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Comparative Analysis of Partial Structures of a Peat Humic and Fulvic Acid Using One- and Two-Dimensional Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

Nuclear magnetic resonance (NMR) resonance integrals obtained from one-dimensional NMR spectra provide semiquantitative contents of humic constituents with limited resolution in structural detail. When supplemented by connectivity information available from homo- and heteronuclear two-dimensional NMR spectra a more reliable assignment of humic substructures becomes available. This is demonstrated with a comparative one- and two-dimensional NMR analysis of a fulvic and a humic acid obtained from *Eriophorum* peat. An example of a detailed analysis of the proton chemical shift region normally attributed to carbohydrates shows substantial contributions from amino acids, amino and desoxy sugars, and highly oxidized aliphatic chains of intermediate length. The very good resolution of structural detail by a combined analysis of all NMR spectra shows that the effect of the fractionation procedure on the composition and chemical structure of humic materials is very significant. The comparison of the partial structures comprising humic acid (HA) and fulvic acid (FA) of the peat humic materials studied indicates that FA is diagenetically downstream of HA, favoring the biopolymer degradation (BD) model of humification.

Humic substances (HS) constitute the most important pool of transient refractory organic carbon in the geosphere. They occur in soil, aquatic, and marine environments and compose an important cross-link of natural and anthropogenic fluxes of carbon, nitrogen, and phosphorus. Humification, with respect to the contribution to the global carbon cycle (20 Pg/yr) is the second important process in turnover after photosynthesis (60 Pg/yr) (Hedges and Oades, 1997). In contrast to biochemical pathways of synthesis, the formation of HS out of the plant and animal debris follows no genetic code and occurs stochastically, governed by kinetic and thermodynamic restraints so that only refractory structures are likely to survive being not susceptible to further microbial and chemical degradation. The stochastic nature of humification is expected to produce an immense structural heterogeneity and polydispersity of HS (MacCarthy and Rice, 1988). As a result, up to date HS are defined purely operationally as the alkali-extractable fraction of soil and solid fuel organic matter. Their most common classification is based on the solubility at different pH values (Stevenson, 1982, p. 36). In general, HS are classified into humic acids (HA), insoluble at pH < 1 to 2, and fulvic acids (FA), soluble across the whole range of pH values. Humic acids represent a higher molecular weight, more condensed fraction of HS, whereas FA are of lower molecular weight and more oxidized than HA. The two most common hypotheses of humification are the biopolymer degradation (BD) and the abiotic condensation (AC) models. The AC hypothesis implies the complete disintegration of the nonliving organic matter as the first stage of humification (Hatcher and Spiker, 1988) and considers FA as precursors of HA. In contrast, the BD hypothesis considers the humification as a slow oxidation, condensation, and decomposition process of plant and animal biopolymers, in which the integrity of the parent materials is not completely destroyed by the microbial degradation (Hedges, 1988; Hedges and Keil, 1999). To assess the validity of both hypotheses, detailed information on the partial structures of HA and FA can be of particular importance.

Nuclear magnetic resonance spectroscopy over last decades has provided key insight into structural details of humic substances (Preston, 1996). Both the polydispersity and structural heterogeneity of HS induce specific effects on the key NMR parameters chemical shift δ, coupling constant J, amplitude A, and resonance width at half height $\Delta\nu_{1/2}$. The heterogeneity of HS causes overlap of resonances in their NMR spectra, resulting in rather broad signal envelopes. Variations in aggregate size, shape (molecular conformation), charge, and mutual interaction of humic molecules affect the NMR relaxation times T_1 and T_2. Large molecular and aggregate size and strong interaggregate interactions induce a low segmental mobility within the humic molecules and lead to slow longitudinal (T_1) and fast trans-

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Abbreviations: A, B, C,...Y, standard one-letter abbreviations for proteinaceous amino acids; AC, abiotic condensation; BD, biopolymer degradation; COSY, correlated spectroscopy; F1, vertical frequency axis in two-dimensional nuclear magnetic resonance spectra (X nucleus–calculated data); F2, horizontal frequency axis in two-dimensional nuclear magnetic resonance spectra (proton-detected data); FA, fulvic acids; HA, humic acids; HMQC, heteronuclear multiple quantum correlation; HS, humic substances; HSQC, heteronuclear single quantum coherence; NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy.

verse (T_2) relaxation (Bovey and Mirau, 1996, p. 353–378). The effects on NMR resonances are a decrease in intensity A and increase in half width $\Delta\nu_{1/2}$, possibly beyond recognition. Differential relaxation within the polydisperse humic matrix interferes with a quantitative NMR analysis, which is based on the determination of resonance position and intensity in both one- and two-dimensional NMR spectra. The extended duration of the two-dimensional NMR experiments makes them more susceptible to effects of variance in relaxation times T_1 and T_2. Owing to extensive signal overlap and to fast relaxation, coupling constants are not commonly used in the analysis of one-dimensional NMR spectra of humic substances. Instead, calculation of partial integrals according to coarse regions of substructures is usually performed (Bortiatynski et al., 1996, p. 55–77; Preston, 1996). This modus of interpretation resembles the state of the art of protein ^{1}H NMR spectra analysis in the late 1950s (Saunders et al., 1957).

Two-dimensional NMR spectroscopy offers several significant advantages in the analysis of the complex humic structure. An increased signal dispersion into two frequency dimensions greatly reduces resonance overlap, especially in the heteronuclear correlated NMR spectra with their large spread of frequency in F1 (vertical frequency axis in two-dimensional NMR spectra [X nucleus–calculated data]) (e.g., ^{13}C) dimension (Croasmun and Carlson, 1996; Cavanagh et al., 1996). In addition, two-dimensional NMR experiments act as filters that emphasize specific forms of binding. So, heteronuclear single bond correlated spectra like heteronuclear single quantum coherence (HSQC) and heteronuclear multiple quantum correlation (HMQC) show exclusively directly bonded pairs of carbon and hydrogen atoms, whereas standard homonuclear correlated spectroscopy (COSY) spectra exhibit geminal (two-bond) and vicinal (three-bond) proton–proton couplings only. The sensitivity of proton detected homo- and heteronuclear two-dimensional NMR spectra in general is much higher than that of X-nuclei one-dimensional NMR spectra. The above effects combined enable a detailed analysis of even the most crowded regions of chemical shift.

Cross peaks in two-dimensional NMR spectra indicate a range of connectivities defined by the kind of NMR experiment performed, allowing a probe of bonding interactions, spatial relationships, and chemical exchange (Croasmun and Carlson, 1996; Cavanagh et al., 1996; Hertkorn et al., 2001). Various aspects of high-resolution two-dimensional NMR spectroscopy of HS have been highlighted in several recent publications. One paper (Chien and Bleam, 1998) focused on the use of NOESY spectra to provide information about spatial arrangement of aromatic and aliphatic units in HS. Another paper (Buddrus et al., 1989) described homonuclear J-resolved spectra of HS. Most of the other research demonstrates that a considerable range of shift-correlated two-dimensional NMR spectra is applicable to the analysis of HS. Schmitt-Kopplin et al. (1998) demonstrated that information from two-dimensional NMR spectra (HMQC and COSY) can reveal structural information about the course of photodegradation of a soil HS. Wang et al. (1998) showed a range of well-resolved two-dimensional NMR spectra (maximum quantum spectroscopy [MAXY], total correlation spectroscopy [TOCSY]) with some preliminary assignments. Haiber et al. (1999) showed HMQC and COSY NMR spectra of ultrafiltrate fractions of an aquatic humic substance, which were differing in cross peak location, but were characterized by limited resolution of structural detail. Kingery et al. (2000) focused on HMQC and TOCSY NMR spectra of a International Humic Substances Society (IHSS) soil HA and tentatively assigned selected HMQC and TOCSY cross peaks to fatty acid derivatives. They demonstrated that additional TOCSY cross peaks could be observed when using DMSO-d_6 instead of D_2O as a solvent and attributed those to CH–NH correlations within amino acid side chains. Fan et al. (2000) performed NOESY, TOCSY, and HSQC NMR spectra for a Mollisol HA treated with the chelating agent Tiron and provided substantial assignment details of peptidic and some carbohydrate cross peaks. They compared these results with TOCSY cross peaks appearing in the acid digest of the HA used. Hertkorn et al. (2001) demonstrated the applicability of a range of homo- (COSY, TOCSY, EXSY) and heteronuclear NMR experiments (HSQC, DEPT-HSQC) for the study of HS structure and provided initial cross peak assignments. It was concluded that the NOESY spectrum in dry DMSO-d_6 could be regarded rather as an EXSY-NMR spectrum, since contributions from chemical exchange significantly outperformed those from spatial relationships.

The objectives of this study are to (i) assess the information content on the partial structures of HS available from the interpretation of single one-dimensional or two-dimensional NMR spectra and from a combined analysis, (ii) identify and compare the partial structures of FA and HA isolated from the same peat source, and (iii) assess the suitability of the biopolymer degradation (BD) and the abiotic condensation (AC) models to explain the process of humification.

MATERIALS AND METHODS

Isolation of the Humic Materials

The peat HA and FA were isolated from *Eriophorum* highland peat (80% *Eriophorum vaginatum*, 10% pine skin, 10% *Sphagnum* moss; 40% decomposition rate) according to the procedure described by Lowe (1992). The homogenized peat sample was debituminized by treating three or four times with a benzene–ethanol mixture (1:1, v/v) and air-dried. The humic fraction was extracted with 0.1 *M* NaOH and desalinated using cation exchangers. To isolate HA, the obtained solution was acidified to pH 2. The precipitated HA were centrifuged, dialyzed and lyophylized. To isolate FA, the supernatant was discharged through the Amberlite XAD-2 resin (Aldrich, Deisenhofen, Germany). The sorbed FA were eluted with 0.1 *M* NaOH, desalinated, and lyophylized.

Elemental Analysis

Elemental analyses were performed on a Carlo Erba (Milan, Italy) Strumentazione 1106 elemental analyzer and calculated on an ash- and water-free basis. The contents of elements

for FA were (%) C = 49.0, H = 4.2, N = 2.0, and O = 44.8; and for HA were C = 55.2, H = 4.3, N = 2.4, and O = 38.1.

Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectra were acquired with a Bruker (Rheinstetten, Germany) DMX 500 NMR spectrometer operating at 500.13 MHz proton frequency. The spectra were recorded on peat HA and FA samples dissolved in 700 μL 0.1 *M* NaOD at concentrations of 25 mg/mL. All proton-detected NMR spectra were acquired with a 5-mm inverse geometry broadband probehead equipped with an actively shielded z gradient coil [90°(^{1}H) = 9.3 μs; 90°(^{13}C) = 9.8 μs]. One-dimensional ^{1}H NMR spectra were recorded using the first increment of the presat-NOESY sequence (acquisition time = 4.68 s, relaxation delay = 1.5 s, mixing time = 0 ms, exponential line broadening = 0.3 Hz). The ^{13}C NMR spectra were acquired with a 5-mm broadband probe, using inverse-gated wideband alternating-phase low-power technique for zero residual splitting (WALTZ-16) decoupling [relaxation delay = 8 s at 90°(^{13}C) = 9.4 μs; 28 672 (FA), 51 968 (HA) scans] with an acquisition time of 250 ms (spectral width = 65360 Hz) and an exponential line broadening of 25 Hz. The duration of relaxation time was set to 8 s on the basis of our previous investigations (Kovalevskii et al., 2000), which showed that time delays of 8 s or longer provided complete relaxation of quaternary carbon nuclei in HS. Hence, ^{13}C NMR spectra acquired under such conditions can be considered as quantitative.

Heteronuclear single quantum coherence [F2 (horizontal frequency axis in two-dimensional NMR spectra [proton-detected data]) (^{1}H): acquisition time = 205 ms at spectral width = 5000 Hz, one bond coupling constant ^{1}J(CH) = 150 Hz, relaxation delay D1 = 1.5 s; number of scans NS = 256; F1 (^{13}C): spectral width = 22 637 Hz (180 ppm), 256 increments] spectra were decoupled [^{13}C: globally optimized alternating-phase rectangular pulses (GARP) = 70 μs] and calculated to a 2k × 512 matrix with typical window functions in F2 (exponential line broadening = 30 Hz) and shifted sine bell (π/6) in F1. Gradient (length = 1 ms, recovery = 450 μs), but not sensitivity enhanced sequences were used for all indirectly detected spectra. The TOCSY spectra (acquisition time = 205 ms at spectral width = 5000 Hz) used composite pulse decoupling sequence (MLEV-17) mixing (70 ms) and solvent non-excitation (3-9-19 binominal pulse sequence; d19 = 222 μs) and 384 TPPI increments. The COSY spectra (2k × 1k matrix) were multiplied by a squared unshifted sine bell in F2 and F1; the TOCSY spectra (2k × 1k matrix) by a squared sine bell, shifted by π/6 in F2 and by a sine bell, shifted by π/6 in F1. Reference for ^{1}H-NMR was the residual HDO resonance at 4.63 ppm and ^{13}C was referenced to external CH_3OH in D_2O (49.00 ppm) at 303 K (for one-dimensional and two-dimensional NMR spectra).

Assignments Used for Calculation of the Model Two-Dimensional Nuclear Magnetic Resonance Spectrum of Humic Substances

The ^{1}H and ^{13}C chemical shift values of the humic constituents indicated in Fig. 7 were obtained from literature values or calculated by the ACD/Labs software (Advanced Chemistry Development, Pegnitz, Germany) NMR Proton and Carbon Predictor, Version 4.0, and put into display with the EXCEL format. The prediction of chemical shift proceeds with sufficient accuracy; this has been tested for a range of model compounds (polyphenols, terpenoid hydrocarbons, and peptides). Peptide chemical shifts of the 20 proteinaceous amino acids were compiled from a database on protein chemical shifts (Wishart et al., 1991, 1995), while the shifts of the other amino acids occurring in humic substances (Sparks, 1995, p. 62–64; Szajdak and Österberg, 1996) were calculated. Reference compounds used for the calculation of the chemical shifts included a softwood lignin (Sakakibara, 1990) and tannin structure (Hemingway and Karchesy, 1989); terpenoid hydrocarbons were composed as a sum of cholesterinacetate and hopane (Ourisson et al., 1979). Lipids used were phosphoenolpyruvate, lipoic acid, and dipalmitoyl–lecithine (Breitmaier and Voelter, 1990, p. 467); amino sugars (Zhang et al., 1997; Jahnel et al., 1998) were composed of the repeating units of various chitin derivatives (Stankiewicz and van Bergen, 1998, p. 154). Heterocyclic nitrogen included porphine as a pyrrol derivative, the four main nucleobases (Kuzyakov, 1997), and indol. Fatty acids and their esters were composed of the saturated long chain, unbranched type, and suberin (Doi, 1990) consisted of a polybutanoic and polypropanoic ester with α-methylene group with respect to carbonyl and an unbranched side chain in the α position to oxygen incremented from C_1 to C_5.

The assignments in Table 6 were given by the Bruker (Rheinstetten, Germany) reference two-dimensional HSQC spectra database SBASE and corroborated by calculation using the ACD predictor (see above), if not specified otherwise.

RESULTS AND DISCUSSION

One-Dimensional Nuclear Magnetic Resonance Spectra

The proton NMR spectra of FA and HA (Fig. 1) at first glance closely resemble each other. Both FA and HA exhibit prominent resonances in the carbohydrate region and the overall lineshape within this region is rather similar with an apparently reduced average linewidth of the individual resonances in FA. A more distinct variation of the resonance pattern is obvious in the

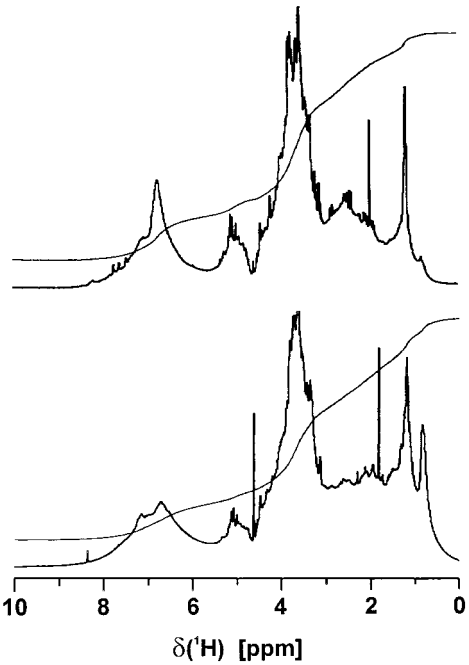

Fig. 1. The ^{1}H nuclear magnetic resonance (NMR) spectrum of the peat fulvic acid (FA) (top) and humic acid (HA) (bottom).

Table 1. Distribution of non-exchangeable hydrogen among different structural groups as determined from ^{1}H nuclear magnetic resonance (NMR) spectra of a peat humic acid (HA) and fulvic acid (FA).

Range of (^{1}H)	Assignment	Proportion of hydrogen in structural groups	
		FA	HA
ppm		%	
10.0–6.0	$C_{ar}H$	18.2	15.6
6.0–4.7	acetal	4.5	6.8
4.6–3.1	CH_nO	45.0	35.6
3.1–1.95	C_f-CH_n	18.1	17.1
1.95–0.5	C_{al}-CH_n	14.2	24.9

region of functionalized aliphatic resonances. The pronounced resonance maximum observed in the aromatic region of FA (6.77 ppm) is also present in the HA spectrum, but is strongly attenuated. Humic acids feature a prominent upfield resonance (0.84 ppm) displayed with much less intensity in FA, probably representative of methyl groups terminating aliphatic units. Strong upfield signals around 1.2 ppm occur both in FA and HA, but with a slightly displaced shift to a higher field in HA, which also appears to be composed of a larger variety of several overlapping resonances.

Integration of the ^{1}H NMR spectra of FA and HA according to specific regions of chemical shift provides the amounts of non-exchangeable protons shown in Table 1. About 25% of the non-exchangeable protons of HA are present in non-substituted aliphatic groups, whereas in FA this value reaches only 14%.

In general, lesser-substituted aromatics and carbohydrates appear to be more relevant constituents of FA than of HA, while unfunctionalized aliphatics are more prominent features of HA. This is confirmed by the appearance of the one-dimensional ^{13}C NMR spectra (Fig. 2).

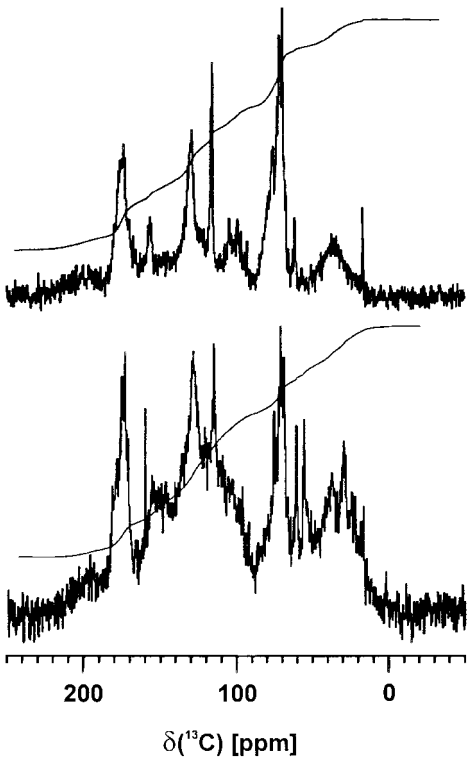

Fig. 2. The ^{13}C nuclear magnetic resonance (NMR) spectrum of the peat fulvic acid (FA) (top) and humic acid (HA) (bottom).

The ^{13}C NMR spectra of FA and HA are more distinctively different than the corresponding ^{1}H NMR spectra. The spectra differ significantly in all regions except the (so called) carbohydrate section ranging from 60 to 90 ppm. Humic acids show a higher fraction of purely aliphatic resonances ($\delta < 50$ ppm) and also more detail and resolution. Strong methoxyl resonances at $\delta = 53$ (methyl esters) and 57 ppm (aliphatic methyl ethers) occur in HA, while in FA only a faint resonance at $\delta = 57$ ppm is visible. The aromatic section within the ^{13}C NMR spectrum of FA exhibits two relatively sharp resonances at $\delta = 117$ and 132 ppm and a relatively narrow phenolic resonance at $\delta = 156$ ppm, while in HA these two high field resonances are superimposed to a relatively broad hump ranging from 95 to 160 ppm. In HA a distinct range of resonances appears downfield of a intensity minimum at 143 ppm in the phenolic region from 150 to 160 ppm. The intensity distribution within the carboxylic region of HA and FA is different. Due to low intensity and dependence on small baseline distortions the integration of the carbonyl region (220–187 ppm) cannot be performed with very good accuracy.

The resonance integrals of seven common spectral regions (Table 2) indicate for HA a reduced content of carboxylic carbons and an enhanced content of phenolic groups. In HA the integral value of the anomeric region is affected by contributions from overlapping high-field aromatic resonances, and therefore there is no good correlation with the CHO carbohydrate integral. As expected, the amount of nonpolar aliphatic and C-H and C-C aromatic carbons is much higher in HA than in FA. The carbohydrate content determined on the basis of the 47- to 90-ppm region is found to be substantially higher in FA. These ^{13}C NMR integral differences agree well with data of elemental analysis, which show a higher content of carbon for HA and a nearly equal content of hydrogen. This uniformity in hydrogen content is due to a compensating effect when in (for example) HA both contents of aromatics (with a low H to C ratio) and pure aliphatics with a high H to C ratio are increased relative to FA. The found content of car-

Table 2. Distribution of carbon among the main structural groups of a peat fulvic acid (FA) and humic acid (HA) as determined from their one-dimensional ^{13}C nuclear magnetic resonance (NMR) spectra.

Range of δ(^{13}C)	Assignment	Proportions of carbon in structural groups	
		FA	HA
ppm		%	
220–187	C=O	5.9	3.8
187–167	(C=O)-X-	14.2	10.8
167–145	C_{ar}-O,N	8.6	10.1
145–108	C_{ar}-C,H	25.3	28.7
108–90	O-C-O	8.4	8.4
90–47	O(N_f)-CH_n	27.4	22.2
47–0	CH_n	10.2	16.0

bohydrates is considered as relatively high for peat (Preston et al., 1989); this is indicative of highland peats.

As seen from Table 2, the relative content of aromatic carbon in HA is 1.15 referred to that of FA, but the ratio of aromatic protons in the ^{1}H NMR spectra of HA and FA is 0.85 (Table 1). That implies that the average degree of nonhydrogen substitution of aromatics in FA is by one quarter less than in HA. From the intensity distribution within the proton NMR spectrum it can be deduced that phenols (and possibly five-membered heterocyclic rings) in FA are on average even less substituted.

Correlated Spectroscopy Nuclear Magnetic Resonance Spectra

The COSY NMR spectrum of FA exhibits more numerous cross peaks than that of HA (Fig. 3). With a few exceptions all of the COSY cross peaks visible in HA also occur in FA. Most of the total cross peak intensity shows up in regions of chemical shift indicated in Table 3.

Fulvic acids show significant cross peak intensity in the region D_C with corresponding cross peaks barely visible in HA. These cross peaks show up in the same region as those of Hα-Hβ in peptides (Fig. 4), but follow a distinctively different pattern. Therefore, they are expected to result not from peptides, but from highly oxygenated side chains of ether and ester type. Correlations in the F1 upfield section originate from α-carbonyl and other functionalized aliphatics while those at higher values of F1 chemical shift are in α positions to heteroatoms. Strong cross peak intensity resulting from intracarbohydrate coupling is found in the regions C_C both in FA and HA.

Although typical HS show a high degree of aromatic substitution (four or five per ring), COSY cross peaks in region E_C indicate the occurrence of less substituted aromatics with vicinally coupled protons (1, 1,2; 1,3; 1,4; 1,2,3; 1,2,4; 1,2,5; and 1,2,3,4) that are characteristic of

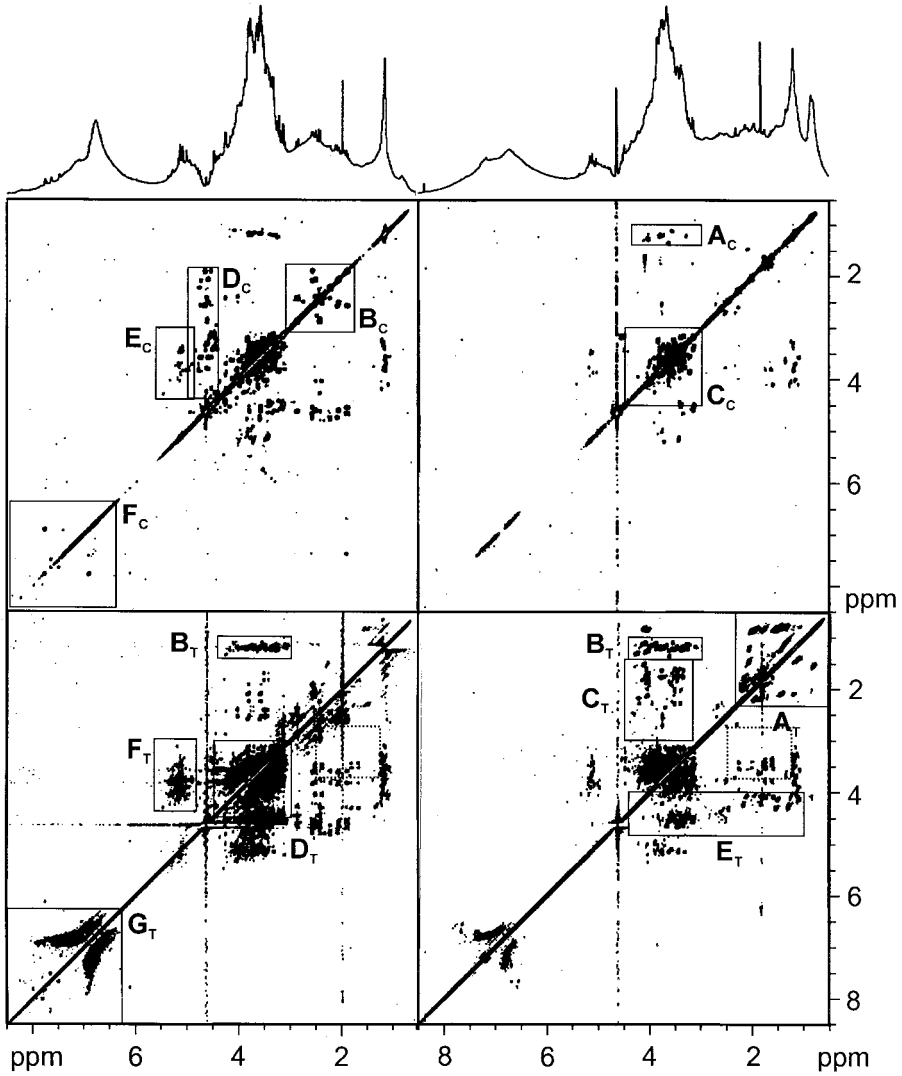

Fig. 3. Correlated spectroscopy (COSY) (top) and total correlation spectroscopy (TOCSY) (bottom: τ_{mix} = 70 ms) nuclear magnetic resonance (NMR) spectra of a peat fulvic acid (FA) (left) and humic acid (HA) (right) with sections of chemical shift indicated in Tables 3 (COSY) and 4 (TOCSY). For reasons of clarity only one set of boxes is shown (exception B_T); the dotted box indicated in the TOCSY spectra has identical chemical shift range to the dotted box within Fig. 4 (see text).

Table 3. Regions of chemical shift in the correlated spectroscopy (COSY) nuclear magnetic resonance (NMR) spectra of a peat fulvic acid (FA) and humic acid (HA).

Region	F2	F1	Assignment	Humic constituent
	ppm			
A_C	4.4–3.0	1.4–1.0	-C-CH-CH-O-	desoxy sugars, ethers, esters
B_C	3.2–1.8	3.2–1.8	-C_f-CH-C_fH-C-	intrafunctionalized aliphatic, β to heteroatoms
C_C	4.5–3.0	4.5–3.0	-CH(O)-CH(O)-	intracarbohydrate, without anomerics
D_C	5.0–4.4	4.4–1.8	-CH(O)-CH-C_f	functionalized aliphatic unit with one heteroatom
E_C	5.6–4.8	4.4–3.0	-O-CH(O)H-CH	intracarbohydrate, with anomerics
F_C	8.5–6.3	8.5–6.3	-C_{ar}H-C_{ar}H-	ortho protons in aromatic rings

plant phenolics and have been found also in materials at early stages of humification (Simpson et al., 1997).

The COSY cross peak intensity per weight unit sample of FA, normalized to an identical number of scans, is considerably larger than that of HA. This less pronounced self-cancellation of antiphase cross peak intensity is expected when individual resonances exhibit larger T_2 values and narrower line widths. Fulvic acids apparently exhibit an extended transverse relaxation time when compared with HA, which is probably caused by the presence of smaller, more mobile aggregates. In region A_C a range of cross peaks is observed only in HA and not found in FA. This is indicative of a class of alkylated carbohydrates occurring only in HA.

Total Correlation Spectroscopy Nuclear Magnetic Resonance Spectra

Both TOCSY NMR spectra of FA and HA (Fig. 4) exhibit a higher number of cross peaks than their respective COSY spectra (Fig. 3, Table 4). Within the region A_T of HA cross peaks indicate about a dozen correlations between the terminal methyl resonance (δ = 0.81 ppm) and aliphatic positions. Analogous cross peaks become visible in the COSY spectra only when calculated with soft truncating window functions in F2 and F1 such as π/2-shifted sine bells; then also aromatic cross peaks in the COSY spectra of FA and HA emerge. The cross peak location closely resembles those of intraaliphatic correlations within amino acids in peptidic bonds (see below). Other cross peaks not visible in COSY spectra show up in region C_T.

These cross peaks correspond to functionalized aliphatic chains of intermediate length ($C_{3–5}$). In contrast to COSY cross peaks, several of these new cross peaks indicate more extended spin systems and can be recognized as ridges of resonances with equal frequencies F1 (or F2). The average number of three or four cross peaks per ridge points toward highly functionalized aliphatic

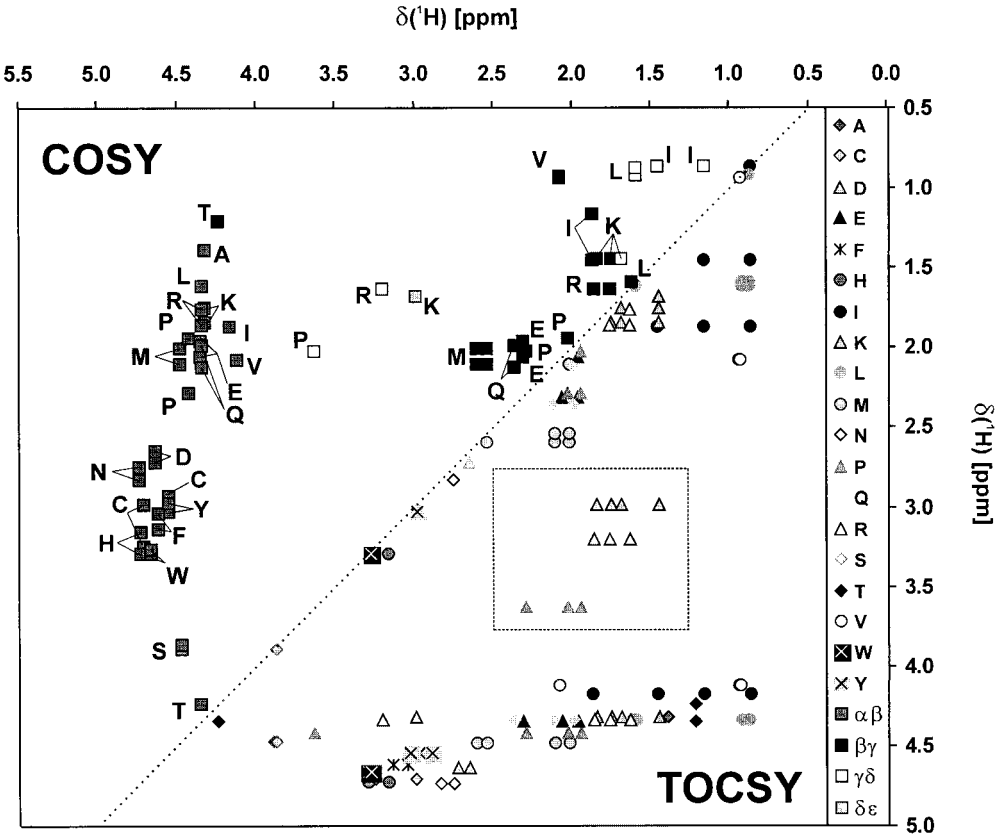

Fig. 4. Correlated spectroscopy (COSY) (upper left half) and total correlation spectroscopy (TOCSY) cross peaks (lower right half) of proteinaceous amino acids in proteins following alanine (A) according to chain position (COSY) and amino acid (TOCSY); see text (Wishart et al., 1995).

Table 4. Regions of chemical shift in the total correlation spectroscopy (TOCSY) nuclear magnetic resonance (NMR) spectra of a peat fulvic acid (FA) and humic acid (HA).

Region	F2	F1	Assignment	Partial structure
	ppm			
A_T	2.3–0.5	2.3–0.5	$-CH_n-CH_n-CH_n-CH_m$	intra aliphatic chains (m = 1–3; n = 1–2)
B_T	4.4–3.0	1.4–1.0	-O-CH-CH-C-	desoxy sugars, ethers, esters
C_T	4.5–3.2	3.0–1.4	$-N,O-CH-CH-CH_f$	functionalized aliphatic chain with one heteroatom
D_T	4.5–3.0	4.5–3.0	-CH(O)-CH(O)-	intra carbohydrate, without anomerics
E_T	4.4–1.0	4.8–4.0	$-(C=O)NH-CH-CH-C_f$	Hα-Hβ in peptides
F_T	5.6–4.8	4.4–3.0	-O-CH(O)-CH(O)-	intra carbohydrate, with anomerics
G_T	8.5–6.3	8.5–6.3	$-C_{ar}H-C_{ar}H-$	ortho protons in aromatic rings

chains with heteroatom substitution terminated by simple and functionalized aliphatics. A range of cross peaks within C_T is found only in HA, but not in FA. Taking into account the improved relaxation characteristics of FA compared with those of HA, these new cross peaks represent structural features characteristic of HA that are virtually absent in FA.

In the region E_T, a series of cross peaks of peptide origin occurs. This region is blanked in the F2 dimension by the non-excitation of the NaOD resonance (with limited bandwidth), but cross peaks are clearly visible along the F1 axis. The location of these peptide cross peaks is quite different from those in the D_C region in the COSY spectra of FA.

Compared with the appearance of the COSY cross peaks in HA, many more (about >30 instead of <10) correlations of anomeric positions (H1 with H2 protons) in carbohydrates are found in the section F_T within the TOCSY spectrum; FA behaves in an analogous manner. The relative intensity of these cross peaks increased when changing the TOCSY mixing time from 35 ms over 75 ms to 100 ms, showing that many of these correlations are characterized by rather small vicinal coupling constants.

In the aromatic region G_T a broad range of cross peaks is found across the whole range of aromatic chemical shifts. Cross peaks with upfield chemical shifts [$\delta(^1H) < 7$ ppm] belong to a class of mono- and doubly oxygenated (ortho and para hydroxyl or methoxyl) rings. Another prominent type of substitution patterns, which is ranging from aliphatic [$\delta(^1H) = 7–7.4$ ppm] up to deshielding ortho carbonyl or carboxyl substitution [$\delta(^1H) > 7.4$ ppm] is observed both in FA and HA. The individual cross peaks present in FA and HA differ, but clearly are indicative of multiple oxygen substitution in conjunction with shift neutral aliphatic and ortho deshielding substituents such as (substituted) carbonyl groups. In general, even while scaled with the relatively low signal intensity from the one-dimensional proton NMR spectra, only marginal cross peak intensity is found in the aromatic systems. This is indicative of highly substituted aromatic systems in HS with only a minor fraction of ortho proton substituted aromatic rings present.

Comparative Analysis of Correlated Spectroscopy and Total Correlation Spectroscopy Spectra

The COSY and TOCSY sequences represent the most important class of homonuclear two-dimensional NMR experiments in which cross peaks arise through the interaction of the J-coupled spins. However, the different mechanisms of magnetization transfer acting in COSY and TOCSY NMR sequences (Croasmun and Carlson, 1996; Cavanagh et al., 1996; Hertkorn et al., 2001) affect cross peak intensity and lineshape in molecularly irregular and polydisperse HS. These proton two-dimensional NMR pulse sequences are intrinsically sensitive but characterized by rather long transfer times (TOCSY: 1/2J; COSY: 1/J) due to small geminal (two bond) and vicinal (three bond) H,H coupling constants ($J < 15$ Hz). This extended duration makes them susceptible to signal loss in the case of fast transverse relaxation. In COSY spectra overlap of antiphase coherence causes an attenuation of cross peak intensity when J is less than or equal to the line width. Thus the disappearance of corresponding FA–HA cross peaks in the COSY relative to TOCSY spectra is thought to be caused by self cancellation of antiphase magnetization at increased linewidth ($\Delta\nu_{1/2} > 10–15$ Hz).

In TOCSY spectra phase sensitive detection of net magnetization transfer and choice of variable mixing times allow differentiation of small and extended spin systems. The TOCSY spectra of HS typically show an enhanced off diagonal cross peak intensity when compared with the respective COSY spectra. However, standard COSY spectra represent vicinal and geminal coupled spin pairs only and frequently produce better resolved cross peaks in the heavily overlapping ''carbohydrate region'' of HS than TOCSY spectra. Under standard conditions COSY cross peaks indicate almost exclusively vicinal couplings in aromatic rings. However, it is expected that many of the cross peaks visible only in TOCSY spectra also represent geminal and vicinal couplings between protons. Clear exceptions are cross peaks within section C_T (Fig. 3), and the intense TOCSY cross peak at F2/F1: 4.1/0.8 ppm, which appears at extended mixing times only. It most likely represents a ^4J long-range correlation between a heteroatom substituted position and a methyl group.

To enable an assignment of the complex spectral patterns within the sections D_C and E_T of the homonuclear two-dimensional NMR spectra, which both represent heteroatom substituted CH groups, Fig. 4 has been assembled from literature data (Wishart et al., 1995). It indicates both COSY and TOCSY cross peaks of the non-exchangeable protons of the proteinaceous amino acids at a chemical shift, when the respective amino acid in proteins is followed by alanine (A). Along the diagonal this figure is split into two sections of equal

size. The upper left half shows the peptidic cross peaks, caused only by geminal and vicinal couplings as observed in the standard COSY spectra. They are classified according to the positions within the amino acid side chain. On the lower right half the respective TOCSY cross peaks are indicated for the complete spin systems excluding aliphatic–aromatic and γε-methionine interactions that are absent due to low transfer efficiency. The TOCSY cross peaks are classified according to the amino acids. The TOCSY spectrum of HA shows most of these peptidic amino acid cross peaks at positions significantly different from the chemical shift values of free amino acids, which are not observed. In the COSY NMR spectrum of HA these cross peaks are strongly attenuated and mostly missing, probably due to self cancellation (see above). Similar patterns in the D_C section of the COSY spectrum of FA, at first glance looking like αβ- and βγ-amino acid correlations (Fig. 3 and 4) occur at significantly displaced chemical shift values. These cross peaks then most probably represent strongly oxidized and functionalized aliphatic chains with no aliphatic CH bonded solely to purely aliphatic units.

The dotted box within Fig. 4 is characterized by TOCSY cross peaks of the long chain amino acids lysine (K), proline (P), and arginine (R). The TOCSY and COSY cross peaks of FA and HA appearing in this section of the spectrum do not follow these patterns and are therefore not expected to originate from peptides but rather from other functionalized aliphatic groups. Of importance is that within this section a few cross peaks appear both in FA and HA, a feature not observed otherwise for noncarbohydrate (and possibly a few aromatic) cross peaks. Some of these cross peaks apparently exhibit identical F1 frequencies and indicate

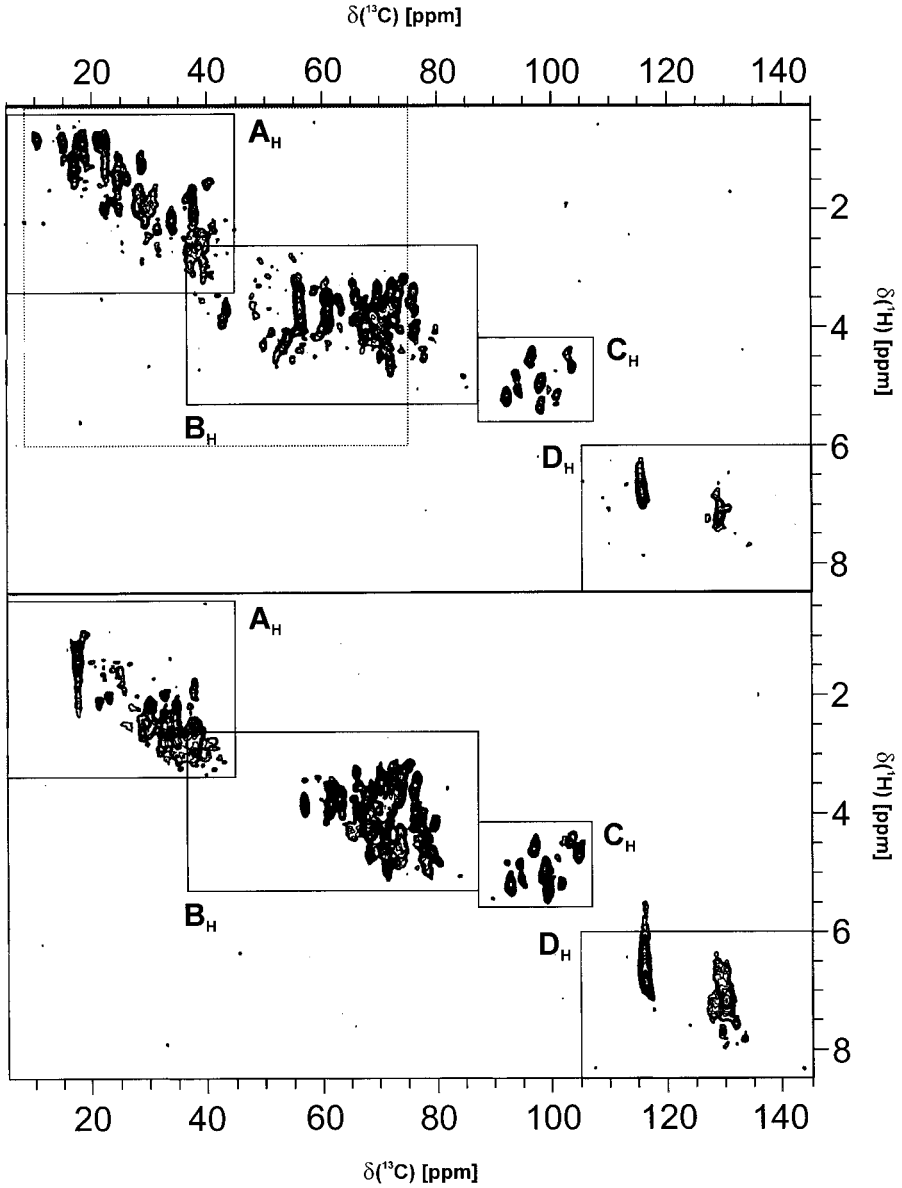

Fig. 5. Heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectra of a peat fulvic acid (FA) (bottom) and humic acid (HA) (top) with sections of chemical shift indicated in Table 5; the dotted box corresponds to the range of chemical shifts shown in Fig. 6.

Table 5. Main regions of chemical shift in the heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectra of a peat fulvic acid (FA) and humic acid (HA).

Region	F2 (^{1}H)	F1 (^{13}C)	Assignment	Partial structure
	ppm			
A_H	3.4–0.4	45–5	C-CH, C_f-CH	aliphatic groups
B_H	5.3–2.6	87–37	X-CH	single heteroatom substituted aliphatics
C_H	5.6–4.2	107–87	O-CH-X	double heteroatom substituted aliphatics (e.g., acetals)
D_H	9.0–6.0	150–105	C_{ar}-H	aromatic and heterocyclic systems

coupled systems mostly of intermediate chain length (C_{3-5}). The range of chemical shift displayed by this series of cross peaks requires a single directly bonded heteroatom and other remotely functionalized groups in the chain (e.g., in oxopropyl groups).

In the carbohydrate region F_T a redistribution of cross peak intensity occurs toward more intense H1–H2 correlations when compared with the COSY section E_C.

The increased ash content of HA may account for some loss of cross peak intensity, especially in the COSY spectra due to increased paramagnetic relaxation and induced aggregation.

Heteronuclear Single Quantum Coherence Spectra

The NMR pulse sequences that correlate directly bonded proton and carbon atoms with respect to their chemical shift show optimum transfer amplitudes in the 5- to 20-ms range [HSQC at $^1J(CH)$ = 150 Hz, 10.9 ms], and their relatively short duration with respect to average T_1 and T_2 relaxation times in humic substances (Preston and Blackwell, 1985; Fründ and Lüdemann, 1989) is more favorable to prevent signal loss in case of fast transverse relaxation. The large spread of frequency in F1 (^{13}C) dimension greatly reduces resonance overlap. These effects combined make HSQC NMR spectra very auspicious candidates to reveal structural information of HS in great detail.

The HSQC NMR spectra of FA and HA (Fig. 5) exhibit a substantial number of cross peaks, which can be divided into four main regions of chemical shift according to Table 5.

The cross peak locations of FA and HA closely correspond to each other in the region C_H. More substantial variation of cross peak location and relative intensity distribution is observed in regions D_H and B_H; while the ^{13}C low field section of B_H ($\delta > 60$ ppm) bears significant resemblance in FA and HA, most of the HA cross peaks at $\delta < 60$ ppm are missing in FA. Within section A_H, the cross peaks in FA and HA follow entirely different patterns. To understand the nature of the differences, the model HSQC cross peaks originating from proteinaceous amino acids in peptide bonds (Wishart et al., 1995) are displayed in Fig. 6, classified according to signal multiplicity and position within the side chain.

The dotted box in Fig. 5 corresponds to the range of chemical shift shown in Fig. 6. Within the section A_H strong resonances of all amino acid methyl positions except methionine (M) show up in HA, but not in FA. With the exception of the β cross peaks of the amino acids tryptophan (W), histidine (H), and cystine (C) almost all of the other β-ε cross peaks occur in HA as well as all of the α cross peaks. However, some additional HA cross peaks observed within the section A_H deviate substantially from the amino acid cross peaks given in Fig. 6. Those in the center of section A_H are likely to originate from terpenoid hydrocarbons, but also lipids, fatty acid derivatives, and aliphatic residues of desoxy sugars appear in this region of chemical shift (Fig. 7).

Despite the considerable nitrogen content of FA, peptidic amino acid cross peaks are apparently missing in FA. Polymethylene (1.2/29 ppm) is prominent in HA but almost absent in FA. Aliphatic methoxy (3.8/57 ppm) is more pronounced in HA than in FA. Within the section B_H a group of upfield ^{13}C cross peaks with chemical shift values around 50 pm and with proton chemical shifts ranging from 3 to 5 ppm not found in FA is present in HA. Among them are strong cross peaks originating from Cα in peptides (Fig. 6), but also more feeble ones appear. Apart from the strong glycine CH-α cross peak (3.8/42 ppm; Hertkorn et al., 2001) these cross peaks are assigned to amino sugar moieties containing a single amino group within a hexose unit according to their characteristic position in the proton and carbon frequencies.

In the section B_H the HSQC spectrum of FA and HA contains a group of cross peaks, assignable to CHOH fragments, usually considered as carbohydrates [δ(^{13}C):

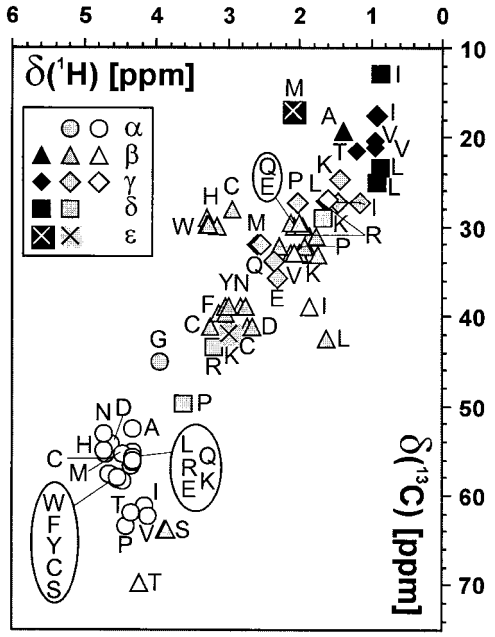

Fig. 6. Heteronuclear single quantum coherence (HSQC) cross peaks of proteinaceous amino acids in proteins following alanine (A), edited according to signal multiplicity (black = methyl, gray = methylene, white = methine) and position within the side chain (Wishart et al., 1995).

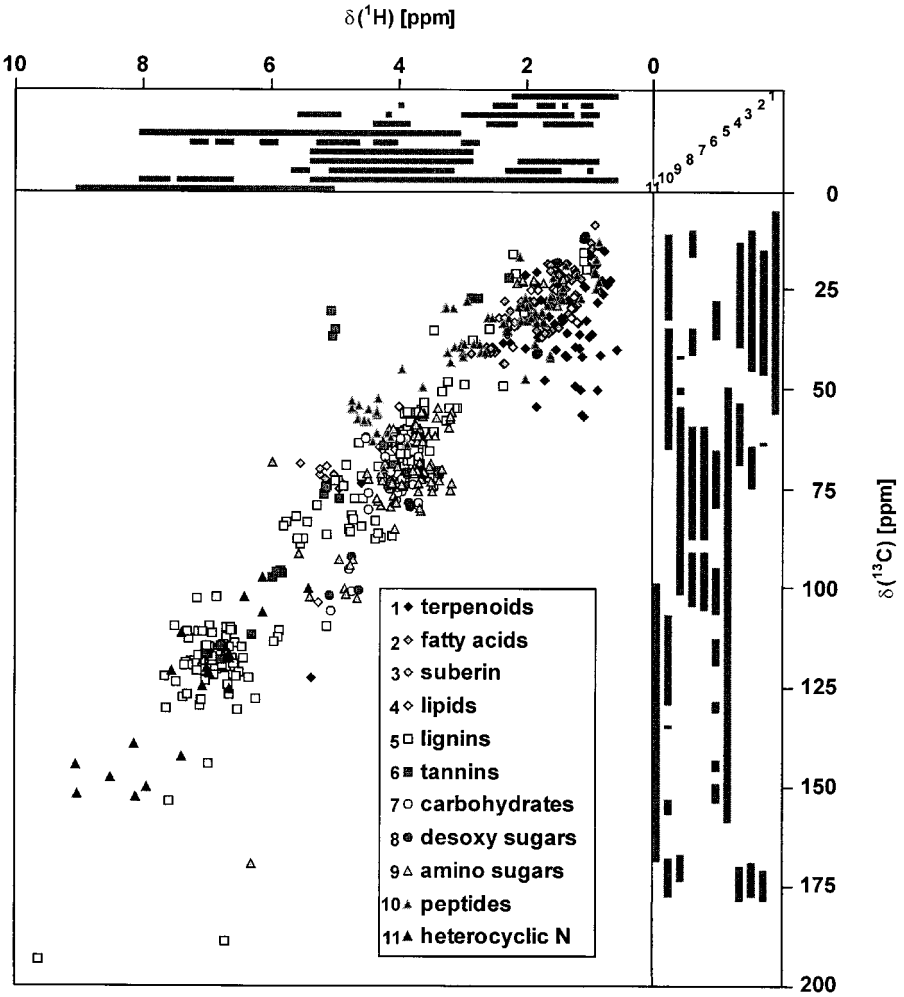

Fig. 7. Chemical shift ranges of eleven humic constituents with respect to their one-dimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and their ^{1}H and ^{13}C heteronuclear single quantum coherence (HSQC) NMR spectra.

67–85 ppm], including a significant cross peak at 3.9/62 ppm, representing C6 in hexoses. This group presents the most significant proportion of the visible resonances in FA and it is accompanied with intense and sharp correlations in the section C_H corresponding to anomeric $(CH)_1$ pairs of the carbohydrates. In the one-dimensional ^{1}H NMR spectrum these signals show partial overlap with CHOH and peptide resonances and the residual water resonance. The presence of the anomeric carbons indicates that the carbohydrates in FA and HA remain mainly intact. The location and intensity of cross peaks within the anomeric region C_H as well as those within the CHOH section of B_H are very similar in the peat FA and HA studied. The tentative assignments provided in Table 6 are based on the location of the HSQC cross peaks within the anomeric region, which show well-resolved resonances.

The positions of the cross-peaks slightly deviate from the published values for the free carbohydrates, since the carbohydrate moieties are bound to the humic core and apparently comprise a part of oligomeric chains. The intense and sharp resonances of rhamnose and fucose C1 carbon atoms are in good agreement with the presence of correlations assigned to CH_3CHO fragments, found in the A_C/B_T section within the COSY/TOCSY spectra of FA and HA. Additional small variations in cross peak location can be attributed to variable contributions from overlapping resonances.

Within the aromatic section two major cross peaks occur: an upfield resonance (6.8/116 ppm) representative of an ortho or para oxygen substituted and a set of a di-ortho aliphatic or hydrogen substituted aromatic rings (7.15/130 ppm). The latter cross peak is split into various resonances, at different positions in FA and HA. Downfield proton resonances [$\delta(^{1}H) > 7.5$ ppm], which are prominent features in the HSQC NMR spectrum of FA, indicate at least one deshielding ortho carbonyl derivative substituent in the ring. In HA a class of upfield ^{13}C resonances at $\delta(^{13}C) < 114$ ppm not visible in FA occurs. Oxygenated aromatic rings, like phenols and oxygenated heterocycles, show a substantial fraction of aromatic carbon upfield resonances.

Comparison of the Experimental and Model Heteronuclear Single Quantum Coherence Spectra of Humic Substances

To support the given detailed assignments, the model HSQC spectrum of HS was calculated using a priori information on the molecular building blocks of HS

Table 6. Proposed assignment of the anomeric region C_H of the heteronuclear single quantum coherence (HSQC) resonances.

T5 FA		T5 HA		
δ(^{13}C)	δ(^{1}H)	δ(^{13}C)	δ(^{1}H)	Tentative assignment
ppm				
91.7	4.83			ribopyranose
92.4	5.19	92.2	5.17	glucuronic acid; xylose; β-glucose in oligomers; terminal (reducing end) α-glucose in oligomers
93.8	4.85	93.7	4.81	oligomers of α-glucose
93.9	5.09	94.1	5.10	fucose; glucose; ribopyranose; galactose
96.6	4.56	96.1	4.58	glucuronic acid; fucose; xylose; mannose; terminal β-glucose in polymers
		96.5	4.51	arabinose; rhamnose; mannose; terminal (reducing end) β-glucose in oligomers
98.0	5.06	97.8	4.99	α-galactose and α-fucose in oligomers; oligomers of α-glucose
98.7	5.36	97.9	5.38	ribopyranose
98.7	5.22	98.0	4.89	lyxose
		98.3	5.23	β-galactose in oligomers
100.5	4.75	99.5	5.07	mannose and fucose in oligomers
101.0	5.19	100.9	5.13	galactose and fucose in oligomers
		100.7	5.31	α-glucose at branch point in polymers
102.0	4.48	102.7	4.44	cellulose fragments†; β-galactose and β-glucose in oligomers
104.3	4.71	103.5	4.67	cellulose fragments†

† Nehls et al., 1994.

deriving from the original natural products: aliphatic and terpenoid hydrocarbons, alkyl aromatics, carbohydrates, fatty acids and their esters, lignin and tannin derivatives (polyphenols), lipids, aromatic nitrogen compounds, peptides and proteins, phenols, and suberins (Kögel-Knabner et al., 1992; De Leeuw and Largeau, 1993; Jahnel et al., 1998; Schulten and Gleixner, 1999).

All of these building blocks are characterized by specific ranges of chemical shift in their one-dimensional ^{1}H and ^{13}C NMR spectra as shown in Fig. 7. In many regions at a given chemical shift significant overlap is observed from at least several constituents both in ^{1}H and ^{13}C NMR spectra. This superposition is more pronounced in the center and the shielded (upfield) sections. This ambiguity precludes an unequivocal resonance assignment according to the humic constituent specified above based solely on the chemical shift in one-dimensional NMR spectra. So, the so called ''carbohydrate region'' of proton chemical shift (Number 7 in Fig. 7) features resonances originating from carbohydrates, lignin side chains, peptides, ethers, and esters. The significant spread in ^{13}C frequency allows a separate identification of several classes of these constituents according to specific ranges within the ^{1}H,^{13}C HSQC NMR spectrum. As an example, CH-α positions of peptides exhibit significant deshielding in proton chemical shift but relative shielding in δ(^{13}C) and show therefore a clustering in a region that is not occupied by other humic constituents. Methoxy resonances from lignin occupy a rather restricted region of average proton chemical shift and are displaced to higher field in the carbon frequency, when compared with peptidic CH-α positions. Compared with normal carbohydrates, CH in α-position to amino groups in amino sugars are significantly shielded both in ^{1}H and ^{13}C frequencies. Phenylpropanoid lignin side chains occupy a chemical shift range between peptidic CH-α and carbohydrate methine positions and another extended region between carbohydrate ring and anomeric resonances. Heterocyclic nitrogen compounds are characterized by maximal downfield shift of ^{1}H and ^{13}C frequencies. Depending on the degree of α, β, and γ carbon substitution, terpenoid hydrocarbons occupy a considerable range of upfield carbon shifts, but retain a most upfield position in the proton frequency. Strong clustering of specific chemical environments is expected for tannins (C ring linkage: 4.8/37 and C5 in A ring: 5.9/96 ppm), methyl ethers (3.8/55 ppm), anomeric positions in carbohydrates (105–96/5–4 ppm), and suberin (2.55/45 ppm), to name a few.

Compared with the appearance of the model HSQC NMR spectrum (Fig. 7), the HSQC NMR spectrum of HA (Fig. 5) is dominated by resonances from a few humic constituents, namely by those of peptides (sections A_H and B_H), carbohydrates (sections B_H and C_H), polymethylene (section A_H), and aliphatic methoxyl (section B_H). In the aromatic section D_H of both FA and HA two major groups of cross peaks of nonprotein origin dominate, and most probably represent an assembly of oxygenated aromatic systems. While aliphatic methoxy and carbohydrate resonances almost exclusively make up the section B_H of FA, the aliphatic section A_H exhibits cross peaks resembling lipid structures. At lower countour levels many more cross peaks appear, which can be traced to other humic constituents.

CONCLUSIONS

The proportion of the various sources of organic matter in humic substances is related to maturity, depositional environment, and degree of degradation (Lu et al., 2000). The combined analysis of several two-dimensional NMR spectra provides superior resolution of structural detail when compared with an exhaustive analysis of one-dimensional NMR spectra alone. Here, differences in structure and composition are more clearly revealed in ^{13}C NMR spectra than in ^{1}H NMR spectra.

The very good resolution of structural detail by a combined analysis of all NMR spectra shows that the effect of the fractionation procedure on the composition and chemical structure of humic materials is very significant.

Both homo- and heteronuclear two-dimensional NMR spectra demonstrate that the carbohydrate structures in FA and HA are remarkably similar, but carbohydrates occupy an increased share within FA. A more detailed resonance assignment is possible starting with an analysis of anomeric cross peaks in the HSQC NMR spectrum.

As visible in the ^{13}C NMR spectrum, the aromatic carbon fractions of FA and HA are clearly different, but the aromatic part of HA contains features found in FA, such as the rather strong resonances at 117 and 132 ppm and the strong HSQC cross peaks derived therefrom that resemble resonances typical of lignin units. The phenolic section in FA appears to be specific of FA and those resonances are not prominent in HA.

The aromatic region D_H in HA and FA is not dominated by cross peaks from aromatic amino acids in peptidic bonds.

However, despite differences in cross-peak location and intensity, a similarity of the basic aromatic substitution patterns of HA and FA is obvious from the two-dimensional NMR spectra: highly substituted aromatic rings dominate, such as multiple oxygenated rings, which induce strong upfield shifts from ortho and para positions in $\delta(^1H)$ and $\delta(^{13}C)$. Alkyl substituted rings generate the strong HSQC crosspeak at δ = 7.2/129 ppm. Cross peaks with strongly deshielded proton resonances [$\delta(^1H) > 7.5$ ppm] are caused by ortho substitution with carbonyl derivatives, which are characterized by a chemical shift anisotropy with strong proton deshielding effect (Günther, 1992, p. 76–91). Cross peaks originating from the broad hump, observed in the ^{13}C NMR spectrum of HA, will be difficult to become detected under standard conditions, since the average degree of proton substitution of aromatic rings within this section will be disproportionally low and so will be the scaling factor of two-dimensional NMR cross peaks. Furthermore, the transverse relaxation within these extended aromatic systems is expected to be fast (Fan et al., 2000).

The composition of the aliphatic part in FA and HA is fundamentally different. Differences in lineshape are already visible from the one-dimensional NMR spectra even when showing almost coinciding amounts of functionalized aliphatic compounds, but the HSQC cross peak location within the whole range of the aliphatic section has almost no correspondence. Fulvic acids are deprived in the typical aliphatic constituents of HA such as polymethylene, peptide side chains, and terpenoid hydrocarbons and do not exhibit readily identifiable components. Fulvic acids also lack other methyl groups in extended aliphatic systems and the aliphatic region is mostly comprised of functionalized aliphatic units. Long chain alkyl and highly branched unsubstituted alkyls are nearly absent in FA; that is, when branching occurs in aliphatic units within FA, a heteroatom or an aromatic ring will be typically not further than two or three bonds away from the terminal, but also from any other aliphatic carbon. The very strong proton NMR signal at δ = 1.17 in FA is not caused by polymethylene (which is present only in HA at δ = 1.22/29.6 ppm) since it is connected to a carbon atom with δ = 18 ppm. This aliphatic resonance shows a series of strong cross peaks in the COSY (A_C) and TOCSY (B_T) spectra, indicating that this resonance could be caused by a series of methylated carbohydrates. A part of the low field ^1H and ^{13}C cross peaks within section A_H (centered around 2.7/37 ppm) is possibly caused by lipids, which then also would produce a share of the cross peaks within section D_C of the COSY spectrum.

Humic acids show prominent peptidic cross peaks both in homo- and heteronuclear two-dimensional NMR spectra and so, a significant fraction of the upfield resonances in the NMR spectra of HA can be attributed to peptide side chains. Terpenoid hydrocarbons, which are characterized by a considerable fraction of isolated methyl groups not coupling to other protons (Buddrus and Lambert, 1995; Lambert and Buddrus, 1996) and carbon resonances shifted to higher field [$\delta(^{13}C) < 25$ ppm; Hertkorn et al., 2001] are found mainly in HA and not in FA. Aliphatic methoxyl resonances, which also do not couple to other protons in standard homonuclear shift correlated two-dimensional NMR spectra exhibit a much stronger cross peak in the HSQC spectrum of HA than in that of FA.

With respect to the chemical environment of nitrogen an analysis of the TOCSY and HSQC spectra reveals pronounced differences in FA and HA even when those are characterized by similar values of nitrogen content and C to N ratio (FA = 28.6, HA = 26.8). Peptidic amino acid cross peaks present in the TOCSY and HSQC NMR spectra of HA are not found in FA. Some HSQC cross peaks of minor intensity in the 3.3/50 range may be representative of amino sugars and are present in HA only. With clear signs of amino sugars and amino acid residues being absent in the NMR spectra the chemical environment of nitrogen in FA is not readily recognizable. In the absence of amino sugars and peptides, amine and heterocyclic nitrogen atoms (indol, pyrrol, and pyridine derivatives) are likely candidates for nitrogen structures in FA.

From the obtained NMR results on the peat humic materials studied, FA appears to be diagenetically downstream of HA in line with the biopolymer degradation (BD) model of humification. Humic acids consist of an aromatic core with some distinct features resembling lignine structures. Both carbohydrates and peptides are part of this structure, possibly enriched at the surface and therefore more mobile and more easily to detect by NMR (Fan et al., 2000). Carbohydrate–lignin complexes (Shevchenko and Bailey, 1996) intimately associated by strong hydrogen bonds may survive partly during degradation; this could explain the close resemblance of carbohydrates observed in FA and HA. Polymethylene and terpenoid hydrocarbons are other aliphatic constituents of HA. In FA both nitrogen and aliphatic structures are more difficult to evaluate on the basis of molecularly characterized structures. Fulvic acids seem to comprise a very far-processed stage of humification.

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REFERENCES

Bortiatynski, J.M., P.G. Hatcher, and H. Knicker. 1996. Humic and fulvic acids. ACS Symp. Ser. 651. Am. Chem. Soc., Washington, DC.

Bovey, F.A., and P.A. Mirau. 1996. NMR of polymers. Academic Press, San Diego, CA.

Breitmaier, E., and W. Voelter. 1990. Carbon-13 NMR spectroscopy. High-resolution methods and applications in organic chemistry and biochemistry. VCH–Wiley, New York.

Buddrus, J., P. Burba, H. Herzog, and J. Lambert. 1989. Quantification of partial structures of aquatic humic substances by one- and two-dimensional solution ^{13}C nuclear magnetic resonance spectroscopy. Anal. Chem. 89:628–631.

Buddrus, J., and J. Lambert. 1995. Isolated paraffinic methyl groups in humic substances. Org. Geochem. 23:269–271.
Cavanagh, J., W.J. Fairbrother, A.G. Palmer, and N.J. Skelton. 1996. Protein NMR spectroscopy, principles and practice. Academic Press, London.
Chien, Y.-Y., and W.F. Bleam. 1998. Two-dimensional NOESY nuclear magnetic resonance study of pH-dependent changes in humic acid conformation in aqueous solution. Environ. Sci. Technol. 32: 3653–3658.
Croasmun, W.R., and R.M.K. Carlson. 1996. Two-dimensional NMR spectroscopy. Wiley-VCH, Weinheim, Germany.
De Leeuw, J.W., and C. Largeau. 1993. A review of macromolecular organic compounds that comprise living organisms and their role in kerogen, coal, and petroleum formation. p. 23–72. *In* M.H. Engle and S. Macko (ed.) Organic geochemistry. Plenum, New York.
Doi, Y. 1990. Microbial polyesters. VCH Verlagsgesellschaft, Weinheim, Germany.
Fan, T.W.-M., R.M. Higashi, and A.N. Lane. 2000. Chemical characterization of a chelator-treated soil humate by solution-state multinuclear two-dimensional NMR with FTIR and pyrolysis-GCMS. Environ. Sci. Technol. 34:1636–1646.
Fründ, R., and H.-D. Lüdemann. 1989. The quantitative analysis of solution- and CPMAS-C-13 NMR spectra of humic material. Sci. Total Environ. 81/82:157–168.
Günther, H. 1992. NMR-Spektroskopie: Grundlagen, Konzepte und Anwendungen der Protonen- und Kohlenstoff-13 Kernresonanz-Spektroskopie in der Chemie. Thieme, Stuttgart, Germany.
Haiber, S., P. Burba, H. Herzog, and J. Lambert. 1999. Elucidation of aquatic humic partial structures by multistage ultrafiltration and two-dimensional nuclear magnetic resonance spectrometry. Fresenius J. Anal. Chem. 364:215–218.
Hatcher, P.G., and E.C. Spiker. 1988. Selective degradation of plant biomolecules. p. 58–74. *In* F.H. Frimmel and R.F. Christman (ed.) Humic substances and their role in the environment. John Wiley & Sons, New York.
Hedges, I.J., and R.G. Keil. 1999. Organic geochemical perspectives on estaruine processes: Sorption reactions and consequences. Mar. Chem. 65:55–65.
Hedges, I.J., and J.M. Oades. 1997. Comparative organic geochemistries of soils and marine sediments. Org. Geochem. 27:319–361.
Hedges, J.I. 1988. Polymerization of humic substances in natural environments. p. 45–58. *In* F.H. Frimmel and R.F. Christman (ed.) Humic substances and their role in the environment. John Wiley & Sons, New York.
Hemingway, R.W., and J.J. Karchesy. 1989. Chemistry and significance of condensed tannins. Plenum Press, New York.
Hertkorn, N., Ph. Schmitt-Kopplin, I.V. Perminova, D. Kovalevskii, and A. Kettrup. 2001. Two dimensional NMR spectroscopy of humic substances p. 149–158. *In* R.S. Swift and K.M. Spark (ed.) Proc. of the 9th Int. Conf. of the Int. Humic Substances Soc. Understanding and Managing Organic Matter in Soils, Sediments and Waters, University of Adelaide, Australia. 21–25 Sept. 1998. IHSS, St. Paul, MN.
Jahnel, J.B., P. Ilieva, G. Abbt-Braun, and F.H. Frimmel. 1998. Aminosäuren und Kohlenhydrate als Strukturbestandteile von refraktären organischen Säuren. Vom Wasser. 90:205–216.
Kingery, W.L., A.J. Simpson, M.H.B. Hayes, M.A. Locke, and R.P. Hicks. 2000. The application of multidimensional NMR to the study of soil humic substances. Soil Sci. 165:483–494.
Kögel-Knabner, I., J.W. de Leeuw, and P.G. Hatcher. 1992. Nature and distribution of alkyl carbon in forest soil profiles: Implications for the origin and humification of aliphatic biomacromolecules. Sci. Total Environ. 117/118:175–185.
Kovalevskii, D.V., A.B. Permin., I.V. Perminova, and V.S. Petrosyan. 2000. Choice of the time of pulse delay for quantitative ^{13}C NMR spectroscopy of humic substances. Bull. Moscow Univ. (Vestnik MGU) Ser. 2 (Chem.) 41:39–42.
Kuzyakov, Y.V. 1997. The role of amino acids and nucleic bases in turnover of nitrogen and carbon in soil humic fractions. Eur. J. Soil Sci. 48:121–130.
Lambert, J., and J. Buddrus. 1996. Quantification of isolated methyl groups in aquatic humic substances by means of ^{1}H and ^{13}C NMR spectroscopy. Magn. Reson. Chem. 34:276–282.
Lowe, L.E. 1992. Studies on the nature of sulfur in peat humic acids from the Fraser River delta, British Columbia. Sci. Total Environ. 113:133–145.
Lu, X.Q., J.V. Hanna, and W.D. Johnson. 2000. Source indicators of humic substances: An elemental composition, solid state ^{13}C CP/MAS NMR and Py-GC/MS study. Appl. Geochem. 15:1019–1033.
MacCarthy, P., and J.A. Rice. 1988. An ecological rationale for the heterogeneity of humic substances. p. 339–345. *In* S.H. Schneider and P.J. Boston (ed.) Proc. of Chapman Conf. on the Gaia Hypothesis, San Diego, CA. 7–11 Mar. 1988. MIT Press, Cambridge.
Nehls, I., W. Wagenknecht, B. Philipp, and D. Stscherbina. 1994. Characterization of cellulose and cellulose derivatives in solution by high resolution ^{13}C-NMR spectroscopy. Prog. Polym. Sci. 19:29–78.
Ourisson, G., P. Albrecht, and M. Rohmer. 1979. The hopanoids. Pure Appl. Chem. 51:709–729.
Preston, C.M. 1996. Applications of NMR to soil organic matter analysis: History and prospects. Soil Sci. 161:144–166.
Preston, C.M., D.E. Axelson, M. Levesque, S. Mathur, H. Dinel, and R.L. Dudley. 1989. Carbon-13 NMR and chemical characterization of particle-size separates of peats differing in degree of decomposition. Org. Geochem. 14:393–403.
Preston, C.M., and B.A. Blackwell. 1985. Carbon-13 nuclear magnetic resonance for a humic and a fulvic acid: Signal-to-noise optimization, quantitation, and spin-echo techniques. Soil Sci. 139:88–96.
Sakakibara, A. 1990. Chemistry of lignin. p. 160. *In* N.-S. Hon and N. Shisraishi (ed.) Wood and cellulosic chemistry. Marcel Dekker, New York.
Saunders, M., W. Wishnia, and J.G. Kirkwood. 1957. The nuclear magnetic resonance spectrum of ribonuclease. J. Am. Chem. Soc. 79:3289–3290.
Schmitt-Kopplin, Ph., N. Hertkorn, H.R. Schulten, and A. Kettrup. 1998. Structural changes in a dissolved soil humic acid during photochemical degradation processes under O_2 and N_2 atmosphere. Environ. Sci. Technol. 32:2531–2541.
Schulten, H.R., and G. Gleixner. 1999. Analytical pyrolysis of humic substances and dissolved organic matter in aquatic systems: Structure and origin. Water Res. 33:2489–2498.
Shevchenko, S.M., and G.W. Bailey. 1996. The mystery of the lignin-carbohydrate complex: A computational approach. THEOCHEM 364:197–208.
Simpson, A.J., R.E. Boersma, W.L. Kingery, R.P. Hicks, and M.H.B. Hayes. 1997. Humic substances, peats and sludges. p. 46–62. *In* M.H.B. Hayes and W.S. Wilson (ed.) Applications of NMR spectroscopy for studies of the molecular compositions of humic substances. Royal Soc. of Chem., Cambridge.
Sparks, L. 1995. Environmental soil chemistry. Academic Press, San Diego, CA.
Stankiewicz, B.A., and P.F. van Bergen. 1998. Nitrogen-containing macromolecules in the bio- and geosphere. ACS Symp. Ser. 707. Am. Chem. Soc., Washington, DC.
Stevenson, F.J. 1982. Humus chemistry. John Wiley & Sons, New York.
Szajdak, L., and R. Österberg. 1996. Amino acids present in humic acids from soil under different cultivations. Environ. Int. 22:3331–3334.
Wang, L., X. Mao, and Y. Yang. 1998. Application of newly-developed ^{1}H NMR techniques to the study of humic acids. Bopuxue Zazhi 15:411–420.
Wishart, D.S., C.G. Bigman, A. Holm, R.S. Hodges, and B.D. Sykes. 1995. ^{1}H, ^{13}C, and ^{15}N random coil NMR chemical shifts of the common amino acids. I. Investigations of nearest-neighbor effects. J. Biomol. NMR 5:67–81.
Wishart, D.S., B.D. Sykes, and F.M. Richards. 1991. Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. J. Mol. Biol. 222:311–333.
Zhang, X., W. Amelung, Y. Yuan, and W. Zech. 1997. Amino sugars in soil of the North American cultivated prairie. Z. Pflanzenernähr. Bodenkd. 160:533–538.