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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 ^{1}H-NMR Spectroscopy

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Preparative high performance size exclusion chromatography (HPSEC) was used to size-fractionate a humic acid (HA) solution with 0.05 M ionic strength before and after having made the humic solution 0.5×10^{-3} M in acetic acid (AcOH). Size-fractions were characterized by pyrolysis-gas-chromatography/mass spectrometry (Pyr-GC-MS) and ^{1}H NMR spectroscopy. Pyr-GC-MS showed that the AcOH treatment altered the distribution of humic molecular components in the size-fractions. The unsaturated alkyl chains were moved from size-fractions of larger molecular-size into those of lower molecular-sizes. Most of the aromatic moieties, which were found in larger molecular-size fractions for the untreated HA, were spread into fractions of lower molecular size after AcOH addition to HA. Carbohydrates, which were undetectable in any fraction of the untreated HA, appeared instead in the pyrogram of the lowest molecular-size and most hydrophilic fraction after treatment with AcOH. Our results suggested that AcOH disrupted the weakly bound association of humic supramolecular structures and HPSEC elution separated size-fractions of different composition without losing humic matter by adsorption on the HPSEC column. The fractions with the largest apparent molecular size were the richest in alkyl chains, thereby suggesting that humic molecules were stabilized into supramolecular associations by multiple weak interactions among apolar groups such as alkyl chains and aromatic moieties. ^{1}H NMR spectra of size-fractions were greatly simplified and more resolved after AcOH treatment. This was attributed to a less complex molecular association in the size separates which provided a larger solubility in the NMR solvent and more favorable relaxation times. Combination of the procedure used here for size-fractionation with NMR and Pyr-GC-MS methods appears to be promising to advance knowledge on the molecular composition of humic substances.

Introduction

The traditional view of humic substances (HS) is that they are polydisperse macromolecular polymers. Common literature reports that their molecular weight may vary from 500 D for some aquatic HS to more than 10^6 D for soil humic acids (*1*). However, there is neither a definite evidence for the polymeric nature of terrestrial humic matter nor agreement between the different methods used to evaluate MW values (*2*).

Current description of humic conformational structure has been adapted to the polymeric view. HS in solution are commonly depicted as polymers (*3*) which are coiled at high concentrations, low pH, and high ionic strength, but they become linear at neutral pH, low ionic strength, and low concentration. An alternative approach describes HS as polymers aggregated in micelle-like or membrane-like structures (*4*−*6*) which are believed to contain inner hydrophobic domains where structural voids may entrap hydrophobic organic compounds and quench their fluorescence activity (*6, 7*). Still within the polymeric understanding, nonlinearity between sorption and desorption of apolar contaminants have been explained with glassy or rubbery regions in the humic matter structure (*8*−*10*).

Using low-pressure size exclusion chromatography, Piccolo et al. (*11, 12*) reported that the absorption peak of humic acids (HAs) was shifted from high to low molecular-size ranges upon organic acids additions before the elution in an alkaline borate buffer. To explain their results they suggested that, instead of being stable polymers, humic substances at neutral or alkaline pHs are supramolecular associations of relatively small heterogeneous molecules held together by weak dispersive forces such as van der Waals, $\pi-\pi$, CH$-\pi$, interactions. Partisans of the traditional polymeric model of HS (*13*) criticized the above results not on the basis of an experimental replication but on theoretical and qualitative interpretations of gel−solute interactions and interferences due to a supposed polymeric form of borate. An experimental replication applying High Performance Size Exclusion Chromatography (HPSEC) was reported by Varga et al. (*14*). They obtained the same results as Piccolo et al. (*11, 12*) and attributed the shift of absorption to lower molecular-size ranges to hydrophobic interactions on the column upon protonation of the humic matter and consequent change in ionic strength. A contribution to size-exclusion chromatography of hydrophobic interactions on the column has been since long recognized in gel-filtration studies of HS (*15*) and other biomolecules (*16*). Moreover, hydrophobic interactions on size-exclusion gels have been often usefully exploited to fractionate humic aggregates in simpler molecular associations which were chemically characterized more easily than for the bulk humic material (*17*−*19*).

In the HPSEC mode, Piccolo et al. (*20*) reproduced the same shift of chromatographic peaks observed earlier by low-pressure gel filtration (*11, 12*) when the pH of humic solutions was adjusted from 7 to only 3.5 by using small amounts ($<0.5 \times 10^{-3}$ M) of a number of monocarboxylic acids. The larger resolution and reproducibility of HPSEC columns in comparison to low-pressure gel-phases (*21*) showed that the alteration of the HPSEC molecular size distribution was progressively enhanced with the number of carbon atoms in

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the organic acids. Conte and Piccolo (*22*), instead of lowering the pH of humic solutions before injection, slightly changed the composition of the HPSEC eluent. A control eluent at pH 7 was modified by addition of $\leq 2.0 \times 10^{-6}$ M of either methanol, HCl, or acetic acid (AcOH) to pH 6.97, 5.54, and 5.69, respectively, without changing the ionic strength of 0.05 M and thereby avoiding the hydrophobic adsorptions which may occur at increased ionic strengths (*15, 16, 23*). Both UV and refractive-index detectors showed major alterations of the humic molecular-size distributions and dramatic reductions of weight-average molecular weights (M_w) in the modified eluents. These results were again interpreted with a disruption of the weak interactions holding together relatively small humic molecules into apparently large molecular sizes and were in line with a supramolecular rather than a polymeric structure of HS. Molecular size reduction due to breaking of ester and other covalent linkages by such low amount of modifiers could not be proposed considering the customary strongly alkaline and acidic conditions in which HS had already undergone during isolation and purification (*22*). Alteration of porosity and thus separation capacity of the HPSEC column due to modified eluents had to be ruled out when elution of well defined and undisputed polymers such as polysaccharides and polystyrenesulfonates, in the same conditions as the HS, showed no changes of either elution profile or retention time (*24, 25*). This mechanism of self-association of humic molecules was advocated to explain results in several recent studies (*26*−*30*).

To view HS in solution as loose self-associations of smaller units instead of polymers implies that humic molecules could then undergo polymerization reactions. Recent HPSEC works (*31, 32*) showed that an oxidative coupling reaction catalyzed by horseradish peroxidase increased significantly the large molecular-size composition of different HAs. Addition of acetic acid to lower the pH of the reacted humic solution before injection in the HPSEC system could no longer shift the material to larger elution volumes in comparison to control as it has been instead regularly observed for the nonpolymerized HS (*11, 12, 14, 20, 22, 33*), thereby suggesting that HS were transformed from loosely bound supramolecular into stable covalently bound polymeric structures.

Due to the chemical complexity of natural HS associations, a fractionation step is necessary to reduce their molecular heterogeneity and reach a better insight of their chemical composition. While the traditional model of polydisperse polymers implies the isolation of several homogeneous humic fractions by size exclusion chromatography, fractions reprocessing showed that smaller sized components are released and isolated fractions would appear to have similar molecular sizes (*34*). This contradiction was evident in studies by poorly resolved low-pressure gel permeation chromatography where the use of concentrated alkaline buffers prevented a sufficient size-exclusion fractionation to allow the chemical identification of fraction components. Except for a few attempts of size-fractionation which provided important chemical information (*17*−*19, 35*−*37*), most of the common knowledge on the chemical characteristics of humic constituents derives from the application of gas-chromatographic, mass-spectrometric, and spectroscopic techniques on either bulk samples or products of degradation reactions (*1*). However, these results are hardly reliable since have been based on either a partial fraction of the total humic sample or afflicted by unpredictable artifacts formed during thermal (*38, 39*) or oxy-reductive reactions (*40*).

The aim of this work was to reduce the chemical heterogeneity of a humic acid by using, for the first time, a HPSEC system in the preparative mode and to investigate the molecular composition of the size-fractions. The fractions were separated before and after addition of AcOH to a HA dissolved in the HPSEC eluent solution in order to maintain a constant ionic strength and characterized by pyrolysis gas-chromatography mass-spectrometry (Pyr-GC-MS) and liquid-state proton NMR spectroscopy (^{1}H NMR).

Materials and Methods

Preparation of Humic Samples. A HA was isolated from a North Dakota lignite as described elsewhere (*20*). This HA resulted 2.70% in ashes, 56% in C, 4% in H, and 2% in N. Oxygen and other elements resulted, then, to be 38% of the total HA. The relative standard deviation measured for elemental analyses did not exceed 2%.

The HA was first suspended in distilled water and titrated to pH 7 with a CO_2-free solution of 0.5 M NaOH by an automatic titrator (VIT 90 Videotitrator, Radiometer, Copenhagen) under N_2 atmosphere and stirring. After having reached the constant pH 7, the solution containing sodium-humates was left under titration for 2 more hours, filtered through a Millipore 0.45 μm, and freeze-dried. Sodium-humates were pretitrated at pH 7 to limit HPSEC interferences (*22*).

The HPSEC mobile phase, a NaCl/NaN_3 (2.89 g.L^{-1}/0.3 g.L^{-1}) solution at pH = 7.0, was used to dissolve the HA to reach a concentration of 0.6 g.L^{-1}. Another batch of HA was similarly dissolved and added with glacial AcOH to lower the pH from 7 to 3.5. The amount of glacial AcOH used to lower pH was 120 μL for each 20 mL of humic solution and corresponded to 10 μg of AcOH per mg of HA. The ionic strength (I) of HA solution was 0.05 M, and the final AcOH concentration of about 0.1×10^{-3} M did not alter the ionic strength of the humic solution. Both HA solutions, treated and untreated with AcOH, were filtered through 0.2 μm nylon filters before HPSEC fractionation.

Preparative HPSEC Separation. A Biosep SEC-S-2000 (600 mm × 21.2 mm i.d.) column preceded by a Biosep SEC-S-2000 Guard Column (78.0 mm × 21.2 mm i.d.) by Phenomenex was used. A Gilson 305 pump, a Gilson autosampler model 231, a Gilson FC205 fraction collector, and a Perkin-Elmer LC-75 UV/vis detector set at 280 nm were used to automatically isolate humic fractions in continuous. A Perkin-Elmer Nelson 1022S was used to automatically record all chromatographic runs. Elution flow rate was set at 1.5 mL.min^{-1}. Due to the lack of proper calibration standards for HS (*22*), protein standards of known molecular weights (205, 64, 20, and 6.0 kD) by Pharmacia (Standard Molecular-Weight Kit) were used to calibrate the column with nominal molecular-weight ranges.

Six fractions of untreated HA were collected during HPSEC separation by changing collecting vials every 10 min within the 60−120 min interval from the start of each chromatographic run. Eight different fractions were, instead, obtained from the HA solution treated with AcOH to pH = 3.5 before injection into the HPSEC system. Fractions were collected from the start of each run as follows: two fractions between 60 and 70 and 70−80 min; a third fraction between 80 and 100 min; fractions 4, 5, and 6 every 10 min from 100 to 130 min; fractions 7 and 8 every 20 min from 130 to 170 min. The reproducibility of the elution profiles was generally excellent. Table 1 shows the molecular weight ranges in which all fractions were collected based on the column calibration by protein standards.

Molecular Weight Determination. Size exclusion chromatograms were evaluated by using Perkin-Elmer-Nelson Turbochrom 4-SEC integration and molecular weight software. A SEC noise threshold of 5 and a filter size of 5 for the Savitzky-Golay smoothing were used. Calculation of weight-

TABLE 1. Nominal Molecular Weights (MW) in Which Size-Fractions Were Separated by Preparative HPSEC from HA Untreated (U) or Treated (T) with Acetic Acid[a]

nominal MW (kD)	fractions from U-HA	fractions from T-HA
205−115	1	1
115−64	2	2
64−36	3	3
36−20	4	3
20−11	5	4
11−6	6	5
6−3.5	NC[b]	6
3.5−1.1	NC[b]	7
1.1−0.34	NC[b]	8

[a] The nominal MW were based on a column calibration obtained with standard proteins. [b] NC = not collected.

averaged molecular weights (M_w) was done by using the following equation (*41*)

$$M_w = \sum_{i=1}^{N} h_i(M_i) / \sum_{i=1}^{N} h_i$$

where M_i and h_i are the molecular weight and the height of the *i*th chromatographic slice in the chromatogram of each sample eluted at volume *i*, respectively. The M_w values, based on 50 consecutive replicates of chromatographic runs for either untreated or AcOH-treated HA samples, were 48635 ± 1094 and 19334 ± 2083 D, respectively.

Evaluation of Carbon Loss during HPSEC Elution. The fractions separated by preparative HPSEC corresponding to 250 mL of the original HA solution (50 HPSEC injections) for both AcOH treated and untreated batches were again gathered together, dialyzed (Spectra/Por 6 dialysis tube, 1 kD of MW cutoff) against deionized water till chloride-free, freeze-dried, and weighed. Mass recovery for both untreated and AcOH-treated HA was more than 98%. The gathered humic fractions were also characterized by Elemental Analyzer. Results for both untreated and AcOH-treated HA gave a carbon content of 56 ± 1.1%, a H content of 4% ± 0.12%, and N content of 2 ± 0.06%. Both mass recovery and elemental composition indicated that the humic material was completely recovered after HPSEC elution and that the addition of slight amount of AcOH did not significantly affect the elemental composition of size-fractions.

Pyrolysis-Gas Chromatography/Mass Spectrometry (Pyr-GC-MS). Samples from either the bulk HA or the different size-fractions were pressed into flattened ferromagnetic wires. Pyrolysis was conducted by inductive heating of the wires to the Curie temperature of 610 °C, held for 5 s, using a Horizon Pyrolyzer. The pyrolysis products were separated by a GC-8060 (Fison's Instruments) equipped with a fused silica column (25 m, 0.32 mm i.d.) coated with ChromPack CP-Sil 5LB (film thickness 0.45 mm), under helium as carrier gas. The initial oven temperature was 40 °C, and then the temperature was raised with a rate of 7 °C/min to 320 °C and held for 20 min. The column end was connected to a EI ion source of a MD800 mass detector from Fison's Instruments (mass range: m/z 45−650; cycle time: 1 s; ionization energy: 70 eV).

^{1}H NMR Spectroscopy. Samples from the original HA and separated size-fractions were studied by liquid state ^{1}H NMR spectroscopy by using a Bruker DPX300 spectrometer operating at 300 MHz on the proton nucleus. Fully deuterated dimethyl sulfoxide (DMSO) was used (0.5 mL) to dissolve 0.5−1.0 mg of humic sample. A 90° pulse, aquisition time of 1.7 s, delay time of 1 s, and 400 scans were applied for spectra acquisition. An exponential multiplication with a line broadening (LB) of 1 Hz was used to transform Free Induction Decays (FID).

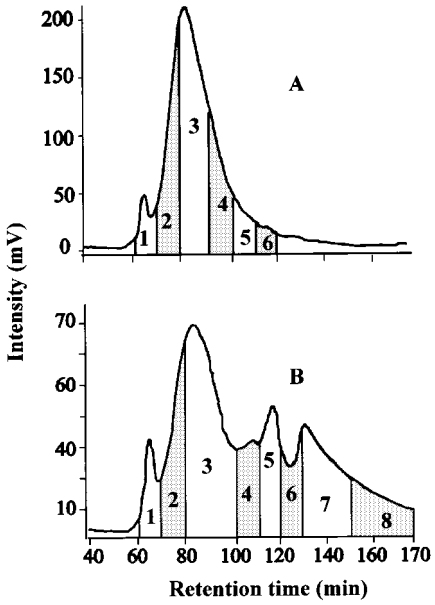


FIGURE 1. Preparative HPSEC chromatograms of A. the untreated HA solution (pH 7) and B. the HA solution treated with acetic acid to pH 3.5 before HPSEC injection.

Results and Discussion

High Performance Size Exclusion Chromatography (HPSEC). The chosen HPSEC eluent had a concentration low enough to minimize hydrophobic interactions with the column (*23, 42*) and was also used to dissolve the HA pretitrated to pH 7 in order to avoid interferences due to changes in ionic strength arising from differences between the injectate and eluent solution (*43*) or from randomly occurring negative charges on the HA (*22*). These attentive conditions for size-fractionation by preparative HPSEC avoided significant losses of humic material by irreversible adsorption on the column. Negligible losses by adsorption on HPSEC columns were also recently reported in similar elution conditions (*44*). Total recovery of humic matter signifies that the entire mass of the bulk HA, whether fractionation was by size-exclusion or other processes, was fully distributed in the size-fractions separated either with or without the AcOH treatment. However, we show that the molecular size distribution for the untreated HA was significantly different from that of the AcOH-treated HA (Figure 1). Treatment of HA with AcOH prior to HPSEC injection produced a chromatogram with peaks which were of lower intensities and spread toward larger retention times (120−180 min) than for the untreated HA. The M_w value of 48635 ± 1094 D for the untreated HA was reduced to 19334 ± 2083 D by the AcOH treatment. These results agree with previous works (*20−22, 33*) which suggested that the weakly bound HS supramolecular structures of apparently large molecular dimension may be disrupted in smaller-sized associations by organic acids. The disruption by lowering pH to 3.5 by AcOH addition is attributed (*20, 21, 33*) to the formation of new inter- and intramolecular hydrogen bonds which are thermodynamically more stable than the hydrophobic interactions which stabilize humic conformations at pH 7. The energy gain resulting from hydrogen bonds formation accounts for the changes in molecular-size distribution shown by the chromatograms. The substantial reduction in peak intensity in the chromatogram of AcOH-treated HA may be explained by the changes in reciprocal orientation of the dipolar moments of neighboring chro-

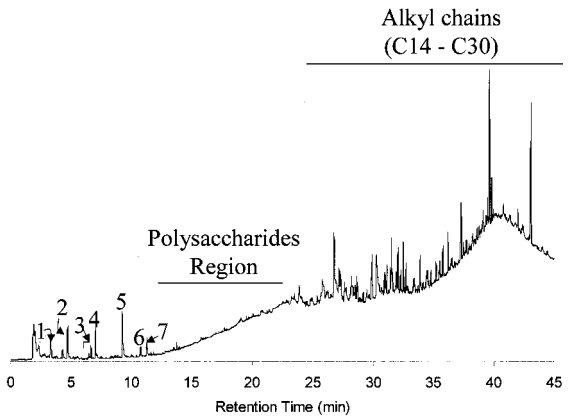


FIGURE 2. Pyr-GC-MS chromatogram of the bulk HA. Further peaks assignment was the following: 1. benzene; 2. toluene; 3. *m*- and *p*-xylene; 4. styrene; 5. phenol; 6. 2-methylphenol; 7. 2-methoxyphenol.

mophores (*21*, *22*, *33*, *45*) following alteration of the humic conformational structure by AcOH.

Pyrolysis-Gas Chromatography/Mass Spectrometry (Pyr-GC-MS). The total ion chromatogram (pyrogram) from Pyr-GC-MS of the bulk HA (Figure 2) is generally similar to HS pyrograms of other studies (*46*, *47*). The seven dominant signals in the first 12 min of retention time were attributed to benzene, toluene, *m*- and *p*-xylene, styrene, phenol, 2-methylphenol, and 2-methoxyphenol, respectively, whereas the peaks appearing after 23 min of retention time were assigned to long alkyl chains. No significant signals were shown in the chromatographic region between 12 and 23 min where compounds deriving from carbohydrates or polysaccharides are commonly found (*48*, *49*).

Within the qualitative and quantitative limitations of the Pyr-GC-MS technique (*38*), the compounds identified in the pyrogram of the bulk HA suggest a rather hydrophobic character for this humic sample. The long alkyl chains may arise from products of thermal degradation causing either decarboxylation or fragmentation of longer chains (*39*). The aromatic compounds may be due to a thermally induced cyclization of aliphatic chains followed by aromatization (*39*) rather than to degradation of more complex aromatic macromolecules such as lignin (*1*).

More information was obtained by Pyr-GC-MS of the six size-fractions for the untreated HA. The first fraction, eluting at about the column void volume (Figure 1), had the largest apparent molecular size (nominal molecular weight between 205 and 115 kD). Its pyrogram signals (Figure 3) were attributed (*50*) to unsaturated alkyl chains (C10−C16), fatty acids (C16−C18), and saturated aliphatic chains (C20−22). The large aliphaticity of this fraction may explain its high molecular size and consequent early elution from the HPSEC column. In fact, the single alkyl components may have preferentially been grouped together in the aqueous medium by the hydrophobic effect (*51*) that reduces the solvation energy of different apolar molecules in solution. This thermodynamical stabilization may produce a supramolecular association with an only apparently large molecular size that is rapidly excluded from the HPSEC column. The pyrogram of the second largest fraction separated from the untreated HA (nominal molecular weight in the 115−64 kD range) suggested a chemical composition intermediate between fractions 1 and 3 (Figure 3). It showed not only both unsaturated C12−C18 and saturated C18−C22 alkyl chains as in fraction 1 but also aromatic moieties similar to those present in the following fraction 3. The pyrogram of the latter fraction (nominal molecular weight between 64 and 36 kD) showed peaks for benzene, toluene, and *m*- and *p*-xylene (peaks 1, 2, and 4 in Figure 3) and 1,2-dimethyl-1-heptene (peak 3), but none for the alkyl chains visible in fraction 1. The predominant aromatic nature of fraction 3 agrees with the large absorption intensity shown by this fraction (80−90 min) in the HPSEC chromatogram (Figure 1). Such absorption may be justified only by conjugated systems of high molar absorptivity such as aromatic compounds. Fraction 4 (nominal molecular weight in the 36−20 kD range) reflects the same aromatic nature as fraction 3 (Figure 3) due to an extended chromatographic tailing (Figure 1).

The pyrogram of fraction 5 (20−11 kD nominal molecular-weight range) revealed the presence of branched olephinic chains (peaks from 5 to 7 in Figure 3) and C14−C16 alkyl chains. Fraction 6, having the lowest molecular size (nominal molecular-weight in the 11−6.0 kD range), produced a pyrogram containing aromatic compounds, unsaturated C12−C18, and saturated C19−C23 alkyl chains (Figure 3). If chemical similarities were evident in pyrograms of fractions

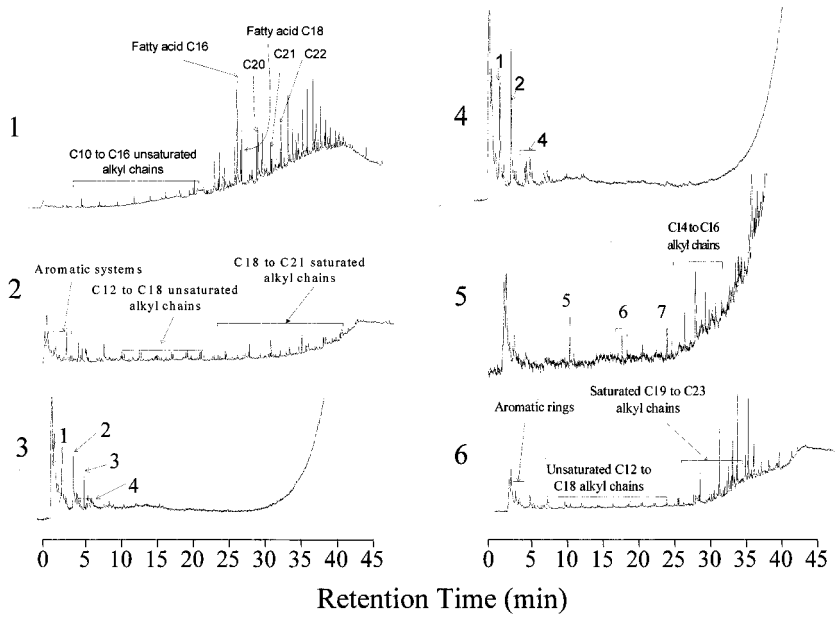


FIGURE 3. Pyr-GC-MS chromatograms of size-fractions from untreated HA (see Table 1 for fractions identification). Further peaks assignment was the following: 1. benzene; 2. toluene; 3. 1,2-dimethyl-1-heptene; 4. *m*- and *p*-xylene; 5., 6., and 7. branched C8−C10 olephinic chains.

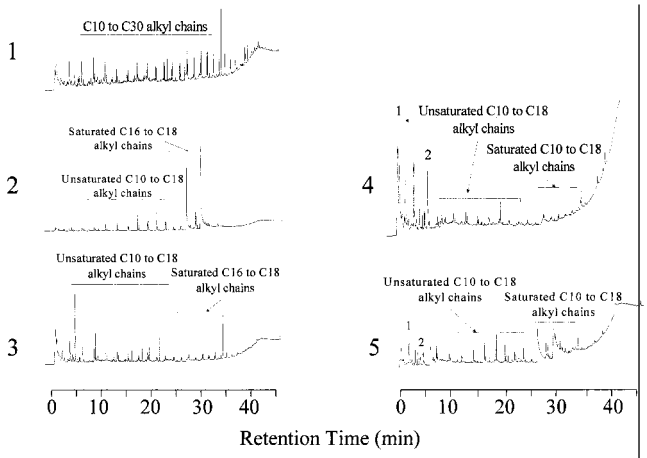


FIGURE 4. Pyr-GC-MS chromatogram of the first five size-fractions from AcOH-treated HA (see Table 1 for fractions identification). Peaks 1 and 2 were attributed to benzene and styrene, respectively.

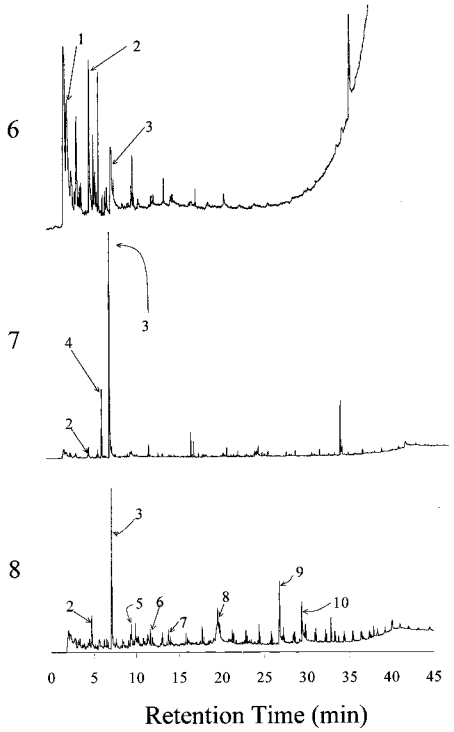


FIGURE 5. Pyr-GC-MS chromatogram of size-fractions 6−8 from AcOH-treated HA (see Table 1 for fractions identification). Further peaks assignment was the following: 1. benzene; 2. toluene; 3. styrene; 4. 1,2-dimethyl-1-heptene; 5. phenol; 6. C11-unsaturated alkyl chain; 7. C13-unsaturated alkyl chain; 8. sugar; 9. C16-fatty acid; 10. C18-fatty acid.

3 and 4 as well as in those of fractions 2 and 6, the distribution and intensity of pyrolysis products from the similar fractions were different (Figure 3). This suggests that the association mode of similar compounds in the various fractions may have produced different molecular sizes and consequently different elution times.

The separation by preparative HPSEC of the AcOH-treated HA produced five fractions (Figure 4) which eluted within the same time intervals as for the untreated HA and three additional fractions (Figure 5) appearing at lower molecular-size intervals. The pyrograms of the first five fractions (Figure 4) were rich in long alkyl chains. The longest chains were present in fraction 1 where pyrolysis products consisted of C10−C30 alkyl chains which may be attributed to *n*-alk-1-enes, and *n*-alkanes (*50*). This fraction appeared to be devoided of the conjugated olephins present in the pyrogram of the size-fraction excluded at the same nominal MW range (205−115 kD) for the untreated HA (Figure 3). However, despite the AcOH treatment, the highly hydrophobic components of fraction 1 may be still tightly assembled in associations of apparently large molecular size by the hydrophobic effect (*51*), thereby causing a rapid exclusion from column. The absence in this first size-fraction of conjugated olephins with large molar absorptivity agrees with the HPSEC chromatogram of the AcOH-treated HA (Figure 1) where the absorption intensity in the 60−70 min range was lower than for the same time interval for the untreated HA. These observations thus suggest that AcOH addition to HA partially disrupted the hydrophobic interactions existing among the original humic components and caused their distribution in more size-fractions (Figure 4) than for the untreated HA (Figure 3). Unsaturated alkyl chains were found in the pyrograms of fractions 2−5 for the AcOH-treated HA. Together with the C10−C18 unsaturated chains, also some saturated alkyl chains were visible as pyrolysis products of these fractions (Figure 4), though their length never exceeded 18 carbon atoms. Despite the chemical similarities in these fractions, the different peaks intensity and distribution in their pyrograms suggested a different association of molecular components. Some aromatic components such as benzene and toluene were also identified in the pyrograms of fractions 4 and 5.

In the absence of any significant humic adsorption on the separating column, the compositional difference between the pyrograms of fractions 1−5 from the AcOH-treated HA and those of fractions 1−6 from the untreated HA may be explained by a disaggregation of the humic heterogeneous suprastructure by the AcOH treatment and rearrangement of its components into smaller and chemically different size-fractions. The unsaturated alkyl chains, which were isolated mainly in fraction 1 for the untreated HA, were spread over a larger range of retention times and recovered in fractions 1−5 for the AcOH-treated HA. The high molar absorptivity of conjugated systems in these unsaturated alkyl chains well explains the fairly large absorption intensity observed for fractions 3, 4, and 5 in the chromatogram of the AcOH-treated HA.

Another effect of the AcOH addition to HA was the shift of aromatic systems, which were mainly recovered in fractions 3 and 4 for the untreated HA (Figure 3), to the larger retention times (120−150 min) of fractions 6 and 7 for the AcOH-treated HA (Figure 5). The main detectable signals of the latter fractions were those of benzene, toluene, and styrene. Fraction 7 showed the presence of 2,4-dimethyl-1-heptene (Figure 5) which had been identified only in fraction 4 for the untreated HA (Figure 3). Fraction 8 for the AcOH-treated HA (Figure 5) contained not only aromatic components such as toluene and styrene but also C11 and C13 unsaturated alkyl chains as well as C16−C18 fatty acids. Interestingly, the pyrogram of this fraction showed peaks attributable to carbohydrates (*49*). Carbohydrates were identified neither in the bulk HA nor in the fractions separated by HPSEC from the untreated HA. A possible explanation is that carbohydrate signals may be masked or altered, during pyrolysis of both the bulk HA and its separated fractions, by their large content of stacked aliphatic and/or aromatic components (*22, 51*). AcOH addition to HA broke apart the humic hydrophobic association and liberated the entrapped hydrophilic compounds. These were separated by the rest of humic materials in smaller and more hydrated fractions which were eluted at the lowest molecular-size intervals such as fraction 8.

^{1}H NMR Spectra. ^{1}H NMR spectra of the bulk HA and its size-fractions from both untreated and AcOH-treated HA are shown in Figures 6−8, respectively. All NMR spectra were separated in high- and low-field signals around the broad

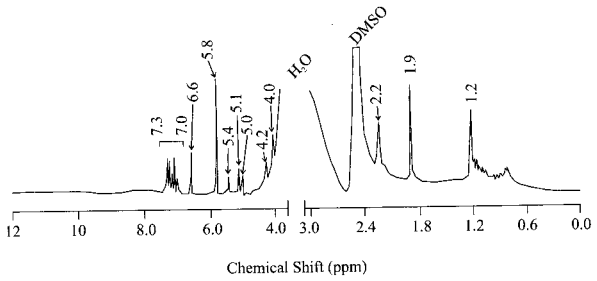


FIGURE 6. ^{1}H NMR spectrum of the bulk HA.

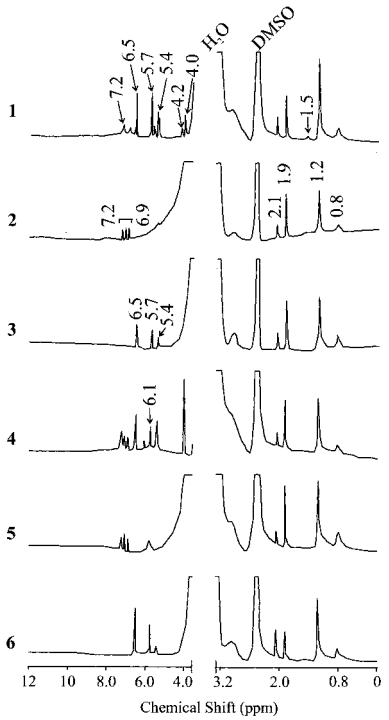


FIGURE 7. ^{1}H NMR spectra of size-fractions separated by the HPSEC from the untreated HA.

resonance of water which was due to the hygroscopicity of deuterated DMSO used to dissolve humic samples. The residual water signal had not been irradiated during acquisition to avoid spectral artifacts. The assignment of ^{1}H NMR signals was based on previous NMR reports for HS (*52*) and general NMR data compilations (*53, 54*).

The ^{1}H NMR spectrum of the bulk HA (Figure 6) shows alkyl components in the 0.5−2.3 ppm region, olephinic compounds between 4.2 and 6.6 ppm, oxygenated systems in the 4.0−4.2 ppm region, and aromatic as well as heterocyclic components in the region between 7.0 and 7.3 ppm. The ^{1}H NMR spectra of size-fractions from the untreated HA (Figure 7) provide more details on the chemical nature of this HA. All size-fractions showed new NMR absorptions at about 1.4−1.5 and 2.1 ppm as compared to the spectrum of bulk HA. The small resonances in the former interval may be attributed to either β-methyl protons in alcohols such as $CH_3CR_1R_2OH$ or β-methylenic protons in amines such as $RCH_2CH_2NR_1R_2$ (*53*). The signal at 2.1 ppm may be assigned to α-methyl protons in ketones and was most intense in the spectra of fractions 5 and 6.

The 4.0−8.6 ppm region in fraction 1 showed the same NMR pattern as for the bulk HA, whereas signals between 5.4 and 6.6 ppm, visible in fractions 1, 3, 4, and 6, were missing in fraction 2. The spectrum of fraction 3 did not show resonances in the 4.0−5.1 ppm and 6.9−8.6 ppm regions. The latter interval is normally attributed to protons in

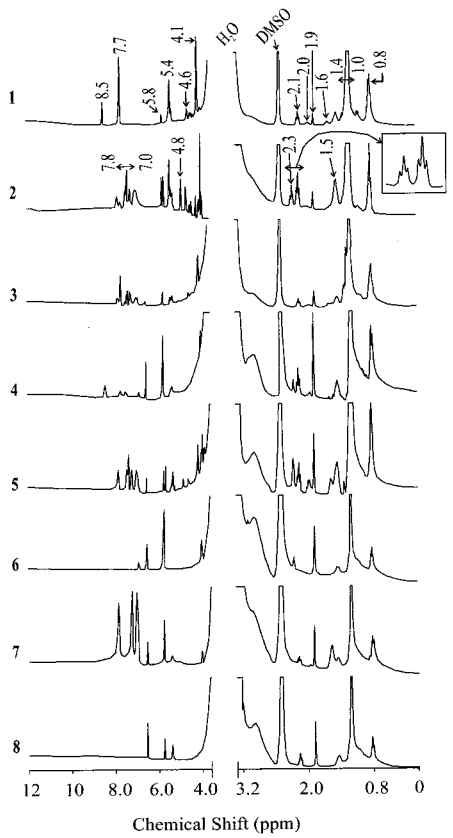


FIGURE 8. ^{1}H NMR spectra of size-fractions separated by HPSEC from AcOH-treated HA.

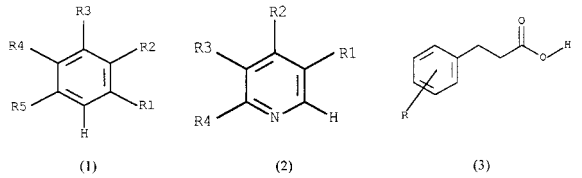
aromatic moieties. This is in contrast with indications from the pyrogram of fraction 3 (Figure 3) and the intensity of the corresponding elution range in the HPSEC chromatogram (Figure 1), which suggested the presence of aromatic compounds in this fraction. This discrepancy may arise from the fact that totally substituted aromatic compounds are not revealed by ^{1}H NMR spectroscopy. Conversely, the same fully substituted aromatic moieties can still be detected by mass spectrometry and show intense absorption during HPSEC elution due to $\pi-\pi^*$ transitions at 280 nm.

The spectrum of fraction 4 is similar to that of fraction 3 except for the signals of aromatic protons (6.9−7.4 ppm region). This suggests that, while both fractions showed aromatic components in their corresponding pyrograms (Figure 3), the resonances for protons in the 6.9−7.4 region are evidence for less substituted aromatic moieties in fraction 4. Fractions 5 and 6, which were eluted at the lowest molecular-size range and were possibly the most polar of all fractions, seemed to contain different amounts of aromatic and olephinic groups. The spectrum of fraction 5 showed three distinct resonances in the aromatic region (6.9−7.4 ppm) and low but broad signals in the region of olephinic protons (5.5−6.9 ppm). In contrast, fraction 6 did not reveal aromatic resonances but only two very sharp signals for olephines centered at 5.6 and 6.5 ppm.

The ^{1}H NMR spectra of the size-fractions from the AcOH-treated HA (Figure 8) appear significantly more resolved than those from the untreated HA (Figure 7), thereby allowing a more detailed chemical assignment to signals. The spectrum of fraction 1 showed not only signals for protons in aliphatic groups next to oxygen and nitrogen atoms and in olephinic systems but also two sharp singlets at 7.8 and 8.5 ppm which were not visible in fraction 1 of the untreated HA. The signal at 7.8 ppm may be due to a proton in highly substituted aromatic systems such as in structure 1, whereas the one at

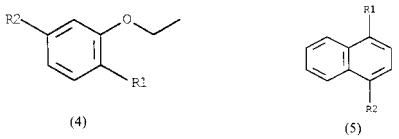
8.5 ppm is unequivocally assigned to a proton in highly substituted heterocyclic systems such as in structure 2.

The spectrum of fraction 2 showed the same signal pattern in the aliphatic region as fraction 1. However, two rather simple triplets were evident at 2.10 and 2.25 ppm in fraction 2, while only one triplet (2.1 ppm) was visible in fraction 1. The triplet systems may be attributed to methylenic protons in benzylic units (2.25 ppm) and α-methylenic protons of carboxylic acid (2.10 ppm). The so-called "roof" effect (*55*), by which the inner peaks between two triplets are slightly enhanced, suggests coupling between these triplets. A structure such as in structure 3 could be responsible for these signals since it includes benzylic methylenes next to another methylene in α to a carboxyl group.



(1) (2) (3)

The 3.5−8.6 ppm region of fraction 2 (Figure 8) showed a resonance profile similar to that of the bulk HA (Figure 6), although more details are visible in this size-fraction. The distinct doublet at 5.8 ppm may be assigned to olephinic protons in a simple molecule such as $HOCH_2C\mathit{H}{=}C\mathit{H}CH_2$-OH, whereas the signals at 4.0, 6.88, 6.91, and 7.25 ppm could be attributed to methylenic protons and to o-, p-, and m-protons in a structure such as in structure 4. Moreover, the signals at 7.8 ppm combined with those at 7.1, 7.2, and 7.45 ppm may suggest a structure such as in structure 5.



(4) (5)

The spectra of fractions 3 and 4 from the AcOH-treated HA were very similar to the spectrum of fraction 2. However, signals at 2.15 and 2.25 ppm and in the 7.0−8.6 ppm region of fraction 4 were reduced in intensity. Furthermore, signals present in the 3.5−4.1 ppm region in fraction 2 were not visible in fractions 3 and 4. The spectra of fractions 5 and 7 were also similar to that of fraction 2. Nevertheless, fractions 5 and 7 showed two additional multiplets at 1.6 ppm attributable to γ-methylenic protons in amines such as $R_1C\mathit{H}_2$-$CR_2R_3CR_4R_5NR_6R_7$ and at 1.95 ppm ascribable to α-methylenic protons in ketones such as $R_1C\mathit{H}_2COR_2$. The spectra of fractions 6 and 8 were similar to each other and, again, to the spectrum of fraction 2 in the interval of aliphatic signals, whereas aromatic protons were absent from spectra of fractions 6 and 8.

The significantly larger resolution in spectra of size fractions from AcOH-treated HA in respect to untreated HA was even more evident in Figure 9 where NMR absolute intensities and relative magnifications for the 0−2.3 ppm interval are compared for some of size-fractions. The absolute areas of the 1.38−0.94 (mainly alkyl $-CH_2-$) and 0.94−0 (mainly alkyl CH_3-) ppm intervals were calculated from spectra of Figure 9 and tabulated in Table 2 as compared to the same ppm regions of the bulk HA. The size-fractions separated from AcOH-treated HA produced NMR signals of higher intensity (e.g.: signal at about 1.2 ppm) than for untreated HA, which were, in turn, generally more intense than for the unfractionated HA. Such findings may be interpreted as an evidence of the effective HPSEC separation of HA constituents into fractions of smaller size and less chemical complexity, for which the solubility of humic samples in the DMSO solvent and consequent NMR signal intensity was enhanced. The AcOH treatment appears to have further deaggregated the hydrophobic clusters which stabilize the humic associations and thereby increased the solubility of the separated fractions in DMSO and, consequently, the intensity of the relative NMR signals. This interpretation

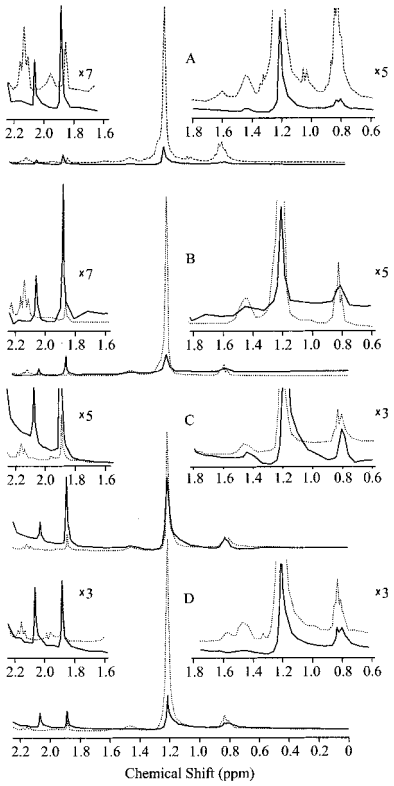


FIGURE 9. Absolute intensities in ^{1}H NMR spectra of size fractions with the same nominal MW collected before (full line) and after (dotted line) AcOH treatment: A. MW = 205−115 kD; B. MW = 115−64 kD; C. MW = 20−11 kD; D. MW11−6 kD.

TABLE 2. Quantitative Evaluation of the 0.94−1.38 ppm Interval in the ^{1}H NMR Spectra of Size-Fractions with the Same Nominal MW Value Obtained before and after AcOH Treatment

	absolute areas (arbitrary units)		absolute areas referred to bulk HA		
samples	1.38−0.94 (ppm)	0.94−0 (ppm)	1.38−0.94 (ppm	0.94−0 (ppm)	CH_2/CH_3
bulk HA	5.70	4.77	1.00	1.00	1.20
Fractions from Untreated HA					
1	14.7	10.4	2.58	2.19	1.41
2	1.29	1.34	0.23	0.28	0.96
3	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
4	9.28	7.99	1.63	1.68	1.16
5	8.46	5.61	1.48	1.18	1.51
6	6.11	4.56	1.07	0.96	1.34
Fractions from Treated HA					
1	42.9	14.1	7.52	2.96	3.04
2	54.9	7.96	9.63	1.67	6.90
3	15.9	5.07	2.78	1.06	3.13
4	8.93	4.33	1.57	0.91	2.07
5	14.0	2.64	2.46	0.55	5.30
6	6.46	3.68	1.13	0.77	1.76
7	10.1	5.75	1.77	1.21	1.75
8	5.97	1.44	1.05	0.30	4.15

[a] ND: not determined.

seems to be supported by the variation of the relative CH_2/CH_3 area ratio (Table 2) which, for the size-fractions of untreated HA, remained similar to that of the bulk HA, whereas it increased substantially for the fractions of AcOH-treated HA. Similarly to what observed for the pyrograms of size-fractions, such variation in the relative content of CH_2 and CH_3 groups suggests a different distribution of alkyl constituents brought about by the AcOH addition to HA.

The new peaks in the 1.8−2.2 and 1.8−0.6 ppm regions appearing after treating HA with AcOH (see magnifications in Figure 9) may again be attributed to a HPSEC separation into smaller, chemically simpler, and more DMSO-soluble size-fractions than for the untreated HA. Moreover, the differences of NMR chemical shifts between size-fractions of AcOH-treated HA and those of untreated HA may be ascribed to the diverse chemical environments in which the responsible protons are immersed and to a consequent different deshielding toward higher δ values of the applied magnetic field.

A further explanation for the higher sensitivity of ^{1}H NMR spectra of simpler and smaller size-fractions separated from the AcOH-treated HA may reside in the changes of proton relaxation times. Both spin−lattice (T1) and spin−spin (T2) relaxation times are related to molecular motions, and the shorter T1 and the longer T2 values, the higher becomes the signal intensity and the lower the resolution (*55*). It is known that T2 are lengthened for compounds in small aggregates (*55*) and slower relaxation enhances NMR sensitivity. In contrast, the chemical complexity of larger size-fractions from the untreated HA and even more from the unfractionated bulk HA prevents fast molecular motions, and the short relaxation times produce less resolved spectra.

Our findings suggest that preparative HPSEC may effectively fractionate a humic material in size-separates of considerably less heterogeneity. Due to automation of HPSEC systems, size-fractions can be reproducibly accumulated in sufficient amount to undergo advanced analytical techniques which become more useful than when applied to the bulk heterogeneous mixture of a HA. The chemical information obtained here on the different size-separates by Pyr-GC-MS and ^{1}H NMR spectroscopy were hardly reached with previous studies on bulk HS. Our results by Pyr-GC-MS and ^{1}H NMR techniques support the interpretation of the disrupting action of organic acids on the humic conformational structure in solution, as previously proposed (*11*, *12*, *20*−*22*, *33*). The size-fractions separated by preparative HPSEC after adding a very slight amount of AcOH to the original HA supramolecular association showed a reduced chemical complexity that allowed clearer distinction among pyrograms and more resolved ^{1}H NMR spectra. The combination of size-fractionation by HPSEC and fraction analysis by Pyr-GC-MS shows that the saturated and unsaturated alkyl chains, which contributed to the apparently large molecular size of a HA, were held together mainly by weak forces and were thus differently distributed in size-separates following AcOH addition to HA. Fractions separated in progressively lower molecular-size ranges maintained a certain hydrophobic character but were increasingly richer in aromatic and more polar groups including carbohydrates. The less chemical complexity of the humic fractions separated by HPSEC after AcOH treatment was also suggested by the larger resolution of the corresponding ^{1}H NMR spectra. Such well resolved spectra were essential in recognizing specific chemical structures of small molecules present in the different humic size-fractions. Further information on the chemical composition of size-fractions is expected by applying ^{13}C NMR spectroscopy, once the required amount is isolated by automated preparative HPSEC.

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