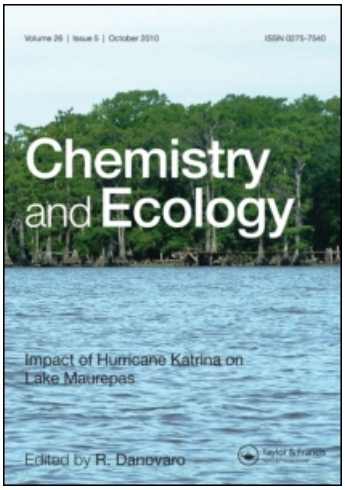

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^{1}H-NMR and ^{13}C-NMR spectroscopy of chernozem soil humic acid fractionated by combined size-exclusion chromatography and electrophoresis

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Humic acid (HA) from chernozem soil was fractionated using combination size-exclusion chromatography–polyacrylamide gel electrophoresis, and three fractions showing distinct electrophoretic mobilities (EM) and molecular sizes (MS) were obtained. Unfractionated HA, all fractions and HA after 7 M urea treatment (HAU) were examined using both solid ^{13}C and ^{1}H in D_2O and DMSO-d_6 NMR. The ratio $C_{\text{ar (165–108 ppm)}}/C_{\text{alk (108–0 ppm)}}$ increased by a factor of 5 from the highest to the lowest MS fraction. This suggests that there is significant heterogeneity in terms of molecular structure and functionality among the various fractions, which differ in both EM and MS. A significant contrast in aromatic range was observed between ^{1}H-NMR spectra of the original unfractionated HA and HAU in DMSO-d_6 solvent. Taking into account that the high MS fraction consists mainly of aliphatic components and the low MS fraction mainly of aromatic components, it is reasonable to suggest that these fractions have different chemical structures. These results are of great environmental importance because different MS fractions might be differently involved in chemical/biological processes in soils.

Keywords: soil humic acid; ^{1}H-NMR and ^{13}C-NMR; PAGE; size-exclusion chromatography

1. Introduction

Soil humic substances (HS), traditionally fractionated into humic acids (HAs) (insoluble in acidic media) and fulvic acids (FAs) (soluble both in alkali and acidic media), are heterogeneous mixtures of the decay products of plant and animal biomass in terrestrial environments. Increasing interest has been given to HS in the past 20 years because they serve as an effective carbon sink, reducing gas emissions and the greenhouse effect, and are solar-energy absorbant. They play an important role in regulating the mobility and fate of plant nutrients and environmental contaminants. Following the absorption of solar radiation, HS initiate a number of photochemical processes, producing radicals and/or other chemical species able to promote the transformation of organic chemicals (pesticides, herbicides and other organic pollutants). HS are composed of an

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array of polar and nonpolar functional groups, including carboxyl, ester, ether, hydroxyl, phenolic, amide, aliphatic and aromatic groups. The detailed molecular structure of HS remains unclear and research has not yet demonstrated convincingly whether HS are cross-linked macromolecules or loosely held aggregates. The fractionation of HS according to electrophoretic mobility (EM) and/or molecular size (MS) is a common procedure in the study of this complex, polydispersed natural mixture. Thus, size-exclusion chromatography (SEC) and ultrafiltration (UF) have been extensively used as rapid and versatile techniques resulting in preparative quantities of HS fractions of differing MS [1]. Electrophoretic methods help in the detailed characterisation of HS, but it is very difficult to relate the electrophoretic results to those of other fractionation methods [2–3].

We have previously [4] developed an effective method for the fractionation of soil humic acids (HAs) based on the combined use of SEC on Sephadex G-75 and polyacrylamide gel electrophoresis (PAGE). This procedure is called SEC–PAGE. The novelty and advantage of this combination rests primarily in the presence of urea, which is added at a level of 7 M in both SEC and PAGE to assist in the rupture of hydrogen bonds and prevents interaction between the fractionated humic material and the solid immobile phase on which the substances are separated [5]. This is accompanied by the disaggregation of HA, which we believe is primarily related to the disruption of hydrogen bonds. The proposed procedure allows separation of primary disaggregated structural humic components, thereby solving some of the key problems occurring in the fractionation of HS, for example, irreversible/reversible chromatographic column adsorption and the influence of PAGE chemical components (e.g. Tris and borate ions) on the composition of HA fractions. Using PAGE in combination with SEC enables preparative amounts of fractions differing in both EM and MS to be obtained.

Using the SEC–PAGE approach, we examined chernozem soil HA from the central European part of Russia. The technique allowed us to separate up to three fractions whose structural chemistry has been studied previously [6–10], but is here examined using both solid- and liquid-state NMR.

NMR spectroscopy is a very powerful tool used successfully to estimate the content and relative abundance of various chemical groups in HS from different environments [11–14]. Moreover, NMR spectroscopy has been the most successful technique in the examination of soil HAs fractionated by different methods [11, 15–18]. However, NMR has not been employed to examine soil HA simultaneously fractionated on the basis of MS and EM.

2. Materials and methods

2.1. *Soil sample and isolation of HA*

Chernozem soil is one of the most fertile found in a considerable region (central and south) of the European and Asian parts of Russia [19]. Our soil sample was taken from the A horizon of a chernozem soil, Kursk region, in the European part of Russia. The methods used to extract and purify HA have been reported elsewhere [7].

2.2. *Chemical analysis*

C, H and N analyses were performed on a Perkin–Elmer 2400 CHN Analyzer, Series II. Values are reported on a moisture- and ash-free basis. The accuracy of the measurements given in Table 1 was 95% or better.

Table 1. Carbon, ash and water weight content (%), atomic ratios (H/C and C/N) of whole chernozem HA and its electrophoretic fractions (obtained on ash- and moisture-free basis), and relative weight of fractions (%).

Sample	C	H/C	C/N	Ash	Water	Relative amount in weight (%)
HA	62.5	0.89	13.3	5.4	9.6	
Fraction A	53.7	1.23	7.8	6.7	10.3	24
Fraction B	57.2	0.97	10.8	10.4	10.7	19
Fraction C+D	57.0	0.99	15.8	9.4	10.2	36
Remaining fractions	n.d.	n.d.	n.d.	n.d.	n.d.	21

Notes: Remaining fractions mostly correspond to a mixture of B and C+D. n.d., not determined.

2.3. *Fractionation of HA*

Fractionation of soil HA by SEC–PAGE (i.e. using PAGE for subsequent testing of SEC aliquots from different sections of the elution profile) has been reported previously [4]. Briefly, 10 mg of HA was dissolved in 7 M urea and loaded onto a Sephadex G-75 (Pharmacia, Sweden) column (1.5 × 100 cm), equilibrated with the same solution. The void (V_o) and total (V_t) column volumes were 47 and 160 mL, respectively. V_o was determined using Dextran Blue 2000. Fractionation ranges for Sephadex G-75 were 80,000 to 3000 for proteins and 50,000 to 1000 for polysaccharides. The flow rate was 15 mL · h^{-1}. The UV-detector (ISCO, USA) was fixed at 280 nm. Column effluent was collected as 2 mL aliquots using a fraction collector (ISCO) and each third aliquot was assayed by PAGE in the presence of denaturating agents. The apparatus was a vertical electrophoresis device (LKB 2001 Vertical Electrophoresis, Sweden) with a gel slab (20 × 20 cm). Electrophoresis was carried out in 10% polyacrylamide gel at room temperature for 1 h at a current intensity of 25 mA. We used 89 mM Tris-borate, pH 8.3, with 1 mM EDTA and 7 M urea as the gel buffer. The sample buffer (0.05 mL) contained 89 mM Tris-borate, pH 8.3, 7 M urea, 1% SDS and 1 mM EDTA. On the basis of PAGE analysis, three fractions (A, B and C+D) of differing EM were obtained from HA. Fractions were dialysed for seven days against distilled water (the cut-off of the dialysis membrane was 5000), lyophilised and used for physical–chemical analyses. The fractionation procedure was repeated 200 times to collect sufficient material for NMR analysis. The chromatographic curves were essentially identical throughout the preparative sequence. The percentage weight distribution of each HA fraction (Table 1) was calculated using the ratio $W_i / \Sigma W_i$, where W_i is the weight of the fraction and ΣW_i is the weight of all HA fractions obtained after SEC fractionation of HA.

2.4. *HA urea treatment*

To investigate the effect of urea treatment on HA chemical structural changes, 200 mg of unfractionated HA was dissolved in 7 M urea for two days, then dialysed for seven days against distilled water (cut-off of the dialysis membrane was 5000), lyophilised and used for further physical–chemical analyses.

2.5. *^{1}H-NMR spectroscopy*

Original unfractionated HA, HA treated with urea and the electrophoretic fractions obtained by SEC–PAGE were all dissolved at a concentration of 2.5 mg · mL^{-1} in alkaline deuterated water (D_2O+0.1 N NaOH) or fully deuterated dimethyl sulfoxide (DMSO-d_6). ^{1}H-NMR spectra were acquired at 400 MHz with a Varian UNITY Plus spectrometer fitted with a multinuclear probe. A 90° pulse programme was used for 1D acquisition of ^{1}H spectra with an acquisition time of 3.744 s, a relaxation delay of 1.0 s and 512 scans.

2.6. *Solid-state ^{13}C-NMR spectroscopy*

Solid-state ^{13}C-NMR spectra were obtained using the basic ramp cross-polarisation pulse programme with two pulse phase-modulated (TPPM) decoupling using SPINAL64 on a 400 MHz Bruker AVANCE II with ^{1}H resonating at 400 MHz and ^{13}C at 100 MHz. Approximately 80 mg of sample was placed in a 4 mm NMR rotor and sealed with a Kel-F cap. Samples were spun at the magic angle (54.7°) with a frequency of 15 kHz, a contact time of 2.0 ms and a 1.5 s recycle delay. Spectra were each acquired using 36,864 scans with a time domain equal to 512 complex data points which was zero filled to a total of 4096 data points. The free induction decay was Fourier transformed with 100 Hz line broadening and the resulting spectra were phased properly. All solid-state spectra were externally calibrated to the glycine standard (176.03 ppm). A direct polarisation with magic angle spinning (DPMAS) spectrum for fraction B was obtained using conditions described by Dria et al. [20].

3. Results

3.1. *HA fractionation*

SEC-fractionation of chernozem HA (Figure 1) revealed the presence of several broad overlapping chromatographic peaks from which we selected, using a fraction collector, several eluates that represent discrete components, denoted as A, B and C+D. PAGE analysis of the total HA is shown in Figure 2 together with PAGE analysis for each individual fraction. Fraction A, corresponds to the excluded peak, formed at the electropherogram start zone which did not move into the PAG. Fraction B forms an intensively coloured zone in the mid part of PAG. The combined fraction C+D forms two main coloured bands of different intensity, having a relatively close EM, on the bottom of the PAG slab. On the basis of fraction elution volumes, we suggest that the MS of electrophoretic fractions are in decreasing order A > B > C + D.

3.2. *^{1}H-NMR spectra*

^{1}H-NMR spectra of the bulk unfractionated HA, HAU and electrophoretic fractions A, B and C+D in D_2O and DMSO-d_6 are shown in Figures 3 and 4. It should be noted that the unfractionated HA, HAU and fractions A, B and C+D all dissolved completely in D_2O. In DMSO-d_6, HA and HAU dissolved completely, fraction B was only partially soluble, and fraction A was insoluble. After centrifugation at 10,000 *g* for 30 min, a brown supernatant of fraction B was used for the ^{1}H-NMR investigation.

On the basis of qualitative analysis, the spectra are subdivided into three main resonance regions, 0.8–3.3, 3.3–4.7 and 6.0–9.0 ppm. In the first region, the peaks are commonly assigned to aliphatic protons. The resonance centred at 0.9 ppm can be assigned to protons of terminal methyl groups of alkyl chains, the resonance centred at 1.3 ppm to protons of methyl groups of highly branched aliphatic structures and methylene groups of alkyl chains, and the peaks in the region 1.4–1.8 ppm to protons on aliphatic carbons which are two or more carbons removed from aromatic rings or polar electronegative functional groups. The small peaks in the region 1.8–3.3 ppm are believed to be protons attached to aliphatic carbons (methyl or methylene groups) which are attached to electronegative functional groups (e.g. carboxyl group or aromatic ring). The second range, 3.3–4.7 ppm, corresponds to a broad resonance assigned to protons on carbons attached to O or N atoms (e.g. the HOC of carbohydrates, methoxyl groups of lignins, amines or amino groups of proteins). Finally, the signals in the 6.0–9.0 ppm range can be attributed to the presence of unhindered aromatic protons, phenols, carboxylic and amide N–H protons.

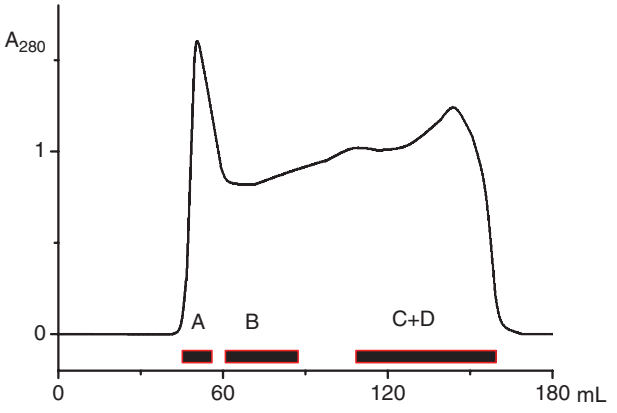

Figure 1. Size-exclusion chromatography of 10 mg chernozem HA on a Sephadex G-75 column (100 × 1.5 cm) using 7 M urea as the eluting system. Black boxes on the *x*-axis show the combined fractions, obtained on the basis of electrophoretic analysis of chromatographic aliquots: fraction A (V_e, 47–55 mL), fraction B (V_e, 60–80 mL) and fraction C+D (V_e, 110–160 mL). Elution volume 55–69 mL contained a mixture of fractions A and B and elution volume 81–109 mL contained a mixture of fractions B and C+D.

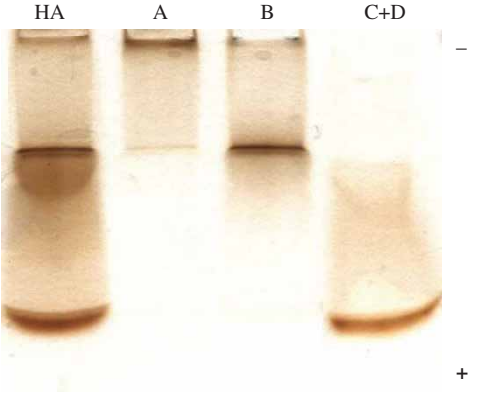

Figure 2. Electrophoretic analysis of the unfractionated chernozem HA and SEC–PAGE fractions: A (V_e, 47–55 mL), B (V_e, 60–80 mL) and C + D (V_e, 110–160 mL).

The ^{1}H-NMR spectra of the chernozem bulk HA in D_2O and DMSO-d_6 exhibit numerous features of soil HAs [12, 21]. The types of resonance signal observed are strongly influenced by the solvent used. Three distinct resonance signals in the 6.9–7.4 ppm region are observed with DMSO-d_6 but are not present in the D_2O spectra (Figures 3 and 4). These are probably from exchangeable hydrogens such as those of phenolic, carboxylic or amidic groups.

The spectrum of fraction A in D_2O shows very weak resonances in the 6.0–9.0 ppm region and several highly resolved signals in the 0.8–4.7 ppm range (Figure 3). In the spectra of fractions B and C+D in D_2O, the resonance intensity in the 6.0–9.0 ppm region is greatly enhanced, especially in fraction C+D where this peak has maximum at 7.9 ppm; fractions A and B display a maximum at 7.4 ppm. There are some noteworthy differences in the ^{1}H-NMR spectra of fractions A, B and C+D in D_2O. First is the relatively higher amounts of aromatic ^{1}H, as denoted by the broad band between 6.5 and 9.0 ppm in fraction C+D. In this fraction, there is proportionally less resonance in the range 3.5–4.5 ppm (carbohydrates, proteins) than is seen in fractions A and B. Fraction A is more enriched in signals for polymethylenic structures (1.3 ppm) than the other two fractions.

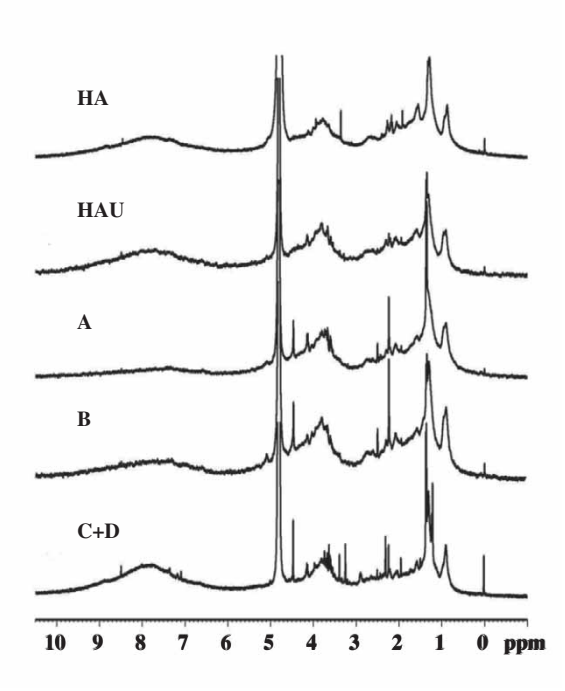

Figure 3. ^{1}H-NMR spectrum, in 0.1 N NAOH/D_2O, of chernozem HA, HAU and electrophoretic fractions A, B and C + D. The peak at 4.8 ppm corresponds to traces of water.

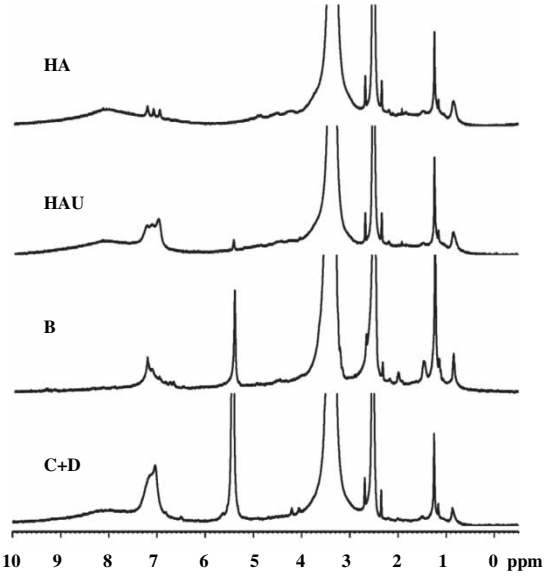

Figure 4. ^{1}H-NMR spectrum, in DMSO-d_6, of chernozem HA, HAU and electrophoretic fractions B and C+D. The peaks at 2.6 and 3.4 ppm correspond to DMSO and traces of water.

The increased presence of polymethylenic signals in fraction A is consistent with previous studies showing higher amounts of long-chain fatty acids and alkanes [9].

Although the ^{1}H-NMR spectra of unfractionated HA and HAU in D_2O show no differences (Figure 3), there is a large contrast between the ^{1}H-NMR spectra of these samples in DMSO-d_6

(Figure 4). Both samples show a broad hump (6.5–9.0 ppm) with a maximum at 8.0 ppm, however, HAU reveals a significantly higher proportion of an overlapping group of peaks between 6.9 and 7.4 ppm. Although we are unsure of a specific assignment for these bands, it is clear that HAU contains a larger proportion of these peaks because urea is expected to rupture H bonds, and the subject peaks, which are not observed in ^{1}H spectra taken in D_2O, are probably from ^{1}H in exchangeable structural units. We suggest that the increased intensity of these signals is due to the rupture of H bonds and the creation of additional exchangeable sites in HA. Urea shows a peak at 5.5 ppm, seen as a small peak in the HAU spectrum. Other signals are observed at 0.9 ppm (methyl groups) and 1.3 ppm (methylene groups). The level of structural details for the spectra in DMSO is much lower that observed for spectra obtained in D_2O. The practical absence of urea in HAU has also been confirmed by ^{13}C-NMR analysis (see below).

The spectrum of fraction B in DMSO-d_6 shows the disappearance of the broad hump between 6.5 and 9.0 ppm, but the appearance of sharp signal at 7.4 ppm with shoulders in the region 7.3–6.9 ppm (Figure 4). We believe that this peak is due to exchangeable amide protons. A sharp signal is observed for urea at 5.5 ppm and the signals for methyl and methylene groups are distinct. The small peak at 1.9 ppm may arise from peptides.

The spectrum of fraction C+D in DMSO-d_6 is similar to that for HAU, but the intensity of the peaks in the 6.9–7.3 ppm region is essentially increased and the sharp peak at 7.4 ppm observed in spectrum B is absent. The high intensity for resonances in the 6.9–7.3 ppm range in the C+D fraction is unusual and probably represents humic compounds that have been disaggregated, perhaps by cleavage of H bonds, releasing molecules with normally exchangeable hydrogens. Such molecules and concomitant structures seem to include phenolic, carboxylic and amide groups. The spectrum obtained in D_2O for this fraction does not show these peaks due to the present exchange. Furthermore, addition of a small amount of D_2O to any of the investigated samples dissolved in DMSO led to complete loss of the signals between 6.9 and 7.4 ppm, indicating that these signals are from exchangeable hydrogens.

3.3. *Solid-state ^{13}C-NMR spectra*

Solid-state ^{13}C-NMR spectra of the bulk untreated HA, HAU and fractions A, B and C+D are shown in Figure 5. Detailed peak assignments and the estimated relative percentage of the peak areas are presented in Table 2. It should be noted that the spectra of unfractionated HA are similar to those of chernozem HA obtained by other researchers [22]. The spectra were obtained using a standard cross-polarisation magic angle spinning (CPMAS) approach and conditions were optimised such that the areas under the peaks are quantitative [20]. The quantitative nature of the CPMAS spectra was verified by examining one of the samples, fraction B, using DPMAS which is considered quantitative. The DPMAS spectrum (not shown) is very similar, if not identical, to the CPMAS spectrum.

In general, the peak areas for CPMAS spectra can be divided into four main regions: aliphatic C (0–108 ppm), aromatic C (108–165 ppm), carboxyl, ester and amide C (165–187 ppm) and carbonyl C (187–220 ppm). It should be noted that the ^{13}C-NMR spectra of bulk untreated HA and HAU show common features for soil HAs [11, 12, 14, 15, 21]. The absence in HAU of a distinct peak at 161 ppm, which is attributed to urea, confirms that most of the urea is removed during dialysation, however, a small portion is known to be retained in the HA sample after dialysis, as shown by ^{1}H-NMR analysis of HAU in DMSO-d_6 (Figure 4).

From Figure 5 it is apparent that the patterns for the ^{13}C-NMR spectra change significantly from fraction A to fraction C+D. This suggests that there is significant heterogeneity in molecular structure and functionality among the various fractions, which differ in both EM and MS. The spectrum of fraction A is dominated by H,C-alkyl and O,N-alkyl carbons. Three intensive peaks have been

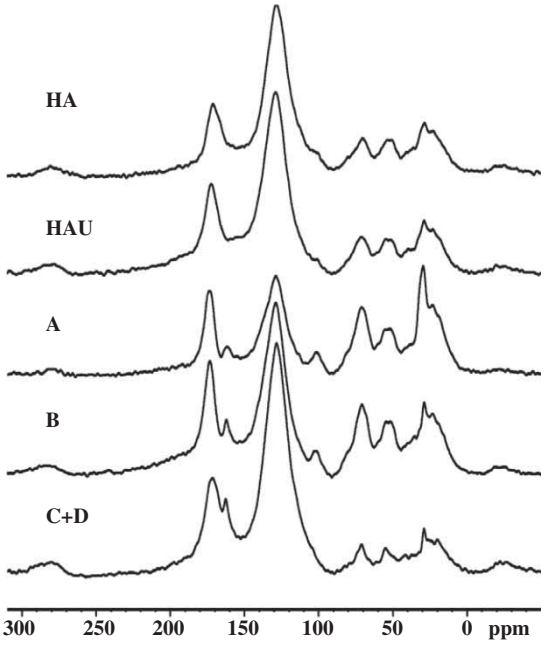

Figure 5. Solid-state ^{13}C-NMR spectrum of chernozem HA, HAU and electrophoretic fractions A, B and C+D.

Table 2. Percentage of carbon in the main structural fragments of chernozem HA and its electrophoretic fractions

Sample	$C_{C=O}$ 220–187 ppm	$C_{COO,CON}$ 187–165 ppm	$C_{Ar-O,N}$ 165–145 ppm	$C_{Ar-H,C}$ 145–108 ppm	$C_{Alk-O,N}$ 108–50 ppm	$C_{Alk-H,C}$ 50–0 ppm
HA	3.4	10.3	7.5	48.7	13.4	16.7
HAU	3.8	12.1	8.2	48.0	12.7	15.2
Fraction A	3.6	10.9	5.8	28.3	23.8	27.6
Fraction B	5.6	13.1	8.0	37.6	17.8	17.9
Fraction C+D	3.9	14.2	9.2	53.5	7.6	11.6

Notes: Percentage of carbon equals the integral intensity of the regions of the ^{13}C-NMR spectra indicated in the columns. Areas have been adjusted to represent a urea-free measurement.

observed in the aliphatic carbon region (0–50 ppm). These three peaks together contributed 27.6% of the total area and can be assigned to methyl (18 ppm), branched aliphatic carbons or methylene groups (23 and 30 ppm). The ^{13}C-NMR spectra coincide with tetramethylammonium hydroxide (TMAH) thermochemolysis results [9], which show that fraction A contained considerably more long chain fatty acids and alkanes than fraction B or fraction C+D.

Within the O-alkyl carbon region (108–60 ppm), the most prominent spectral features are well-defined peaks at $\sim$ 70 ppm (ordinarily assigned to carbons in CH(OH) groups, ring carbons of polysaccharides and ether-bonded aliphatic carbon) and 100 ppm (anomeric carbon in polysaccharides). Note that the 100 ppm peak exists only as a shoulder in the spectrum for bulk untreated HA. This means that fraction A is relatively enriched in carbohydrates compared with the HA sample. The other peak observed in the alkyl region (50–60 ppm) is assigned to either methoxyl carbons (56 ppm normally) or the α-amino-substituted carbon in peptides. The relative intensity of this peak is greatest in fraction A, perhaps because it is known to contain the largest proportion of amino acids [6]. We expect that methoxyl groups from lignin-derived structures, identified from TMAH thermochemolysis data [9], are also important constituents of fraction A.

The percentage of aromatic carbon (108–165 ppm) is greatly decreased in fraction A compared with the HA sample (Table 2). A distinct peak appears at 161 ppm in all fractions except HA and HAU. This signal is likely to be from a small admixture of urea introduced during SEC separation of HA in 7 M urea. The contribution of urea to the spectra was determined by integration and the remaining spectral areas were adjusted to exclude this peak from relative area calculation (Table 2).

In fraction B, the percentage of both alkyl and O,N-alkyl carbon is essentially decreased compared with fraction A, but the percentage of aliphatic carbon remains more than in the unfractionated HA (Table 2). The aromatic character of HA becomes pronounced in fraction C+D, where the O-alkyl carbon peak at 100 ppm disappears and alkyl carbons between 0 and 50 ppm are considerably less intense than in unfractionated HA, HAU and fractions A and B.

In the unfractionated HA and all HA fractions, a distinct peak appears in the 165–187 ppm range, which is commonly assigned as carboxylic, ester and amidic carbons and partially includes quinones. Essentially, the intensity of this peak does not change in HA and all fractions. Taking into account the amino acid content of fractions A, B and C+D, which is 13.2, 7.9 and 2.3%, respectively [6], we assume that this peak is mostly due to carbonyl carbon from peptide bonds (fraction A) and carboxylic protons (fraction C+D). Some confirmation of this may be the small shift of this peak in fraction C+D in comparison with fractions A and B.

The percentage of carbonyl carbon (220–187 ppm) does not essentially change in fractions A, B and C+D compared with the HA and HAU samples, although some increase was noted in fraction B.

4. Discussion

Several approaches have been used to improve the resolution of NMR spectra by further fractionation of soil HAs. For example, some researchers used preparative SEC to reduce the chemical heterogeneity of soil HA, producing simplified ^{1}H-NMR spectra [18] and using ^{13}C-NMR to show a trend of increasing of aromatic moieties with decreasing MS [11, 15]. This same trend has been noted in detailed interpretations of ^{13}C-NMR spectra of soil HA fractions obtained by ultrafiltration [16, 17]. However, NMR investigations for soil HA fractionated on the basis of differences in electrophoretic behaviour have never been reported. This study provides such data and gives us insights into how MS and EM affect HA structure.

^{1}H-NMR spectra in D_2O and DMSO-d_6, and solid ^{13}C-NMR spectra, show significant differences in the distribution of chemical compounds between soil HA fractions, obtained using the combined SEC–PAGE method (Figures 3–5, Tables 1 and 2). These data are supported by the H/C atomic ratio (Table 1), UV-visible spectra [7], TMAH thermochemolysis data [9] and amino acids content [9], which indicate that fraction A, the lowest EM fraction, is considerably more aliphatic than fractions B and C+D. Moreover, the ratio $C_{ar\ (165-108\,ppm)}/C_{alk\ (108-0\,ppm)}$, or aromaticity, increases by a factor of 5 from fraction A to fraction C+D (Figure 6). This is greater than the corresponding ratio between high and low MS fractions obtained by other researchers using SEC or UF techniques for soil HA fractionation, in which the aromaticity changed by only a factor of 3 or less [15–17]. The selective enrichment of aromatic or aliphatic structural components in soil HA fractions, observed here may perhaps explain the greatly enhanced fluorescence and photoinductive activity of fraction C+D [8, 23]. It should be noted that the fractions differ greatly in terms of their solubility in DMSO. Fraction C+D, like the unfractionated HA, was completely dissolved in DMSO, whereas fraction A was practically insoluble and fraction B dissolved only incompletely. The data confirm that there are differences in the chemical structure of the fractions obtained.

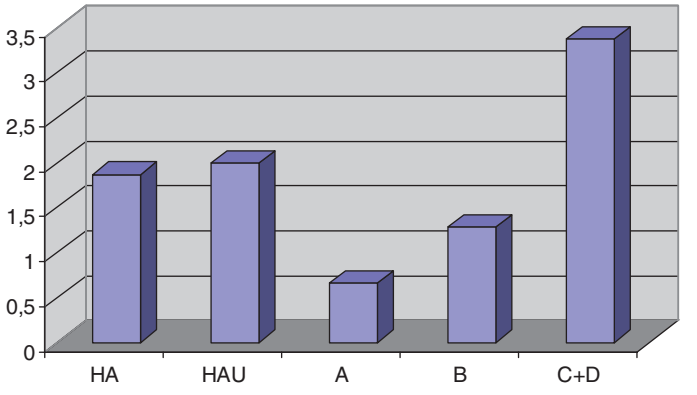

Figure 6. Ratio $C_{ar\ (165\text{-}108\ ppm)}/C_{alk\ (108\text{-}0\ ppm)}$ for chernozem HA, HAU and electrophoretic fractions A, B and C+D.

5. Conclusions

The data obtained are relevant to the continuing discussion of whether HS are randomly coiled macromolecules or micellar structures [24]. Taking into account that high-MS fractions A and B consist mainly of aliphatic components, portions of which (e.g. fatty acids) possess amphiphilic character, it is reasonable to suggest that these compounds are capable of organising into micellar structures. However, the more aromatic, hydrophilic and photochemically active low-MS fraction C+D [8, 23] seems to connect with the humic core through hydrogen bonds. These results are of great environmental importance because different fractions might be differently involved in chemical/biological processes in soils.

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References

[1] P.J. Janoš, *Separation methods in the chemistry of humic substance*, J. Chromatogr. A 983 (2003), pp. 1–18.
[2] J.M. Duxbury, *Studies of the molecular size and charge of humic substances by electrophoresis*, in *Humic Substances II*, M.H.B. Hayes, P. MacCarthy, R.L. Malcolm and R.S. Swift, eds., John Wiley, Chichester, 1989, pp. 593–620.
[3] P. Schmitt-Kopplin, A.W. Garrison, E.M. Perdue, D. Freitag, and A. Kettrup, *Capillary electrophoresis of humic substances. Facts and artifacts*, J. Chromatogr. A 807 (1998), pp. 101–109.
[4] O.A. Trubetskoj, O.E. Trubetskaya, G.V. Afanas'eva, O.I. Reznikova, and C. Saiz-Jimenez, *Polyacrylamide gel electrophoresis of soil humic acid fractionated by size-exclusion chromatography and ultrafiltration*, J. Chromatogr. A 767 (1997), pp. 285–292.
[5] A. Wallqvist, D.G. Covell, and D. Thirumalai, *Hydrophobic interactions in aqueous urea solutions with implications for the mechanism of protein denaturation*, J. Am. Chem. Soc. 120 (1998), pp. 427–428.
[6] O.E. Trubetskaya, O.A. Trubetskoj, G.V. Afanas'eva, O.I. Reznikova, L.F. Markova, and T.A. Muranova, *Amino acids distribution in soil humic acids fractionated by tandem size exclusion chromatography–polyacrylamide gel electrophoresis*, Environ. Int. 24 (1998), pp. 573–581.
[7] O.A. Trubetskoj, O.E. Trubetskaya, O.I. Reznikova, and G.V. Afanas'eva, *Weight and optical differences between soil humic acids fractions obtained by coupling SEC–PAGE*, Geoderma 93 (1999), pp. 277–287.
[8] C. Richard, O.E. Trubetskaya, O.A. Trubetskoj, O.I. Reznikova, G.V. Afanas'eva, J.-P. Aguer, and G. Guyot, *Key role of low molecular size fraction of soil humic acids for fluorescence and photoinductive activity*, Environ. Sci. Technol. 38 (2004), pp. 2052–2057.
[9] C. Saiz-Jimenez, B. Hermosin, O.E. Trubetskaya, O.I. Reznikova, G.V Afanas'eva, and O.A. Trubetskoj, *Thermochemolysis of genetically different soil humic acids and their fractions obtained by tandem size exclusion chromatography–polyacrylamide gel electrophoresis*, Geoderma 131 (2006), pp. 22–32.

[10] S. Sanchez-Cortes, G. Corrado, O.E. Trubetskaya, O.A. Trubetskoj, B. Hermosin, and C. Saiz-Jimenez, *Surface-enhanced Raman spectroscopy of chernozem humic acid and their fractions obtained by coupled SEC–PAGE*, Appl. Spectrosc. 60 (2006), pp. 48–53.
[11] R.L. Wershaw, D.J. Pinckney, E.C. Llaguno, and V. Vicente-Beckett, *NMR characterization of humic acid fractions from different Philippine soils and sediments*, Anal. Chim. Acta 232 (1990), pp. 31–42.
[12] R.L. Malcolm, *The uniqueness of humic substances in each of soil, stream and marine environments*, Anal. Chim. Acta 232 (1990), pp. 19–30.
[13] I. Kögel-Knabner, *Analytical approaches for characterizing soil organic matter*, Org. Geochem. 31 (2000), pp. 609–625.
[14] P.G. Hatcher, K.J. Dria, S. Kim, and S.W. Frazier, *Modern analytical studies of humic substances*, Soil Sci. 166 (2001), pp. 770–794.
[15] R.S. Swift, R.L. Leonard, R.H. Newman, and B.K.G. Theng, *Changes in humic acid composition with molecular weight as detected by C-13 nuclear magnetic-resonance spectroscopy*, Sci. Total Environ. 118 (1992), pp. 53–61.
[16] E. Tombácz, *Colloidal properties of humic acids and spontaneous changes of their colloidal state under variable solution conditions*, Soil Sci. 164 (1999), pp. 814–824.
[17] I. Christl, H. Knicker, I. Kögel-Knabner and R. Kretzschmar, *Chemical heterogeneity of humic substances: characterization of size fractions obtained by hollow-fibre ultrafiltration*, Eur. J. Soil Sci. 51 (2000), pp. 617–625.
[18] A. Piccolo, P. Conte, E. Trivellone, and B. Van Lagen, *Reduced heterogeneity of a lignite humic acid by preparative HPSEC following interaction with an organic acid. Characterization of size-separates by Pyr-GC-MS and H-1-NMR spectroscopy*, Environ. Sci. Technol. 36 (2002), pp. 76–84.
[19] M.M. Kononova, *Soil Organic Matter*, Pergamon Press, Oxford, 1966.
[20] K.J. Dria, J.R. Sachleben, and P.G. Hatcher, *Solid-state carbon-13 nuclear magnetic resonance of humic acids at high magnetic field strengths*, J. Environ. Quality 31 (2002), pp. 393–401.
[21] W.L. Kingery, A.J. Simpson, M.H.B. M.A. Hayes, M.A. Locke, and R.P. Hicks, *The application of multidimensional NMR to the study of soil humic substances*, Soil Sci. 165 (2000), pp. 483–494.
[22] V.D. Tikhova, M.M. Shakirov, V.P. Fadeeva, and M.I. Dergacheva, *Specific features of humic acids from various soils as studied by thermal analysis and C-13 NMR*, Russian J. Appl. Chem. 71 (1998), pp. 1229–1233.
[23] O.E. Trubetskaya, O.A. Trubetskoj, G. Guyot, F. Andreux, and C. Richard, *Fluorescence of soil humic acids and their fractions obtained by tandem SEC–PAGE*, Org. Geochem. 33 (2002), pp. 213–220.
[24] R. Sutton and G. Sposito, *Molecular structure in soil humic substances*, Environ. Sci. Technol. 39 (2005), pp. 9009–9015.