Cross polarization, magic-angle spinning ^{13}C nuclear magnetic resonance spectroscopy of soil humic fractions

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Abstract—Cross polarization, magic-angle spinning ^{13}C nuclear magnetic resonance spectroscopy was used to characterize humic fractions isolated from different soils. The humic acid fractions are more aromatic than the humin fractions, probably due to the higher polysaccharide content of humins. However, fulvic acid fractions are more aromatic than the corresponding humic acid and humin fractions. These results can be interpreted in terms of the isolation procedure, because the high affinity of Polyclar AT for phenols results in higher aromaticities as compared with other isolation methods (e.g. charcoal).

Key words: organic structure determination, aromaticity, fulvic acids, polysaccharides

INTRODUCTION

Nuclear magnetic resonance (NMR) techniques have been applied in various ways to obtain information about humic substances. Most of these techniques involve NMR measurements of hydrogen (^1H) and carbon (^{13}C) nuclei on liquid samples (Stuermer and Payne, 1976; Sposito *et al.*, 1978; Gonzalez-Vila *et al.*, 1978; Ogner, 1979; Hatcher *et al.*, 1980, 1981a, b). Conventional NMR techniques have in the past required liquid samples, and typically only small fractions of most of the humic substances can be dissolved under conditions for which one can be confident of retaining the structural integrity of the compounds in the sample. Humic substances are extremely complex organic materials, and a wide variety of different types of aliphatic carbons (straight-chain, branched, cyclic, etc.) and aromatic carbons (unsubstituted, substituted, condensed, etc.) are expected to contribute to the NMR spectra. For this reason, spectra often are devoid of much fine structure, so the potential resolution advantage of liquid-solution NMR is often not realized with humic samples.

Recently, ^{13}C NMR techniques using cross polarization (CP) and magic-angle spinning (MAS) have been applied to solid samples of humic fractions by Hatcher *et al.* (1980, 1981a, b). They demonstrated the usefulness of the CPMAS ^{13}C NMR technique for the structural analyses of humic and fulvic acids by showing that aromatic carbon signals can be distinguished clearly from those of carboxyl, ether, methoxyl and aliphatic carbons and that carbon aromaticity can be measured quantitatively. A major advantage of the direct solid-sample ^{13}C NMR

approach is that the NMR measurements are made directly and nondestructively on either unaltered samples or on *entire* samples resulting from a prescribed fractionation technique and can provide valuable information on the carbon distribution between aromatic and nonaromatic structures in the samples.

A CPMAS ^{13}C NMR study of humic acids from soils of different climatic regions was recently accomplished by Hatcher *et al.* (1981b). The authors demonstrated the presence of significant quantities of aliphatic structures that generally were not observed by chemical oxidation studies; and, consequently, the derived aromaticities of the samples were lower than those previously estimated by permanganate oxidation. It appeared to us to be of interest to progress in humus characterization by studying the main humic fractions (humic and fulvic acids and humin) isolated from different acid, neutral, and alkaline soils.

MATERIALS AND METHODS

The soil samples used in this study were taken from the A horizons of a Typic Rendoll, Typic Xerorthent and two Typic Xerochrept(s) on different parent rocks (granite and shale, respectively). All the soils were located in the northern part of the province of Huelva, southwestern Spain. Some properties and characteristics of the soils are reported in Table 1.

Each sample was air-dried and was powdered to pass a 2 mm sieve. Before extraction, plant debris was removed by flotation. Batches of 400 g of the sieved sample were extracted with cold 1:1 solution of 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ and 0.1 M NaOH under N_2 . The extraction was repeated with fresh solvent until no appreciable amounts of humic acid (HA) could be removed (about 9 treatments). The extracts were centrifuged at 10,000 g, acidified to pH 2 with HCl, and the precipitated humic acid was removed by centrifugation. It

Table 1. Some properties and characteristics of the soils

Soil	pH(H ₂ O)	C(%)	N(%)	Altitude	Vegetation
Santa Olalla (Typic Xerochrept on granite)	5.6	3.5	0.4	480	Prairie of graminaceous, <i>Medicago</i> and <i>Trifolium</i>
Puerto Moral (Typic Xerochrept on shale)	6.8	1.2	0.1	575	<i>Quercus ilex</i> , <i>Lavandula</i> sp., <i>Cistus crispus</i> , <i>C. salicifolius</i>
Higuera Sierra (Typic Rendoll)	7.7	3.3	0.3	512	<i>Cistus albidus</i> , <i>C. ladaniferus</i>
Cañaveral Leon (Typic Xerorthent)	5.6	6.9	1.2	720	<i>Quercus coccoloba</i> , <i>Cistus albidus</i> , <i>C. salicifolius</i>

was dissolved again in 0.1 N NaOH, centrifuged at 15,000 *g* to remove mineral residues, then acidified and washed with 0.1 N HCl until colorless. After a de-ashing treatment with a dilute HF/HCl-solution (5 ml 48% HF + 5 ml 36% HCl + 990 ml H₂O) for 24 h and subsequent centrifugation, it was repeatedly washed with 0.1 N HCl, dialyzed against distilled water for one week and freeze-dried.

From the supernatant solution after the HA precipitation, the fulvic acid (FA) was isolated by Polyclar AT (polyvinyl-pyrrolidone from Serva, Heidelberg). The Polyclar was pretreated by washing several times with 0.1 N HCl and water; then the fulvic acid was adsorbed on this Polyclar by filtration and recovered afterward by elution with 0.1 N NaOH (Goh, 1970). The eluate was filtered and adjusted to pH 4, and then the FA was precipitated by adding FeCl₃. The precipitate was washed several times to remove chloride and then treated with Amberlite IR 120 in the H⁺ form by shaking to solubilize the FA at a pH of about 2.5-3. To isolate humin, the soil sample obtained after extraction with pyrophosphate-NaOH was repeatedly washed with distilled water and treated afterward several times with the HF/HCl solution for 24 hr. Then the sample was washed until the water reached a pH of 6, and extracted two times with 0.5 N NaOH. The alkaline extract was treated as described earlier for the HA, but without the de-ashing procedure.

The ¹³C CPMAS NMR spectra were run at 37 MHz with 150 MHz proton decoupling on a NT-150 spectrometer modified for solids experiments and

utilizing a home-built probe. Samples ranging in size from about 90 to 600 mg required from 40,000 to 80,000 scans of spectral accumulation. All spectra were determined using 1-ms contact times and 1-sec repetition times. The samples were spun at about 4 kHz at the magic angle. A Hartmann-Hahn match was established with both ¹³C and ¹H RF fields of about 45 kHz.

RESULTS AND DISCUSSION

Some chemical characteristics of the humic fractions examined are presented in Table 2. The parameters are similar to those reported for other humic fractions (Martin *et al.*, 1977; Saiz-Jimenez *et al.*, 1979), and fall within the ranges and means reported for humic fractions from soils from widely differing climatic zones (Schnitzer, 1977).

The apparent carbon aromaticities summarized in Table 2 were estimated by integration of the ¹³C NMR spectra. Intensity between 160 ppm and 95 ppm was assigned to aromatic carbons. For these purposes, as the intensities of spinning sidebands in the spectra employed are comparable to noise, they were neglected. Small, but significant errors in these apparent aromaticities might be expected because of contributions of polysaccharide resonances in the assigned aromatic carbon region (*vide infra*); these errors should be partially compensated by the neglect of spinning sideband intensities. Of course, these estimated aromaticities, as well as any other attempts to extract quantitative interpretations from the ¹³C

Table 2. Analytical characteristics of the soil humic fractions

Samples	C (%)	H (%)	N (%)	O (%)	Ash (%)	Atomic H/C	Apparent carbon aromaticity (%) ^a
<i>Humic acids</i>							
Typic Xerochrept (granite)	52.76	5.97	4.37	36.90	5.6	1.4	26
Typic Xerochrept (shale)	49.95	5.87	4.21	39.97	15.11	1.4	25
Typic Rendoll	55.50	5.55	4.23	34.72	5.71	1.2	34
Typic Xerorthent	54.55	5.58	3.65	36.22	4.17	1.2	26
<i>Fulvic acids</i>							
Typic Xerochrept (granite)	49.75	4.35	2.72	43.18	14.67	1.0	39
Typic Xerochrept (shale)	44.72	4.42	2.18	48.68	14.84	1.2	41
Typic Rendoll	49.64	3.79	2.01	44.56	10.93	0.9	42
Typic Xerorthent	46.90	4.11	1.44	47.55	4.24	1.0	44
<i>Humins</i>							
Typic Xerochrept (granite-1)	56.56	7.13	4.30	32.01	1.89	1.5	20
Typic Xerochrept (granite-2)	58.39	7.22	5.25	29.14	11.94	1.5	24
Typic Rendoll	51.87	5.89	5.45	36.79	6.06	1.4	31

^aAromaticity derived from ¹³C CPMAS measurements, as described in text, ±2% (e.g. 26 ± 2% signifies 24-28%).

Fig. 1. ^{13}C CP/MAS NMR spectra of humic acids.

NMR intensities, are subject to the uncertainties associated in general with deconvoluting overlapping peaks and with variations of pertinent relaxation parameters with structure. Nevertheless, while keeping these uncertainties in mind, it has been well established that semiquantitative comparisons of ^{13}C NMR spectral intensities can yield valuable insights into chemical structural relationships among organic geochemical solids.

Assignment of peaks in ^{13}C NMR spectra of humic substances and related materials has been discussed by several authors (Gonzalez-Vila *et al.*, 1978; Hatcher *et al.*, 1980, 1981a, b; Wilson, 1981). In this paper, assignments are mainly based on spectral examination of well-defined compounds (aliphatic hydrocarbons, fatty acids, proteins, polysaccharides, lignins, phenolic polymers, etc.) and extrapolation to humic substances, which are also known to contain these specific kinds of structures (Martin *et al.*, 1977, 1979; Saiz-Jimenez and de Leeuw, 1984a, b). ^{13}C -chemical shift measurements in the solid state are probably accurate to ± 3 ppm, and unless there are unusual paramagnetic environments, chemical shifts should be similar to those expected for compounds in solution.

Hatcher *et al.* (1981b) reported that the CPMAS ^{13}C NMR spectra of humic acids, major signals are observed for aromatic carbons (130 ppm) and carboxyl carbons (175 ppm). Other major signals are those of unsubstituted aliphatic carbons (0–50 ppm), methoxyl carbons (55 ppm), ether and carbohydrate carbons (60–72 and 106 ppm), oxygen-substituted aromatic carbons of phenols (150 ppm) and carbonyl carbons (170–200 ppm) (with carboxyl carbons in the range of about 170–180 ppm).

The CPMAS ^{13}C NMR spectra of the humic acids from the soils herein reported (Fig. 1) are qualitatively similar, but different in some details from those presented by Hatcher *et al.* (1981b). In fact, they show a dominant peak at 33 ppm, characteristic of methylene carbons in long-chain hydrocarbons (Lindeman and Adams, 1971), followed in intensity by a carboxyl peak at 173–176 ppm. Model compounds like fatty acids (Johnson and Jankowski, 1972) show peaks of methyl carbons between 23 and 34 ppm, and a peak of carboxyl carbons at 180 ppm. The presence of shoulders at 15 and 25 ppm in some of these humic acids together with the above-mentioned resonances suggest that fatty acids (and aliphatic hydrocarbons) might be important con-

tributors to these signals. Furthermore, fatty acids and aliphatic hydrocarbons have been isolated from humic acids in various amounts (Schnitzer and Neyroud, 1975; Saiz-Jimenez and de Leeuw, 1984a, b, 1986).

Recently, Preston *et al.* (1982) reported that in *p*-benzoquinone polymers incorporating amino acids and peptides (glycine, alanine, glycylglycine), bands for amino acid carbons occur between 41 and 53 ppm, with additional bands at 15 and 16 ppm for methyl groups in alanine derivatives. The spectrum for benzoquinone incorporating casein shows many protein bands from 11 to 67 ppm. However, in the humic acids of the present study no definite bands in this region were observed, but only a broad band centered at about 33 ppm, as reported above.

Oxygen-substituted and nitrogen-substituted aliphatic carbons have resonances between 50 and 110 ppm. Because the nitrogen content of humic acid is low (3.65–4.37%) compared with the oxygen content (34.72–39.97%), contributions from N-substituted aliphatic carbons are expected to be small. Thus, the peak at 56–67 ppm is most probably that of methoxyl carbons. Lignins present a prominent, rather sharp peak at about 56 ppm due to aryl OCH₃ resonances (Bartuska *et al.*, 1980; Maciel *et al.*, 1981), and lignin derivatives and lignin degradation products have been reported to constitute a part of the complex humic macromolecules (Martin *et al.*, 1979; Saiz-Jimenez *et al.*, 1979; Saiz-Jimenez and de Leeuw, 1984a, b, 1986).

Polysaccharides (e.g. cellulose) show an intense peak at about 75 ppm that is associated with resonances of the carbons numbered 2, 3, and 5; further peaks at about 66, 86 to 90, and 106 ppm are assigned to the 6, 4, and 1 carbons, respectively (Atalla *et al.*, 1980; Earl and VanderHart, 1980). The peaks at about 73–75 and 105–106 ppm in the humic acid spectra agree well with those of cellulose. Because complex polysaccharide patterns have been observed in the pyrolysis mass spectra of these humic acids (Saiz-Jimenez *et al.*, 1979) and because evidence of such materials has been extensively reported (Saiz-Jimenez and de Leeuw, 1986) peaks in the region between 60 and 110 ppm could be assigned to polysaccharide carbons.

Hatcher *et al.* (1980, 1981a, b) reported that unsubstituted and alkyl-substituted aromatic carbons resonate between 120 and 130 ppm in humic samples, and that a peak at about 150 ppm is related to oxygen-substituted aromatic carbons of phenols. Furthermore, Bartuska *et al.* (1980) pointed out that in lignins, peaks at about 132–134 ppm can be identified with the C₁ resonance, and the intense peaks at 147–150 ppm belong to C₃ and C₄ resonances in the typical lignin unit. Peaks at 111 to 114 ppm can be assigned to C₂ resonances. C₆ resonances would also occur in the resonance band between about 117 and 133 ppm, the individual contributions depending on the detailed substitution

pattern on the ring. The spectra of humic acids show shoulders at about 118–119 ppm and peaks at 130–132, and 151–153 ppm, which likely correspond to the carbon resonances of the lignin building blocks. As discussed above, lignin derivatives can commonly be identified in humic acid degradation studies (Martin *et al.*, 1979; Saiz-Jimenez *et al.*, 1979; Saiz-Jimenez and de Leeuw, 1984a, b) in variable amounts. More condensed structures like polycyclic aromatic hydrocarbons would also have ¹³C resonances in the 110–160 ppm region if present in the molecules of humic acid.

Although Preston *et al.* (1982) reported that nitrogen substitution on aromatic carbons in *p*-benzoquinone compounds incorporating amino nitrogen results in ¹³C NMR bands around 125–128 and 155 ppm, it appears that the resonances at about 153 ppm in humic acids cannot be assigned to this nitrogen substitution, because peptides or proteins are linked through the free amino group of the terminal amino acid of the protein chain to the phenolic rings (Haider *et al.*, 1965; Flaig *et al.*, 1975; Saiz-Jimenez *et al.*, 1979). This C–N moiety would represent a negligible part of the total content of an average soil humic acid molecule; hence, the ¹³C NMR intensity associated with this C–N moiety would be very small in comparison with C₃ and C₄ resonances of typical lignin units, widely represented in soil humic fractions, as pointed out by Saiz-Jimenez and de Leeuw (1986).

¹³C NMR spectra of podzol fulvic acids have been previously reported by Gonzalez-Vila *et al.* (1978) and Hatcher *et al.* (1981b). However, in the first study, several difficulties were found in recording the spectrum, which shows broad and poorly resolved resonances and high baseline noise. These can be due to several factors, such as the molecular complexity of the sample, the high viscosity of the solution, and to inherent limitations of the techniques employed. Hatcher *et al.* (1981b) presented a better fulvic acid spectrum by using the CPMAS technique, which results in much improved signal/noise ratios and with only a relatively minor loss in resolution in the solid-sample spectrum. This spectrum can be compared with those presented herein.

Fulvic acid spectra shown in Fig. 2 are very similar to those reported by Hatcher *et al.* (1981b). The main peaks are those reported above for humic acids, but with substantial intensity differences. In fact, the major peak was in these cases associated with carboxyl carbons at 176 ppm. Only for the Typic Xerochrept (granite) fulvic acid did the peaks of the aliphatic (35 ppm), carbohydrate (75 ppm) and aromatic (131 ppm) carbons attain intensity similar to that of the carboxyl carbons. One important feature of the fulvic acid spectra is the appearance of minor peaks in various regions. Thus, in the region of aliphatic carbons, peaks at 33–35 and 45 ppm and shoulders at 24–25 and 39 ppm are evident; these peaks indicate the existence of a random mixture of

Fig. 2. ^{13}C CP/MAS NMR spectra of fulvic acids.

paraffinic structures, producing an unresolved or badly resolved mixture of peaks for which it is difficult to assign specific aliphatic structures. Such assignments would be especially difficult because of the complex interplay of α , β and γ carbon-substitution shifts, resulting in a situation in which a great deal of overlap occurs (Hatcher *et al.*, 1980).

As fulvic acids have lower nitrogen contents (Table 2) than do humic acids, a possible contribution of resonance intensity for amino acid carbons to the 0–50 ppm region does not appear probable. Furthermore, Saiz-Jimenez and de Leeuw (1984b) found no significant nitrogen-containing compounds among the pyrolysis products of these fulvic acids, which adds support to this statement.

Saiz-Jimenez and de Leeuw (1984b) pointed out that soil polysaccharides constitute one of the most important components of soil fulvic acids, which in addition contain microbially oxidized lignin units, fatty acids and dialkyl phthalates. The importance of the complex polysaccharide fraction (which contains ten or more major sugar units) is stressed in the fulvic acid spectra by the appearance of many shoulders or

small peaks and two major resonances; these features agree well with those reported for cellulose (Atalla *et al.*, 1980; Earl and VanderHart, 1980). In fact, small peaks or shoulders at about 64, 79–82, 86, 89, 100–103 ppm, in addition to the prominent peaks at 75–76 and 107–108 ppm, are strongly suggestive of polysaccharide carbon resonances. Furthermore, the polysaccharide carbon chemical shift region accounts for roughly one-third of the total spectral area, less that of the area of carboxyl carbons, which indicates that roughly one third of the carbon in fulvic acid molecules can be accounted for as polysaccharide. This conclusion agrees with previous fulvic acid structural studies (Saiz-Jimenez *et al.*, 1979; Saiz-Jimenez and de Leeuw, 1984b).

The aromatic region of the fulvic acid ^{13}C spectra displays similar resonances to those of humic acids, with a major peak at 131 ppm and a minor peak at 151 ppm. Further, several shoulders in the 110–160 ppm region are observed. As stated above for humic acids and because an important part of the fulvic acid pyrolysis products was identified as lignin-derived units (Saiz-Jimenez and de Leeuw, 1984b), it

Fig. 3. ^{13}C CP/MAS NMR spectra of humins.

can be concluded that most of the resonances in the aromatic region can reasonably be assigned to lignin fragments or microbially oxidized lignin residues.

In the ^{13}C spectra of the fulvic acids, resonances between 190 and 200 ppm, although still small, are more conspicuous than in the spectra of humic acids. In this region one finds resonances of carbonyl carbons from aldehydes and ketones, which indicates the presence of minor amounts of these functional groups. Schnitzer (1977) has reported that fulvic acids have more oxygen-containing functional groups than humic acids, including ketonic carbonyls.

Humin spectra are shown in Fig. 3. For humin extraction two different procedures were adopted. Procedure 1 consisted, as described in the section on "Materials and Methods", of the complete extraction of humic and fulvic acids, which usually requires about 9 treatments, resulting in humin-1. Procedure 2 involves only 3 treatments for removal of humic and fulvic acids, which results in about 80–90% removal of the total alkali-soluble fractions. This indicates that a part of the humic acid fraction could be coextracted and incorporated into a humin-2 fraction. This second procedure is less tedious and

time-consuming; however, as denoted in Fig. 3, no major differences were observed between the ^{13}C NMR spectra of the humins isolated by the two procedures. Nevertheless, humin-2 seems to contain slightly higher carbohydrate, aromatic and carboxyl carbon contents, which implies a less exhaustive organic carbon removal than is accomplished by procedure 1.

Interestingly, the ^{13}C spectra of the humins display a close similarity to those of the humic acids, with no major differences in carbon resonance positions. This indicates that humins might be described simply as humic acids that are in some way "agglomerated", e.g. collectively bonded to clay minerals (compare Figs 1 and 3). In fact, it has been reported (Saiz-Jimenez and de Leeuw, 1986) that humins are similar to humic acids, but that the former generally contain high amounts of polysaccharides. It appears that humin itself does not contain such high amounts of polysaccharides, but that both fractions are complexed with clays, including interlamellar adsorption with montmorillonite. Destruction of clays by HF:HCl treatments releases both fractions, which are coextracted with alkaline agents. Because of

the intimate mixture of polysaccharides and humic substances in soils, there is coprecipitation of polysaccharides during the extraction procedures of humic fractions.

Hatcher *et al.* (1981b) reported that aromaticity in humic fractions ranged from 35 to 92%, with humic acids being more aromatic than the single fulvic acid sample analysed, which exhibited the lowest aromaticity. In the present study, humic acids and humins were found to have very similar aromaticities, ranging from 20 to 34% (Table 2). In general, humic acids were found to be more aromatic than humins, probably due to the higher contribution of polysaccharides in humins. However, in contrast with the data reported by Hatcher *et al.* (1981b), fulvic acids in the present work appears to be more aromatic than the corresponding humic acid or humin isolated from the same soil (Table 2). This is also indicated by the atomic H/C ratios. These contrasting results can be explained in terms of the procedures followed for isolating the fulvic acids. In our case fulvic acids were isolated by using Polyclar AT. This polyvinyl pyrrolidone polymer preferentially retains the polyphenols, partially excluding the polysaccharide fraction that is usually present in the extract. As Saiz-Jimenez *et al.* (1979) have demonstrated, the method of isolation of fulvic acids determines the chemical composition of the molecules. Fulvic acids isolated by charcoal adsorption exhibited higher polysaccharide and lower lignin components than corresponding preparations isolated with Polyclar AT. Therefore, the high affinity of Polyclar AT for phenols, as indicated by its use to remove polyphenols from wines and beers, may explain the high apparent aromaticities of the fulvic acid preparations herein reported.

Polysaccharides are bound by hydrogen bonding or by covalent bonds to humic species, or may be entrapped within coiled humic molecules; and consequently, removal of polysaccharides from humic materials is a major problem in purification of soil humic fractions. According to the isolation and purification procedures, the humic fractions will be more or less enriched in accompanying soil macromolecules, and aromaticities will strictly depend on the specific procedure chosen. Polyclar AT purification appears to be the more efficient method for partially removing polysaccharides from soil extracts, and, at the same time, the more adequate method for recovering soil polysaccharides, as pointed out by Saiz-Jimenez and de Leeuw (1984b).

Acknowledgements—The authors gratefully acknowledge the use of the facilities of the Colorado State University Regional NMR Center, funded by National Science Foundation Grant Number CHE-8208821, and partial support of this work by the Colorado State University Experiment Station and by the CAICYT of Spain.

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