Anal. Chem. **1999,** *71,* 4719−4726

Gas-Phase Hydrogen/Deuterium Exchange Reactions of Fulvic Acids: An Electrospray Ionization Fourier Transform Ion Cyclotron Resonance Mass Spectral Study

T. Solouki,*,† M. A. Freitas,‡ and A. Alomary†

Department of Chemistry, University of Maine, Orono, Maine 04469, and CIMAR, National High Magnetic Field Laboratory, 1800 East Paul Dirac, Tallahassee, Florida 32310

A gas-phase hydrogen/deuterium (H/D) exchange reaction technique to determine the number of active hydrogens (NOAH) in fulvic acid ions using electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry is described. First, fulvic acid precursor ions are isolated by stored waveform inverse Fourier transform dipolar excitation. Second, ion−molecule reactions of the selected fulvic acid ions with neutral H/D exchange reagent gases are monitored. The number of incorporated deuterium isotopes in the product ion provides the NOAH for the precursor ion. Previously characterized northern hardwood (NHFA) and red spruce (NCFA) fulvic acid samples were analyzed in this study. Selected ions of both fulvic acid samples undergo H/D exchange with D_2O, ND_3, and CD_3OD reagent gases. The extent of H/D exchange of the fulvic acid ions increases with reagent gas basicity, $D_2O < CD_3OD < ND_3$. For the first time, we were able to count the NOAH of selected fulvic acid molecules. The average maximum NOAH for NCFA and NHFA ions at *m*/*z* region 700−1000 Th is ∼7−9. For example, the singly charged NCFA positive ions at *m*/*z* 800 Th contain eight active hydrogens. There is no significant difference between NOAH for NCFA and NHFA. The proton affinities of fulvic acid ions at *m*/*z* range of 600−1000 Th do not vary significantly.

The primary goal of this study is to develop mass spectral methods for structural analysis of fulvic acids. Fulvic acids are found in soil and water, and they are traditionally defined according to their solubilities in water.[1] Binding and complexation of fulvic acids with environmentally significant substances such as pesticides, polychlorinated biphenyls, polyaromatic hydrocarbons, and metals are of considerable interest. Conventional separation and fractionation techniques cannot be used to isolate individual molecular components of fulvic acids.[2] Hence, the study of humic substances is limited to investigating bulk properties of this diverse group.[3]

Previous Studies. In previous studies, fulvic acids have been treated as homogeneous mixtures of polymers and thus detailed chemical information for these species, at the molecular level, is limited. The early ion exchange work,[4] ion-selective potentiometric,[3,5−7] acid−base,[8] and fluorescence quenching[9] titrations have provided useful chemical information on the complex mixtures of fulvic acids in solution. Recently, new emerging synchrotron-based techniques such as soft X-ray spectroscopy and spectromicroscopy have been used to study element-specific functional groups of natural organic molecules as well as their aggregate structures.[10] Other sophisticated techniques also have been employed to address the difficulties with fulvic acid analysis. For example, NMR, ESR, and fluorescence techniques have been used to study humic materials and their environmental impact, e.g., transport, fate, and chemical speciation of pollutants.[2,9,11−19]

Recently, Patterson and co-workers utilized fluorescence polarization to probe structural variations of NHFA and NCFA upon metal complexation.[19] Although fluorescence methods provided useful data about the reactivity of these geomacromol-

† University of Maine.

‡ CIMAR.

(1) Gaffney, J. S., Marley, N. A., Clark, S. B., Eds. *Humic and Fulvic Acids*; ACS Symposium Series 651; American Chemical Society: Washington, DC, 1996.

(2) Dixon, A. M.; Larive, C. K. *Anal. Chem.* **1997**, *69*, 2122−2128.

(3) Khan, S. U. *Soil. Sci. Soc. Am. Proc.* **1969**, *33*, 851−854.

(4) Schnitzer, M.; Hansen, E. H. *Soil Sci.* **1970**, *109*, 333−340.

(5) Bresnahan, W. T.; Grant, C. L.; Weber, J. H. *Anal. Chem.* **1978**, *50*, 1675−1679.

(6) Saar, R. A.; Weber, J. H. *Anal. Chem.* **1980**, *52*, 2095−2100.

(7) Lövgren, L.; Hedlund, T.; Ohman, L. O.; Sjoberg, S. *Water Res.* **1987**, *21*, 1401−1407.

(8) Backes, C. A.; Tipping, E. *Water Res.* **1987**, *21*, 211−216.

(9) Ryan, D. K.; Weber, J. H. *Anal. Chem.* **1982**, *54*, 986−990.

(10) Martinez, A.; Hussain, Z.; Warwick, A. *Book of Abstracts*, National Meeting of the American Chemical Society, Boston, MA, August 23−37 1998; ENVR 005.

(11) Cook, R. L.; Langford, C. H.; Yamdagni, R.; Preston, C. M. *Anal. Chem.* **1996**, *68*, 3979−3986.

(12) Nanny, M. A.; Bortiatynski, J. M.; Hatcher, P. G. *Environ. Sci. Technol.* **1997**, *31*, 530.

(13) Cook, R. L.; Cooper, H. L. *Environ. Sci. Technol.* **1998**, *32*, 719−725.

(14) Kim, J.-E.; Fernandes, E.; Bollag, J.-M. *Environ. Sci. Technol.* **1997**, *31*, 2392−2398.

(15) Senesi, N.; Schnitzer, M. *Soil Sci.* **1977**, *123*, 224−234.

(16) Cabaniss, S. E.; Shuman, M. S. *Anal. Chem.* **1988**, *60*, 2418−2421.

(17) Plankey, B. J.; Patterson, H. H. *Environ. Sci. Technol.* **1988**, *22*, 1454.

(18) Plankey, B. J.; Patterson, H. H.; Cronan, C. C. *Anal. Chim. Acta* **1995**, *300*, 227−236.

(19) Lakshman, S.; Mills, R.; Fang, F.; Patterson, H.; Cronan, C. *Anal. Chim. Acta* **1996**, *321*, 113−119.

10.1021/ac990185w CCC: $18.00
Published on Web 09/10/1999

ecules and kinetics of their metal complexation reaction,[17,18] the organic constituents involved and the nature of the binding sites are still poorly understood. The microscopic nature of the reaction mechanisms is only indirectly accessible by these conventional approaches. For instance, in all of the kinetic studies of fulvic acids, an assumed average molecular weight is used to calculate the molar concentrations of these species. Obviously, more reliable studies of fulvic acids require detailed knowledge about the true molecular identities of these acidic geomacromolecules. To minimize the difficulties associated with the analyses of such a diverse group of colloidal material, hollow-fiber ultrafiltration techniques have been used in tandem with other analytical tools.[20] However, size-fractionated samples are still complex mixtures of hundreds of different molecules.

Mass Spectrometry. Mass spectrometry is a powerful technique for direct molecular characterization of complex species and has been used extensively to analyze humic substances.[21,22] Novotny and co-workers compared the calculated number-average (M_n) molecular weight of five fulvic acid samples from low-power laser desorption ionization (LDI) and Fourier transform ion cyclotron resonance (FT-ICR) MS with gel filtration chromatography (GFC) and vapor pressure osmometry (VPO). Due to ion fragmentation, the calculated M_n molecular weights from LDI results were lower than GFC and VPO results for all five samples.[23] Recently, we used FT-ICR mass spectrometry coupled with matrix-assisted laser desorption (MALDI)[24,25] and electrospray ionization (ESI)[26] to analyze humic and fulvic acids.[27,28] FT-ICR offers ultrahigh mass resolving power, high mass accuracy, high sensitivity, multistage MS^n and ion storage capabilities.[29−35] The combination of the ion storage and high-resolution ion isolation capabilities of FT-ICR[36,37] offers a powerful approach for analyzing complex samples.

H/D Exchange Reactions. The gas-phase hydrogen/deuterium (H/D) exchange method is a suitable approach to probe complex structures. Most of the early H/D experiments, including the methods that are based on the mass spectrometric measurement of the extent of hydrogen/deuterium exchange, were conducted in solution.[38−41] Hunt and co-workers demonstrated that CH_3OD and ND_3 could be used as the chemical ionization (CI) reagent gases to count the number of "active hydrogens".[42] Smith and co-workers reported the use of thermal energy H/D exchange ion−molecule reactions to probe conformational differences of multiply protonated gas-phase proteins.[43] Other experiments have elegantly demonstrated the use of H/D exchange reactions in the gas phase for structural elucidation of biological molecules.[44−52]

To date, structural identities of fulvic acids remain unknown. For example, prior to this work, the number and/or the type of active hydrogens for individual fulvic acid species has not been directly/experimentally discerned. In this paper and for the first time, we describe the use of gas-phase H/D exchange ESI FT-ICR mass spectrometry technique to determine the number of active hydrogens (NOAH) (i.e., O−H and N−H, S−H, and COO−H) for fulvic acid species. In addition, we present data on two different fulvic acid samples and report the NOAH for a selected number of fulvic acid species. It is worth noting that experimental limitations prohibit the use of direct solution-phase H/D exchange reactions to probe the number of active hydrogens for *neutral* fulvic acid molecules. In other words, the lack of suitable analytical techniques to separate fulvic acid samples to their individual components makes it impossible to detect mass shifts (due to the deuterium incorporation) for individual molecules. Therefore, the complex nature of the fulvic acid mixture, in terms of the molecular weight range and the molecular weight proximity of present species in each sample, necessitates high-resolution isolation of selected species prior to H/D exchange reactions. To date, such a narrow mass-selective isolation of fulvic acid species can only be achieved for charged species in the gas phase. Careful isolation of the specific ions from various m/z regions is accomplished by the use of stored waveform inverse Fourier

(20) Gaffney, J. S., Marley, N. A., Clark, S. B., Eds.; *Humic and Fulvic Acids*; ACS Symposium Series 651; American Chemical Society: Washington, DC, 1995; Chapter 3, pp 26−40.
(21) Saiz-Jimines, C.; Leeuw, J. W. d. *J. Anal. Appl. Pyrolysis* **1986**, *9*, 99−119.
(22) Abbt-Braun, G.; Frimmel, F. H.; Schulten, H. R. *Water Res.* **1989**, *23*, 1579−1591.
(23) Novotny, F. J.; Rice, J. A.; Weil, D. A. *Environ. Sci. Technol.* **1995**, *29*, 2464−2466.
(24) Karas, M.; Bachmann, D.; Bahr, D.; Hillenkamp, F. *Int. J. Mass Spectrom. Ion Processes* **1987**, *78*, 53.
(25) Karas, M.; Hillemkamp, F. *Anal. Chem.* **1988**, *60*, 2299−2301.
(26) Whitehouse, C. M.; Dreyer, R. N.; Yamashita, M.; Fenn, J. B. *Anal. Chem.* **1985**, *57*, 675−679.
(27) Fievre, A.; Solouki, T.; Marshall, A. G.; Cooper, W. T. *Energy Fuels* **1997**, *11*, 554−560.
(28) Solouki, T.; Fievre, A.; Marshall, A. G.; Cooper, W. T., 45th ASMS, Palm Springs, CA, June 1−5 1997; p 941.
(29) Köster, C.; Castoro, J. A.; Wilkins, C. L. *J. Am. Chem. Soc.* **1992**, *114*, 7572−7574.
(30) Buchanan, M. V.; Hettich, R. L. *Anal. Chem.* **1993**, *65*, 245A−259A.
(31) McIver, R. T. J.; Y. Li, Y.; Hunter, R. L. *Int. J. Mass Spectrom. Ion Processes* **1994**, *132*, L1−L7.
(32) Williams, E. R.; Henry, K. D.; McLafferty, F. W. *J. Am. Chem. Soc.* **1990**, *112*, 6157−6162.
(33) Solouki, T.; Emmett, M. R.; Guan, S.; Marshall, A. *Anal. Chem.* **1997**, *69*, 11−63−1168.
(34) Solouki, T.; Paša-Toliç, L.; Jackson, G. S.; Guan, S.; Marshall, A. *Anal. Chem.* **1996**, *68*, 3718−3725.
(35) Solouki, T.; Marto, J. A.; White, F. M.; Guan, S.; Marshall, A. G. *Anal. Chem.* **1995**, *67*, 4139−4144.
(36) Marshall, A. G.; Wang, T. C. L.; Ricca, T. L. *J. Am. Chem. Soc.* **1985**, *107*, 7893−7897.
(37) Wang, T. C. L.; Ricca, T. L.; Marshall, A. G. *Anal. Chem.* **1986**, *58*, 2935−2938.
(38) Englander, S. W.; Kallenbach, N. R. *Q. Rev. Biophys.* **1984**, *16*, 521−655.
(39) Englander, S. W. *Science* **1993**, *262*, 848−849.
(40) Katta, V.; Chait, B. T. *J. Am. Chem. Soc.* **1993**, *115*, 6317−6321.
(41) Miranker, A.; Robinson, C. V.; Radford, S. E.; Aplin, R. T.; Dobson, C. M. *Science* **1993**, *262*, 896−900.
(42) Hunt, D. F.; McEwen, C. N.; Upham, R. A. *Anal. Chem.* **1972**, *44*, 1292−1294.
(43) Winger, B. E.; Light-Wahl, K. J.; Rockwood, A. L.; Smith, R. D. *J. Am. Chem. Soc.* **1992**, *114*, 5897−5898.
(44) Gur, E. H.; Koning, L. J. d.; Nibbering, N. M. M. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 466−477.
(45) Grad, E.; Green, M. K.; Bregar, J.; Lebrilla, C. B. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 623−631.
(46) Suckau, D.; Shi, Y.; Beu, S. C.; Senko, M. W.; Quinn, J. P.; F. M. Wampler, I.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 790−793.
(47) Wood, T. D.; Chorush, R. A.; Wampler, F. M., III; Little, D. P.; O'Connor, P. B.; McLafferty, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2451−2454.
(48) Dookeran, N. N.; Harrison, A. G. *J. Am. Soc. Mass Spectrom.* **1995**, *6*, 16−26.
(49) Heck, A. J. R.; Jørgensen, T. J. D.; O'Sullivan, M.; Raumer, M. V.; Derrick, P. J. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1255−1266.
(50) Freitas, M. A.; Hendrickson, C. L.; Emmett, M. R.; Marshall, A. G. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1012−1019.
(51) Wyttenbach, T.; Bowers, M. T. *J. Am. Soc. Mass Spectrom.* **1998**, *10*, 9−14.
(52) Campbell, S.; Rodgers, M. T.; Marzluff, E. M.; Beauchamp, J. L. *J. Am. Chem. Soc.* **1995**, *117*, 12840−12854.

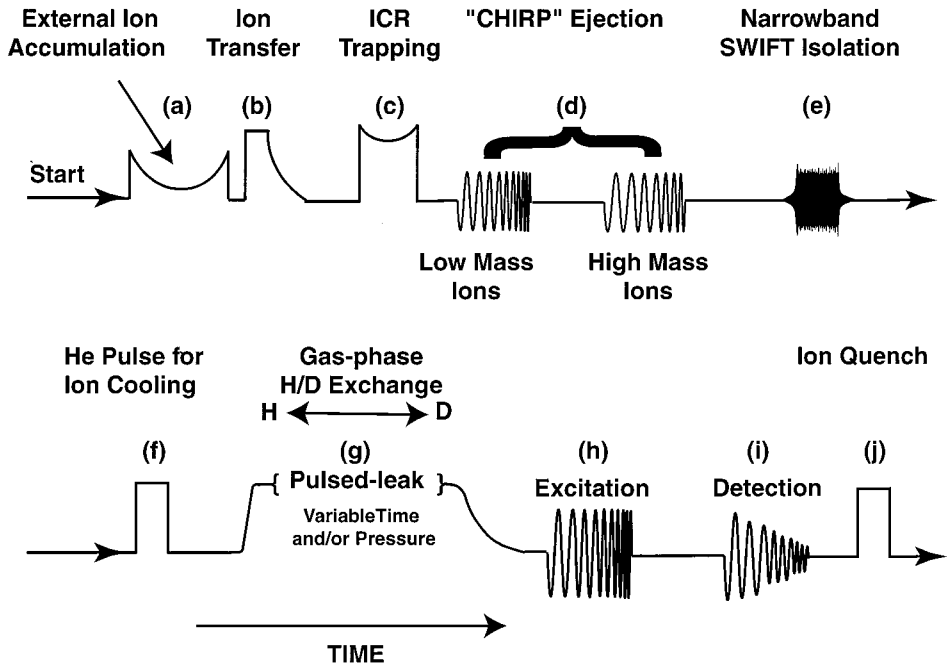

Figure 1. Experimental event sequence for an ESI FT-ICR hydrogen/deuterium exchange reaction of selected ions. First, the parent fulvic acid ions (a) are accumulated externally, (b) transferred into the ICR cell, and (c) trapped. Then, a combination of (d) CHIRP frequency sweep and (e) SWIFT dipolar excitation is used to isolate selected ions. A helium gas pulse (f) is used to relax the isolated ions prior to the introduction of (g) H/D exchange reactant gas via a pulsed-leak valve. A normal sequence of (h) dipolar frequency sweep excitation and (h) direct broad-band mode detection is used to analyze the product ions. At the end of each experiment, (j) all trapped ions are ejected from the ICR cell.

transform (SWIFT) dipolar excitation.[36,53] We report the gas-phase H/D exchange results for SWIFT isolated northern hardwood fulvic acid (NCFA) and red spruce fulvic acid (NHFA) ions with D_2O, ND_3, and CD_3OD reagent gases. To minimize the undesirable effect of ion fragmentation of fulvic acid species, we used ESI to produce intact ions.[27]

EXPERIMENTAL SECTION

Sample Preparation. Previously characterized Northern Soil fulvic acids, namely, NHFA and NCFA samples were provided by Dr. H. H. Paterson.[18] D_2O {D, 99%}, ND_3 {D, 99%}, and CD_3OD {D, 99.8%}were purchased from Cambridge Isotope Laboratories (Andover, MA). All other solvents were purchased from commercial sources and used without further purification. The ESI FT-ICR fulvic acid samples were prepared by dissolving 1 mg of fulvic acid sample in a 50:50 methanol/water solution (1 mg/mL, 0.25% acetic acid).

Electrospray Ionization FT-ICR Mass Spectrometry. ESI FT-ICR mass spectra were acquired with a home-built FT-ICR mass spectrometer equipped with a 9.4-T superconducting magnet.[54,55] Details of the instrumental configuration of the ESI FT-ICR MS system have been published elsewhere.[50,54] Briefly, submillimolar solutions of fulvic acid samples were infused from a fused-silica microelectrospray ionization needle (50 μm i.d.)[56] at 300−500 nL/min flow rate. A 30 cfm rotary pump (Varian, Lexington, MA) and three hybrid turbodrag pumps (Balzers, Hudsun, NH) provide the differential pumping of the vacuum system to maintain an operating base pressure of $\sim 6 \times 10^{-9}$ Torr inside the ICR cell.

Typically, 1.8 kV was applied to the needle and the heated capillary current was set at ~3.5 A. The electrosprayed ions pass through a 1-mm-diameter skimmer prior to their entrance into a first 60-cm-long, rf-only linear octupole ion guide. A second octupole, 200 cm in length, guides the ions into a 9.4-cm-diameter cylindrical (~30.4-cm-long) open-ended three-section Penning trap.[57] We applied a direct current voltage to each end cap of the first octupole to allow ion accumulation for ~10−50 s inside the linear ion trap (Figure 1a). Then, by appropriate ion gating, we guided the ions into the ICR cell (Figure 1b). Trapping dc voltages were varied between 4 and 8 V to allow a sufficient number of fulvic acid ions to remain in the ICR cell. Once the ions were trapped inside the ICR cell (Figure 1c), a combination of "CHIRP" frequency sweep[58] and SWIFT dipolar excitation[36,37] was used to isolate a specific ion or a set of ions. The sequence of broad-band ejection of low- and high-mass ions (Figure 1d) followed by narrow-band SWIFT isolation (Figure 1e) was necessary to cleanly isolate the target analyte ions. To reduce the ion fragmentation and/or minimize the effect of translational and vibrational excitation on H/D exchange rates (as a consequence of ion heating during ion isolation events),[52] the isolated ions were allowed to relax for 60 s. During the ion relaxation event, helium gas was pulsed into the vacuum chamber to a pressure of $\sim 6 \times 10^{-6}$ Torr

(53) Chen, L.; Wang, T. C. L.; Ricca, T. L.; Marshall, A. G. *Anal. Chem.* **1987**, *59*, 449−454.
(54) Senko, M. W.; Hendrickson, C. L.; Pasa-Tolic, L.; Marto, J. A.; White, F.; Guan, S.; Marshall, A. G. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1824−1828.
(55) Solouki, T.; Reinhold: B. B.; Costello, C. E.; O'Mally, M.; Guan, S.; Marshall, A. G. *Anal. Chem.* **1998**, *70*, 857−864.
(56) Emmett, M. R.; Caprioli, R. M. *J. Am. Soc. Mass Spectrom.* **1994**, *5*, 605−613.
(57) Beu, S. C.; D. A., J. L. *Int. J. Mass Spectrom. Ion Processes* **1992**, *112*, 215−230.
(58) Comisarow, M. B.; Marshall, A. G. *Chem. Phys. Lett.* **1974**, *26*, 489−490.

(Figure 1f) to assist ion thermalization. To ensure prolonged pressure reproducibility inside the ICR cell, the neutral reagent gases (e.g., ND_3 or D_2O) were introduced into the vacuum system via a pulsed-leak valve (Figure 1g).[50,59] In the absence of neutral reagent gases, background pressure was at $\sim 6 \times 10^{-9}$ Torr. A Granville Philips model 274 ion gauge (Boulder, CO) was used to monitor the pressure throughout all experiments. During the reagent gas-pulse event, rectangular pressure profiles were observed and the partial pressures of the neutral reagents in the cell region remained constant (Figure 1g).

The extent of H/D exchange from the neutral reagents to fulvic acid ions was controlled by varying the reagent gas partial pressure inside the ICR cell (up to 1×10^{-5} Torr), and ion−molecule reaction time (up to 600 s reaction delay). Immediately following the H/D reaction delay, a 120 s pump-down delay was inserted to ensure that ion excitation and detection occurred at a low and consistent pressure. The pressure during ion excitation and ion detection was typically $\sim 2 \times 10^{-8}$ Torr. The trapped product ions were excited (Figure 1h) by dipolar frequency sweep excitation[58] (40−280 kHz at a sweep rate of 300 Hz/μs) and detected (Figure 1i) in the direct broad-band mode (64K or 32K data points and 300-kHz Nyquist bandwidth). At the end of each experiment, all trapped ions were ejected from the ICR cell (Figure 1j).

All mass spectra were constructed from a single time domain data set, by truncating the time domain transient signals after 32Kword data points. Fourier transformation of the resulting time domain signal with one zero fill, baseline correction, and Hamming apodization followed by magnitude calculation and frequency-to-mass conversion yielded the ESI FT-ICR mass spectra. We used an Odyssey data station (Finnigan Corp., Madison, WI) to control all of the experimental parameters. The MIDAS software was used for data analysis.[60]

RESULTS AND DISCUSSIONS

The primary goal of this study was to develop a gas-phase method to determine the number of active hydrogens in fulvic acid species. There are two problems in using conventional solution-phase titration to determine an average NOAH for fulvic acids. First, the molecular weights of fulvic acids are not accurately known and hence the exact molarity of fulvic acid solutions cannot be determined. Second, and more importantly, a complex mixture of fulvic acid sample such as NHFA or NCFA cannot be separated into its individual components; for example, solution-phase titration of the acidic hydrogen in a fulvic acid sample yields an average value for the entire population.[61]

In this section, we present results that demonstrate the utility of high-resolution SWIFT ion isolation[36,37] in FT-ICR H/D exchange experiments of fulvic acids. The use of SWIFT isolation and ion−molecule H/D exchange reactions enable the unambiguous determination of "active hydrogens"[42,44,52] for selected species within a complex mixture of molecules. All of the mass spectra reported herein are in the positive-ion mode.

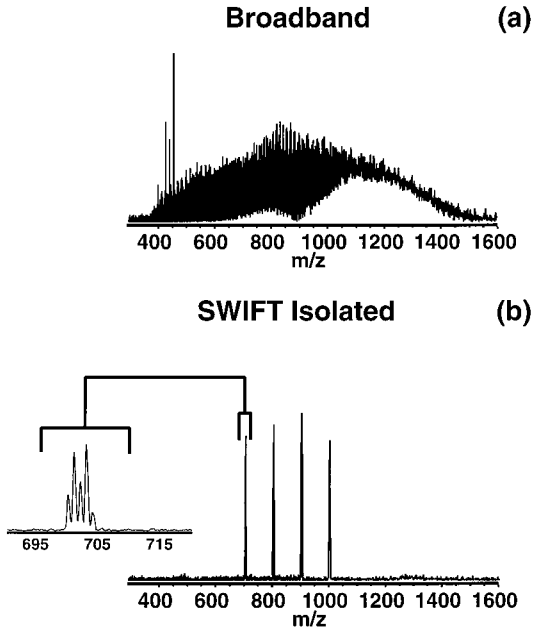

Figure 2. A representative single-scan (32Kword data points) positive-ion mode ESI FT-ICR MS of NHFA sample (a) before and (b) after SWIFT isolation of *m/z* regions 700−705, 800−805, 900−905, and 1000−1005 Th.

We selected the previously characterized NHFA and NCFA samples for our studies.[18] Previously, Patterson and co-workers utilized fluorescence techniques to investigate the complex formation kinetics of aluminum with NHFA and NCFA. In the pH range of 2.4−3.6, NHFA and NCFA were found to contain two kinetically distinguishable components, which define two types of average aluminum binding sites.[18] In this paper, we report the ion−molecule reactions between various deuterium exchange reagent neutrals (D_2O, CD_3OD, ND_3) with NHFA and NCFA ions. The deuterium isotope is one mass unit heavier than the hydrogen isotope and therefore an exchange or incorporation of a single deuterium shifts the m/z value of a singly charged molecule by one unit. Assuming that heteroatom bonded hydrogens are labile to exchange with the above reagents, the NOAH can be determined by comparing the m/z values of parent molecular ions before and after H/D exchange reactions.

A representative single-scan (32Kword data points) ESI FT-ICR mass spectrum of NHFA is shown in Figure 2a. The pH of electrosprayed fulvic acid solutions was ∼2.8−3.0. The ESI mass spectrum in Figure 2a is similar to previously reported results; i.e., ions are observed at essentially every m/z value in the range $400 \leq m/z \leq 1600$.[27] Changes in the m/z values of species present in NHFA, as a consequence of solution and/or gas-phase hydrogen/deuterium exchange, cannot be measured from a single mass spectrum. In other words, the m/z values for deuterium-exchanged species would overlap with the m/z value of other nonreactive or slow-reacting species. Hence, it would not be possible to distinguish the deuterium-exchanged species from the other species. Moreover, due to the experimental variations in the observed ion abundance for different species, it would not be possible to determine an average mass shift for the entire mass spectrum (i.e., from the comparison of natural and deuterium-exchanged mass spectra). One way to eliminate the interference from the neighboring ions is to monitor exchange rates of isolated

(59) Jiao, C. Q.; Ranatunga, D. R. A.; Vaughn, W. E.; Freiser, B. S. *J. Am. Soc. Mass Spectrom.* **1996**, *7*, 118−122.
(60) Drader, J. J.; Shi, S. D.-H.; Freitas, M. A.; Hendrickson, C. L.; Marshall, A. G. 46th ASMA, Orlando, FL, May 31−June 4, 1998; p 499.
(61) Stumm, W.; Morgan, J. J. *Aquatic Chemistry*, 3rd ed.; John Wiley & Sons: New York, 1996.

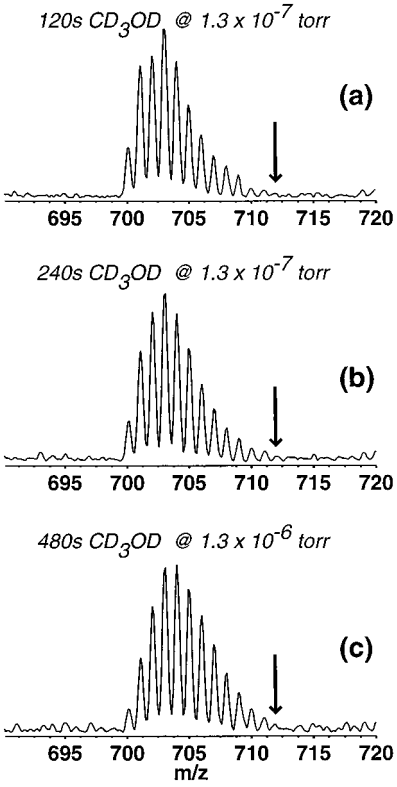

Figure 3. ESI FT-ICR single-scan mass spectrum of NHFA after SWIFT isolation of the selected ions followed by H/D exchange reaction in the presence of CD_3OD (a) at 1.3×10^{-7} Torr for 120 s, (b) at 1.3×10^{-7} Torr for 240 s, and (c) at 1.3×10^{-6} Torr for 480 s. Only the expanded *m*/*z* region 690−720 Th is shown in (a−c).

species separately. To study the relation between deuterium uptakes and fulvic acid molecular weights, we isolated ions at four m/z regions. Figure 2b shows the SWIFT isolated ESI FT-ICR mass spectrum of NHFA. We used a combination of CHIRP ejection sweeps (Figure 1d) and a narrow-band SWIFT radial ejection (Figure 1e) to remove ions of all but some selected m/z ratios, viz., $700 \le m/z \le 705$, $800 \le m/z \le 805$, $900 \le m/z \le 905$, and $1000 \le m/z \le 1005$. We chose an arbitrary m/z isolation width of 5 Th to study the differences within and between the isolated species of various fulvic acid samples. The 5 Th isolation width yields acceptable signal-to-noise ratios (S/N >20:1) and allows the isolation of comparable species by reducing ion abundance errors associated with high-resolution ion isolation. For example, narrow isolation of ions from such complex mixtures as fulvic acid samples reduces the S/N ratio (see Figure 6). The inset in Figure 2b shows the expanded $700 \le m/z \le 705$ region. Careful selection of the CHIRP ejection sweeps and a narrow-band SWIFT radial ejection yields cleanly isolated mass spectrum (Figure 2b).

In the following sections, we present data from the deuterium/hydrogen exchange reactions of isolated fulvic acid ions with CD_3OD, ND_3, and D_2O reagent gases.

Time and Pressure Variations. To select the optimal reagent gas pressure and reaction time for all subsequent H/D exchange reactions, we performed several experiments. We wanted to determine the minimum time and pressure necessary for complete incorporation of deuterium isotopes to all available active sites.

Figure 3a shows the expanded $690 \le m/z \le 720$ Th region of the NHFA after 120 s of H/D exchange in the presence of CD_3OD reagent gas at a constant pressure of 1.3×10^{-7} Torr. Figure 3a shows that the deuterium incorporation into the isolated species, changes the appearance of the original mass spectrum (see Figure 2b). For example, deuterium incorporation into the isolated singly charged fulvic acid ions ($700 \le m/z \le 705$ Th from Figure 2b) has shifted the masses of the original species to new values in the $700 \le m/z \le 712$ Th range. Notice that the m/z shifts of 1, 2, 3, ... Th upon deuterium incorporations, vis-à-vis for example, 0.5, 1.0, 1.5, ... Th *for doubly charged ions*, confirm that the isolated ions are indeed singly charged molecules. Although an absolute NOAH for each species within the m/z 700−705 Th cannot be determined from Figure 3a alone, it is clear that at least some ions have exchanged up to seven active hydrogens (i.e., 712−705 = 7). Notice that after 120 s at 1.3×10^{-7} Torr CD_3OD a portion of the ions at m/z 700 Th has not exchanged any hydrogens. The similar results observed for other three regions ($800 \le m/z \le 805$, $900 \le m/z \le 905$, and $1000 \le m/z \le 1005$, for example, see Figure 4a) are not shown here. Figure 3b shows the expanded $690 \le m/z \le 720$ region of the NHFA after 240 s of H/D exchange in the presence of CD_3OD reagent gas at a constant pressure of 1.3×10^{-7} Torr. The 2-fold increase of allowed reaction time (Figure 1g) from 120 s in Figure 3a to 240 s in Figure 3b (i.e., 2-fold increase in the number of ion−molecule collisions) does not change the appearance of the mass spectrum significantly. Notice that the maximum NOAH remains the same from Figure 3a to b. Assuming a polarizability of $\alpha = 3.28 \times 10^{-24}$ cm^3 for methanol neutral molecules,[62] in 240 s and at a pressure of 1.3×10^{-7} Torr, each fulvic acid ion at $m/z \sim 700$ Th has had over 174 collisions with the CD_3OD neutral molecules.[63] Figure 3c shows the expanded $690 \le m/z \le 720$ Th region of the NHFA after 480 s of H/D exchange in the presence of CD_3OD reagent gas at a constant pressure of 1.3×10^{-6} Torr (number of ion−molecule collisions >3489). Again, the NOAH does not change from Figure 3b to c. Notice that even after 480 s reaction time at 1.3×10^{-6} Torr CD_3OD some ions (e.g., ions at m/z 700 Th) do not exchange any hydrogens.

The H/D exchange reactions of fulvic acid ions with neutral CD_3OD are relatively "slow"[52] (i.e., a successful H/D exchange reaction requires multiple ion−molecule collisions). For example, at 1.3×10^{-7}, we did not observe significant H/D exchange between fulvic acid ions and CD_3OD for reaction delay times of <10 s (data not shown). The maximum NOAH does not change from 120-s H/D exchange time and 1.3×10^{-7} Torr (Figure 3b) to 480-s H/D exchange time and 1.3×10^{-6} Torr; at least some fulvic acid ions have exchanged up to seven active hydrogens (i.e., 712 − 705 = 7).

In addition to the maximum NOAH, it is possible to extract high mass accuracy and ion abundance data from ESI mass spectra before and after H/D exchange (e.g., Figures 2b and 3, respectively) and calculate the average NOAH. For example, the average mass (M_{av}) for any isolated region containing x number

(62) Miller, K. J.; Savchik, J. A. *J. Am. Chem. Soc.* **1979**, *101*, 7206−7213.
(63) Langevin, P. *Ann. Chim. Phys.* **1905**, *5*, 245.

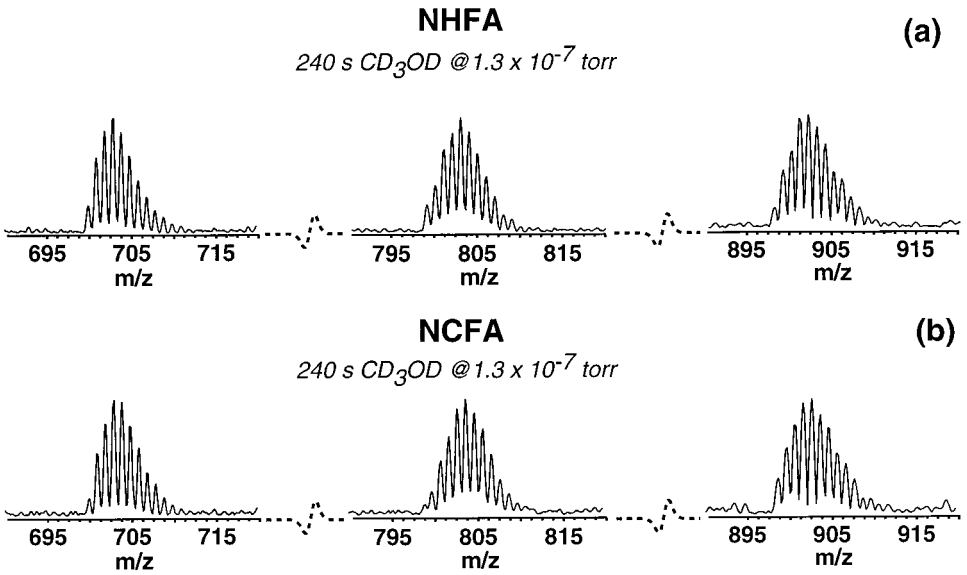

Figure 4. ESI FT-ICR single scan mass spectra of (a) NHFA and (b) NCFA after SWIFT isolation of the *m*/*z* regions shown in Figure 2b followed by gas-phase hydrogen/deuterium exchange for 240 s at 1.3 × 10^{-7} Torr CD_3OD. The horizontal dashed lines indicate that the expanded *m*/*z* regions belong to the same mass spectrum.

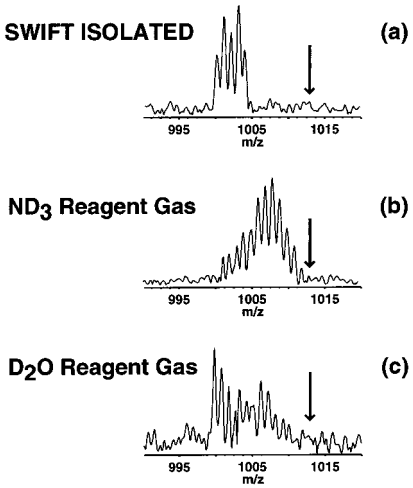

Figure 5. The expanded *m*/*z* region 990−1020 Th from ESI FT-ICR single-scan mass spectra of NCFA. The (a) original SWIFT isolated ions at *m*/*z* 900−905 Th react with ND_3 and D_2O at 1.3 × 10^{-6} Torr for 120 s to produce the deuterium incorporated product ions shown in (b) and (c), respectively.

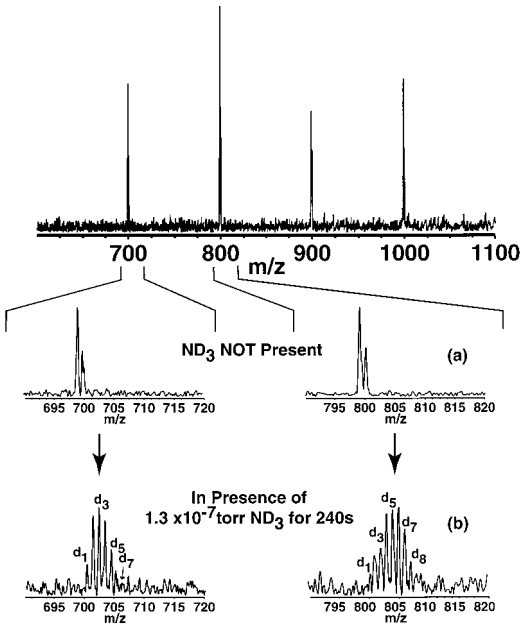

Figure 6. A narrow isolation of multiple ions of NCFA at *m*/*z* 700, 800, 900, and 1000 Th. Expanded views of the selected ion in regions 690−720 and 790−820 Th before and after H/D exchange with ND_3 for 240 s at 1.3 × 10^{-7} Torr are shown in (a) and (b), respectively. The labeled peaks d_1−d_8 represent the number of incorporated deuterium isotopes in the selected fulvic acids ions.

of ions can be calculated using the following equation

$$M_{av} = \sum_{n=1}^{x} I_n M_n / \sum_{n=1}^{x} I_n$$

where I_n and M_n are relative ion abundance and mass-to-charge ratio for each ion, respectively. The difference between M_{av} before and M_{av} after deuterium exchange reaction yields the average NOAH. However, relative ion abundance variations, associated with the ion isolation steps within each mass spectrum and from one experiment to the next, limit the accuracy of this procedure. For instance, the relative ion abundance of various isolated species in Figure 2b (before H/D exchange reaction) may not be the same for the precursor ions that reacted with CD_3OD to produce the product ions observed in Figure 3. Therefore, in this section, we limit our discussion to the maximum NOAH. One way to eliminate the uncertainties associated with the ion isolation steps is to utilize a multiple remeasurement approach for mass analyses.[32,34] A second approach to eliminate the relative ion abundance variations in H/D exchange reaction of fulvic acids is to utilize narrow mass isolation (species within a single *m*/*z*). Application of the second approach to eliminate relative ion abundance variations is described in Figure 6a and b.

Comparison of NHFA and NCFA Samples. Parts a and b of Figure 4 show the expanded *m*/*z* regions of ESI FT-ICR mass spectra after 240-s H/D exchange at 1.3 × 10^{-7} Torr CD_3OD for

NHFA and NCFA selected ions, respectively. The dashed lines in Figure 4 indicate that the expanded m/z regions belong to the same mass spectra. At the selected m/z regions shown in Figure 4, the number of incorporated deuterium isotopes for NHFA and NCFA is the same. Both NHFA and NCFA results show increased NOAH for H/D exchange reactions with the molecular weight of fulvic acid ions. For example, at the m/z isolation ranges of 700−705 Th and 900−905 Th, at least some fulvic acid ions have exchanged up to seven (i.e., 712−705 = 7) and eight (i.e., 913−905 = 8) active hydrogens, respectively. At an m/z range of 1000−1005 Th, the number of active hydrogens is about nine (data for CD_3OD not shown; see Figure 5b). The mass spectra in Figure 4 suggest that the proton binding behavior of NCFA and NHFA ions at m/z range of 600−1000 Th (at pH ∼2.8−3.0) is similar; these results are consistent with the previous studies that illustrated the similarities of proton binding behavior of the two fulvic acids.[18]

Figure 5 shows ESI FT-ICR mass spectra of NCFA before and after H/D exchange with ND_3 and D_2O reagent gases. The SWIFT isolated NCFA ions at the m/z range of 1000−1005 Th (Figure 5a) react with ND_3 neutral molecules (at 1.3×10^{-6} Torr for 120 s) to yield the mass spectrum shown in Figure 5b. Clearly, the mass spectrum in Figure 5b shows that deuterium isotopes are incorporated in the mass-selected fulvic acid ions of Figure 5a. A comparison of parts a and b in Figure 5 reveals that at least some fulvic acid ions have exchanged up to nine hydrogens (i.e., ∼1014−1005 = 9). Figure 5c shows an ESI FT-ICR mass spectrum of NCFA ions after 120-s H/D exchange with D_2O neutral molecule at 1.3×10^{-6} Torr. Note that after 120-s reaction delay some fulvic acid ions have not exchanged any hydrogens with D_2O neutral molecules at 1.3×10^{-6} Torr. The H/D exchange reaction kinetics may be influenced by gas-phase ion conformations and differences in gas-phase basicities between the deuterium exchange reagent and the fulvic acid ions as well as other properties of the reactants.[52] The comparison between parts b and c of Figure 5 suggests that there may be more than one type of active hydrogen in the fulvic acid ions studied herein. The observed variations for the three H/D exchange reagent gases may be due to other reasons such as the presence of more than a single molecule within the isobaric peaks, the presence of structural isomers, or the existence of different H/D exchange mechanisms. Additional studies are needed to establish the mechanism of fulvic acid H/D exchange reactions. These results confirm the polyfunctionality of fulvic acids and are consistent with the previous NMR, solution-phase kinetic data, and solution-phase alkalimetric titration results.[18,61] To obtain the NOAH for individual ions directly form the raw mass spectral data, we performed higher resolution ion isolation H/D exchange experiments as shown in Figure 6.

Figure 6 shows the high-resolution isolation of ions at m/z 699, 799, 899, and 999 Th. The expanded m/z regions from 690 to 720 and 790 to 820 Th show that in addition to the desired species (m/z 699 and 799 Th) ions at m/z 700 and 800 Th are also present. A narrow isolation of multiple ions in a large m/z range and a single mass spectrum requires high-resolution SWIFT. In high-resolution SWIFT isolation experiments, the S/N ratio is inversely proportional to the ion isolation m/z width, i.e., the S/N ratio decreases with increase in the resolution of SWIFT ion isolation. A sufficient S/N ratio is required to monitor an H/D exchange reaction of a single species. For example, formation of various product ions from a single precursor ion upon deuterium incorporations reduces the S/N ratio for the final mass spectrum. To retain acceptable S/N ratios (>3) for the final product ions (Figure 6b) we employed the same conditions/parameters that were used to obtain the mass spectrum in Figure 6a. In other words, it is possible to eject the unwanted ions and enhance ion isolation resolution of the precursor species; however, isolation of a single m/z ion reduces the S/N ratio of the product ion mass spectrum (e.g., S/N ratio for d_7 and d_8 in Figure 6b of <3). The expanded regions for NCFA ions from m/z 690 to 720 and 790 to 820 Th before and after reaction with ND_3 are shown in Figure 6a and b, respectively. Data in Figure 6 suggest that, on average, the NOAH increase with the fulvic acid mass-to-charge ratio. For example, the average number of active hydrogens ($\Delta M_{av} = [M_{av}]_{\text{Figure 6b}} - [M_{av}]_{\text{Figure 6a}}$) for the molecules in 700 Th and the 800 Th regions are ∼3.8 and ∼4.4, respectively; this trend is consistent with the observed maximum NOAH. For example, ions at m/z 700 and 800 Th region exchange up to seven and eight deuteriums with ND_3, respectively. The subscripts in labeled peaks d_1, d_2, ..., d_8 of Figure 6B, represent the number of incorporated deuterium isotopes in the selected fulvic acids ions of Figure 6a (i.e., the NOAH). The results from Figure 6 are consistent with the findings of Figures 3−5. For example, in Figure 6, it is confirmed that the maximum NOAH for the selected NCFA ions at m/z ∼700 Th is at least seven.

Although numerous studies have relied on modeling to predict the polyelectrolytic nature of fulvic and/or humic acids,[64−66] the exact structures of fulvic acids or the numbers of various functional groups per molecule are not known. For example, on the basis of ^{13}C NMR data and titrimetric results, Leenheer et al. proposed three structural models "(A−C)" for fulvic acid molecules from the Suwannee River. The molecular weights of these structural models are 566, 688, and 774; the corresponding number of proposed active hydrogens for these species are 4, 7, and 7, respectively.[67] Moreover, based on one of these three structural models for Suwannee River fulvic acid, viz., structure A in Leeheer et al.,[67] and additional NMR data, Shin and Moon proposed an "average" structure for fulvic acid extracted from topsoil of the Okchun Basin (Republic of Korea). The proposed "average" structure of Okchun-FA has a molecular weight of 817 with seven labile hydrogens.[68] It is interesting to note that the NOAH from the experimental H/D exchange results compare closely to the proposed number from the previous studies. However, our experimental results are based on the interrogation of a selected set of species whereas the theoretical models were based on the bulk properties of fulvic acid samples. Clearly, the ESI FT-ICR H/D exchange results complement other spectroscopic and titrimetric methods and offer additional information. Moreover,

(64) Wershaw, R. L. *J. Contam. Hydrol.* **1986**, *1*, 29−45.
(65) Bartschat, B. M.; Cabaniss, S. E.; Morel, F. M. M. *Environ. Sci. Technol.* **1992**, *26*, 284−294.
(66) Marinsky, J. A.; Reddy, M. M.; Ephraim, J. H.; Mathuthu, A. S. *Anal. Chim. Acta* **1995**, *302*, 309−322.
(67) Leenheer, J. A.; Wershaw, R. L.; Reddy, M. M. *Environ. Sci. Technol.* **1995**, *29*, 393−398.
(68) Shin, A. S.; Moon, H. *Soil Sci.* **1995**, *161*, 250−256.

data from ESI FT-ICR H/D exchange experiments can provide structural details for any selected fulvic acid molecule.

CONCLUSIONS AND FUTURE DIRECTIONS

We have shown that the selective ESI FT-ICR MS method coupled with the gas-phase H/D exchange technique offers a powerful method to probe the chemistry of humic substances at the molecular level.

Fulvic acid ions undergo H/D exchange with all the reagent gases used in this study. Results from D_2O, CD_3OD, and ND_3 suggest that these reagents can be used to differentiate various active protons in fulvic acid ions. For the time scale of our experiments, the extent of H/D exchange of the protonated fulvic acids increases with reagent gas basicity, $D_2O < CD_3OD < ND_3$ (i.e., deuterium uptake of fulvic acid ions is most efficient with ND_3 molecules). *For the first time, using the ESI FT-ICR H/D exchange reactions, we are able to determine NOAH for various fulvic acid molecules.* The average maximum NOAH for NCFA ions at m/z range 600−1000 Th is ∼7−9. There are no significant differences between NOAH for NCFA and NHFA at pH ∼2.8−3.0. The NCFA ions at m/z of 700 and 800 Th contain ∼7−8 active hydrogens. Two general observations can be made from the exchange data with D_2O, CD_3OD, and ND_3. First, the proton affinities of fulvic acid ions at m/z range of 600−1000 Th do not vary significantly. Second, reagent gases with different proton affinities can be used to (a) distinguish between various active protons and (b) estimate proton affinities of fulvic acid ions. Previously, we have shown that ultrahigh-resolution and exact mass measurement[27] as well as CID and IR/MPD MS^n capabilities[28] of FT-ICR can be used for structural determination of fulvic acids. We plan to employ similar techniques and monitor the H/D exchange behavior of possible components in an isobaric ion peak and/or determine molecular formulas of fulvic acids and their fragment ions (before and after H/D exchange reactions) based on accurate mass measurement alone. A current limitation for performing such experiments on fulvic acid samples is the difficulty associated with isolating and maintaining a sufficient number of selected ions for the H/D exchange experiments in the ICR cell. Future studies will address these issues. We are currently using H/D exchange reactions to study the details of metal complexation with various fulvic acids.[69] We also plan to explore the effects of pH and ionic strength on the H/D exchange and fulvic acid complexation reactions.

(69) Solouki, T.; Patterson, H.; Alomary, A. Submitted to *Environ. Sci. Technol.*

ACKNOWLEDGMENT

This work was supported in part by the Regular Faculty Research Fund from the University of Maine in Orono (UM5-4-26157) and the NSF National High-Field FT-ICR Mass Spectrometry Facility (CHE-94-13008) in Tallahassee, FL. The authors thank Professor A. G. Marshall and the NHMFL staff. We gratefully acknowledge the support of NHMFL for providing a copy of the MIDAS FT-ICR data analysis software. We thank Professors C. S. Cronan and H. H. Patterson for their interest in this work and for providing the fulvic acid samples used in this study.

Received for review February 17, 1999. Accepted July 19, 1999.

AC990185W