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Effects of acid hydrolysis on the ^{13}C NMR spectra of humic substances

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Summary Two humic and one fulvic acid were hydrolyzed with hot 6 *M* HCl and a combination of 12 *M* and 0.5 *M* H_2SO_4. Effects of acid hydrolysis on the chemical structures of the humic materials were assessed by comparing liquid-state ^{13}C NMR spectra of hydrolyzed with those of unhydrolyzed humic substances. Hydrolysis with 6 *M* HCl was found to be more efficient for removing proteinaceous materials and carbohydrates than was hydrolysis with 12 *M*-0.5 *M* H_2SO_4. The latter appeared to better preserve the structural integrity of the humic materials, especially in the lower aliphatic region (0–50 ppm). Hydrolysis with the two acids increased the aromaticity of the humic materials, in part by removing proteinaceous components and carbohydrates. It also lowered the CO_2H content of two out of three humic preparations, probably because of acid decarboxylation. The data presented demonstrate the great potential of ^{13}C NMR spectroscopy for structural research on humic materials.

Introduction

The application of carbon-13 (^{13}C) Nuclear Magnetic Resonance (NMR) spectroscopy to structural investigations of humic substances is a relatively new technique[8,9,10,11,14,15,19,26], which makes possible direct observations of the macromolecular carbon skeletons and examinations of the gross structures of the heterogenous polymers. ^{13}C NMR spectra of humic material published so far contain a number of signals which have not yet been assigned to specific structural components. This applies especially to proteinaceous and carbohydrate constituents. Thus, the major objectives of this investigation were: (a) to remove proteinaceous components by hydrolysis with hot 6 *M* HCl; (b) to remove carbohydrates by hydrolysis with a combination of 12 *M* and 0.5 *M* H_2SO_4; and (c) to compare ^{13}C NMR spectra of hydrolyzed with those of unhydrolyzed humic substances in order to arrive at a more comprehensive interpretation of their ^{13}C NMR spectra. Secondary objectives of our research were to examine possible effects of acid hydrolysis on the aromaticity of these materials and on the quantitative distribution of carboxyl groups.

Materials and methods

Origin, extraction, purification and analytical characterizations of HA and FA samples

The humic materials used in the present investigation were: (a) a HA extracted from the Ah horizon (0–25 cm; 6.1% organic C; 0.5% total N; pH = 6.4, soil/water ratio 1:2.5) of the Beaverhills soil, a Chernozem from Central Alberta; (b) a HA extracted from the A horizon (0–15 cm; 3·7% organic C; 0.3% total N; pH = 6.3) of the Bainsville clay loam, an Orthic Humic Gleysol from the Central Experimental Farm at Ottawa, Ontario; and (c) a FA extracted from the Bh horizon (15–22 cm; 4.2% organic C, 0·1% total N; pH = 4.0) of the Armadale soil, a Podzol from Prince Edward Island. The methods used for the extraction, fractionation and purification of the HA's and the FA as well as for the subsequent elemental and functional group analyses were the same as those described previously[7,23]. Analytical data for the HA's and the FA are shown in Table 1.

Carbohydrates in the humic preparations were determined by the phenol-sulfuric acid method[15] after refluxing each sample (at about 100°C) with 0.5 *M* HCl for 5 hours. Amino acid-N in the HA's and FA was determined, after hot 6 *M* HCl hydrolysis for 24 hours on an amino acid analyzer[22]. Moisture was determined by heating portions of each sample at 105°C for 24 hours and ash by igniting samples at 750°C for 4 hours. E_4/E_6 ratios were determined by dissolving 2.0 mg of HA and 5.0 mg of FA in 10 ml of 0.1 *M* $NaHCO_3$ solution and measuring absorbances at 465 and 665 nm. The ratio of the optical densities at the two wavelengths was the E_4/E_6 ratio[7].

The methoxyl content was determined by the Zeisel method[24]. For hydrolysis with HCl, 500 mg of HA and FA was refluxed with 50 ml of 6 *M* HCl for 24 hours. Insoluble residues were separated from supernatants by filtration through sintered glass funnels and washed thoroughly with distilled water until free of Cl^-. The residues were then dried over P_2O_5 in a vacuum desiccator at room temperature and analyzed by ^{13}C NMR spectroscopy. The procedure used for the hydrolysis of the HA's and FA

Table 1. Analytical characteristics of HA's and FA (on a moisture- and ash-free basis)

Characteristics	Chernozem HA	Gleysol HA	Podzol FA
C(%)	56.4	55.3	50.9
H(%)	5.5	5.4	3.3
N(%)	4.1	4.6	0.7
Amino acid N(%)	1.4	1.6	0.2
S(%)	1.1	0.9	0.3
O (%)	33.0	33.8	44.8
OCH_3 (meq/g)	1.0	1.2	0.2
Total acidity (meq/g)	6.6	6.4	12.4
CO_2H (meq/g)	4.5	3.5	9.1
Phenolic OH (meq/g)	2.1	2.5	3.3
Ketonic C=O (meq/g)	2.5	1.8	2.5
Quinonoid C=O (meq/g)	1.9	2.0	0.6
Carbohydrates (%)	5.7	5.4	8.2
E_4/E_6 ratio	3.8	4.2	7.1
Ash (%)	0.9	0.6	2.0

with H_2SO_4 was that of Cheshire and Mundie[4]. Briefly, 500 mg of HA and FA was allowed to stand with 2.5 ml of 12 *M* H_2SO_4 for 16 h at room temperature. Following this, the acid was diluted to 0.5 *M* by the addition of distilled water and the mixture refluxed (near 100°C) for 5 h. After cooling the residue was separated by centrifugation, thoroughly washed with distilled water and dried over P_2O_5 at room temperature. Portions of the residues were subjected to ^{13}C NMR analyses.

^{13}C NMR spectroscopy

^{13}C NMR spectra were recorded at 62.83 MHz, using a Bruker WM 250 spectrometer and 10-mm sample tubes. Samples of HA's, FA's, and their hydrolyzed residues (90 to 100 mg) were made up in 2–3 ml of 0.5 *M* NaOD (D_2O plus 5 *M* NaOH), gently shaken for several hours, centrifuged and filtered before use. Practically 100% of the humic materials was soluble under these conditions. Chemical shifts were measured relative to sodium 3-trimethylsilyl-propionate-2,2,3,3,-d_4 (TSP). Spectra were usually obtained without sample spinning; 100,000 to 250,000 scans were accumulated.

Spectra were obtained using inverse-gated decoupling[5]; that is, the decoupler was off except during the acquisition time of 0.1 sec. Samples were run with a 45° pulse and total interpulse delay of 0.6 Sec. The inverse-gated decoupling produces decoupled spectra, while suppressing the nOe (nuclear Overhauser enhancement) effect, which can cause intensity distortions if the enhancement is not the same for all carbons. (It also avoids sample heating during the acquisition).) The pulse widths and delay times were chosen to reduce distortions due to nOe effects and lack of complete relaxation of all carbons[1,14, 17]. The free induction decays (FID's) were multiplied by a decaying exponential ("sensitivity enhancement"), which reduces the contribution of the tail end of the FID, for which the noise content is relatively high[13]. After Fourier transformation, a small baseline correction was applied to some of the spectra. The error for chemical shifts was ±0·5 ppm.

Results

^{13}C NMR spectra of HA's

The ^{13}C NMR spectrum of the Chernozem HA (Fig. 1A) exhibits several distinct peaks in the aliphatic (0–105 ppm), aromatic (105–165 ppm) and carboxyl (165–190 ppm) regions. The peaks at 16.5, 21.1, 25.0, 27.1 and 31.3 ppm are most likely due to aliphatic carbons in alkyl chains. The peak at 16.3 ppm is characteristic of terminal methyl groups, and that at 31.3 ppm of $(CH_2)_n$ in long alkyl chains, although other alkyl carbons may occur at this chemical shift[8,12]. The peak at 40.2 ppm may include contributions from both alkyl carbons, and from amino acid carbons, such as glycine (α-carbon, 42.6 ppm), arginine (δ 41.5 ppm), lysine (ε 40.0 ppm)[2]. In the 50–105 ppm region aliphatic carbons substituted by oxygen and nitrogen are usually observed. The peaks at 52.6 and 58.5 ppm may be due to -OCH_3 although the methoxyl content of the unmethylated HA is small (Table 2). Amino acids may also contribute in this region; for example, the α-carbons of arginine, glutamic acid, and lysine, all found in HA's[3,18] occur at approximately 55 ppm. In view of the relatively high N content of the HA (Table 1), one would certainly expect amino acid carbons to contribute to the intensity in the 50–70 ppm region. The HA also contains carbohydrates (Table 1) which generally give rise to signals around 60–65 ppm (C_6), 70–80 ppm (C_2-C_5 ring carbons) and 90–105 ppm (anomeric carbons)[2,8], so that the peak at 63.5, and the broader resonances at 73.4 and approximately 105

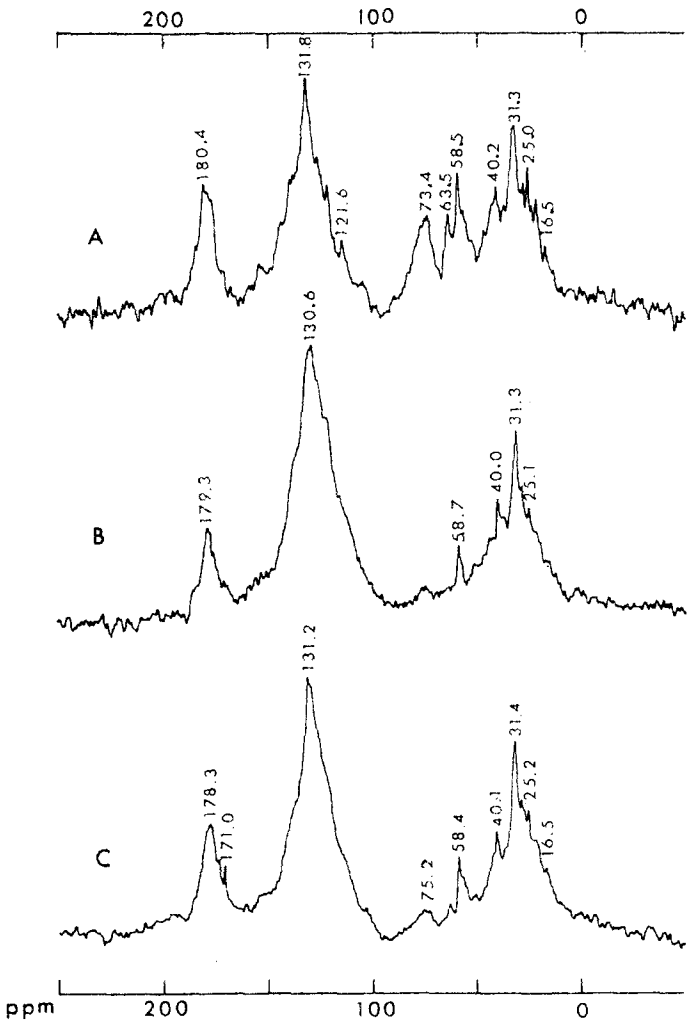

Fig. 1. ^{13}C NMR spectra of: **A** Chernozem HA; **B** Chernozem HA hydrolyzed with 6 *M* HCl; **C** Chernozem HA hydrolyzed with 12 *M*-0.5 *M* H_2SO_4.

ppm in Fig. 1A are probably due to carbohydrates. The region from approximately 110 to 165 ppm may be assigned to aromatic carbons. Although olefinic carbons also produce signals in this region, they do not appear to be present in humic materials in significant amounts[21].

The aromatic region contains a relatively sharp maximum at 131.8 ppm. Hatcher *et al.*[9] also observed a major peak for a peat HA at 130.0ppm. They assigned this resonance to ring carbons in which the ring was not substituted by strong electron donors such as oxygen and nitrogen. Alkyl benzenes are typical components which would yield such resonances[2,8], as would unsubstituted aromatic C-H. Thus, it appears that in the HA, a large proportion of the

Table 2. Effects of methylation on the analytic characteristics of the Mollisol HA and the Spodosol FA

Characteristic	HA		FA	
	b*	a**	b*	a**
C(%)	56.4	57.7	50.9	57.1
H(%)	5.5	6.1	3.3	5.3
N(%)	4.1	4.7	0.7	0.5
S(%)	1.1	0.5	0.3	1.8
0(%)	32.9	31.0	44.8	35.3
OCH_3 (%)	1.0	16.3	0.2	27.2

b* before methylation, a** after methylation

aromatic carbon is not substituted by O and N. The small peak at approximately 155 ppm in Fig. 1A indicates the presence of 0- and N-substituted aromatic C groups (phenolic OH or aromatic NH_2). The broad peak near 180 ppm is due to C of carboxyl groups; carboxylic acids as well as amides and esters would contribute to this peak.

In general, the spectrum of the HA in Fig. 1A (and similar spectra of HA's and FA in Figs. 2A and 3A) exhibit the good signal/noise ratio routinely available at high field (250 MHz for protons). A large number of distinct peak maxima can be observed and correlated with the structural information provided by chemical techniques. However, the spectra still have very limited resolution, and this may be attributed to several factors. The first is the natural complexity of humic materials, which do not have an easily-defined structure or repeating unit, and contain carbons in many different environments. The presence of paramagnetic organic free radicals and metal ions would also cause line broadening; a further cause may be the relatively slow motion (long correlation times) of these large polymeric molecules in solution. It has also been suggested that hydrophobic regions of HA's and FA's may be poorly solubilized in NaOH, so that these portions may exist in a micellar or solid-like phase, removed from contact with the solvent[6,25].

The ^{13}C NMR spectrum of the Chernozem HA which has resisted hydrolysis with hot 6 *M* HCl is shown in Fig. 1B. The spectrum is simpler than that in Fig. 1A, with distinct maxima remaining only at 18.7, 25.1, 31.3, 40.0 and 58.7 ppm in the aliphatic region. The ^{13}C NMR spectrum of Chernozem HA which has "survived" hydrolysis with 12 *M*-0.5 *M* H_2SO_4 (Fig. 1C) is very similar to that of

the same HA following hydrolysis with 6 *M* HCl (Fig. 1B). In the aliphatic region, small signals can be observed at 14.4, 25.0, 31.0, 54.0 and 58.7 ppm. The persistence of distinct peaks at 58.7 ppm in Figs. 1B and 1C indicates that this band in the spectrum of the original HA (Fig. 1A) is due to methoxyl carbons (see also Table 1). Resonances at 56.2, 63.5 and 73.4 ppm in Fig. 1A are no longer present in the spectra of the hydrolyzed HA's (Figs. 1B and 1C). Chemical analyses for amino-N and carbohydrates showed that hydrolysis with the two acids had removed most of the proteinaceous components and carbohydrates from the acid-treated HA's. On the basis of these observations, and from data published in the literature[2], we can now assign the following bands in Fig. 1A: 56.2 ppm (amino acids), 63.5 ppm and 73.4 ppm (carbohydrates). One striking

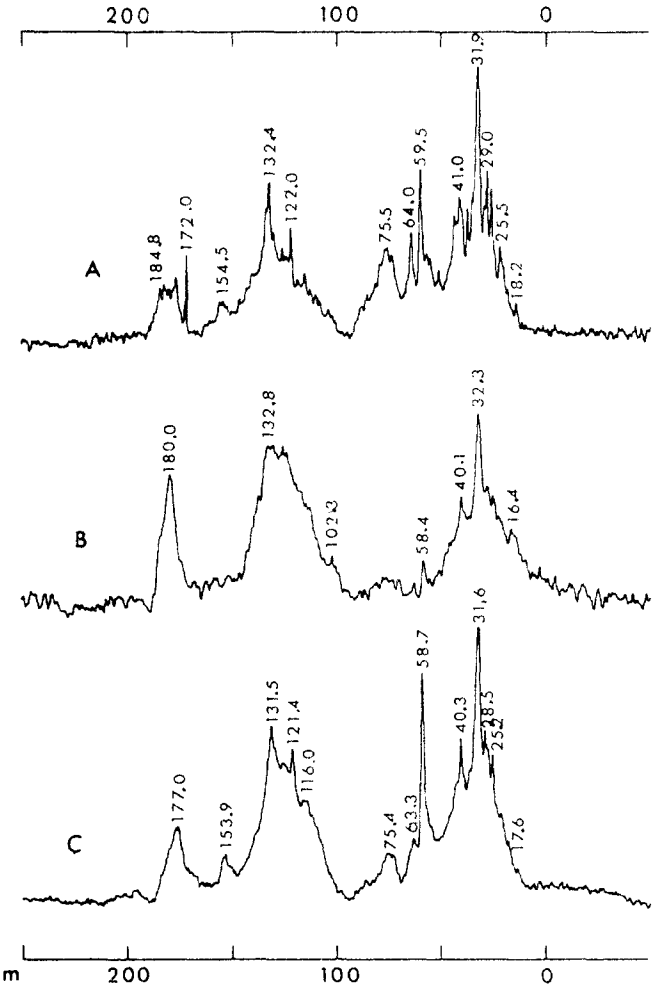

Fig. 2. ^{13}C NMR spectra of: **A** Gleysol HA; **B** Gleysol HA hydrolyzed with 6 *M* HCl; **C** Gleysol HA hydrolyzed with 12 *M*-0.5 *M* H_2SO_4.

observation is that the relative intensities of the aromatic regions in Figs. 1B and 1C are much greater than that in Fig. 1A, although the position of the maximum remains near 130 ppm. Aromaticities were computed by expressing areas due to aromatic C (105–160 ppm) as percentages of the total areas (0–160 ppm) but omitting contributions from carboxyl carbons (160–180 ppm). For the unhydrolyzed Chernozem HA (Fig. 1B), the HCl-hydrolyzed Chernozem HA (Fig. 1B) and the H_2SO_4-hydrolyzed Chernozem HA (Fig. 1C), aromaticities calculated in this manner are 41, 62 and 53%, respectively. Acid hydrolysis also appears to reduce the intensity of the carboxyl region (170–185 ppm) in Figs. 1B and 1C relative to that in Fig. 1A. Area integrations of the carboxyl regions in Figs. 1A, 1B and 1C show CO_2H contents of 4.5, 3.0 and 3.1 meq/g respectively. Thus, aside from lowering the CO_2H content by removing amino acids and carbohydrates, the two strong acids also decarboxylate some aromatic CO_2H groups. Increases in aromaticities in the hydrolyzed HA's, as compared to the original HA, can be explained as being due in part to the removal of proteinaceous components, carbohydrates, other adsorbed organics and metals. These findings are in line with previous observations made in this laboratory[20] that hot acid hydrolysis "purifies" humic materials and so provides more homogeneous materials for subsequent investigations.

To establish whether the information obtained by ^{13}C NMR spectroscopy on the Chernozem HA also applied to other HA's, ^{13}C NMR spectra were obtained for a Gleysol HA before and after acid hydrolysis. The spectrum of the unhydrolyzed Gleysol HA (Fig. 2A) is similar to that of the Chernozem HA (Fig. 1A). For example, major resonances near 21, 25, 27, 31 and 40 ppm, all due to aliphatic carbons, occur in both spectra. This is also true for signals near 56, 59, 64 and 74–76 ppm from amino acids, amino sugars, methoxyls and carbohydrates, respectively, aromatic C resonances near 132 ppm, and signals due to CO_2H groups, amides and esters, between 172 and 185 ppm. The peaks in Fig. 2A are unusually sharp, due most likely to the very low ash content of the HA (0.6%). The many well defined signals in the 115–156 ppm region indicate the presence of aromatic rings with many different types of substitutions. Of special interest are the many resonances in the CO_2H-C (172–185 ppm) region, which suggests the occurrence in this HA of at least several different types of CO_2H groups. Overall, the spectrum in Fig. 2A provides a large amount of fine structural information on the HA, which demonstrates the great potential of ^{13}C NMR for structural research on humic materials.

The ^{13}C NMR spectrum of the Gleysol HA following hydrolysis with HCl is shown in Fig. 2B. This spectrum resembles that of the HCl-hydrolyzed Chernozem HA (Fig. 1B), showing many of the same or similar resonances in the aliphatic, aromatic and CO_2H-C regions. Most striking are the prominent resonances near 32 ppm characteristic of alkyl $(CH_2)n$, 40 ppm (aliphatic C), 58 ppm (OCH_3), 130–132 ppm (aromatic C), and 180 ppm, (CO_2H-C). Hot acid hydrolysis removed a number of peaks between 50 and 80 ppm. due to amino

acids, and carbohydrates (compare Figs. 2A and 2B). In fig. 2B aromatic carbon resonances span a wide chemical shift range from 115 to 156 ppm, indicating a variety of substituents on the aromatic rings. At the lower end of the aromatic range (between 115 and 130 ppm) most of the aromatic rings are highly protonated. Resonances between 130 and 147 ppm suggest substitution of some of the aromatic protons by carbon. The small but distinct peaks at 154.5 and 155.9 ppm are probably due to carbons bonded to phenolic OH groups.

The spectrum of the Gleysol HA which has resisted hydrolysis with H_2SO_4 is shown in Fig. 2C. The lower aliphatic region (0–50 ppm) in this spectrum is very similar to Fig. 2A (the unhydrolyzed Gleysol HA). Compared to hydrolysis with HCl (Fig. 2B), hydrolysis with H_2SO_4 (Fig. 2C) appears to better preserve structures rich in alkyl groups (see signals near 27, 29, 34, and 37 ppm, also near 32 and 59 ppm). Hydrolysis with H_2SO_4 also reduces the intensities of signals near 43, 50, 55, 56, 64 and 74–76 ppm due to amino acids and carbohydrates, respectively. A comparison of Figures 2A with SB and 2C shows that hydrolysis with HCl is more effective for removing amino acids and carbohydrates from the HA than is hydrolysis with H_2SO_4. Another point of interest is that hydrolysis with H_2SO_4 improves the definition of aromatic resonances at 112, 116, 121, 126 and 132 ppm, and of phenolic carbons (at 154 ppm).

As is the case with the Chernozem HA, aromaticities increase as a result of hydrolysis with HCl and H_2SO_4. Thus, the aromaticities of the untreated Gleysol HA (Fig. 2A), the HCl-hydrolyzed Gleysol HA (Fig. 2B) and the H_2SO_4-hydrolyzed Gleysol HA (Fig. 2C) are 36, 50 and 42%, respectively. In contrast to the Chernozem HA, the CO_2H content of the acid-hydrolyzed Gleysol HA's is higher (2.7 and 2.2 meq/g) than that of the unhydrolyzed Gleysol HA (2.0 meq/g).

^{13}C NMR spectra of FA's

The ^{13}C NMR spectrum of FA (Fig. 3A) consists of a number of aliphatic resonances in the 20–50 ppm region, followed by signals from amino acids, amino sugars and carbohydrates between 50 and 85 ppm. The presence of aromatic carbons is indicated by a broad region of intensity with a maximum at 130–133 ppm. The preponderence of CO_2H groups (see Table 1) is confirmed by the strong signals between 171 and 182 ppm in Fig. 3A. Fewer sharp signals are observed in the NMR spectrum of FA (Fig. 3A) than in those of HA's (Figs. 1A and 2A), possibly because of more H-bonding in and greater structural complexity of the FA.

The spectrum of the HCl-hydrolyzed FA (Fig. 3B) shows a strong and well defined signal at 32.6 ppm, possibly due to CH_2 in alkyl chains, and smaller aliphatic signals at 40.7 and 59.0 ppm, the latter probably due to -OCH_3. Most of the resonances between 43 and 80 ppm, prominent in Fig. 3A and due to amino acids and carbohydrates, are no longer present in Fig. 3B, although a weak, broad resonance, due to residual carbohydrate remains at 74.7 ppm. The

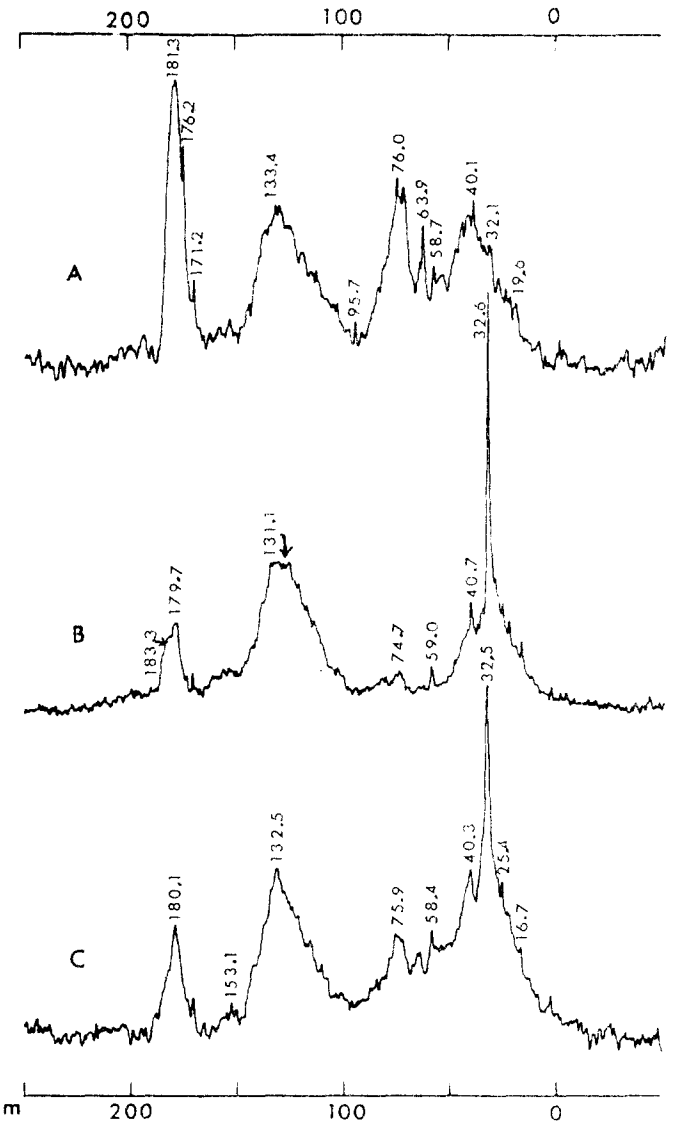

Fig. 3. ^{13}C NMR spectra of: **A** Podzol FA; **B** Podzol FA hydrolyzed with 6 *M* HCl **C** Podzol FA hydrolyzed with 12 *M*-0.5 *M* H_2SO_4.

spectrum of the FA which has resisted hydrolysis with H_2SO_4 is shown in Fig. 3C. The structural integrity of the aliphatic components appears to be better maintained in the H_2SO_4-hydrolyzed FA than in the FA hydrolyzed with HCl (compare Figs. 3B and 3C, also Figs. 2B and 2C). There are indications in Fig. 3C that not all amino acids and carbohydrates were removed. The relative intensity of the aromatic signals (105–160 ppm) in the two hydrolyzed FA's is greater than that of the unhydrolyzed FA. The corresponding aromaticities are: 32%

(unhydrolyzed FA), 52% (HCl-hydrolyzed FA), and 41% (H_2SO_4-hydrolyzed FA).

Judging from the relative intensities of the CO_2H-C signals, the hydrolyzed FA's contain fewer CO_2H groups than does the unhydrolyzed FA.

Discussion

The data presented herein show that hydrolysis with 6 *M* HCl is more efficient for removing proteins and carbohydrates from humic acids and fulvic acid than is hydrolysis with 0.5 *M*-12 *M* H_2SO_4. From the disappearance of peaks in the ^{13}C NMR spectra it is possible to assign signals to protein and carbohydrate components. Peaks (or chemical shifts) near 43, 50, 55 and 56 ppm are due to amino acids, peptides or proteins, whereas peaks at 64, 73 and 105 ppm arise from carbohydrates. It is also possible to observe effects of acid hydrolysis on the aromaticity and CO_2H content of the humic materials. Thus, one can obtain valuable information on the chemical composition and structure of humic materials from ^{13}C NMR spectra. This method is clearly of considerable importance for the characterization of humic materials. Since each of the humic materials examined originates from genetically different soil profiles, one can assume that the results presented in this paper will also apply to many other soils. The data suggest that ^{13}C NMR could be used to examine effects of different agricultural practices, treatments and seasonal variations on the protein, carbohydrate and humic content of soil extracts. With further developments in solid-state ^{13}C NMR spectroscopy one can look forward to analyses of whole soils rather than extracts, so that more precise information on the major organic soil components will be obtainable. But even at its current state of development, ^{13}C NMR spectroscopy, both in the liquid and solid states, is an important and exciting analytical tool for soil scientists.

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