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DIFFUSION COEFFICIENTS AND MOLECULAR WEIGHT DISTRIBUTIONS OF HUMIC AND FULVIC ACIDS DETERMINED BY FLOW FIELD-FLOW FRACTIONATION

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ABSTRACT

The ability to characterize molecules whose physical and chemical properties are intimately linked to their diffusion coefficients and molecular weight is important to further understanding of chemical transport in the environment. Flow field-flow fractionation (flow FFF) was used to obtain separations of water-soluble macromolecules of varying molecular weight, including polystyrene sulfonates and humic substances. The separation occurs due to differing diffusion rates for chemical species of differing molecular weight in aqueous solution. Flow FFF uses fluid flow as the mechanism of separation. A model that yields liquid phase diffusion coefficients as a function of molecular weight was utilized to determine molecular weights from degree of separation. Separations of polystyrene sulfonates, a humic acid, and two fulvic acids of known molecular weight were accomplished using flow FFF. The separations obtained were used to develop a relationship between flow FFF separation and species molecular weight. Separations were obtained for humic and fulvic acids of unknown molecular weight.

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INTRODUCTION

Field-flow fractionation (FFF) is a rapidly developing class of analytical techniques used for the separation of macromolecules. The separation techniques comprising FFF are less than thirty years old (1). FFF is applicable for macromolecules having effective molecular weights of 10^3-10^{18} (2). FFF is a general class of separation techniques that includes thermal FFF, sedimentation FFF, electrical FFF, and flow FFF (3,4). The separation techniques depend on the physiochemical parameters of macromolecules, such as size, charge, or molecular weight (2). Conversely, from the separations achieved, the respective physiochemical parameter of the macromolecule can be calculated.

Flow FFF is the most universally applicable of the FFF techniques (5). Separations in the flow FFF system depend on molecular diffusivity which is, in turn, related to molecular weight or size (6). Flow FFF has been used to achieve molecular weight separations of polystyrene particles, colloidal silica, viruses, and proteins (3,7,8,9). Flow FFF is characterized by laminar flow through a long, narrow channel. A semipermeable membrane is placed along one of the channel floors parallel to the channel (longitudinal) flow. A cross flow, perpendicular to the channel flow, is used to induce molecular weight separations. The cross flow enters the channel opposite to the membrane and exits the channel through the membrane. Macromolecules are injected into the channel flow. The channel flow is diverted away from the channel as the macromolecules enter the channel. The cross flow moves the species toward the membrane. The membrane is impermeable to the macromolecules but allows the liquid cross flow to pass through. Thus, the macromolecules accumulate along the membrane. Steady state is reached when the cross flow driving force is counteracted by diffusion of the components back into the channel stream. The channel flow is redirected through the channel, and the macromolecules flow out of the channel along the parabolic channel flow. The composition of the channel flow is most commonly determined by an ultraviolet (UV) detector.

Diffusion coefficients of the species in the channel determine how far back into the channel the species move during the time the cross flow is acting on them. The diffused distance determines which segment of the parabolic flow moves the species out of the channel. Because diffusion coefficients are related to molecular weight, larger molecules will accumulate closer to the membrane, thus placing them in the low velocity region of flow; while smaller molecules will diffuse into regions of higher velocity and, thus, will be driven out of the channel quicker. This gives rise to different emergence times for molecules of differing size. From the various emergence times, diffusion coefficients are calculated. By using this method to calculate diffusion coefficients of known molecular weight species, a calibration curve is obtained. Diffusion coefficients of macromolecules of unknown molecular weight are calculated by comparing the emergence times with the calibration curve. The molecular weights of the species are then determined.

The research presented in this paper focused on determining the diffusion coefficients and molecular weights of dissolved organic carbon (DOC) macromolecules by flow FFF. DOC is operationally defined as the fraction of total organic carbon that passes through a 0.45 μm glass fiber filter, and it is composed of a variety of organic compounds in various oxidation states (10). Two types of water soluble humic substances were considered, humic and fulvic acids. Humic substances are organic compounds; brown-to-black in color, and found in soils, sediments, and waters. Humic acids are water soluble only above a pH of two while fulvic acids are soluble at all pHs (11). A third type of water-insoluble humic substance is humin. Humic substances contain a small amount of sulfur, 1 to 2 percent nitrogen, and many oxygen-containing functional groups (predominantly carboxyl, phenolic, and methoxyl) (11). The properties of humic substances are determined to a large degree by these functional groups. Humic and fulvic acids have a strong binding capacity for trace metals (11,12,13). Additionally, the water soluble humic substances may form aggregates that can solubilize nonpolar organic compounds such as pesticides, polychlorinated

biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) (11,12,13,14). Due to their binding capacity, humic substances are involved in the fate and transport of contaminants in the environment. Humic substances are also implicated in the formation of by-products, such as trihalomethanes (THMs), during water treatment by chlorination (15). Humic substances are present in almost all terrestrial and aquatic systems. This, combined with their reactive nature, makes them important components of environmental studies.

THEORETICAL

The theory of flow FFF has been extensively developed in several articles (2,3,4,5,16). Flow FFF is characterized by flow within a channel whose length (L) is much greater than its width (w) [L > > > w] allowing application of an infinite parallel plate model.

A dimensionless length term, λ, represents the ratio of solute layer thickness (l) to channel width (w) within the flow FFF channel. The layer thickness (l) is a function of the molecular diffusion coefficient, D, such that

$$l = \frac{D}{U} \tag{1}$$

where U is the particle drift velocity induced by the cross flow field. U is equal to the volumetric flow rate of the cross flow in cm^3/sec divided by the channel breadth and length in cm (V_c/bL). Substitution of Equation 1 into the definition for λ gives

$$\lambda = \frac{l}{w} = \frac{D}{Uw} \tag{2}$$

In order to compare the differences in emergence times, a retention relationship is developed. While flow FFF is not a chromatographic technique, the chromatographic term of "retention" is oftentimes used interchangeably with

the term "emergence" to signify the length of time that the species stay within the channel.

The retention relationship, called the retention ratio (R), is the ratio of the mean downstream zone velocity, V, to the mean stream velocity, $<v>$,

$$R = \frac{V}{<v>} = \frac{6<v>\lambda\left[\coth\frac{1}{2\lambda} - 2\lambda\right]}{<v>} = 6\ \lambda\ \left[\coth\frac{1}{2\lambda} - 2\lambda\right] \qquad (3)$$

where R is the retention ratio of the components (7). The retention ratio can also be expressed as a ratio of times

$$R = \frac{t_o}{t_R} \qquad (4)$$

where t_R is the emergence time of the species and t_o is the void time of the channel. The value of t_o is equal to bwL/V, where b is the channel breadth and V is the channel volumetric flow rate in cm^3/sec. This represents the residence time of the channel flow within the channel itself. The combination of Equations 3 and 4 gives

$$R = \frac{t_0}{t_R} = 6\ \lambda\left[\coth\frac{1}{2\lambda} - 2\ \lambda\right] \qquad (5)$$

At high cross flow velocities, λ approaches zero and the term $\coth(1/2\lambda)-2\lambda$ goes to one. The resulting retention relationship becomes

$$R = \frac{t_0}{t_R} = 6\ \lambda \qquad (6)$$

From the emergence times of the components, the channel and cross-flow rates, and the channel dimensions, a value for λ can be obtained.

$$\lambda = \left(\frac{t_o}{6\ t_R}\right) = \left(\frac{b\ w\ l}{6\ V\ t_R}\right) \qquad (7)$$

A value for D is obtained from Equation 8.

$$D = \lambda U w = \left(\frac{t_0}{6\ t_R}\right)\left(\frac{V_c}{bL}\right)(w) \quad (8)$$

Diffusion coefficients are related to molecular weight for linear random-coil polymers by

$$D = \frac{\alpha}{M^{\beta}} \quad (9)$$

where M is the molecular weight of the diffusing species and α and β are constants for a given polymer in a designated solution (6). The diffusion coefficient varies inversely with molecular weight; thus, small particles diffuse to a greater distance into the channel while larger particles remain closer to the membrane. The placement of the smaller particles in the higher velocity region of the laminar flow distribution gives an emergence time (t_R) that is less than that of the larger particles. According to Equation 9, a logarithmic plot of the diffusion coefficient versus molecular weight (M) gives a relationship that can be used to calculate the unknown molecular weight of linear random-coiled polymers from their emergence times under specific flow FFF conditions. The constants of Equation 9 are determined by the observed relationship. The log/log model was used by Benincasa and Giddings (17) for sodium salts of polystyrenesulfonate ranging in molecular weight from 6,500 to 690,000 and by Beckett et al. (11) for humic substances and polystyrene sulfonate molecular weight standards ranging from 4,000 to 100,000.

Separations in flow FFF depend upon the existence of a laminar flow profile. To verify that the flow within the channel was laminar, a Reynolds number, Re, was calculated. The equation for a Reynolds number for flow FFF (18) is

$$Re = \frac{U_o w}{2\nu} \quad (10)$$

where U_o is taken as a constant cross flow velocity in cm/sec, and ν is the

kinematic viscosity of the carrier fluid in cm^2/sec. The kinematic viscosity of water was used to calculate a Reynolds number of 0.0022 for the system studied. The error in the expression for R of Equation 6 is less than 1 percent when Re is less than 0.03 (18). Thus, the flow is laminar.

EXPERIMENTAL

Flow FFF Apparatus and Procedures

The flow FFF channel, Model F-1000, was purchased from FFFractionation, Inc. (Salt Lake City, UT). A Hewlett-Packard (San Fernando, CA) Model 1090M liquid chromatograph delivered the channel flow at 0.6 mL per minute; while the cross flow was generated at a rate of 3.1 mL per minute using a pump, Model DQP-1, purchased from Dionex (Sunnyvale, CA). The flow FFF channel essentially takes the place of the analytical column in the HPLC system. The channel dimensions in the mylar spacer were 28.5 cm (tip-to-tip length) x 2 cm (breadth) x 0.0508 cm (width). The membrane consisted of polypropylene-backed polysulfone, type PM10F, manufactured by Amicon (Beverly, MA), having a molecular weight cutoff of 10,000. The membrane, exclusive of the channel area, was coated with silicone (Dow Corning Corp. USA, Midland, MI).

Samples were loaded with a 50 μL syringe into a Rheodyne manual injector (Rheodyne Corporation, Cotati, CA) having a 20 μL sample loop. The Hewlett-Packard 1090M diode array detector was used to monitor components flowing from the channel in milliabsorbance units (mAU). The wavelengths monitored were 254 nm and 270 nm with a reference at 450 nm and a bandwidth of 4 nm. A Hewlett-Packard ChemStation controlled the channel pumping system and the detector, including data collection and processing.

Data analysis was initiated when the sample was injected. Fifteen seconds were allowed for the injected specimen to be deposited in the channel. At that

time, the carrier flow was diverted from the channel by manually switching two 3-way valves simultaneously, thereby allowing only the cross flow to continue through the system. The flow path remained in this configuration for two minutes to allow the species to attain steady state within the cross flow field. At the end of two minutes, the carrier flow was redirected through the channel. Pressure fluctuations were closely monitored and adjusted using pressure gauges (Ashcroft, Stratford, CT) and pressure regulators (Optimize Technologies, Portland, OR). The pressure of the system was maintained at 2.2 ± 0.15 atm on the channel flow and 2.45 ± 0.15 atm on the cross flow. Channel and cross flow rates were determined before and after each injection.

Reagents

Two carrier phases were examined. A carrier liquid consisting of 0.05M tris(hydroxymethyl)aminomethane, 0.0268M nitric acid and 0.00308M sodium azide, in HPLC-grade water (Fisher Scientific, Fair Lawn, NJ), was found to be unsuitable as the detection limit of the lower molecular weight polystyrene sulfonate standards was significantly increased. A more suitable carrier phase was composed of HPLC-grade water containing a surfactant, FL-70, 0.05 percent (Fisher Scientific) and sodium azide, 0.03 percent, having a resulting pH of 7. The lower detection limit of the 1800 MW polystyrene in the FL-70 carrier could be due to the lower system pressure encountered. At the higher pressure of the tris system, the smaller molecular weight species could be forced through the membrane during the relaxation time. Additionally, the tris carrier fluid could alter the charge of the membrane surface thereby altering the repulsion of the charged species.

The water-soluble macromolecules of known molecular weight used as reference standards were sodium salts of polystyrene sulfonate (PSS), supplied by Polysciences, Inc. (Warrington, PA); Nordic and Suwanee fulvic acids; and Nordic humic acid, supplied by the International Humic Substances Society (IHSS, Golden, CO). Fulvic and humic acids of unknown molecular weight were

supplied by Aldrich Corporation (Milwaukee, WI), Fluka Chemical Corporation (Ronkonkoma, NY), the IHSS, and Dr. G.K. Stearman (19). Each standard and sample was dissolved in the carrier solvent and filtered through a 0.45 μm Acrodisc filter (Fisher Scientific). Dissolved organic carbon (DOC) analyses (20) determined concentrations to be approximately 1 mg/mL.

RESULTS AND DISCUSSION

The environmental applications of FFF for separation of aquatic particulates and macromolecules have primarily been explored by Beckett, Giddings, and coworkers (11,12,13,21,22). Three different FFF techniques -- sedimentation FFF, steric FFF, and flow FFF -- were applied to the characterization of environmental samples. Steric FFF was used to analyze silt-sized (1-60 μm) sediment particulate matter (12). Sedimentation FFF was applied to the characterization of colloidal-sized particles (0.06-0.6μm) (12,21,23), and the study of pollutant-colloid interactions (22). Flow FFF was used to examine dissolved organic fulvic and humic acids (11,12,13). Molecular weight distributions of dissolved organic matter extracted from both aquatic and terrestrial sources were established. Number average and weight average molecular weights were determined. Some highly concentrated natural samples were injected directly without pretreatment. The research presented in this paper emulates the published procedures of Beckett and Giddings for fractionation and determination of the diffusion coefficients, molecular weight, and polydispersity of fulvic and humic acids.

Figure 1 illustrates an overlay of the fractograms obtained with the polystyrene sulfonates of 1800, 5400, and 8000 nominal molecular weight showing that emergence times are related to molecular weight. These fractograms demonstrate excellent baseline resolution between the peaks caused by perturbations due to sample injection and channel flow diversion, and the peak

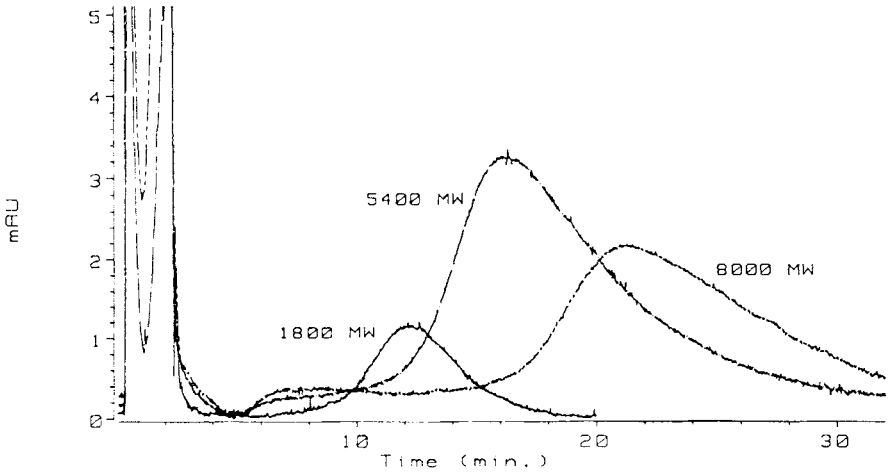

FIGURE 1. Flow FFF fractograms of polystyrene sulfonates.

due to the macromolecules. The ability to analyze solutes having molecular weights much lower than the molecular weight-cutoff of the membrane is attributed to charge repulsion between the sample and the membrane. Both are negatively charged in the systems examined in this research.

Overlays of the fractograms for pairs of fulvic and humic acids collected from the same geographic sites are shown in Figure 2A-D. The fractograms illustrate that the fulvic acids are lower in molecular weight and are less polydisperse than the corresponding humic acid. Similar conclusions were reached by Beckett et al. (11). The fractograms illustrated were obtained at 270 nm. The detector response at 270 nm was generally less noisy than that recorded at 254 nm.

Diffusion coefficients, D_p, were calculated at the peak maxima according to Equation 8, for the polystyrene sulfonate, and organic carbon standards and samples and are reported in Tables 1 and 2. The emergence times were read from the fractograms averaged over multiple injections at the peak maximum. The time required for the sample to reach the channel, attain the steady state

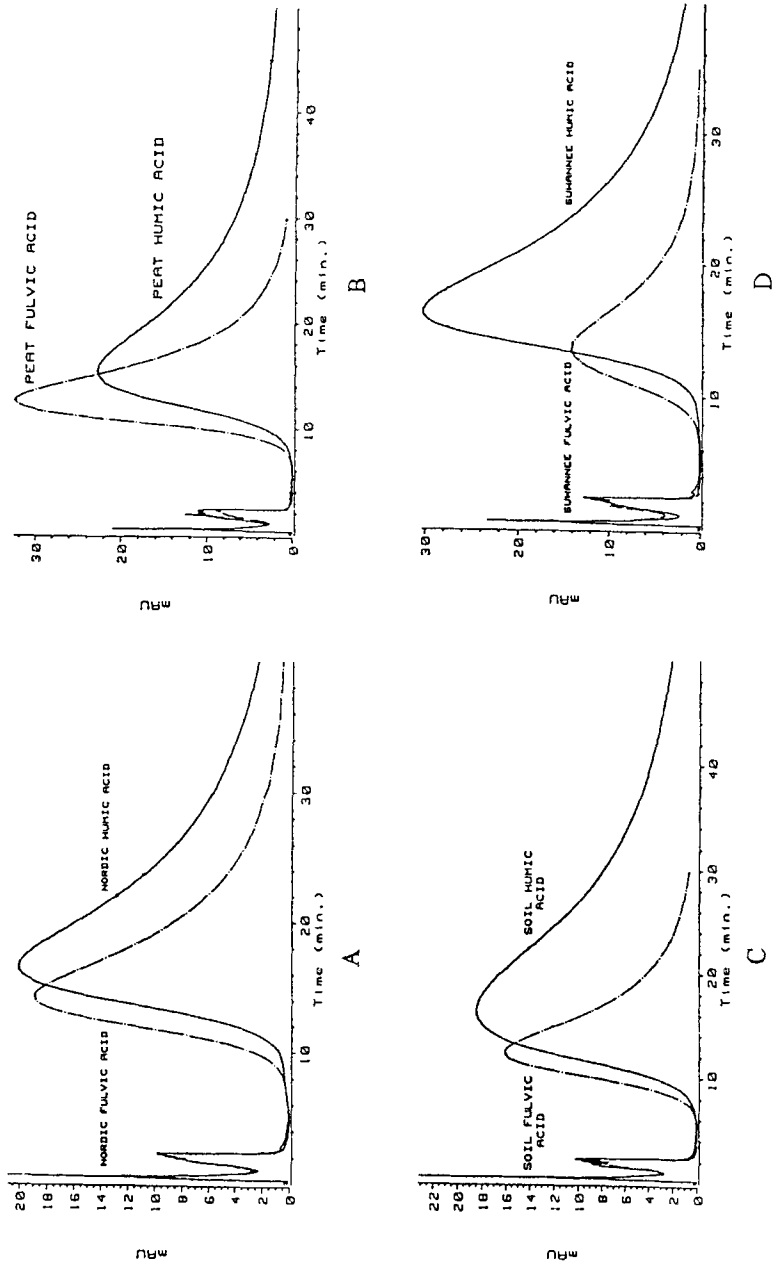

FIGURE 2. Flow FFF fractograms of fulvic and humic acids collected from the same geographic sites, denoted: (a) Nordic, (b) Peat, (c) Soil, and (d) Suwanee.

Table 1. Calibration Standards

	Reported M_w	Reported M_n	Measured Diffusion Coefficient,[a] D_p(x 10^6) (cm ^{2}/sec)	Measured Diffusion Coefficient,[b] D_w (x 10^6) (cm ^{2}/sec)	Measured Diffusion Coefficient,[b] D_n (x 10^6) (cm ^{2}/sec)	Calculated M_w[c] (% difference)	Calculated M_n[d] (% difference)	Calculated M_p[e] (% difference)
Suwanee River Fulvic Acid	1340	1060	3.441	3.135	2.868	1372 (2.4%)	1119 (5.4%)	
Nordic Fulvic Acid	2137	1827	3.256	2.802	2.451	2083 (2.6%)	1666 (9.2%)	
Nordic Humic Acid	3264	2272	2.670	2.239	1.912	3286 (0.7%)	2374 (4.4%)	
Polystyrene Sulfonate, 1800	1800 (nominal)		3.635	3.784	3.567			1732 (3.8%)
Polystyrene Sulfonate, 5400	5400 (nominal)		2.676	2.545	2.391			5562 (3.0%)
Polystyrene Sulfonate, 8000	8000 (nominal)		2.089	2.049	1.923			7906 (1.2%)

[a]Calculated from Flow FFF Results of Peak Maximum, Equation 8
[b]Calculated by GPC software, Equations 11 and 12
[c]Calculated from the Calibration of D_w and M_w, $R^2 = 0.998$
[d]Calculated from the Calibration of D_n and M_n, $R^2 = 0.950$
[e]Calculated from the Calibration of D_p and nominal molecular weight, $R^2 = 0.998$

Table 2. Diffusion Coefficients and Molecular Weight Distributions of Fulvic and Humic Acids Determined by Flow FFF

Sample	Diffusion Coefficients ($x10^6$) (cm^2/sec)			Molecular Weight		Polydispersity	
	D_p	D_w	D_n	M_w	M_n	M_w/M_n	Peak Width at Half Height (sec)
Soil FA	3.897	3.427	3.097	748*	819*	0.9134	555
Peat FA	3.756	3.350	3.081	911*	840*	1.0857	495
Stearman 1-2	3.108	2.683	2.185	2338	2016	1.1593	660
Stearman 22	3.153	2.649	2.212	2409	1980	1.2165	684
Stearman 25A	2.999	2.545	2.100	2633	2127	1.2377	735
Stearman 3-2	2.980	2.538	2.095	2647	2134	1.2406	705
Stearman 18	2.934	2.507	2.074	2714	2162	1.2553	750
Stearman 21	2.904	2.472	2.023	2789	2229	1.2510	720
Stearman 10	2.798	2.466	2.041	2801	2205	1.2704	819
Stearman 14	2.878	2.445	2.053	2846	2190	1.2996	690
Stearman 17	2.858	2.442	1.993	2854	2268	1.2580	645
Stearman 57	2.819	2.417	2.033	2907	2216	1.3118	615
Stearman 9	2.750	2.353	1.938	3043	2340*	1.3006	750
Summitt HA	2.947	2.331	1.324	3090	3146*	0.9822	555
Peat HA	2.904	2.312	1.420	3131	3020*	1.0366	570
Stearman 1A	2.709	2.302	1.938	3153	2340*	1.3474	705
Suwanee HA	2.752	2.301	2.009	3153	2247*	1.4032	570
Fluka HA	2.785	2.298	1.915	3161	2371*	1.3332	765
Aldrich HA	2.679	2.292	1.959	3172	2313*	1.3713	660
Leonardite HA	2.838	2.263	1.389	3235	3062*	1.0566	594
Soil HA	2.735	2.196	1.244	3378*	3253*	1.0388	765

*extrapolated from calibration equation

diffused location, and move from the channel outlet to detection (162.5 seconds) was subtracted from the emergence time at the peak maximum. The void time of the channel was calculated to be 289.6 seconds. Therefore, values for λ were calculated, Equation 7, and diffusion coefficients estimated, Equation 8.

D_p was related to the unadjusted retention time by a cubic equation. The cubic relationship provided calibration between these two parameters as input to the gel permeation chromatographic (GPC) software available on the ChemStation. In GPC, molecular weights decrease with increasing retention time while the opposite is true for flow FFF. However, in flow FFF the diffusion coefficients of the molecular weight species decrease with increasing retention time. The correlation of diffusion coefficients with time was supplied to the software instead of data for molecular weights. Two quantities denoted D_w and D_n were calculated according to Equations 11 and 12.

$$D_w = \frac{\Sigma(Area_i \cdot D_i)}{\Sigma\ Area_i} \tag{11}$$

$$D_n = \frac{\Sigma\ Area_i}{\Sigma(Area_i / D_i)} \tag{12}$$

A plot of the distribution of diffusion coefficients was produced rather than a plot of molecular weight distribution. The applicability of the approach was substantiated by the excellent correlation between the D_w obtained by the FFF data and the reported M_w of the humic substances provided by the IHSS. The standards were estimated with less than a 3 percent difference (Table 1). A linear fit between D_w and M_w demonstrated a higher correlation coefficient than the log/log model of Equation 9. The nominal molecular weights of the PSS standards were better correlated with the diffusion coefficients measured at the peak maximum, i.e., D_p. Unlike Beckett et al. (11), a single relationship correlating the PSS and humic substances was not identified.

In an analogous manner, the diffusion coefficients denoted D_p, D_w, and D_n were determined by flow FFF for twenty-one unknown fulvic and humic acid

samples (Table 2). The M_w and M_n estimates of molecular weight were interpolated separately from the linear equations established using standard humic substances. The weight-average molecular weight estimates for three of the samples (the soil fulvic and humic acids, and the peat fulvic acid) were extrapolated from the calibration equations. The fulvic acids were found to have approximately 70 percent lower molecular weights than the humic acids derived from the same geographic site. The difference in the molecular weight of the aquatic humic acid and the soil humic acid was not significant.

Two measures of polydispersity are reported; the calculation of M_w/M_n and the measured peak width at half height. In two instances, for the soil fulvic acid and the Summit humic acid, the values of M_w/M_n were determined to be less than one. The fractograms for each of these samples displayed nearly Gaussian behavior and the M_w and M_n estimates are very nearly equal producing a polydispersity of nearly one. Also, the correlation was less accurate for the prediction of M_n. Data collection for these fractograms was concluded at 40 to 50 minutes. In a few cases, that is for those samples having higher molecular weight components, this may have resulted in low estimates of molecular weight.

Comparison of the humic acid molecular weight with soil sample depth and tillage effect is summarized in Table 3. Molecular weight estimates for humic acids extracted (19) from soil in till and no till situations for three agronomic crops (corn, cotton, and soybeans) and various cover crops in no till practices (no cover, vetch, wheat, and crimson clover) were made. No conclusions regarding the tillage effect on molecular weight of the humic acids could be reached. However, the molecular weight of the humic acids was observed to decrease with soil sample depth.

CONCLUSIONS

The use of flow field-flow fractionation to characterize the diffusion coefficients and molecular weight distributions of polystyrene sulfonates and

Table 3. Comparison of Soil Sample Depth and Tillage Effect on Humic Acid Molecular Weight

Sample	Description	M_w	M_w/M_n
Stearman 57	corn, till, no cover, 0-2 cm depth	2907	1.31
Stearman 1A	corn, no till, no cover, 0-2 cm depth	3153	1.35
Stearman 17	cotton, till, no cover, 0-1 cm depth	2854	1.26
Stearman 18	cotton, till, no cover, 1-2 cm depth	2714	1.26
Stearman 21	cotton, no till, no cover, 0-1 cm depth	2789	1.25
Stearman 22	cotton, no till, no cover, 1-2 cm depth	2409	1.22
Stearman 9	cotton, no till, vetch, 0-1 cm depth	3043	1.30
Stearman 10	cotton, no till, vetch, 1-2 cm depth	2801	1.27
	cotton, no till, vetch, 0-3.8 cm depth	2338	1.16
Stearman 14	cotton, no till, wheat, 1-2 cm depth	2846	1.30
Stearman 3-2	cotton, no till, crimson clover, 0-3.8 cm depth	2647	1.24
Stearman 25A	soybean, no till, no cover, 0-2 cm depth	2633	1.24

humic and fulvic acids was demonstrated. Only limited extrapolation of the calibration curve was necessary in this research. The weight-average molecular weights of the standard humic and fulvic acids were determined within a 3 percent difference. Fulvic acids were found to have molecular weights approximately 70 percent lower than their corresponding humic acids. The weight-average molecular weight of humic acids extracted from soils sampled under agronomic crops was demonstrated to decrease with soil sample depth. Diffusion coefficients were readily determined from the data obtained by flow FFF. Converting this information into molecular weight distributions is more difficult. The information gained regarding the diffusion coefficient may ultimately be more useful than determination of molecular weight in environmental studies of chemical fate and transport. The advantage of this separation process is that only physical forces are acting on the specimen. The technique is nondestructive. When the molecular weight fractions have been obtained, no chemical changes of the species have been made; the species remain intact. This is a great advantage if further study of the fractionated components is to be conducted.

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