

C-13 Nuclear magnetic resonance spectra of soil humic fractions and fungal melanins

F. J. GONZALEZ-VILA, F. MARTIN and C. SAIZ-JIMENEZ
Centro de Edafología y Biología Aplicada del Cuarto, C.S.I.C., Apartado 1052, Sevilla

H. LENTZ
Gesamthochschule Siegen, Fachbereich 8, Postfach 210209, D-5900 Siegen

H. D. LÜDEMANN
Institut für Biophysik und physikalische Biochemie, D-8400 Regensburg

C-13 nuclear magnetic resonance has been extensively used to study biopolymers such as proteins, nucleic acids, polysaccharides and lignins. Recently the technique has been applied to the investigation of humic substances by several authors, e.g. NEYROUD and SCHNITZER (1974), STUERMER and PAYNE (1976), GONZALEZ-VILA et al. (1976), WILSON and GOH (1977 a, b) and GONZALEZ-VILA et al. (1978). In the study of humic molecules several difficulties are found in recording the spectra, which may show broad and poorly resolved resonances. These can be due to several factors, such as the extreme molecular complexity of the samples with many different types of C-13 atoms, the high molecular weight, the high viscosity of the solution, the presence of free radicals which interact with C-13 nuclei causing line broadening or changes in chemical shift, and to insufficiently developed technique.

GONZALEZ-VILA et al. (1976, 1978) reported well resolved C-13 resonance spectra for soil humic fractions and fungal melanins. In the present communication the spectra of a vertisol humic acid, a podzol fulvic acid and two fungal melanins from *Aspergillus niger* and *Eurotium echinulatum* are compared.

The C-13 spectra were taken in 5% W/W solutions in 0.1 N NaOD. A Varian XL-100-15 spectrometer operating at 25.2 MHz was used to obtain the spectra by the Fourier transform mode with a fluorine field/frequency lock and full proton decoupling. 250 blocks of 1000 transients were accumulated in the long-term-averaging mode. The resolutions of the spectra were ± 10 Hz (approximately 0.5 ppm). Chemical shifts are reported in ppm downfield from an external TMS capillary. No attempts to correct for magnetic susceptibility effects were made.

Figure 1 shows the spectra of the humic fractions and the fungal melanins, where maximal baseline noise is indicated by two horizontal bars in the last spectrum. In all the spectra prominent resonances in the 10-100 ppm range can be discerned, they are characteristic of aliphatic amino acid, aliphatic side chain carbons and carbohydrate carbons. The broad resonances in the 10-60 ppm region

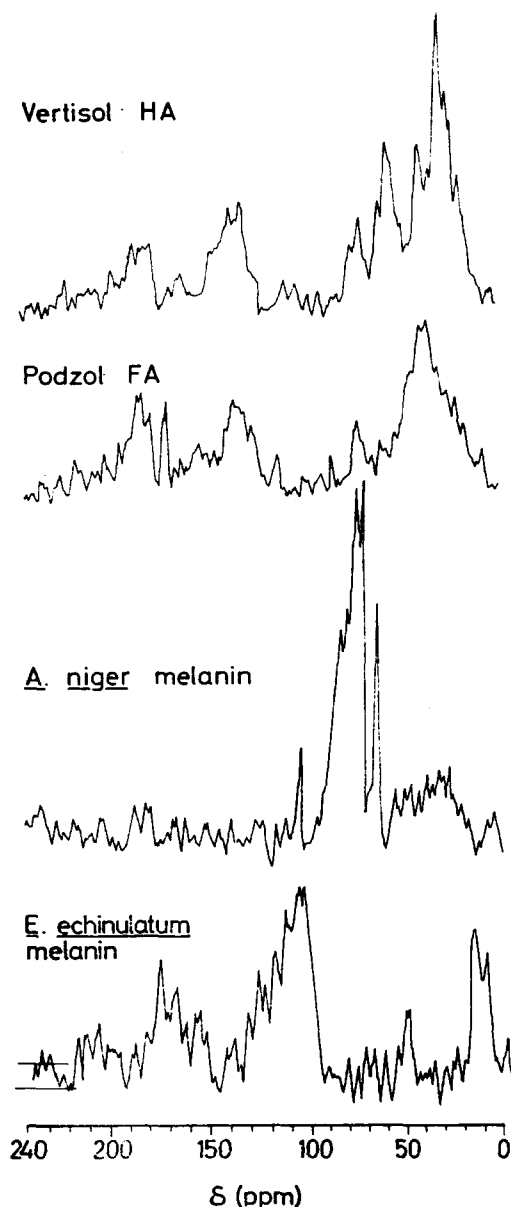


FIG. 1. — C-13 NMR spectra of soil humic fractions and of fungal melanins. Intensities in arbitrary units. δ is the chemical shift in ppm on the TMS scale. The approximate baseline noise is indicated by two horizontal bars in the spectrum at the bottom.

are characteristic of aliphatic moieties, which are present in the soil humic fractions and the *Eurotium echinulatum* melanin. However, the *Aspergillus niger* melanin spectrum shows sharp and distinct resonances in the 60-100 ppm range, which are characteristic of carbohydrates.

Resonances in the 100-160 ppm region are due to aromatic and hetero-aromatic constituents, these being more prominent in the *Eurotium echinulatum* melanin than in the soil humic fractions and low in the *Aspergillus niger* melanin.

Resonances in the 160-240 ppm region are evident in all samples, except in the *Aspergillus niger* melanin, which presents poorly developed resonances. Carboxylic, quinones and other oxygen-containing groups contribute to this region.

Table 1 shows the centers of the signals obtained for the presented spectra. These signals may have more resolution; however, the low signal to noise ratio only allows the safe assignment of the resonances listed in the table.

TABLE 1. — $C-13$ chemical shift of humic fractions and fungal melanins.

δ	Vertisol HA	Podzol FA	<i>A. niger</i> melanin	<i>E. echinulatum</i> melanin	
210				210	aldehydes ketones
200					
190					COOH esters amides
180	180	180	180	170	
170		170			aromatic and heteroaromatic rings
160					
150					
140					
130	135	135		135	
120		115		120	carbohydrates
110	104		104		
100					
90					
80			80		
70	75	75	75	75	aliphatic amino acids and side chains
60			70		
50	55		65	50	
40	38	38	40		
30	28				
20					10
10					

In complex substances like melanins and soil humic acids, one has to consider another complication. The molecules might consist of rigid and mobile regions in which only the carbon-13-nuclei of the flexible parts possess sufficiently long transversal relaxation times, T_2 , to be observable in a high resolution spectrum. Consequently, it is hazardous to derive absolute concentration for a certain class of carbon atoms from the intensities of the signals in different samples or to deduce from the absence of a signal the absence of a specific chemical structure. ^{13}C MNR spectra may thus only be used for the positive assignment of certain structural elements in a heterogenous polymer.

The *Aspergillus niger* melanin shows a spectrum different from the other samples studies here, in which the prominent resonances are due to carbohydrate carbon atoms. This agrees with previous work where it was demonstrated that 52.5 per cent of the molecule is made up from carbohydrates (SAIZ-JIMENEZ et al., 1978). The *Eurotium echinulatum* melanin spectrum has prominent signals in the aromatic and functional groups regions, even more intensive than in the soil humic fractions. Phenols and anthraquinones were demonstrated to contribute in the build up of this melanin (SAIZ-JIMENEZ et al., 1975) which might explain these intense resonances.

Humic and fulvic acids show similar spectra with differences in the region of aliphatic carbons; the sharp resonance at 28 ppm in humic acid is due to aliphatic side chains.

In comparing the spectra of soil humic fractions and fungal melanins the differences are evident, showing that distinct types of C-13 atoms may contribute to these molecules. The results suggest that even though soil humic fractions and fungal melanins resemble each other in its elementary composition, IR spectra and other characteristics, the chemical structures may be different.

In a recent publication, WILSON and GOH (1977) refer to a previous paper (GONZALEZ-VILA et al. (1976) stating that although we have found aromatic signals in the C-13 resonance spectra, their preparations, without aromatic resonances, should contain essentially only « core » humic material, and the « core » is neither of a polynuclear aromatic nature nor is lignin-like, but is a random mixture of high molecular weight polymers with only a relatively small aromatic content. We cannot agree with these statements, because in our works on the chemical structure of humic substances we found many indications for an aromatic « core ». Thus, we have recently studied the humic acids isolated from different soil types by pyrolysis-gas chromatography-mass spectrometry and found that protein, polysaccharide and lignin pyrolysis products are the main contributors to the pyrograms. The humic acids were hydrolysed with 6 N HCl for three periods of 24 hours and the hydrolysed residues or « core » were pyrolysed. In this case the pyrograms do not show evidence of pyrolysis products derived from proteins, polysaccharides or lignins, but alkylbenzenes, alkyl-naphthalenes, alkylfluorenes, phenols, indenes, benzofuranes, and only minor amounts or traces of alkanes and olefins (MARTIN et al., 1977, 1978). These results together with SCHNITZER'S (1977) indicate an aromatic nature for the soil humic acid « core ».

The C-13 NMR technique seems to be worthwhile in providing structural information on soil humic fractions and fungal melanins, however, more basic works on model compounds and less complex fractions are needed to improve the assignment of the resonances.

REFERENCES

- GONZALEZ-VILA F. J., LENTZ H. and LÜDEMANN H. D.: *Biophys. Biochem. Res. Comm.*, 72, 1063 (1976).
GONZALEZ-VILA F. J., SAIZ-JIMENEZ C., LENTZ H. and LÜDEMANN H. D.: *Z. Naturforsch.*, 33c, 291 (1978).

- MARTIN F., SAIZ-JIMENEZ C. and CERT A.: *Soil Sci. Soc. Am. J.*, 41, 1114 (1977).
MARTIN F., SAIZ-JIMENEZ C. and CERT A.: *Soil Sci. Soc. Am. J.*, 42 (in press) (1978).
NEYROUD J. A. and SCHNITZER M.: *Can. J. Chem.*, 52, 4123 (1974).
SAIZ-JIMENEZ C., HAIDER K. and MARTIN J. P.: *Soil Sci. Soc. Am. Proc.*, 39, 649 (1975).
SAIZ-JIMENEZ C., MARTIN F. and CERT A.: *Soil Biol. Biochem.*, 10 (in press) (1978).
SCHNITZER M.: *Soil Organic Matter Studies*, vol. II, IAEA, Vienna, 117 (1977).
STUERMER D. H. and PAYNE J. R.: *Geochim. Cosmochim. Acta*, 40, 1109 (1976).
WILSON M. A. and GOH K. M.: *Plant Soil*, 46, 287 (1977).
WILSON M. A. and GOH K. M.: *J. Soil Sci.*, 28, 645 (1977).

SUMMARY. — The Chemical structure of soil humic acids, fungal melanins and soil fulvic acid was investigated by C-13 nuclear magnetic resonance. The spectra obtained can be roughly divided into four parts. Between 10 and 60 ppm intense signals assigned to alkyl chains appear in all substances. From 60 to 100 ppm prominent bands of polysaccharides only can be discerned in the spectrum of *Aspergillus niger* melanin. Aromatic carbon bands located between 100 and 160 ppm are strong in the *Eurotium echinulatum* melanin and soil humic substances. Functional groups appear between 160 and 210 ppm. The results presented show the applicability of the C-13 NMR method to structural investigations of humic substances.

RÉSUMÉ. — La structure chimique des acides humiques et fulviques du sol et de mélanines de champignons a été recherchée par spectroscopie de MNR de C-13. A peu près, on peut diviser les spectres obtenues en quatre parties. Entre 10 et 60 ppm il apparaît des signaux intenses dans toutes les substances qui correspondent à des chaînes aliphatiques. Depuis 60 ppm jusqu'à 100 ppm il n'apparaît que des bandes prominents de polysaccharides dans le spectre de la mélanine d'*Aspergillus niger*. Les bandes de C aromatique localisées entre 100 et 160 ppm sont fortes dans la mélanine d'*Eurotium echinulatum* et dans les substances humiques du sol. Les groupes fonctionnels apparaissent entre 160 et 210 ppm. Ces résultats montrent la possibilité d'application de la spectroscopie de NMR de C-13 pour des recherches sur la structure de substances humiques.

ZUSAMMENFASSUNG. — Die chemische Struktur von Huminsäuren, Melaninen aus Pilzkulturen und von einer Fulvinsäure wurde durch C-13 NMR untersucht. Die Spektren können grob in vier Abschnitte aufgeteilt werden. Für alle untersuchten Substanzen zeigen die Spektren zwischen 10 und 60 ppm intensive Signale, die zu Alkylketten gehören. Von 60 bis 100 ppm treten deutliche Banden von Polysacchariden nur im Spektrum des *Aspergillus niger* Melanins auf. Aromatischer Kohlenstoff zeigt sich zwischen 100 und 160 ppm bei den Humussubstanzen und *Eurotium echinulatum* melanin.

Zwischen 160 und 210 ppm werden funktionelle Gruppen angezeigt. Die Resultate demonstrieren die Anwendbarkeit der C-13 NMR Spektroskopie für Strukturuntersuchungen von Humussubstanzen.

RESUMEN. — La estructura química de ácidos húmicos y fúlvicos de suelos y melaninas de hongos fue investigada mediante espectroscopia de

NMR de C-13. Los espectros obtenidos pueden dividirse aproximadamente en cuatro partes. Entre 10 y 60 ppm aparecen en todas las sustancias intensas señales asignadas a cadenas alifáticas. Desde 60 a 100 ppm solo se observaron bandas prominentes de polisacáridos en el espectro de la melanina de *Aspergillus niger*. Las bandas de C aromático localizadas entre 100 y 160 ppm son intensas en la melanina de *Eurotium echinulatum* y en las sustancias húmicas del suelo. Los grupos funcionales resuenan entre 160 y 210 ppm. Los resultados presentes muestran la aplicabilidad de la espectroscopia de NMR de C-13 para investigar la estructura de sustancias húmicas.

RIASSUNTO. — La struttura chimica degli acidi umici e fulcivi dei suoli e melanine dei funghi furono ricercate per l'uso della spettroscopia NMR di C-13. Gli spettri ottenuti possono dividersi approssimativamente in 4 parti: tra 10 e 60 ppm appaiono in tutte le sostanze segnali intensi riconducibili alle catene alifatiche. Tra 60 e 100 ppm si evidenziano solo bande prominenti di polisaccaridi nello spettro delle melanine di *Aspergillus niger*. Le bande di carbonio aromatico localizzate tra 100 e 160 ppm sono intense nella melanina di *Eurotium echinulatum* e nelle sostanze umiche del suolo. I gruppi funzionali si notano tra 160 e 210 ppm. I risultati ottenuti mostrano l'applicabilità della spettroscopia di NMR di C-13 per le ricerche delle sostanze umiche.