (100)	207 (70)		183 (65)		139 (100)	367 [2M − H] (60)
7.	coumarin-3-carboxylic acid [531-81-7]	190	191 (10)	213 (100)	234 [M + 2Na − H] (20), 447 [2M + 3NA − 2H] (15), 659 [3M + 4Na − 3H] (40)	189 (25)		145 (100)	401 [2M + 2Na − 2H] (10)
8.	galacturonic acid [91510-62-2]	212			217 [M + Na − H$_2$O] (100), 249 [M + 37] (15)	211 (2)	193 (100)		409 [2M − Na] (10)
9.	1,3,5-cyclohexanetricarboxylic acid, 95% cis,cis,cis [16526-68-4]	216	na			215 (100)			107 [M − 2H]$^{-2}$ (50), 237 [M + Na − 2H] (20)
10.	cis,cis,cis-1,2,3,4-cyclopentanetetracarboxylic acid [3786-91-2]	246		269 (100)	515 [2M + Na] (5)	245 (100)			122 [M − 2H]$^{-2}$ (90), 267 [M + Na − 2H] (30)
11.	tetrahydrofuran-2,3,4,5-tetracarboxylic acid [26106-63-8]	248		271 (10)	285 [M + 37] (100)	247 (100)			261 [M + CH$_2$] (50)
12.	pyromellitic acid [89-05-4]	254	na			weak			
13.	chlorogenic acid [327-97-9]	354	355 (2)	377 (100)		353 (100)			707 [2M − H] (10), 191 [?] (10)
14.	lasalocid acid [25999-20-6]	590		617 (15)	377 (100), 635 [M + 2Na − H] (20), 595 [M + Na − H$_2$O] (20), 577 [M + Na − 2H$_2$O] (20)	589 (100)			
15.	glycyrrhizic acid [140-86-3]	822	weak						273 [M − 3H]$^{-3}$ (100), 421 [M + Na − 3H]$^{-3}$ (25)

[a] na, not analyzed.
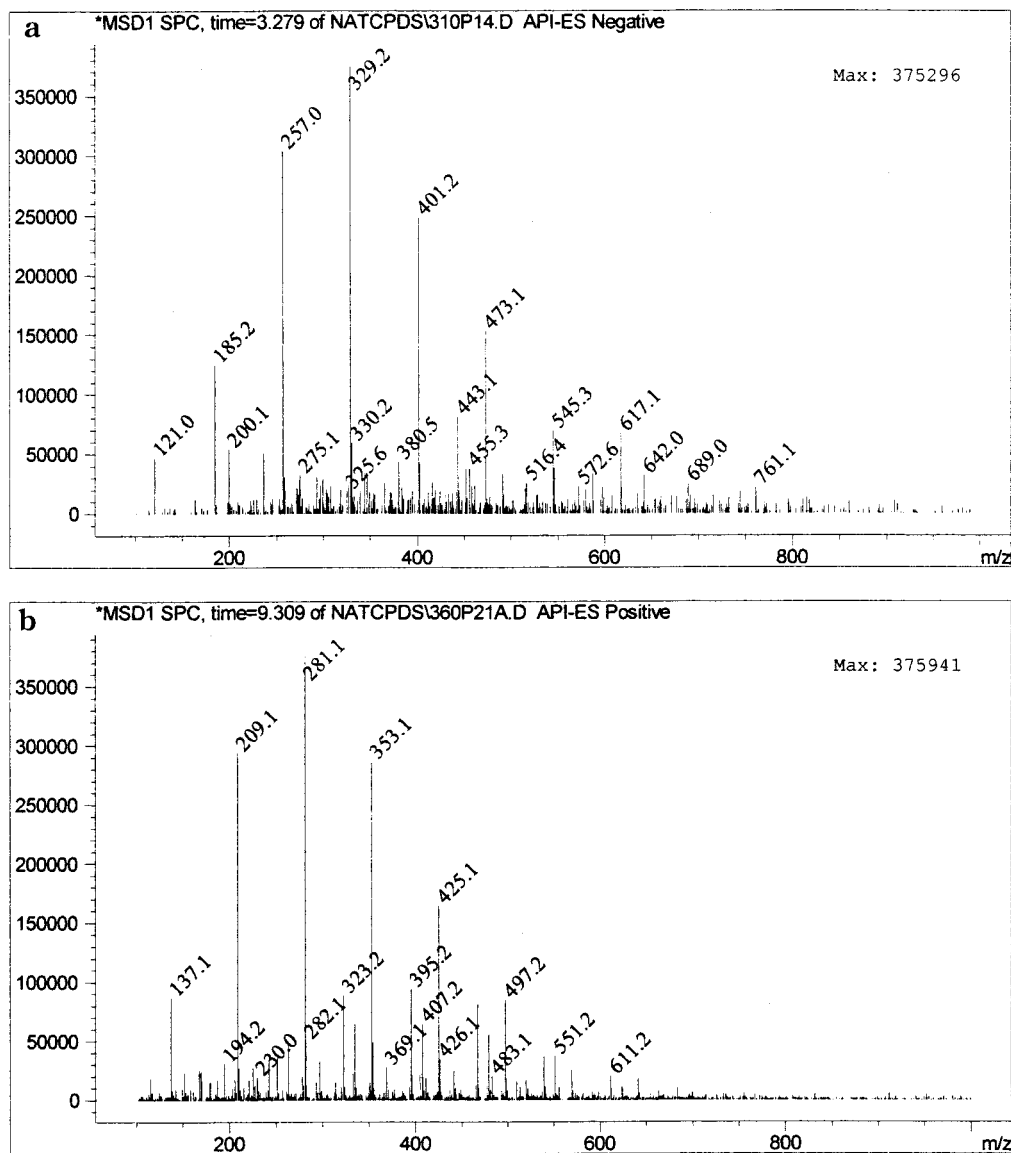
Figure 2. Poly(acrylic acid) (average MW 2000) electrospray spectra in (a) negative ion mode (b) positive ion mode.

Under different solvent conditions the spectra changed, as has been previously reported.[19] With only water in the sample and mobile phase, caproic acid ionized to produce [M − H]⁻ ions, but for THTCA, the [M − H]⁻ ion was only 5% of caproic acid [M − H]⁻. It appears that electrospray conditions with water did not result in optimum ionization of THTCA. For caproic acid, water produced fewer ions (450K) with a signal-to-noise ratio (S/N) of 5.0. For the same standard solution, with 25/75 water/acetonitrile as the mobile phase, more [M − H]⁻ ions were produced (980K) but S/N was 3.8 due to high background noise. With 25/75 water/methanol as the mobile phase, ionization was high (900K), and deprotonated ions were produced in equal amounts for both caproic acid and THTCA, with some background noise, to produce S/N of 2.3. Despite lower S/N, the 25/75 water/methanol mobile phase produced the best conditions for production of primarily deprotonated molecular ions, our goal for fulvic acid characterization.

To investigate the applicability of electrospray ionization to greater molecular weight poly(carboxylic acid)s, two standards

(19) Temesi, D.; Law, B. *LC-GC* **1999**, *17*, 626.

of poly(acrylic acid), Aldrich 19,202-3, average MW 2000, and Aldrich 19,203-1, average MW 5000, were analyzed. The mass spectra for the 2000 average MW poly(acrylic acid) standard is shown in Figure 2. The observed mass distribution was much less than 2000 average MW, and the mass distribution in positive mode differed by essentially a sodium adduct [M + Na]⁺ from the negative mode spectra [M − H]⁻. Almost all the major ions were related by a difference of 72, the monomer weight of [−CH₂−CH(COOH)−], and the major ion series had one water loss. Examination of the minor ions revealed oligimer series that had two, three, and four negative charges as shown in Figure 3. The doubly charged oligimer series began near 400 Da, the triply charged oligimer series began near 800 Da, and the quadruply charged series began near 1030 Da. These data suggest that charges must be separated by 8−10 carbon atoms (for this linear polymer) to form stable multiply charged species. If negative charges in carboxyl groups are closer than this minimum spacing, charge neutralization occurs by protonation, anhydride formation with water loss, and/or decarboxylation. All of these charge neutralization processes were observed in the low molecular
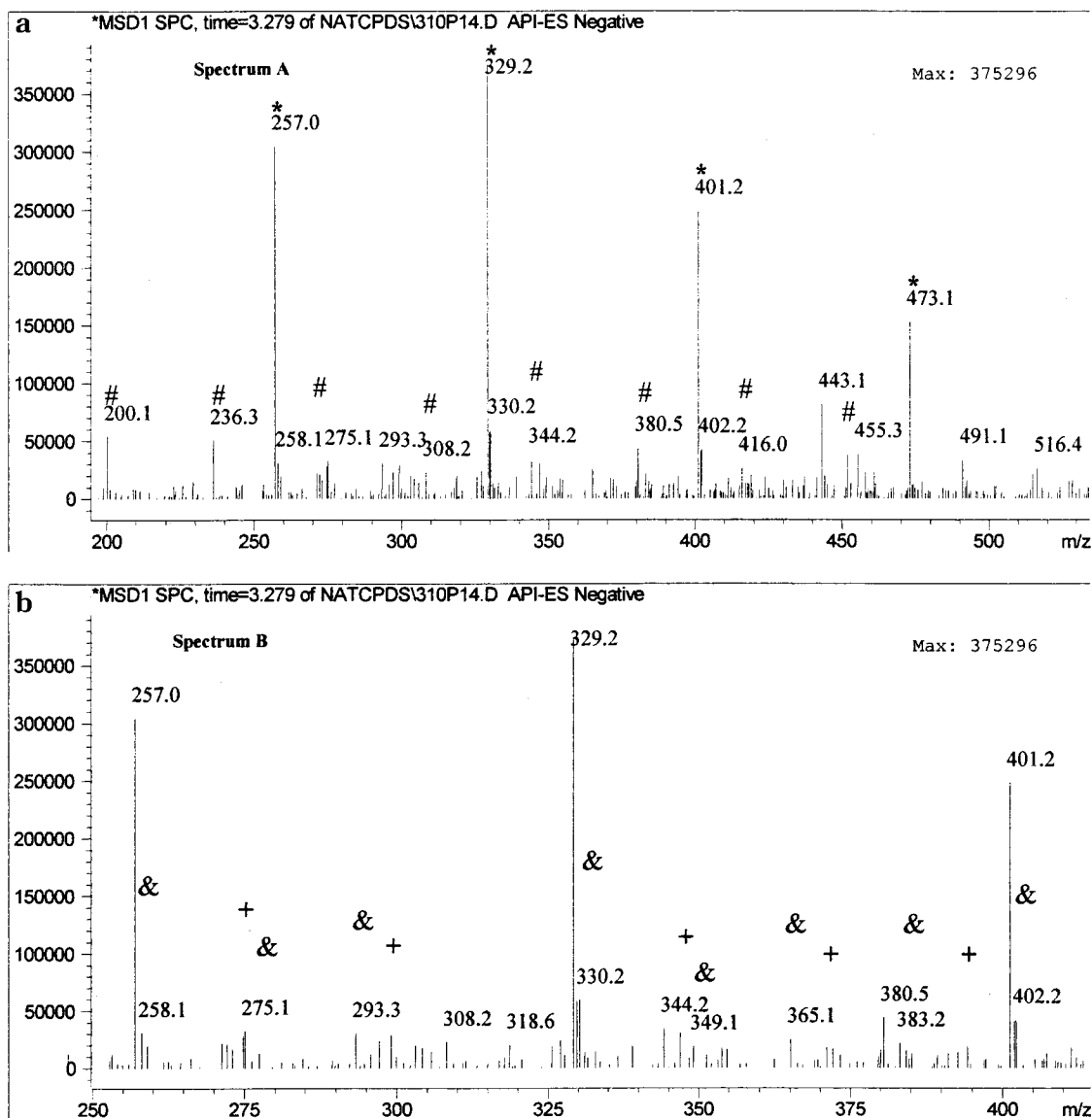
Figure 3. Poly(acrylic acid) (average MW 2000) electrospray spectra with negative ion detection showing single (* = $[(M - H^+) - H_2O]^{-1}$)- and double (\# = $[(M - 2H^+) - H_2O]^{-2}$)-charged ions (spectrum A), and triple- (+ = $[(M - 3H^+) - H_2O]^{-3}$)- and quadruple (& = $[(M - 4H^+) - H_2O]^{-4}$)-charged ions (spectrum B).

weight standards in Table 1. The mass spectra (not shown) of the 5000 average MW poly(acrylic acid) are very similar to the spectra of Figure 2 except the abundance of minor ion oligimer series was much greater because of the greater size of the oligimers and the greater abundance of multiply charged ions.

The poly(acrylic acid) results indicated there might be a limitation in the detection of large (>400 Da) poly(carboxylic acid)s. Therefore, lasalocid acid (590 Da) and glycyrrhizic acid (822 Da) were run under previously described optimum conditions in the negative mode. Only lasolocid acid (with only one carboxyl group) gave a well-defined [M − H] ion. Glycyrrhizic acid gave predominately the triply charged species (Table 1). These results with well-defined standards confirm the finding that the higher molecular weight poly(carboxylic acid) molecules form multiply charged species with the number of charges dependent on the number of carboxyl groups and the molecular spacing between these groups. These results clearly show the cause for the low molecular weight bias in ESI/QMS determinations of molecular

weight distributions of humic substances with either negative or positive ion detection.

ESI/Ion Trap Multistage Tandem MS of Model Compounds. ESI/MST/MS of various model compounds were conducted under a variety of conditions and only the dominant fragmentation pathways of parent ion (MS/MS) and certain product ions (multiple MS) will be discussed. For parent ion fragmentations, water loss [M − H − H₂O]⁻ was the dominant ion detected for *cis,cis,cis*-1,3,5-cyclohexanetricarboxylic acid, *cis,-cis,cis,cis*-1,2,3,4-cyclopentanetetracarboxylic acid, tetrahydrofuran-2r,3t,4t,5c-tetracarboxylic acid, and 2,4,6-trihydroxybenzoic acid. For the aliphatic, alicyclic carboxylic acid standards, water loss resulted from the formation of cyclic five-membered-ring anhydrides between adjacent carboxyl groups in the cis configuration. Water loss for the 2,4,6-trihydroxybenzoic acid, which is a major product of flavonoid degradation,[20] probably results from loss of

(20) Nordstrom, C. G. *Soumen, Kemistilehti* **1968**, *B 41*, 351−353.
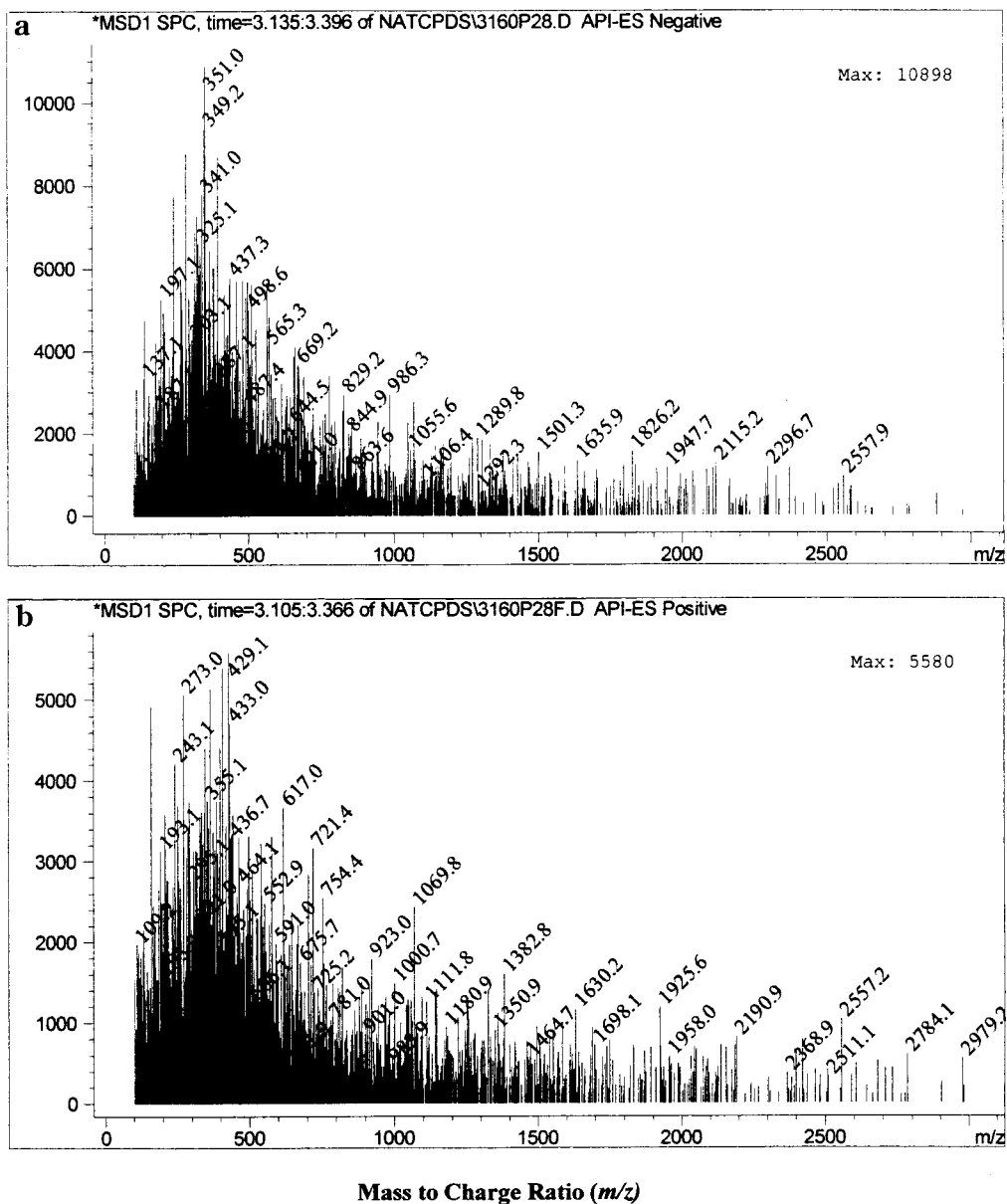
Figure 4. Electrospray spectra of Suwanne River fulvic acid in (a) negative ion mode and (b) positive ion mode.

OH from the aromatic ring which leads to a benzyne ring fragment.

Carbon dioxide loss $[M - H - CO_2]^-$ by decarboxylation from the parent ion was the dominant loss for coumarin-3-carboxylic acid, 3,4-dihydroxybenzoic acid, and 2-hydroxyphenylacetic acid. Pyromellitic acid (1,2,4,5-tetracarboxybenzene) produced the major ion at $[M - H - 88]^-$ (two CO_2 losses) and another fragment resulting from three CO_2 losses. Simultaneous loss of both water and carbon dixode from the parent ion were observed in the MS/MS spectra of tetrahydrofuran-2r,3t,4t,5c-tetracarboxylic acid and cis,cis,cis-1,3,5-cyclohexanetricarboxylic acid, which indicates that multiple fragmentation pathways are possible for some compounds.

Chlorogenic acid, which is an ester of caffeic acid with quinic acid, was cleaved at the ester linkage with the negative charge remaining on the quinic acid acid fragment for the MS/MS spectra. This ester cleavage fragmentation for chlorogenic acid was also observed in the spectra for the ion trap mass spectrom-

eter, whereas the quadrupole mass spectrometer detected only the parent ion. For fulvic acids with intramolecular ester linkages that are common in tannins, ester cleavage may prevent the detection of parent ions when ion trap mass spectrometry with this ion source is used.

For the multiple MS (MS ≥ 3) of the product ions of the standards, additional water and carbon dixode losses were observed until no aliphatic alcohol or carboxylic acid functional groups remained. Cyclic anhydrides produced by water loss from adjacent carboxyl groups were relatively stable but fragmented, losing carbon dioxide and carbon monoxide when rf fragmentor voltages were increased. The lactone ester in coumarin-3-carboxylic acid lost CO (28 Da) from the decarboxylated product ion at m/z 145. Phenol groups in 2-hydroxylphenylacetic acid and 3,4-dihydroxybenzonic acid were also stable, as indicated by ions at m/z 107 for the former and m/z 109 for the latter. The quinic acid fragment (m/z 191) from chlorogenic acid produced successive losses of three waters and one carbon dixode to give a stable
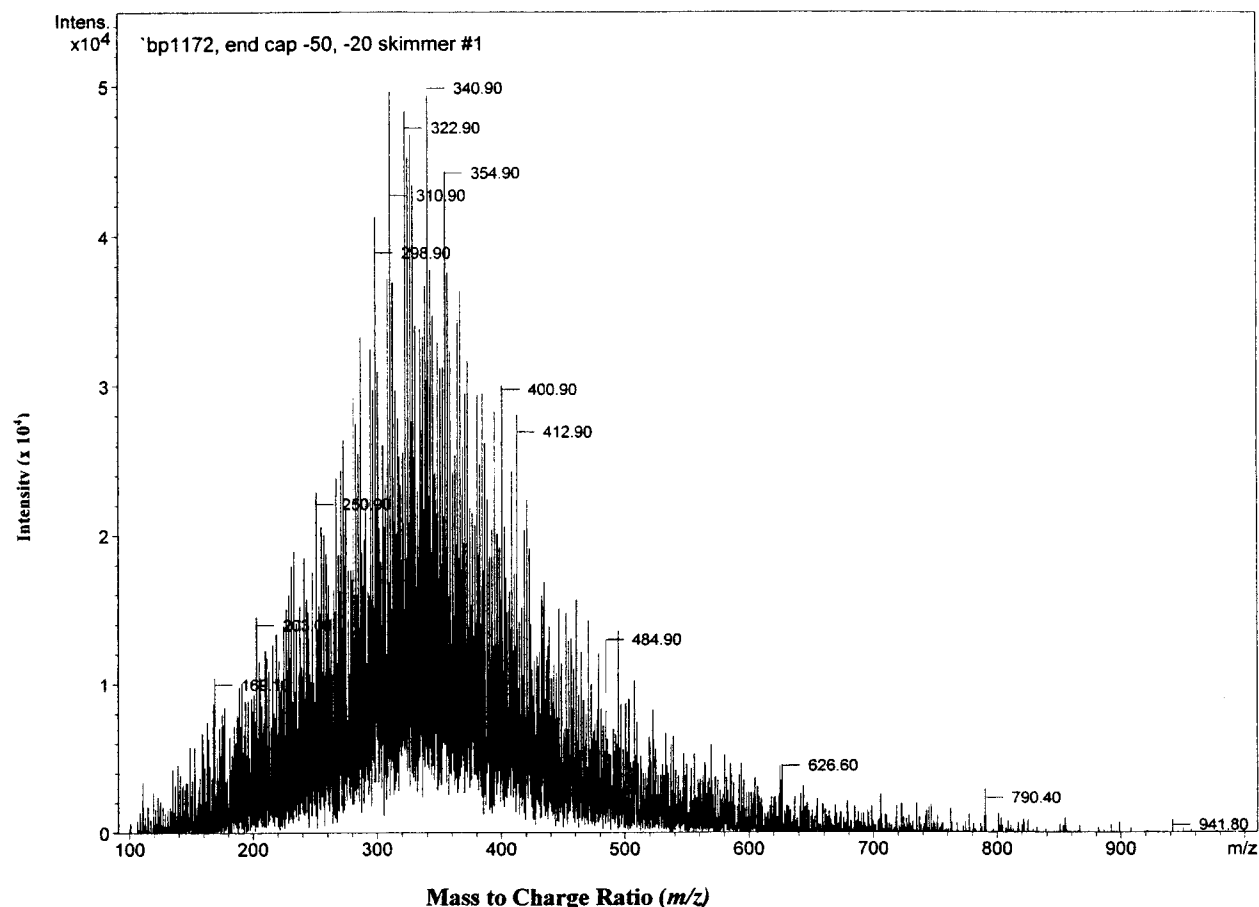
Figure 5. Electrospray ionization/ion trap MS spectrum of Suwanee River fulvic acid. The sample was directly infused into the ESI interface in negative ionization mode at 5 μL/min. ESI source conditions were scan range of m/z 100−1000, nebulizer pressure 35 psi, drying gas temperature 350 °C, drying gas flow 12 L/min, capillary end cap voltage 3.25 kV, end plate voltage 2.75 kV, capillary exit voltage −50 kV, and skimmer 1 voltage −20 V.

fragment at m/z 111 that did not fragment. Benzene rings and aliphatic alicyclic rings of the poly(carboxylic acid) model compounds did not fragment; however, the tetrahydrofuran ring was cleaved at the ether group in the MS/MS/MS spectrum of the m/z 229 product ion.

The observed fragments of the model compounds for the ESI/MST/MS were similar to mild pyrolysis reaction products of these compounds with water loss resulting from dehydration of alcohols and formation of cyclic anhydrides.[21] Decarboxylation proceeded to complete loss of the carboxyl group if stable cyclic anhydrides were not formed. If more appropriate standards had been run, water loss might be observed from the formation of certain lactones and aryl ethers. Ring strain seemed to be an important factor in water loss. Water loss was not observed for pyromellitic acid and 2-hydroxyphenylacetic acid, where moderately strained five-membered rings (a cyclic anhydride and lactone) could be formed, whereas five-membered cyclic anhydrides readily formed if adjacent carboxyl groups were attached in the cis configuration in aliphatic rings, which minimizes ring strain. A six-membered cyclic anhydride readily formed with *cis,cis,cis*-1,3,5-cyclohexanetricarboxylic acid.

ESI/Quadrupole MS of Fulvic Acid. Samples of Suwanee River fulvic acid were analyzed by flow injection electrospray

ionization quadrupole mass spectrometry under the same conditions (with the extended mass range to 3000) that produced predominantly deprotonated molecular ions $[M - H]^-$ for the carboxylic acid standards. Spectra produced under negative ionization are shown in Figure 4a and under positive ionization are shown in Figure 4b. Figure 4b resembles the positive molecular weight distribution shown for Suwanee River fulvic acid using different electrospray instrumentation.[7] As found for the carboxylic acid standards and the poly(acrylic acid), the positive ion spectrum differs by about the mass of a sodium ion from the negative ion spectrum. Although the extent of sodium adducts or fragmentation is unknown, spectra from the two polarities of ionization match well.

Number-average (M_n) and weight-average (M_w) molecular weight distributions of the Suwannee River fulvic acid based on ion intensity distributions were calculated according to accepted methods.[22] The M_n by negative ESI averaged 591 m/z (standard deviation (SD) of 11, $n = 8$), and by positive ESI averaged m/z 617 (SD of 12, $n = 8$). The weight average (M_w) by negative ESI was m/z 914 (SD of 28, $n = 8$), and by positive ESI was m/z 936 (SD of 28, $n = 8$). Both positive values differ from the negative by about the mass of a sodium ion. This is independent confirmation of minimal additional adduct formation in positive ion mode

(21) Bracewell, J. M.; Haider, K.; Larter, S. R.; Schulten, H.-R. In *Humic Substances II. In Search of Structure*; Hayes, M. H. B., MacCarthy, P., Malcolm R. L., Swift, R. S., Eds.; J. Wiley and Sons: New York, 1989; pp 182−222.

(22) Wershaw, R. L.; Aiken, G. R. In *Humic Substances in Soil, Sediment, and Water*; Aiken, G. R., McKnight, D. M., Wershaw, R. L., Eds.; J. Wiley and Sons: New York, 1985; pp 477−492.
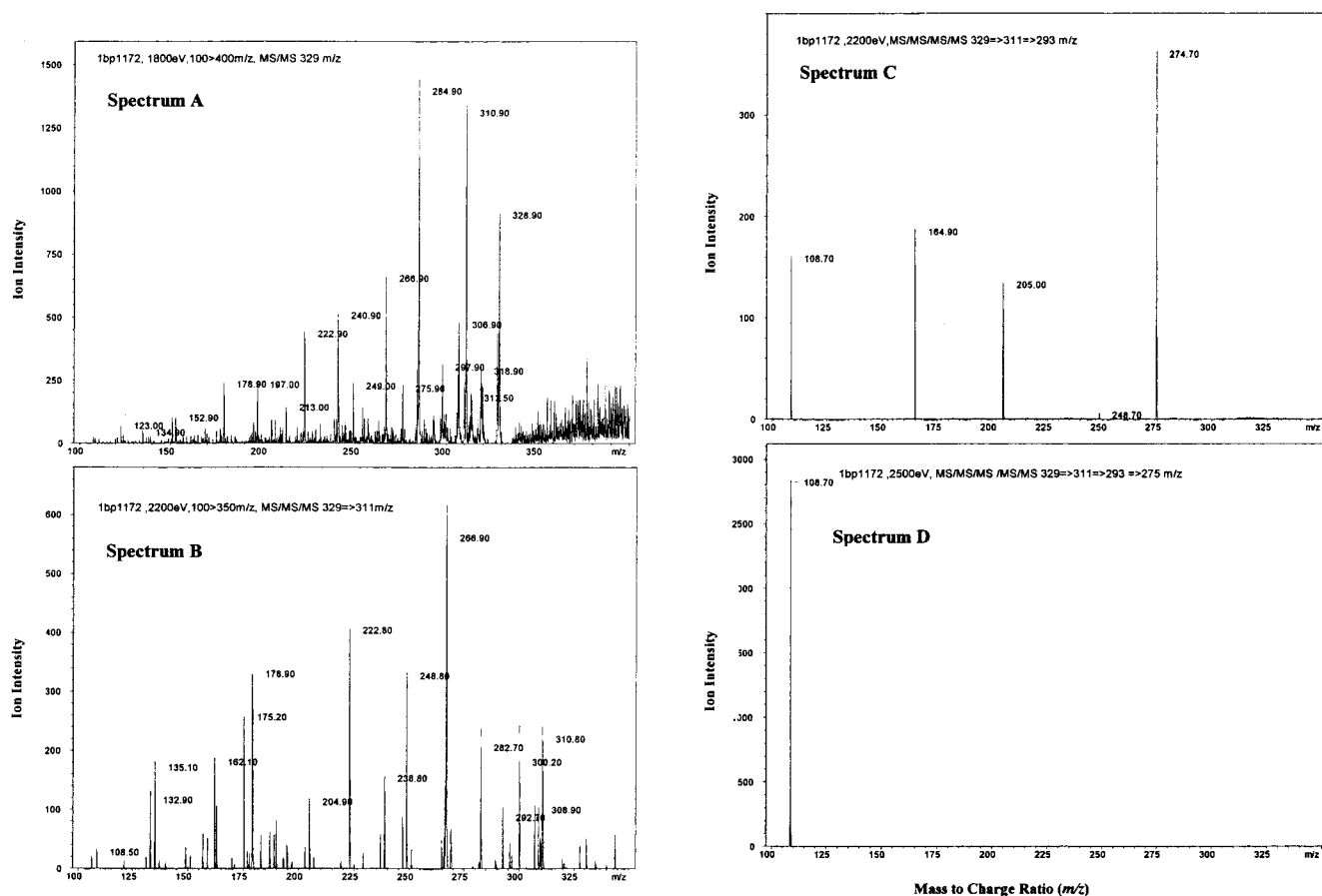
Figure 6. The averaged (n = 20) profile spectra representing a series of MS^n experiments (n = 2−4) which follow the loss of water (m/z 18) from the previous experiment, m/z 329 $\Rightarrow$ 311 $\Rightarrow$ 293 $\Rightarrow$ 275. The MS^5 fragmentation of the m/z 275 produced only one product ion at m/z 109, which is not amenable to further fragmentation. Source conditions were nebulizer pressure 35 psi, drying gas temperature 325 °C, drying gas flow 12 L/min., capillary end cap voltage 3.25 kV, end plate voltage 2.75 kV, capillary exit voltage −50 kV, and skimmer 1 voltage −20 V.

or fragmentation in negative ion mode for this mixture of structures. On the basis of our experience with the wide variety of related standards, the possibility of formation of sodium adducts or fragmentary losses is greater in positive mode.

The molecular weight distributions determined by ESI/QMS compare moderately well with various independent molecular weight determinations. Published M_n values for Suwannee River fulvic acid are 470 (acid form)−1060 (K$^+$ salt form) by equilibrium ultracentrifugation,[15] 623−965 (acid form) by vapor pressure osmometry,[23] 645−816 (acid form) by low-angle X-ray scattering,[23] and 1390 (Na$^+$ salt form) by gel permeation chromatography.[24] Published M_w values are 1110 (acid form)−1340 (K$^+$ salt form) by equilibrium ultracentrifugation[15] and 2080 (Na$^+$ salt form) by gel permeation chromatography.[24] When comparing these results, it is important to note that mass spectrometric determination is the only mass-specific measurement of fulvic acid molecular weights. The other methods suffer from assumptions of size to mass estimates, inclusion of metal cations and hydration spheres, molecular density estimates, and specific UV detector errors. The M_w values determined by ESI/QMS appears to deviate to lower

mass more than the M_n values in this comparison with other methods, and this deviation is likely related to multiply charged species at higher masses.

Although there appeared to be a peak at every mass, there was a definite dominance of odd- over even-mass ions, as noted by Brown and Rice.[5] The odd/even ion predominance calculated in a manner analogous to the carbon preference index[25] in the 250−500 Da mass range, was determined to be 2.0 for negative ESI and 2.8 for positive ESI. On the basis of the data from standards under these ionization conditions, we assume the ions represent [M − H]$^-$ in negative mode and [M + H]$^+$ or [M + Na]$^+$ in positive mode, from even molecular weight molecules. Nitrogen occurs on the average (based upon a M_n of 800 Da) once every 2.5 molecules in the Suwannee River fulvic acid,[26] which may indicate that the even ions in the negative and positive ESI spectra may contain a nitrogen atom. However, even ions can also result from doubly charged species.

ESI/Ion Trap Multistage Tandem MS of Fulvic Acid. The ESI/MST/MS spectrum of the Suwannee River fulvic acid is shown in Figure 5. The ion distribution in this figure is less

(23) Aiken, G. R.; Brown, P. A.; Noyes, T. I.; Pinckney, D. J. In *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*; Averett, R. C., Leenheer, J. A., McKnight, D. M., Thorn, K. A., Eds. *U. S. Geol. Surv. Water−Supply Pap.* **1994**, *No. 2373*, 89−98.

(24) Chin, Y.-P.; Aiken, G. R.; Danielsen, K. M. *Environ. Sci. Technol.* **1997**, *31*, 1630−1635.

(25) Tissot, B. P.; Welte, D. H. *Petroleum Formation and Occurrence*; Springer-Verlag: Berlin, 1984; p 101.

(26) Thurman, E. M.; McKnight, D. M., In *Humic Substances in the Suwannee River, Georgia: Interactions, Properties, and Proposed Structures*; Averett, R. C., Leenheer, J. A., McKnight, D. M., Thorn, K. A., Eds. *U. S. Geol. Surv. Water−Supply Pap.* **1994**, *No. 2373*, 203−204.

skewed to the high molecular weight (M_n = 354 m/z, M_w = 383 m/z) than is the ion distribution determined by the ESI/quadrupole MS present in Figure 4. It is possible that cleavage of labile ester linkages in the ion trap, as was shown for chlorogenic acid, resulted in the relative reduction in high molecular weight ions in the spectrum of Figure 5.

MS/MS with negative ion detection was conducted on 32 parent ions in Figure 5 ranging in mass from 279 to 537 Da. All of the parent and product ion spectra were dominated by odd-mass ions. The most intense product ion for 31 of the parent ion fragmentations was the $[M - H - CO^2]^-$ ion. For the 537 Da ion, the parent ion was the most intense followed by the $[M - H - CO_2]^-$ ion in intensity. As the molecular weight increased, the number of the ions observed in the MS/MS spectra increased, suggesting greater mixture and molecular complexity. Successive losses of two to five units of 44 (CO_2) from the parent ion were observed with an average CO_2 unit loss of 3.4. An initial $[M - H - H_2O]^-$ loss was also a significant ion in most of the MS/MS spectra, and a secondary CO_2 loss series was observed that was derived from the $[M - H - H_2O]^-$ ion. This secondary CO_2 series had either the same or one less CO_2 loss as compared to CO_2 losses from the parent ions.

The molecular ion at m/z 329 was selected for multiple MS characterization because of its relatively high mass intensity near the median of the molecular ion distribution in Figure 5. The multiple MS spectra of the parent ion $[M - H]^-$, the product ion at $[M - H - H_2O]^-$ (m/z 311), the product ion at $[M - H - 2H_2O]^-$ (m/z 293), and the product ion at $(M - 3H_2O]^-$ (m/z 275) are presented in Figure 6.

The MS/MS spectrum of the 329 molecular ion is likely a mixture of compounds and isomers, with the number of peaks and the complexity of the spectrum reflecting the mixture characteristics. However, as the number of MS stages of the product ion increases, the spectra become increasing simple until only one product ion at m/z of 109 is detected which cannot be further fragmented. The water loss series was selected for multiple MS characterization to apply some of the knowledge gained about water loss pathways from multiple MS of the standards. The simplicity of the fragmentation pattern for the m/z 293 ion (Figure 6, spectrum C) and the similarity of this fragmentation pattern to cyclic anhydrides derived from poly(carboxylic acid) standards in Figure 1 suggests that the m/z 293 ion is derived from a single compound in the complex fulvic acid mixture. The ESI/MST/MS characterization of poly(carboxylic acid) standards that form cyclic anhydrides indicated that both anhydride formation and fragmentation are very specific to molecular structure configuration. This molecular-level information is the most significant finding of this study and has not been previously demonstrated.

A hypothetical fragmentation pathway involving alcohol dehydration and anhydride formation and neutral water loss that leads to the m/z 293 product ion is presented below:
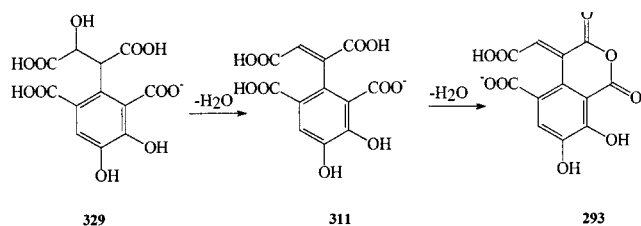
A hypothetical fragmentation pathway of the m/z 293 product ion of spectrum C of Figure 6 is presented below:
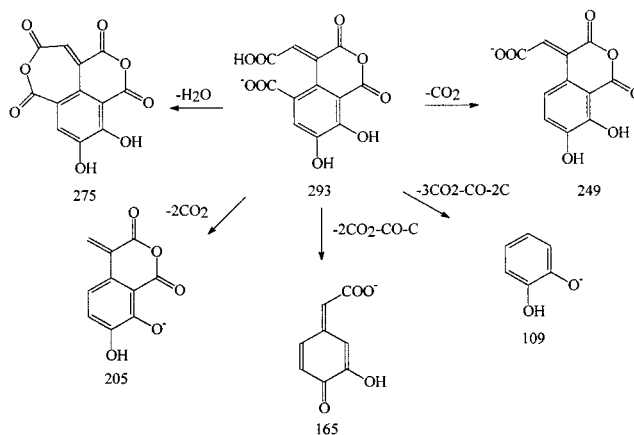
Water loss in these fragmentation pathways is based upon alcohol dehydration and formation of cyclic anhydrides whose rings are sufficiently large to avoid ring strain. Structures involving water loss by lactone ester formation were not presented because lactone esters spontaneously form in hydroxy acids in aqueous solutions prior to MS analyses.[27] Previously formed lactone esters were also not detected by characteristic CO loss as was found for courmarin-3-carboxylic acid. Water loss and CO_2 loss were not detected in the multiple mass spectrum of the m/z 275 product ion (spectrum D) as they were in the m/z 329 (spectrum A), 311 (spectrum B), and 293 (spectrum C) spectra. The negative ions formed by fragmentation of the cyclic anhydrides also involve neutral loss of CO and elemental carbon in addition to water and carbon dioxide losses. These fragmentation pathways are speculative and need to be confirmed with appropriate synthesized standards.

CONCLUSIONS

The findings of this report show the analytical power of ESI/MST/MS, which first resolves complex fulvic acid mixtures into groups of identical-mass parent ions followed by additional MS stages of resolution of product ions whose fragmentation patterns can be traced to more specific fulvic acid molecules. For the potential of ultimate structural analyses of fulvic acid components to be fully realized, much additional work with chemical standards, synthesis of hypothesized structures for confirmation, and ESI/high-resolution mass spectrometry to obtain elemental composition are needed.

ESI/MST/MS appears to have the sensitivity and specificity for molecular structural analyses of specific fulvic acid compounds with the limitation of labile ester linkages that are cleaved during the analysis. ESI/MS production and detection of singly charged species (which is the fundamental assumption in calculation of molecular weight distributions) also appears to be limited, at least in the case of polycarboxylic fulvic acids and model compounds, to molecules of 400 Da or less in molecular mass. Poly(carboxylic

(27) Antweiler, R. C. In *Organic Substances and Sediments in Water. Humics and Soils*; Baker, R. A., Ed.; Lewis Publishers: Chelsea, MI, 1991; Vol. 1, pp 163−177.

acids) greater than 400 Da with charge separations greater than eight singly bonded carbons produce multiply charged species which give a low bias to molecular weight measurements by ESI/MS.

Although unambiguous compound ESI/MST/MS spectra cannot be unequivocally assigned to individual ions, the structural information that can be derived from these spectra is a substantial improvement in understanding the molecular structure of fulvic acid components. Structure-specific models derived from ESI/MST/MS data will be crucial in determining the mediating behavior of naturally occurring material in organic and inorganic environmental processes.