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Aggregation and Disaggregation of Humic Supramolecular Assemblies by NMR Diffusion Ordered Spectroscopy (DOSY-NMR)

DANIELA ŠMEJKALOVÁ AND
ALESSANDRO PICCOLO*

Dipartimento di Scienze del Suolo, della Pianta, dell'Ambiente e delle Produzioni Animali, Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy; Centro Interdipartimentale di Ricerca per la Spettroscopia di Risonanza Magnetica Nucleare (CERMANU), Via Università 100, 80055 Portici, Italy

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Diffusion ordered nuclear magnetic resonance spectroscopy (DOSY-NMR) was applied to a number of fulvic (FA) and humic (HA) acids of different origin. Spectral separation achieved by DOSY based on diffusion coefficients (D), and correlated to molecular sizes by calibration standards, showed that carbohydrates had the largest molecular size in FA, whereas alkyl or aromatic components were the most slowly diffusing moieties in HA. At increasing concentrations, these components had invariably lower D values in DOSY spectra for all humic samples, thereby indicating an aggregation into apparently larger associations, whose increased hydrodynamic radius was confirmed by viscosity measurements. When humic solutions were brought from alkaline to acidic pH (3.6), components diffusivity detected by DOSY increased significantly, suggesting a decrease of aggregation and molecular size. A general comparison of HA and FA molecular sizes was achieved by multivariate statistical analysis. While a larger extent of aggregation and disaggregation was observed for HA than for FA, no aggregation was detected, under similar conditions, for a true macropolymeric standard. Such difference in diffusion between a polymeric molecule and humic samples, is in line with the supramolecular nature of humic matter. The possible formation of humic micelles was also investigated by both changes of diffusivity in DOSY spectra and shift of ^1H NMR signals. Except for HA of peat and soil origin, revealing a self-assembling in micelle-like structures at the 4 mg mL⁻¹ concentration, no other humic sample showed evidence of critical micelle concentration (cmc) up to 20 mg mL⁻¹. These results indicated that DOSY-NMR spectroscopy is a useful technique to evaluate components of different molecular size in natural humic superstructures.

Introduction

Detailed knowledge of molecular sizes of humic substances (HS) is essential for understanding their physical–chemical properties and environmental role. Ultrafiltration (1, 2), dynamic light scattering (3), size exclusion chromatography (4, 5), viscosimetry (6), fluorescence correlation spectroscopy

(7), electrospray–mass-spectrometry (8, 9), and other analytical methods (10) were employed to estimate molecular shape and size of HS. However, mainly because of the large structural heterogeneity of HS and lack of model standards, a generalized consensus on the molecular nature of HS has been attained only recently (11–13).

Traditional concepts which originated from the supposed observations of large molecular weights (14, 15), postulated that HS comprised polymeric macromolecules (16). Despite the lack of direct evidence and existing alternative results (17), such macropolymeric view became common place in literature. New findings gathered during the past decade and based on different techniques were not consistent with the macropolymeric view and rather suggested humic substances to behave as supramolecular associations (11–13). This alternative indicates the large molecular weights of HS to be only apparent and resulting from self-assembling of relatively small and heterogeneous humic molecules in large supramolecular conformations, which are stabilized mainly by weak dispersive forces, such as van der Waals, π – π , and CH– π (11).

Diffusion ordered NMR spectroscopy (DOSY) unquestionably represents a powerful tool for structural studies of aggregation of natural organic matter (12, 18). DOSY is a pulsed field gradient NMR spectroscopy that enables measurement of translational diffusion of dissolved molecules. Except for the overall molecular size and shape, the magnitude of diffusion coefficient provides direct information on molecular dynamics, including intermolecular interactions (19, 20), aggregation, and conformational changes (10, 21). Moreover, DOSY processing is a particularly suitable technique for complex samples because it provides a direct correlation of translational diffusion to the chemical shift in the second dimension and hence a prior separation of mixture components is not required (22, 23).

Previous diffusion results reported that variations of humic molecular sizes may be related to the original source from which humic matter was isolated (10). Moreover, DOSY diffusion measurements were successfully elaborated by spectrally separating alkyl, aromatic, and sugar components of organic matter based on their diffusion (24, 25). Our aim was to apply DOSY to probe the diffusion behavior of a number of fulvic (FA) and humic acids (HA) from different origins and, in particular, investigate their aggregation, disaggregation, and, possibly, formation of micelle structures.

Materials and Methods

Humic Substances. Two fulvic acids (FA) and seven humic acids (HA) were isolated as previously described (9) from peat soil (Dystric Histosol, Tuscany, Italy) FA PEAT, HA PEAT; volcanic soil (Typic Fulvudand, Lazio, Italy) FA VICO, HA VICO; arable soil (Haplic Luvisol, Denmark) HA DAK; Rio Negro basin (Hydromorphic soil, Brazil) HA RIO NEGRO; Lignite 1 (North Dakota) HA LIG; Lignite 2 (Hungary) HA HUNG; and oxidized Sulcis Coal (Sardinia, Italy) HA COX. The HA were purified and freeze-dried as customary (9), whereas the FA were adsorbed on an acid-conditioned XAD-8 column, and then eluted from column by a 1 M NaOH solution. The eluted FA were neutralized, dialyzed against water, and freeze-dried.

Solution-State DOSY-NMR Spectroscopy. Solution-state NMR spectra were obtained on a Bruker Avance 400 MHz instrument operating at a proton frequency of 400.13 MHz, equipped with a 5 mm Bruker inverse broadband probe. All spectra were elaborated by Bruker Topspin 1.3 (Bruker Biospin). Both freeze-dried humic samples (1–5, 10, and 20

* Corresponding author phone: +39 081 253 91 60; fax: +39 081 253 91 86; e-mail: alessandro.piccolo@unina.it.

mg), and the same amount of polystyrene sulfonate (PSS, Polymer Standard Service, Germany) having molecular weight of 6780 Da, were dissolved in 0.4 mL of 0.1 M NaOH and diluted with 0.6 mL of deuterated water (D₂O). A total sample volume of 1 mL, having pH of 12.8 ± 0.1 , was reached in each case. Samples were transferred to 5 mm NMR quartz tubes fitted with Doty susceptibility plugs. ¹H NMR spectra were referenced to the chemical shift of solvent, resonating at 4.8 ppm. The ¹H 90° pulse was calibrated using HOD signal, and both 1D and 2D experiments were acquired under saturation of water signal. For 1D experiments, presaturation was achieved with a 52 dB attenuation of a 60W amplifier for a period of 2 s, and a watergate 3-9-19 pulse train with gradients was applied for acquisition of 2D spectra. 2D-DOSY diffusion-ordered spectra were obtained using a stimulated echo pulse sequence with bipolar gradients (STEBPGP). Scans (240) were collected using 2.5 ms sine-shaped pulses (5 ms bipolar pulse pair) ranging from 0.674 to 32.030 G cm⁻¹ in 32 increments, with a diffusion time of 100–200 ms, and 8 K time domain data points. Apodization was made by multiplying data with a line broadening of 1.0 Hz, spike suppression factor of 1.0, maximum interactions number set to 100, noise sensitivity factor of 2, and number of components set to 1. All humic samples (10 mg mL⁻¹) and the 6780 Da PSS (10 mg mL⁻¹), were again subjected to ¹H NMR and 2D-DOSY analyses after the addition of 17 μL of deuterated glacial acetic acid (CD₃COOD) per 1 mL of original alkaline solution. In all cases, the pH was lowered to pH 3.6 ± 0.1 . The chemical shift variation for determination of critical micelle concentration (cmc) was evaluated on the signal resonating at 1.83 ppm in the ¹H NMR spectra.

Results and Discussion

Molecular Characteristics. The relative ¹³C distribution in the different NMR spectral regions from CPMAS-¹³C NMR spectra of all samples is reported in Supporting Information (SI) Table S1. As expected, both FA (FA VICO and FA PEAT) were rich in carbohydrate (93–44 ppm), anomeric (113–93 ppm), and carboxyl C (200–161 ppm). Lower, but still significant amount of carbohydrate signals was observed for HA of soil and peat origin (HA VICO, HA DAK, HA RIO NEGRO, and HA PEAT). Interestingly, HA VICO and HA PEAT together with the lignite samples, HA LIG and HA HUNG, possessed the highest amounts of alkyl C (44–0 ppm). Relative abundance of aromatic and phenolic carbons (161–113 ppm), sometimes referred to as the index of aromaticity, was largest for HA from oxidized coal (HA COX), followed by lignite samples (HA LIG and HA HUNG). As a consequence, these three humic samples were recognized to be highly hydrophobic, while the rest of humics having lower aromaticity was found to be relatively more hydrophilic. The abundance of polar (hydrophilic) and apolar (hydrophobic) C were evaluated as HI/HB ratio (SI Table S1).

The different distribution of polar and apolar components can be expected to influence the hydrodynamic volume of dissolved humic molecules. In fact, it can be expected that the hydrodynamic size of a humic material rich in polar components will be kept small when dissolved in aqueous solutions due to stabilization exerted by hydration water. Conversely, more hydrophobic materials self-associate in large dimensions and become separated from water, thereby decreasing the total free energy of the dissolved system (26, 27). Since the diffusion behavior of molecule in a solution is related to molecular conformation, and hence to molecular volume, the HI/HB ratio (SI Table S1) is one of the structural parameters that is expected to influence the diffusion behavior of FA and HA.

Aggregation. Figure 1 shows the diffusivities, and molecular sizes of humic samples dissolved at pH 12.8 at a concentration ranging from 1 to 20 g mL⁻¹. Diffusion

coefficients (*D*) were related to the apparent molecular sizes (*M_w*) according to the relationship between molecular weight and diffusivity of known standards (SI Figure S1), as shown earlier (28). Since the major advantage of 2D DOSY is that differences in *D* can be observed as a function of chemical shift, diffusivities were extracted using three spectral regions corresponding to various structural components present in HS. The regions were (i) 9.0–5.0 ppm due to the presence of protons on aromatic rings, nitrogen heteroatoms and amides, (ii) 4.4–2.9 ppm corresponding to proton signals on CH₃, CH₂, or CH carbons directly bonded to O, N, or carbohydrates, and (iii) 2.9–0.0 ppm containing remaining aliphatic signals, including protons on alkyl carbons which are two or more carbons away from an aromatic ring or polar functional group (29).

A decrease in diffusion (Figure 1), and, consequently, an increase in molecular size, was observed with increasing concentration for all samples in each of the three chemical shift regions (9.0–5.0, 4.4–2.9, and 2.9–0.0 ppm). The increase of molecular size was more significant for HA than for FA. Moreover, the smaller decrease of diffusivity for FAs was comparable to the diffusion behavior of polystyrene sulfonate (PSS) standard (Figure 1) and was in good agreement with earlier results (28).

The PSS are commercially available as size calibration standards and widely used in size-exclusion studies of humic matter. To our knowledge, there is no restriction of concentration range regarding the application of PSS as molecular size standards. However, according to the Stokes–Einstein (SE) equation (30):

$$D = \frac{kT}{6\pi\eta r_H} \quad (1)$$

where *k* represents the Boltzmann constant (1.38×10^{-23} JK⁻¹), *T* is the absolute temperature, *η* is the solvent viscosity, and *r_H* hydrodynamic radius of a spherical particle, the increase of molecular size of PSS at high concentrations (Figure 1) could have been caused by an increase in solution viscosity. Similar theoretical explanation was given for the diffusion of maltodextrin polysaccharides with increasing concentration (28). In fact, the SE equation applies mostly to diluted solutions (30, 31), where the viscosity of solvent corresponds to the overall viscosity of solution. Conversely, at high concentrations the solute contribution to the solution viscosity cannot be any longer neglected and may result in substantial violation of the SE equation (32).

The shear viscosities of the most diluted (1 mg mL⁻¹) and the most concentrated solutions (20 mg mL⁻¹) of the PSS standard, FA and HA samples, as well as the corresponding hydrodynamic radii are reported in SI Table S2. As assumed, the viscosity of diluted solutions was not different from that of solvent (*η* = 1.032×10^{-3} Pa s), whereas a slight increase was observed for PSS, HA VICO, and HA LIG at 20 mg mL⁻¹. Calculation of hydrodynamic radius (SI Table S2) indicated an enhancement in molecular diameter for both FA and HA in more concentrated solutions, and thus confirmed aggregation of FA and HA into larger entities. Conversely, it excluded any size change for the PSS standard. In that case, the observed increase in molecular size shown in Figure 1 was only apparent and just a result of increased solution viscosity.

Besides the influence of increasing concentration, the largest size-components with slowest diffusion varied for different samples (Figure 1). The carbohydrate region appeared to have the slowest diffusion in FA, whereas the largest size in HA DAK, and, to some extent, also in HA PEAT and HA VICO was observed for alkyl components. Aromatics showed the slowest diffusion in HA HUNG, HA RIO NEGRO, HA LIG, and HA COX. An example of 2D DOSY projection for each of the three groups of samples is shown in Figure 2, including corresponding slices (*A*, *B*, and *C*), that indicate

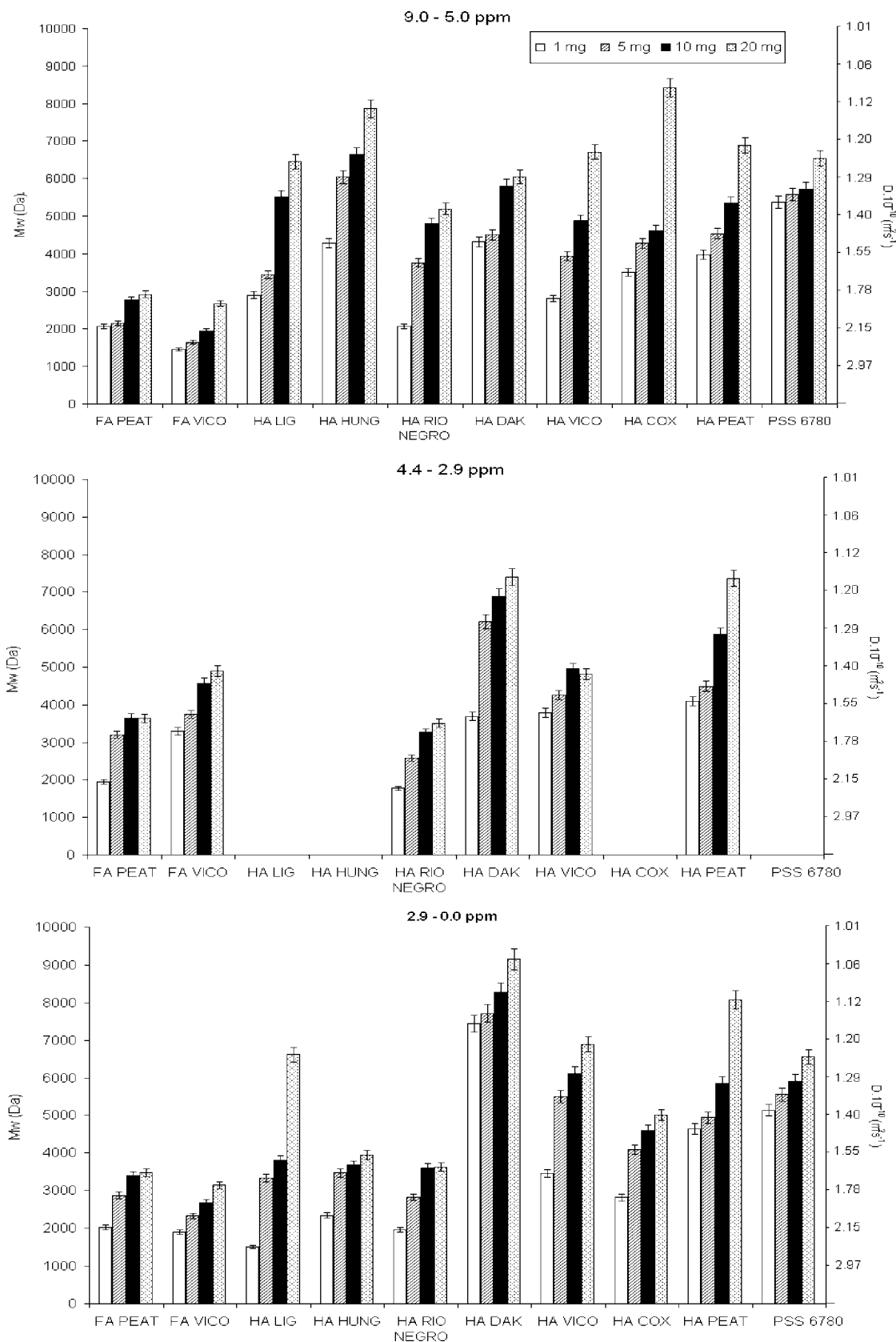


FIGURE 1. Diffusion coefficients (D) and molecular sizes (M_w) of fulvic (FA) and humic acids (HA) of different origin, and polystyrene sulfonate (PSS), dissolved at pH 12.8 at various concentrations (1, 5, 10, and 20 mg mL^{-1}). VICO, PEAT, DAK, and RIO NEGRO refer to soil extracts, HUNG and LIG to lignite material, and COX to oxidized coal origin.

^1H chemical shift profiles at different diffusion. The proton slices still comprise many overlapping resonances reflecting the complex composition of FA and HA. Slice A was cut

through the most negative log D value, and thus describes the largest size components in each sample. In contrast, slice C, taken through the least negative log D , is related to the

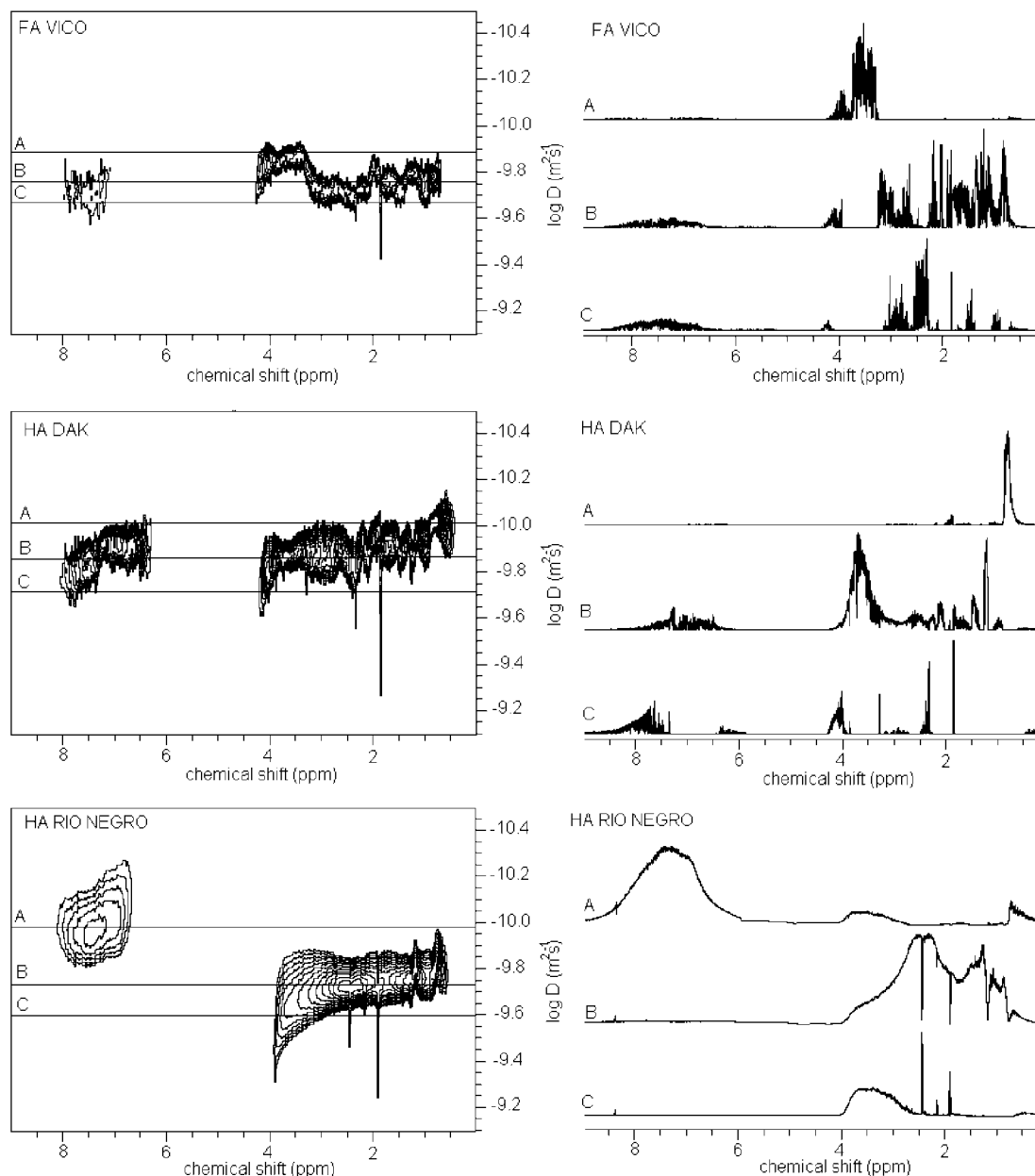


FIGURE 2. 2D-DOSY projection of FA VICO, HA DAK, and HA RIO NEGRO dissolved at pH 12.8 at a concentration of 20 mg mL⁻¹. Slices A, B, and C refer to ¹H chemical shift profiles taken through different levels of diffusion axis, where corresponding molecular size decreased in order: slice A > slice B > slice C.

smallest size moieties. The smallest size components consisted of aromatics and partially alkyls in the case of FA VICO, while they were mainly aromatics in HA DAK, and carbohydrates in HA RIO NEGRO. The different diffusivities of aromatic, carbohydrate, and alkyl components also suggest that humic matter consists of heterogeneous and independent species, which are not linked in macro-polymeric entities, where all constituents should reveal a similar diffusion.

To generalize the visualization of diffusion results (Figure 1), these were analyzed by principal component analysis (PCA). PCA is an unsupervised multivariate statistical method used for the recognition of similarities and/or dissimilarities among samples whereas reducing the high amount of original variables into just a few principal components retaining high percentage of the original information (33). In the resulting PC plot (SI Figure S2), the two extracted principal components, PC1 and PC2, covered 90% of the total sample variance. Positive scores on PC1 and PC2 in the second quadrant were

correlated with high molecular size obtained for aromatic signals (9.0–5.0 ppm), while positive scores on PC1 and negative scores on PC2 in the third quadrant were correlated with high molecular size for the 4.4–0.0 ppm region. As a result, PC2 indicated which components (aromatics or carbohydrates/alkyls) diffused more slowly and thus were larger in molecular size. In fact, the separation of HA HUNG, HA RIO NEGRO, HA LIG and HA COX from the rest of samples along PC2 in SI Figure S2 is in agreement with previous discussion. Furthermore, PCA revealed additional information regarding overall molecular sizes of humics by positioning the samples along PC1. In fact, the smallest hydrodynamic radii, and, thus, the smallest molecular sizes were found for FA VICO and FA PEAT, closely followed by HA RIO NEGRO. A larger hydrodynamic radius was observed for HA LIG, HA COX, HA HUNG, and HA VICO, and the largest ones for HA PEAT and HA DAK (SI Table S2). Hence, it is noteworthy that the most hydrophilic samples, FA PEAT and FA VICO (see HI/HB in SI Table S1), revealed the smallest molecular sizes,

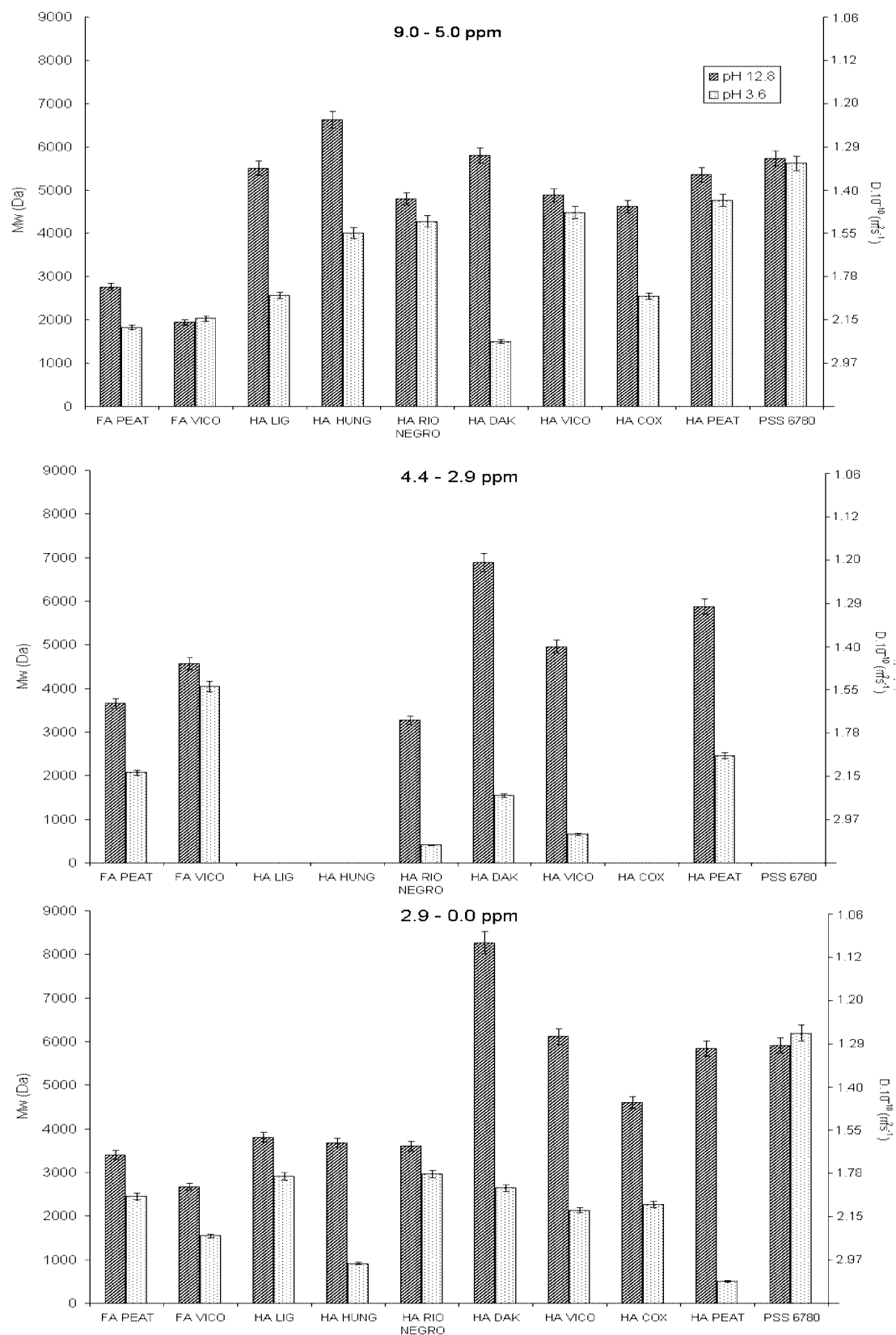


FIGURE 3. Diffusion coefficients (D) and molecular sizes (M_w) of fulvic (FA) and humic acids (HA) of different origin, and polystyrene sulfonate (PSS) at a concentration of 10 mg mL^{-1} brought to pH 3.6 by acetic acid treatment. Assignments of humic fractions are as in Figure 1.

whereas the most hydrophobic samples, such as HA COX, did not show the largest hydrodynamic volumes. This can be explained by the tendency of hydrophobic, and concomitantly, highly aromatic humic structures, to repel

hydration water from their molecular associations, and lower the hydration volume of their conformations (4).

Specific attention should be paid to the position of PSS in the PC score plot (SI Figure S2). Unlike humic samples,

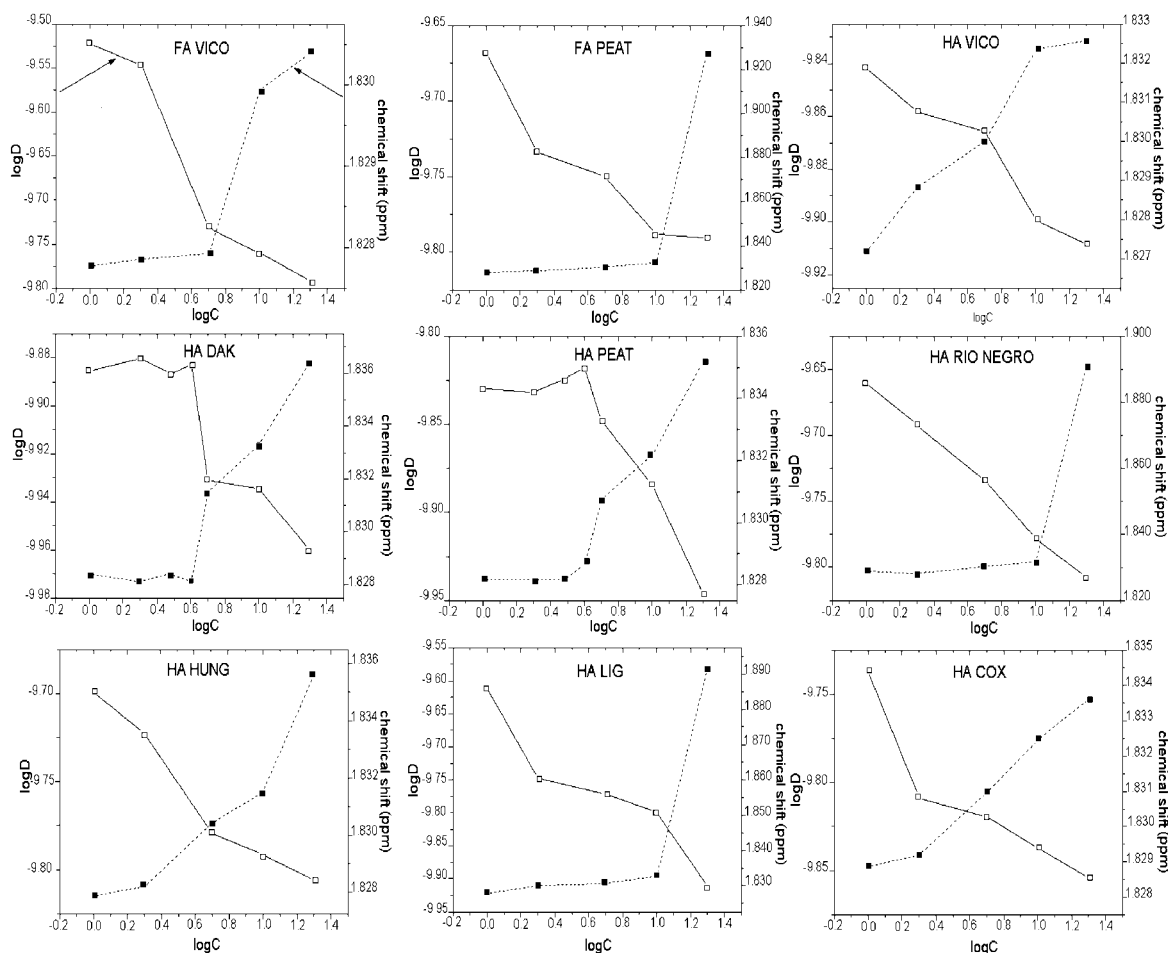


FIGURE 4. Influence of concentration on diffusion coefficients (D , straight line) of alkyls and carbohydrates (4.4–0.0 ppm), and chemical shift (dashed line) of a signal at 1.83 ppm. Assignments of humic fractions are as in Figure 1.

PSS is placed on the borderline that differentiates humic samples on the basis of predominant large components. This indicates that ^1H NMR signals of PSS were detected in both regions (9.0–5.0 and 4.4–0.0 ppm), and their observed diffusion coefficients were identical, as it would be expected for any cross-linked macromolecule. SI Figure S2 also suggests that the PSS standard had lower molecular size than HA DAK aggregates and larger molecular size than the rest of the humic samples.

Disaggregation. Previous findings by size-exclusion chromatography showed that humic aggregates can be disrupted into smaller molecular dimensions by treatment with small amounts of organic acids (4, 11). This process may be naturally occurring in the biosphere, since low molecular weight organic acids excreted by plant roots into soil (34) are believed to disrupt bulk humus into small molecular-size humic components, which may be in turn adsorbed through membrane carrier system and affect plant growth by hormone-like activity (35, 36).

Here, the alkaline pH of humic solutions (10 mg mL⁻¹) was reduced to pH 3.6 by acetic acid addition and the resulting changes in diffusion recorded (Figure 3). To overcome viscosity changes after acetic acid addition, calculation of corresponding molecular sizes was based on a reconstructed calibration (SI Figure S1) using the same standards as for alkaline conditions, but brought to pH 3.6. Acidification of humic solutions resulted in all cases in a significant decrease of molecular sizes for alkyl and carbohydrate moieties (Figure 3). For instance, the carbohydrates were lowered to less than 700 Da in HA RIO NEGRO and HA VICO, and the alkyls displayed diffusivities corresponding to less than 1000 Da in

HA HUNG and HA PEAT, and were below 3000 Da for the rest of humic samples. Lower but still remarkable reduction was obtained for signals resonating in the aromatic region. An exception was FA VICO, where the aromatics had similar diffusivity regardless of the acetic acid treatment. In fact, neither FA VICO nor FA PEAT did undergo such a significant disaggregation of carbohydrates and alkyl moieties as HA samples. This agrees with the more polar nature of FA (see HI/HB in SI Table S1), and their already low aggregation status in aqueous solutions (4, 37).

The observed values of molecular sizes after disaggregation are somewhat larger than those obtained in an earlier work (28). This inconsistency mainly resulted from lower concentration (5 mg mL⁻¹) and different set of calibration standards used in the aforementioned study. In fact, the calculated molecular weights under no circumstances represent the absolute sizes of humic molecules, since they were indirectly derived as an approximation of the hydrodynamic volumes of selected standards, which do not behave like HS.

At alkaline pH, the intramolecular hydrogen bondings are disrupted and humic molecules become fully dispersed. Thus, prior to acidification, the aggregated humics could have been stabilized only by weak hydrophobic interactions (11). Since acetic acid treatment did not cause significant changes in solution viscosity (data not shown), and neither affected the diffusion behavior of covalently bonded PSS (Figure 3), the disaggregation of humic material must have been mainly due to the disruption of weak non covalent interactions, such as van der Waals, π - π , and CH- π . This is in line with our previous findings that suggested HS in solution to form loosely bound supramolecular conforma-

tions of self-associating molecules (4). The collapse of humic conformations into smaller molecular associations was explained by an increased thermodynamic stability due to the formation of intra- and intermolecular hydrogen bonds between protonated acidic functions and complementary oxygen-containing groups present either in humic molecules or organic acids (11).

Micellization. Molecules containing both hydrophilic and hydrophobic parts undergo self-aggregation and under certain conditions may form weakly stabilized aggregates, called micelles (38). The concentration at which micelles form is the critical micelle concentration (cmc), and is connected to changes in a number of physical parameters, including translational diffusion. Moreover, changes in molecular conformation affect the resonating frequencies of involved nuclei at NMR scale (39). For common surfactant systems, i.e.: sodium dodecyl sulfate, it is known that diffusion coefficient and chemical shift of all signals remain constant up to the cmc (SI Figure S3), whereafter their values begin to vary (39, 40).

To determine whether the FA and HA of this work reached a pseudo cmc at a range of concentration between 1 and 20 mg mL⁻¹, additional DOSY experiments were performed for samples dissolved at 2, 3, and 4 mg mL⁻¹. Diffusion experiments were not conducted at concentrations lower than 1 mg mL⁻¹, due to low sensitivity of NMR spectroscopy for diluted humic solutions.

Biplots showing the dependence of diffusivity and proton chemical shifts of alkyls and carbohydrates (4.4–0.0 ppm) on the concentration of humic material are shown in Figure 4. Apparently only two samples, namely HA DAK and HA PEAT, showed a micelle-like behavior, by losing a constant diffusion after a certain concentration. The alkyls and carbohydrates (as well as aromatics, data not shown) of both these HA seemed to begin a spontaneous aggregation after reaching the concentration of 4 mg mL⁻¹. However, the diffusion values for aromatic, carbohydrate, and alkyl region were not the same (Figure 1), thereby suggesting that they were not concomitantly involved in the same micelle-like structure.

An explanation for the lack of constant diffusion in the remaining humic samples may reside in the fact that, regardless of their amphiphilic nature, their hydrophobic components might be too short in chain length or present in a too low amount to form micelles. The lack of constant diffusion in most samples suggested an incipient aggregation already at low concentrations. In fact, the aggregation seemed to be limited to a spontaneous stacked-like association of hydrophobic (mainly aromatic) moieties, without ever reaching a proper cmc (41). However, such conformational arrangements would still function in water as solubility-enhancing agents for hydrophobic molecules, and, to some extent, lower the surface tension of solution, thereby being in line with the well-known “surfactant” properties of HS (17, 42).

This work has shown that DOSY-NMR spectroscopy is a useful technique for the separation of components of different molecular size present in HS. The spectral separation provided information about the slowest diffusing component in each humic material and showed that this component can be either aromatic-, carbohydrate- or alkyl-like. Increasing humic concentration resulted in an enhancement of molecular size for all humic components, as shown for different NMR spectral regions, and indicated self-association of humic molecules. Aggregation into larger molecular entities was more significant for HA than for FA and was only apparent for PSS. Furthermore, a modification of humic solutions with acetic acid resulted in a progressive alteration of the diffusion behavior, and led to reduction of molecular size for all humic samples, whereas it did not alter that of PSS. The observed

disruption of humic aggregates with acidification by DOSY-NMR spectroscopy, further supports the present understanding that HS are associations of relatively small molecules held together mainly by weak dispersive forces. In addition, DOSY spectra also indicated that most of humic samples do not form pseudomicelles. A micelle-like behavior was observed only for two humic samples, and the apparent critical micelle concentration was, in both cases, established to be greater than 4 mg mL⁻¹.

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Supporting Information Available

Investigation on CPMAS-NMR spectroscopy, shear viscosities, hydrodynamic radii, calibration curves at alkaline and acid pH, PCA score plot for all humic samples, and determination of cmc of SDS. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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