¹³C-NMR STRUCTURAL FEATURES OF SOIL HUMIC ACIDS AND THEIR METHYLATED, HYDROLYZED AND EXTRACTED DERIVATIVES

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(Received June 8, 1982; accepted after revision October 6, 1982)

ABSTRACT

González-Vila, F.J., Lüdemann, H.-D. and Martin, F., 1983. ¹³C-NMR structural features of soil humic acids and their methylated, hydrolyzed and extracted derivatives. *Geoderma*, 31: 3–15.

¹³C-NMR spectra of aqueous solutions of three humic acids (1 Typic Chromoxerert (HA-V), 1 Humic Haplorthod (HA-P), and 1 Inceptisol (HA-D)) are presented. The influence of chemical modifications (hydrolysis with 6N HCl, diazomethane methylation, and organic solvent extraction) of the humic substances and their spectra is studied. The spectra of the various humic substances show significant differences.

Methylation of the compounds introduces changes in the aromatic and aliphatic (sp³) region of the spectra. Acid hydrolysis removes residual carbohydrate and amino acid signals from the spectra, whereas solvent extraction has the most pronounced influence upon the aliphatic high field region of the spectra, and shows also, that a significant amount of the paraffinic carbons is bound covalently to the aromatic nuclei of the compounds.

INTRODUCTION

¹³C-NMR studies of humic substances at natural abundance have increased markedly during the past few years since the publication of the first definite report on ¹³C-NMR spectra of humic and fulvic acids by Gonzalez-Vila et al. (1976). Both conventional high-resolution NMR and newly developed NMR techniques, including solid-state NMR, widely used in basic coal research (Retcofsky and Link, 1978), have yielded more or less unresolved humic substance ¹³C-spectra (Stuermer and Payne, 1976; Wilson and Goh, 1977; Wilson et al., 1978; Ruggiero et al., 1979; Newman et al., 1980; Worobey and Webster, 1981; Hatcher et al., 1980a, b, 1981; Wilson et al., 1981). The poor resolution is due to a combination of different factors such as the extremely complex chemical characteristics of the samples, the high molecular weight and polydispersity, the presence

of paramagnetic ions and free radicals and the low solubility in common NMR solvents.

Soil humic acids are assumed to be highly cross-linked aromatic polymers of high molecular weight with covalent carbon-carbon-, ether-, and ester-linkages connecting the substituted aromatic moieties (Schnitzer and Khan, 1972). Groups of similar particles are associated by weak covalent and non-covalent bonds to "homogeneous" aggregates, which in unfractionated humic substances form large mixed aggregates (Wershaw et al., 1977). Carbohydrates, lignin derivatives, proteins and lipids have been reported as components of humic and fulvic acid preparations. It is thus difficult to characterize the chemical structure of humic substances because, they could present a complex mixture. This presumed complexity raises considerable difficulties in the detailed interpretation of the ^{13}C -NMR spectra.

Part of the non-humic components associated with humic acids can be removed by boiling the acidic aqueous solutions, apparently without changing the chemical characteristics associated with humic acids (Cheshire et al., 1967). Riffaldi and Schnitzer (1973) pointed out that acid hydrolysis provided more uniform material for subsequent structural investigations, and the same might occur after an exhaustive extraction with a sequence of organic solvents. Methylation, on the other hand, has been proposed (Haworth, 1971; Schnitzer and Khan, 1972) as a chemical treatment to remove the intramolecular hydrogen bonds that could associate aromatic moieties. In addition, methylation has the advantage of providing new information on the distribution of the environments of hydroxyl groups by ^{13}C -NMR spectroscopy (Snape et al., 1982).

The prime purpose of the work described in the present paper is the application of ^{13}C -NMR spectroscopy to unmodified and chemically modified (hydrolysis with 6N HCl, methylation and exhaustive extraction with organic solvents) samples of humic acids isolated from different soils. It is hoped that these experiments lead to a better understanding of the humic acid structure as seen by ^{13}C -NMR spectroscopy.

MATERIAL AND METHODS

Samples and preparation

A number of field and analytical characteristics of the soil samples from which humic acids were extracted are given in Table I. Methods used for the extraction, separation and purification of the humic acids, and for ultimate and functional group analysis were as described previously (Martin et al., 1977). Table II shows the elementary composition and functional group analysis of the humic acids.

The compounds were acid-hydrolyzed twice under N_2 in 6N HCl solution in a sealed tube at 105°C for 8 h. The humic acid residues were washed

TABLE I

Origins and some characteristics of soil samples

Sample	Horizon	Depth (cm)	Soil series	Order	Geographical origin	pH	C (%)
V	Surface	0-20	Utrera	Typic Chromoxerert	Sevilla (Spain)	7.8	1.0
P	B _h	20-30	Ribadeo	Humic Haplorthod	Lugo (Spain)	3.3	6.6
D	Surface	0-10	Coto Donana	Inceptisol	Huelva (Spain)	3.4	6.4

TABLE II

Analytical characteristics of humic acids

Sample	C* (%)	H* (%)	N* (%)	O* (%)	Ash* (%)	Total acidity (meq./g)	Carboxyls (meq./g)	Phenolic hydroxyls (meq./g)	Methoxyls (meq./g)
HA-V	57.7	5.6	5.8	30.9	1.5	5.7	3.1	2.6	0.4
HA-P	59.4	4.2	3.3	33.1	0.7	10.5	4.2	6.4	0.4
HA-D	57.1	4.5	3.4	35.0	1.1	6.0	3.3	2.7	0.3

*Dry, ash-free basis.

and dialysed until free of chloride ion and freeze-dried. The weight loss on acid hydrolysis was 40% (HA-V) and 38% (HA-P), respectively.

For methylation the humic acids were suspended in methanol and methylated with diazomethane to a constant methoxyl content. Methylation increased the methoxyl content in the HA-V from 0.4 to 17.5%, in the HA-P from 0.4 to 19.3% and from 0.3 to 15.6% in the HA-D.

The samples of HA-V and HA-P to be extracted with organic solvents, were prepared by Soxhlet extraction for 24 h each with the following sequence of solvents (100 ml): hexane, benzene, ethyl acetate and methanol. After each extraction the solvents were removed at 40°C by applying a reduced pressure of 0.1 Pa. The ¹³C spectra show that this procedure was successful.

¹³C-NMR spectroscopy

Solutions of the samples were prepared for ¹³C-NMR analysis by dissolving 200 mg of substance in 3 cm³ 0.5M aqueous NaOH. More concentrated solutions have higher viscosities which sharply decrease the spectral resolution. High-resolution spectra were recorded in 12 mm diameter tubes on a Varian XL-100-15 FT spectrometer operating at 25.2 MHz for ¹³C; proton-carbon couplings were removed by broad-band noise de-

coupling. A sweep width of 10 kHz and an acquisition time of 0.2 s with no pulse delay were chosen. The spectra shown result from 300,000 transients. The resolution of the spectra is ± 10 Hz (approx. ± 0.5 ppm). The spectrometer was locked to an external F-19 lock. Shift values are given in the TMS scale, referenced to a coaxial internal neat TMS capillary. No attempts to correct for magnetic susceptibility effects were made.

RESULTS AND DISCUSSION

The ^{13}C -NMR spectra of the three humic acids investigated (HA-V) from Typic Chromoxerert, (HA-P from Humic Haplorthod and HA-D from Inceptisol) are reproduced in Fig. 1. They show several broad but clearly resolved peaks throughout the 0-200 ppm range. The broadness of the NMR resonance is due to the presence of paramagnetic species, presumably free organic radicals, which were also found in preliminary ESR-experiments at g -values typical for free carbon radicals. These radicals may be also responsible for the signals in the region of negative chemical shifts. The ash content, though low, may also cause a pronounced peak broadening due to the additional relaxation induced by paramagnetic ions.

A tentative assignment of the various absorption signals in the ^{13}C -NMR spectra of the humic acids from a comparison with literature data (Stothers, 1972; Breitmaier and Voelter, 1974; Maekawa et al., 1979) is given in

Fig. 1. ^{13}C -NMR spectra of the humic acid samples.

Table III. A discrete peak is observed at about 175 ppm in the HA-V and HA-D spectra, which is assigned to carboxylate entities, but may also contain contributions from carboxyl, amide-, and ester-carbons. According to Hatcher et al. (1980a) the carboxyl groups could be partly derived from polyuronic acid moieties associated with polysaccharides. A distinct peak for carboxyl carbons is found at a lower field (180 ppm) in the HA-P spectrum, which exhibits also above the baseline noise a peak at 235 ppm attributed to other carbonyl functions, such as ketones and quinones, which have been identified by other analytical methods and account for 3 meq./g in a "model" humic acid presented by Schnitzer, 1978. The broad absorption envelope in the 110–160 ppm region of the spectra, not found by some other authors (Neyroud and Schnitzer, 1974; Wilson and Goh, 1977), is evidence for the presence of aromatic units commonly found by degradative methods in the humic acids. Recent investigations (Wilson et al., 1981a, b) of the solid state ^{13}C -NMR spectra, with proton cross-polarisation and magic angle spinning, of whole unextracted soils showed a contribution of 15 to 37% of aromatic carbons to the spectra. Although olefinic sp^2 -carbons could contribute to the signals in this region, there is no definite evidence for their presence in humic substances. In this context it might be of interest that acid-base treatment of carbohydrates yields

TABLE III

Tentative assignment of absorption bands in the ^{13}C -NMR spectra of the humic acids

HA-V	HA-P	HA-D	Shift range (ppm)	Possible assignments
(maximum peaks; ppm)				
175	236 175	180	190–160	Carbonyl, carboxyl, amide, ester carbons
150	148 145		160–145	Oxygen or nitrogen functional groups substituted aromatic carbons/bound to hydrocarbon substituted groups excluding CH_3 groups
	133	132	145–130	Bridgehead and alkyl substituted aromatic carbons/internal carbons of condensed aromatic compounds
125	128	125	130–110	Unsubstituted aromatic carbons/ $\text{C}_{\text{Ar}}\text{-H}$ carbons ortho to $\text{C}_{\text{Ar}}\text{-O}$
122		120		
103	103	103	110–90	Anomeric carbons of polysaccharides
73		71	90–55	Substituted aliphatic carbons: OH , oxygen and nitrogen substituted carbons in polysaccharides and amino acids/aliphatic ethers other than methoxy or ethoxy groups
71		60		
60		55		
52				
	48		55–0	Unsubstituted aliphatic carbons: CH_2 carbons in long chain saturated hydrocarbons/ C , CH , CH_2 bound to aromatic rings/ $-\text{CH}_2$ -carbons in alicyclic and ethyl groups/aliphatic carbons of peptides/methyl carbons
29	33			
23	25	25		
15	18	15		

aromatic compounds which could represent model monomers for humic substances (Worobey and Webster, 1981).

The resonances in the aromatic region resemble more the spectra of coal derivatives than those of lignin materials; the latter exhibit a set of well-resolved peaks assigned to aromatic carbon atoms in the para-hydroxy-phenyl propane structural units with distinct chemical shifts depending on the substituents in the aromatic ring, on the C- α substituents and on the fact of whether the phenolic hydroxyl group is alkylated or not (Lüdemann and Nimz, 1973). In their basic studies of coal structures, Bartle et al. (1975) and Maekawa et al. (1979) proposed a number of assignments for the aromatic carbon resonances. The ^{13}C -signals in the 148–160 ppm region are assigned to aromatic carbon bound to the strongly electron-withdrawing OH group and other heteroaromatic carbons.

In the range 129–148 ppm, sp^2 carbons with aliphatic carbon substituents and internal carbons of condensed aromatic compounds are observed. Signals in the 118–129 ppm region are assigned to aromatic C–H resonances and those in the 108–118 ppm region to aromatic C–H ortho to aromatic C–O groups.

According to these assignments there are several distinct spectral features in the aromatic region of the samples under study. In the spectra of HA-V and HA-P, discrete peaks are observed for oxygen-substituted aromatic carbons (150–148 ppm), though chemical analysis gives a similar content of OH phenolic groups (Table II) for the three humic acids. It is, however, hazardous to decide from the absence of peaks at about 150 ppm in the spectrum of HA-D that phenolic groups are absent in this latter sample, because it has been shown previously that aromatic carbons bound directly to an oxygen will show, depending on the chemical nature of the residual ring carbons, signals with chemical shifts varying between 130 and 160 ppm (Lüdemann and Nimz, 1974).

All humic acid spectra show pronounced aromatic signals between 128 and 120 ppm, suggesting the presence of non-substituted benzene rings. Minor carbon resonances below 120 ppm may be assigned to aromatic carbons carrying hydrogen ortho to aromatic C–O in the spectra of the three samples.

There are therefore few signals above 130 ppm, and hence the majority of the aromatic carbons appear to be protonated, although substituted aromatic carbons are also present to a small extent in the three humic acids.

Major differences occur in the aliphatic carbon region (0–100 ppm). The relative contributions from unsubstituted aliphatic hydrocarbons, carbohydrates, amino acids and ether carbons differ from sample to sample. Because, the signals from a random mixture of paraffinic carbons in different configurations overlap appreciably, it is problematical to assign the peaks found within the 0–50 ppm region.

The aliphatic structures which may contribute to the carbon resonances

in this range vary from dicarboxylic acids (41.4 ppm, α -C in malonic acid to 22 ppm, β -C in glutaric acid), obtained commonly by degradative methods, to lipids of various origin or to amino acids. Only the peaks near 30 ppm and 15 ppm have been assigned to methylene carbons and terminal methyl groups, respectively, in long-chain aliphatic constituents. Each spectrum presents a distinct set of peaks or shoulders in this range, confirming the lack of a common paraffinic structural component. In the 55–110 ppm range oxygen and nitrogen substituted carbons are observed. Carbohydrates, ether linkages, O-heterocycles and methoxyl groups are expected to be the major contributors to the peaks found in this region of the spectra, whereas the contribution from amino acid derivatives and unhydrolysed peptides should be small because of the low nitrogen content of the samples studied here (Table II).

The soil carbohydrates are a mixture of a large number of various polysaccharides. The "average" soil polysaccharide is of high complexity and closely associated with the humic matrix (Ogner, 1981). With the exception of the resonance near 80 ppm typical for carbons involved in glycosidic bonding to the anomeric position of the adjacent residue, the ^{13}C -spectra of the humic acids show signals with chemical shifts characteristic of carbohydrate polymers (Perlin and Hamer, 1979), between 100 and 110 ppm for the anomeric carbons (C-1), between 58 and 65 ppm for carbons of primary alcohol groups and around 75 ppm for the remaining secondary carbons. The peaks at 55–58 ppm and 72 ppm could alternatively be assigned to methoxyl groups and ether-bound aliphatic carbons. The pattern of peaks in this aliphatic region also seems to be different for each sample.

Quantitative determinations of the different organic-carbon-containing fragments would provide important information on the structural make-up of humic substances. Attempts to characterize this distribution by using destructive techniques have been seriously questioned (Maximov et al., 1977). ^{13}C -solution spectra can be used for the quantitative analysis of heterogeneous polymers only with great precaution. The presence of paramagnetic impurities, the dependence of the nuclear Overhauser effect on the correlation time of the single carbon atoms, a broad distribution of relaxation times and the possibility of a wide variety of relaxation mechanisms may lead to major differences between the recorded signal intensity and the relative concentrations of the different classes of carbon atoms. Van der Hart and Retcofsky (1976) showed that the intensities in solid-state ^{13}C -NMR spectra of coal products obtained by cross-polarization combined with the magic angle spinning technique relate quantitatively to the chemical composition. The solid-state spectra of humic materials obtained by Hatcher et al. (1980b) are quantitatively similar to the solution spectra, the latter being slightly better resolved. The relative areas of the peaks in both solid or solution spectra are nearly the same, and therefore quantitative measurements can be made from either spectra.

After these considerations are taken into account, Table IV gives an estimation of the amounts of different types of carbons in the humic substances by ^{13}C -NMR spectroscopy.

TABLE IV

Amounts (%) of the various C atoms in the humic acids as derived from the ^{13}C -spectra

C atom (range ppm) samples	Carboxyls (170–185)	Aromatic C (110–160)	Aliphatic C (0–50)	Unaccounted area (polysaccharichs, amino acids, methoxyls, ketones, etc.)
HA-V	3	30	27	40
HA-P	5	20	38	35
HA-D	7	24	32	38

Although the results must be viewed with caution and should be compared carefully with other information about the chemical structure of these materials, some conclusions can be drawn.

(a) NMR overestimates the carboxyl group contents determined by wet chemical methods for HA-P and HA-D but agrees well with the same value for HA-V. In the two former cases this discrepancy probably stems from the contribution of groups such as amides and esters to the carboxyl content calculated by direct integration of the region between 185 and 160 ppm in the ^{13}C -NMR spectra.

(b) The aromaticity decreases in the order HA-V > HA-D > HA-P, in accord with the characteristics of the soils from which the humic acids were extracted and the results of degradative methods (Martin and Gonzalez-Vila, 1981).

(c) The content of aliphatic material is relatively high. This result supports previous claims (Anderson and Russell, 1976; Grant, 1977), necessitating a reduction of the older estimates for the content of aromatic substances in humic materials.

The structural nature of humic materials is still controversial. Some workers claim the predominance of aromatic units in humic substances (Wilson et al., 1978; Ruggiero et al., 1979), whereas other groups have shown that many humic extracts contain largely aliphatic structures (Stuerner and Payne, 1976; Ogner, 1979; Newman et al., 1980; Hatcher et al., 1980a). Several factors such as the extraction technique (Worobey and Webster, 1981), the purification method and the origin of the humic material (Wilson and Goh, 1981) are responsible for the discrepancies found because the various groups are working with basically different materials. It would thus seem well to follow a suggestion by McCarthy (1976) and to establish a reference collection of well-defined humic materials as well as to adopt common methods of study in order to facilitate interlaboratory comparisons.

Figs. 2, 3 and 4 show the ^{13}C -NMR spectra of chemically altered humic acids. For comparison, the spectra of the unmodified materials are included.

The methylated samples (Figs. 2a, 3a and 4a) show spectra different from those of the original samples. The reduction in hydrogen bonding caused by the replacement of the protons in hydroxyl-groups with CH_3 -groups results in a lowering of the aggregation. This is clearly seen in the aromatic region of the spectra, where the methylated samples show a splitting of the original broad envelope into several discrete peaks. This splitting results from the influence of the methylation upon the chemical shifts of the aromatic carbons and from the reduction of the hydrogen-bonding which leads via a loss of aggregation to longer spin-spin-relaxation times and thus to better resolution of the spectra.

Fig. 2. ^{13}C -NMR spectra of the humic acid extracted from a Typic Chromoxerert (HA-V) before and after methylation (a), hydrolysis with 6N HCl (b) and exhaustive extraction with organic solvents (c).

As a consequence of the methylation the signal of the ester carbonyl increases in intensity and shifts slightly, particularly in the methylated HA-P and HA-D (Figs. 3a and 4a). It is known (Wershaw and Pinckney, 1979) that weakly acidic phenolic groups and alcoholic groups which are

Fig. 3. ^{13}C -NMR spectra of the humic acid extracted from a Humic Haplorthod (HA-P) before and after methylation (a), hydrolysis with 6N HCl (b) and exhaustive extraction with organic solvents (c).

Fig. 4. ^{13}C -NMR spectra of the humic acid extracted from an Inceptisol (HA-D) before and after methylation (a) and hydrolysis with 6N HCl (b).

found in humic acids, together with carboxylic groups and highly acidic phenolic groups, will not react with diazomethane. This is consistent with the presence of polyhydroxyl bands from polysaccharides in the methylated samples.

Methylation must also modify the spectral region of the aliphatic carbons. The new signals at 60 ppm (Fig. 4a) and 50–52 ppm (Figs. 2a and 3a) observed in comparing the spectra of unmethylated and methylated humic acids, have been assigned to OCH_3 of aromatic ethers with two adjacent alkyl or ring substituents and OCH_3 of COOCH_3 , respectively (Ogner, 1979; Snape et al., 1982). In addition the bands at 54–56 ppm due to aromatic ethers, with at most one adjacent alkyl or ring substituent, increase in the methylated HA-V and HA-P spectra (Figs. 2b and 3b).

The modifications introduced by methylation are well understood in the ^{13}C spectra of methylated humic acids, and these assignments may thus help in the interpretation of the differences found between degradation products of unmethylated and methylated humic substances (Schnitzer and Khan, 1972).

The ^{13}C spectra of the residues obtained after acid hydrolysis of HA-V and HA-P are reproduced in Figs. 2b, 3b and 4b. As expected, both spectra lack the signals from carbohydrates and amino acids, whereas the resonances in the aromatic region are more pronounced than in the original samples. Only weak signals appear for the residual unsubstituted aliphatic carbons of the residual moieties. The spectra also show a reduction in the intensity of the signals in the $\text{C}=\text{O}$ and COOH areas.

The ^{13}C spectra of the residues of HA-V and HA-P obtained after extraction with a sequence of organic solvents are shown in Figs. 2c and 3c. Because paraffinic compounds have been removed from the samples by this treatment, the signals of 0–55 ppm in the ^{13}C spectra must be assigned to the aliphatic carbon atoms covalently bound to the aromatic structures. Compared to the spectra of the hydrolyzed samples, the spectra of the residues after extraction with solvents are better resolved and show resonances from the polysaccharide moieties.

Both hydrolysis and extraction treatments have been recommended as "purification" procedures prior to structural studies of humic materials. It is still questionable whether these pretreatments in addition do modify substantially the natural humic fraction, especially the aromatic nuclei. So far this open question cannot be decided from the results of the ^{13}C -NMR spectroscopy. Even so, the technique appears useful in observing and assessing changes produced by chemical treatments.

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