



Effect of Land Management on Humic Acid Spectra Generated Using ^{13}C -NMR Spectroscopy

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ABSTRACT

Liquid ^{13}C -NMR (Nuclear Magnetic Resonance Spectroscopy) spectra of HA (humic acid) extracted from a Hanslope Soil, Writtle College, UK under five types of land management: grassland under permanent pasture on 5 y ley before stocking; grassland under permanent pasture sown with red clover a y before stocking; grassland under permanent pasture treated with N-fertilizer a y before stocking; arable land under barley and deciduous woodland showed four major fragments: the aliphatic (alkyl C) region between 10 – 48 ppm; the carbohydrate (o-alkyl C) region between 49 and 110 ppm; the aromatic (aryl C) region between 111 and 165 ppm and the carboxyl region between 166 and 195 ppm. The study also revealed carboxyl conspicuous spectra between 150 and 200 ppm. The highest concentration of humic acid was in deciduous woodland, 55.2 mg g^{-1} , and least in permanent pasture on a 5 y ley before stocking, 33.8 mg g^{-1} . Since soil organic matter (SOM) influences soil structure and fertility, humic substances effects bioavailability of elements and chemical compounds in the environment. The degradation of humic substances are slow. Hence, they do not appear to be a major direct source of nutrients – carbon, nitrogen, phosphorus and sulphur.

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Introduction

The influences of SOM on soil structure and on soil fertility are well recognized. Horth et al (1988) evaluated the effects of humic substances on the bioavailability of elements, and chemical compounds in the environment. They state that, since the degradation of humic substances is slow, they do not appear to be a major direct source of nutrients (C, N, P and S) for soil biota, except for some highly specialized organisms, mainly fungi, which can utilize humic substances as a source of energy. However, the release of the above nutrients has been recognized to be more important in aquatic systems where humic substances are likely to be transformed more rapidly due to processes such as photoalteration. The most useful of the physic-chemical procedures for studying the structure of humic substances has focused on molecular size, shape and charge characteristics (Hayes, 1991). For such studies it has been desirable to work with molecules that are relatively homogenous with respect to sizes and shapes. The application to the study of humic substances of liquid and solid state NMR have been discussed by Wershaw (1985); Malcolm (1989) and Steelink et al (1989). Progress in the use of liquid and solid state NMR analyses now make it possible to obtain reasonably quantitative and well resolved spectra for humic substances. The availability of cross polarization magic angle spinning (CPMAS) ^{13}C -NMR has done much to improve resolution and application of this procedure to the study of humic substances in the solid state (Hayes, 1991)

There is no evidence to suggest that there is genetic or biological control of the synthesis of humic substances. Hence, they lack regularity in the sequencing of the molecules which compose the macro molecules. Further intra-molecular and inter-molecular associations can take place within and between humic and other macro-molecules to give semblance of secondary and tertiary structure. However, there are no rigid regularities between such associations. Although associations can form

between humic substances and other organic and inorganic materials such associations are invariably random, and are not necessarily a part of any biological function which humic substances may have. Hence humic substances do not meet any of the criteria for structure as they apply to proteins (Hayes, 1991).

Two broadly based processes are considered to give rise to humic substances. The first of these is the “degradative process” involves the biological transformation of refractory organic macro-molecules such as lignins, paraffinic substances, cutins, melanins, suberins and other substances which give rise to humic substances (Nip et al; 1986; Hayes, 1991). Polysaccharides and proteins form readily available substrates for microorganisms, but these too give rise to humic substances, although the origins of such substances are more likely to be from the micro-organisms which proliferate on the labile substrates, rather than from the substrates themselves.

There is also the possibility that amino acids and peptides released from proteins and sugars, and oligosaccharides released from polysaccharides can undergo “browning” reactions to give rise to humic substances. Quinones from oxidized phenols are also known to give rise to humic-type substances. Such pathways are part of the second or synthetic process, for the genesis of humic substances (Hayes, 1991; Maillard, 1912 as cited by Hayes, 1985 introduced the “browning” reaction or the “melanoidin” theory when they observed monomeric reducing sugars, such as glucose, could condense with amino acids such as glycine to form brown macromolecular substances. The spectra generated in current work have added more to this discipline as data is lacking in various types of terrestrial and aquatic ecosystem. The pristine woodland and permanent pastures on various leys and nutrient enrichment in this study is very expository.

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Materials and Methods

Site description

Writtle College, Essex, UK is located approximately 68 km east of London (51° 44'; 0° 26' E, 32 OD). The soil at Writtle College belongs to Hanslope Soil Series of Chalky Boulder Clay Parent material. Five types of land management were evaluated: arable land under barley, grassland under permanent pasture, one on 5 y ley before stocking; the second sown with red clover a y before stocking, the third treated with N-fertilizer a y before stocking and a pristine deciduous woodland. Each site had four replicate sampling points which were revisited on each sampling occasion.

Laboratory methods

Extraction and purification of humic acid (HAs)

Ground dried soil passed through a 0.5 mm sieve was mixed with deionised water in a ratio of 1:1 (w/v). This was equilibrated to pH 1.2 using HCl (1M). The solution volume was adjusted with HCl (0.1 M) to provide a final concentration of 1 g dry sample 10 ml⁻¹ liquid. The suspension was shaken in an orbital shaker for 1 h at room temperature. This was followed by the separation of the supernatant from the residue by centrifugation at 3000 rpm for 10 min. The soil residue was mixed with the same volume of deionised water and then neutralized to pH 7 using NaOH (1 M). Na₄P₂O₇ (sodium pyrophosphate) concentration in NaOH (0.1 M, pH 13) was added to the soil residue under an atmosphere of N₂ for 2 h with continuous stirring. The supernatant was collected by centrifugation at 12,000 rpm for 10 min. The supernatant was then immediately acidified with HCl (6 M) to pH 1.2 and allowed to set for 22 h. The precipitated HAs were separated by centrifuging at 12,000 rpm for 10 min. The HAs were redissolved in a minimum volume of NaOH (0.1 M) and the solution was placed in 3.5 KD molecular weight cut-off dialysis tubing (Medicell Ltd, UK) and left to dialyse against deionised water for 64 h with daily change of water. The solution was dried by rotary evaporation and HAs collected (Hayes, 1985)

Fractionation of humid acids (HAs)

For the molecular weight fractionation of HAs samples were prepared according to the above protocol. The HAs were separated into 2 fractions using disposable ultrafiltration units (0.5 ml Vivaspin) with molecular weight cut-off membranes of 30 KD. A NaOH solution (0.1 M) prepared in UHP water was used to wash and reconstitute volumetrically the retentate present in the ultrafiltration unit comprising fraction 1 (HAs >30KD). Subsequently fraction I and II (HAs <30 KD) were analysed by calculating the relative concentration of HAs with molecular weights greater and/or less than 30 KD (fraction I and II) respectively, by relating the maximal absorbance values obtained at 451.5nm to the maximal absorbance at the same wavelength of the original non-fractionated sample (Hayes, 1985).

Examination of humic acid using ¹³C-NMR spectroscopy

This was based on methods described by Wilson (1987, 1989, 1990). Sample tubes (5 mm) were used to obtain C-NMR from a liquid state spectrophotometer (Model JNM-Ex 270 supplied by JEOL, Ltd, UK). The samples for NMR examination were prepared by dissolving HAs (80 mg) in NaOD (sodium deroxide) (0.1 M, 0.8 ml). Spectra were obtained by running samples under the following conditions Pulse delay (0.56 s) Acquisition time (0.16 s) Pulse angle (45°) Under these conditions, and using a broadening factor of 50 Hz about 90,000 spectra were obtained. The -5 ppm to + 210 ppm range were examined.

Results and Discussions

Humic acid spectra generated using the ¹³C-NMR Spectroscopy. Figure 1 shows the five spectra obtained following ¹³C-NMR of humic acids (HA) extracted from the soil under various land management

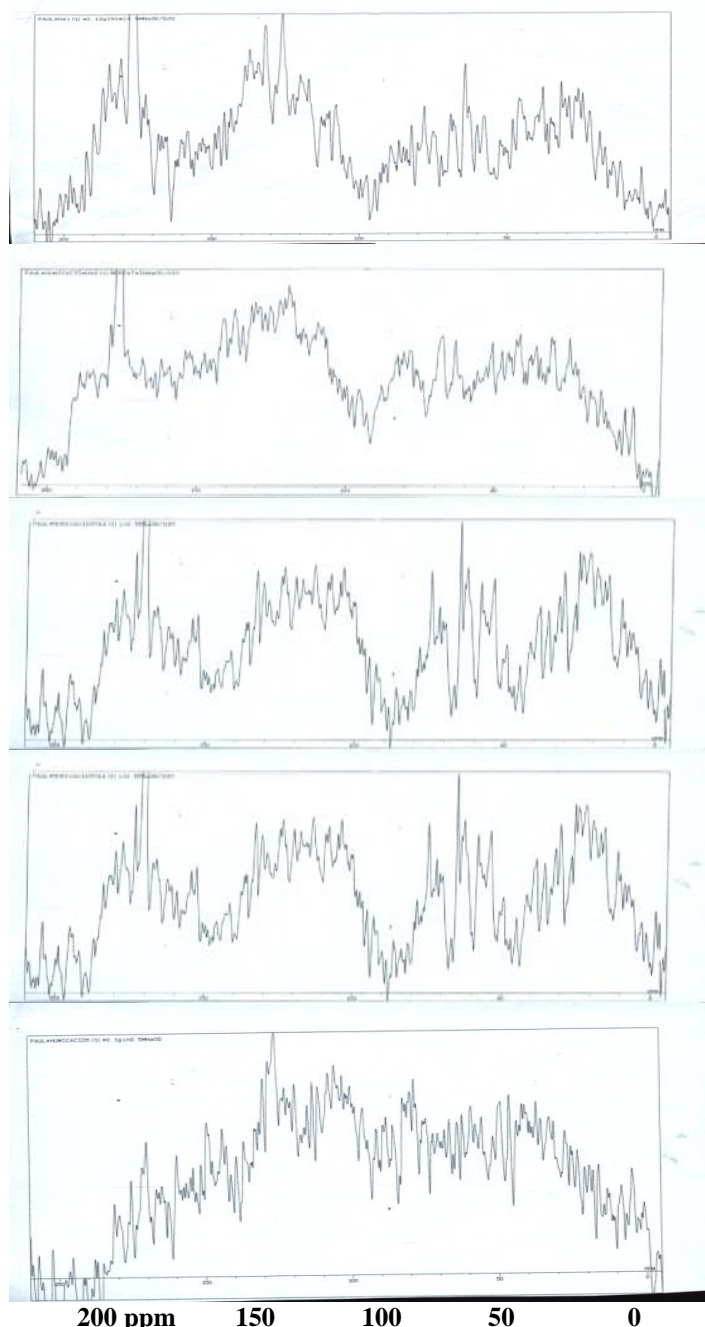


Fig 1. Liquid ¹³C-NMR spectra of HA extracted from (1) - grassland under permanent pasture on 5 y ley before stocking; (2) grassland under permanent pasture sown with red clover a y before stocking; (3) grassland under permanent pasture treated with N fertilizer a y before stocking; (4) arable land under barley and (5) deciduous woodland

For convenience these spectra which were all measured within the range - 5 ppm to +210 ppm is divided into four areas for analysis. The aliphatic region (alkyl C) ranges between 10 ppm and 48 ppm. The ten major peaks in this region which are those around (in ppm) -2.5, 5, 12.5, 20, 25.5 (CH₂); 35.5 {(CH₂)_n}; 40, 45.2, 48.1, 49.8 corresponds to alkyl groups of amino acids and alkyl substituted aromatics which were observed in all five spectra. The carbohydrate (o-alkyl C) region lies between 49 and

110 ppm. The major peaks are similar to all four spectra except that of spectra 5 where peaks appear both quantitatively and qualitatively greater. Those peaks were specifically found at (in ppm); 55 (methoxyl C), 60, 65.7 (CH₂OH); 72.1, 77.9 (CH(OH), 83.7, 90.2, 95.2, 98.8. The aromatic (aryl C) region lies between 111 and 165 ppm. The peaks that were commonly found in this range are related to the presence of numerous C with aliphatic substituents and/or internal C of condensed aromatic compounds. The second largest peak in all the five spectra was observed in this region. Further the largest peak in all spectra (with the exception of the deciduous woodland was found in the carboxyl region which is defined approximately between 166 and 195 ppm. This first distinct peak in the spectra is further highlighted in Fig. 2

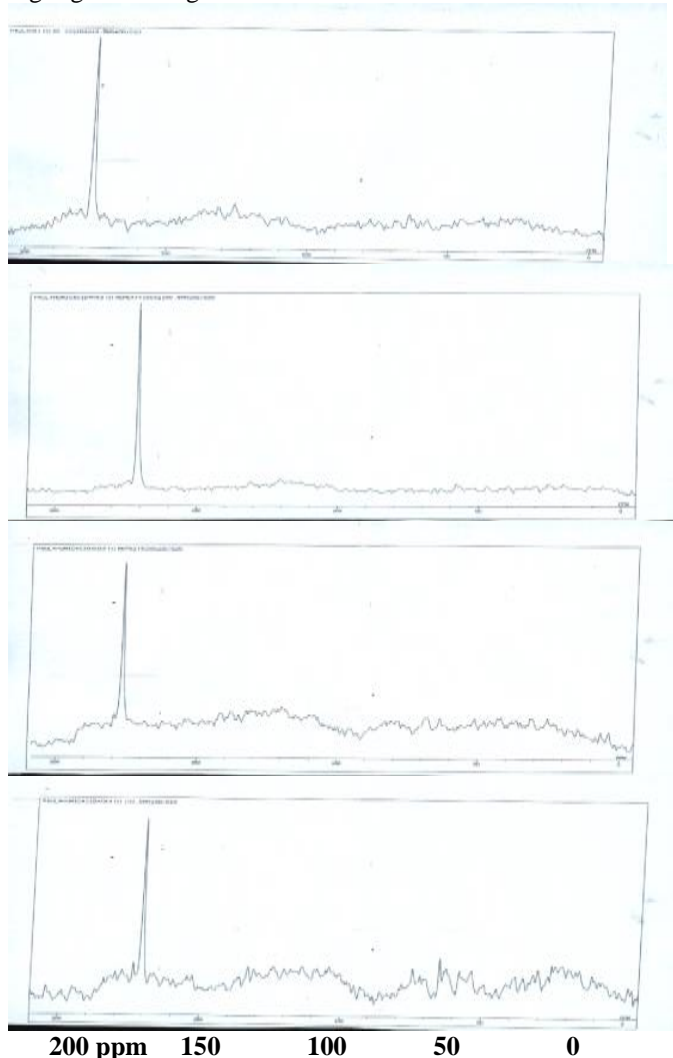


Fig. 2: Liquid ¹³C-NMR spectra of HA extracted from soil under (1) grassland under permanent pasture on 5 y ley before stocking; (2) grassland under permanent pasture sown with red clover a y before stocking; (3) grassland under permanent pasture treated with N fertilizer a y before stocking; (4) arable land under barley. Deciduous woodland was unavoidably omitted due to lack of carboxyl C spectra as shown in Fig 1.

Humic acid (HAs) production

The concentration of humic acid in soils from deciduous woodland was the highest (55.2 mg g⁻¹), followed by that from grassland under permanent pasture treated with N fertilizer a y before stocking (49.6 mg g⁻¹). The lowest concentration of humic acid was in soils from grassland under permanent pasture on 5 y ley before stocking (33.8 mg g⁻¹). The overall mean

humic acid for all types of land management was 44 ± 9.6 mg g⁻¹ (Fig. 3)..

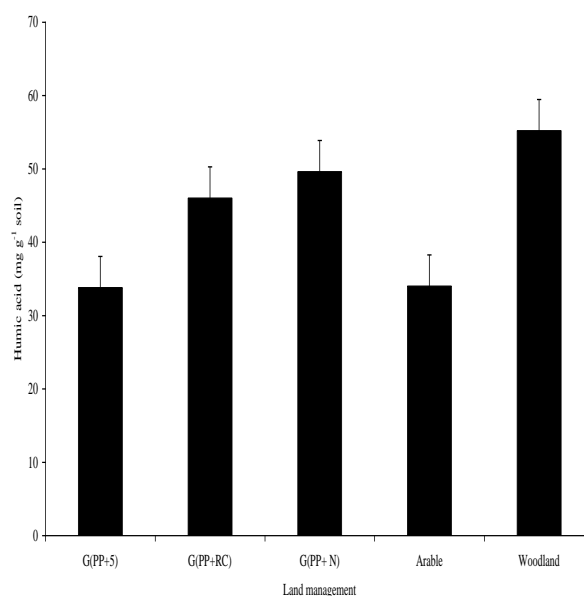


Fig. 3. Quantitative comparison of the amount of soil humic acid (HA) – mg g⁻¹ from grassland under permanent pasture on 5 y ley before stocking; grassland under permanent pasture sown with red clover a y before stocking; grassland under permanent pasture treated with N fertilizer a y before stocking; arable land under barley and deciduous woodland. Each value is mean of 4 replicates x 12 months. Bars represent standard error of means.

Nuclear magnetic resonance spectroscopy is a non-destructive technique that provides comparisons of the composition of humic substances from different sources and environments. Thus, under the condition applied herein ¹³C-NMR spectra of the HAs extracted from soils under various land management appeared very similar with all major peaks found at similar positions, with the exception of the carboxyl C peak which was not observed in the same position or which was conspicuously absent in HA samples from soils of deciduous woodland. Pasture grazing and cropping activities which change soil vegetation and architecture more frequently than is observed in deciduous woodland which has remained untilled or grazed for decades and where the only source of OM is leaf litter and shrubs. Nevertheless, the aliphatic (alkyl C), carbohydrate (o-alkyl C) and aromatic (aryl C) moieties were all identified in each land management type. It has also been observed that small spectral differences may be difficult to distinguish using ¹³C-NMR spectroscopy due to the high complexity of the signals/peaks present in such a spectrum (Hayes, 1985).

Oka et al. (1969) made the first proton NMR measurement on an underivatized humic acid. They studied three different peat humic acid samples extracted with a solution consisting of 1% sodium hydroxide, 3% sodium acetate and 1.8% pyrophosphate. The spectra of the three humic acids were very similar, each spectrum consisting of a series of broad bands. In general, the assignments are in agreement with later studies except for the region between 4 and 5.5 ppm which Oka et al (1969) and Lakatos and Meisel (1978) assigned to lactone protons. However, other workers have assigned this to exchangeable protons (Ruggiero et al, 1979c). In Ruggiero et al (1979a,b,c) this peak disappear upon methylation of hydroxyl and carboxyl groups Ludemann et al (1973) and Lentz et al (1977) used proton NMR to study a number of soil humic acid

fractions dissolved in D₂O. The spectra had broad bands in the following regions: 1 – 2.5 ppm, 3.8 ppm and 6.5 – 8.5 ppm. An intense DOH peak was present at about 5.2 ppm. The aliphatic region from 1 to 2.5 ppm was composed of several broad bands: a single methoxyl band was present at 3.8 ppm and a single very broad band was present in the aromatic region. They calculated the percentage of aromatic protons to the total protons by integration under the peaks. The highest percentage of aromatic protons was 35% and the lowest was 19%.

Ruggiero and co-workers (1978, 1979a,b; 1981) conducted a series of ¹H and ¹³C-NMR studies on underivatized, derivatized and degraded humic and fulvic acids. They were the first to make concerted effort to eliminate the strong exchangeable proton absorption band whose chemical shift is a function of relative concentration of carboxylic acid and alcohol hydroxyl groups in the sample. The exchangeable proton generally obscures the region between approximately 4 and 5 ppm but it can also distort the aromatic region. The yield of HA in these sites especially deciduous woodland compares favourably with the yields for humic acid (HA) and fulvic acids (FA) extracted by different extractants from a H⁺-exchanged sapric Histosol as reported by Hayes (1985). The basic solvent systems, 0.5 NaOH and 2.5 M ethylenediamine (EDTA) extracted more humic substances from the H⁺-exchanged organic soil than the other solvents. Because of the highly alkaline conditions which prevailed (the pH of the soil suspension in the aqueous EDA system was 12.6) all the acid groups in the macromolecule were dissociated and the repulsion of charge gave the fully expanded random coil structure. Thus, the anionic and polar sites could be readily solvated with water molecules. The yields of humic substances (humic acids plus fulvic acids) were slightly higher from the exhaustive extractions with aqueous EDA when compared with those extracted with NaOH (for the exhaustive extractions of soil samples were repeatedly equilibrated with solvent until the supernatant solutions had negligible colour. The data reported by Hayes (1985) suggest that less humic acid and more fulvic acid substances were extracted with EDA when compared to that extracted with NaOH. However, it was found that considerable amounts of the substances classified as fulvic acids (components remaining in solution when the aqueous EDA extracts were acidified to pH 1) were precipitated during dialysis against distilled water. This would indicate that some humic acids were solubilized by EDA salts formed on acidification and these acids were precipitated as the salts were lost during dialysis. The use of aqueous solution of NaOH for the extraction of humic substances was first described by Achard (1786) as cited by Hayes (1985). Such solutions have been the solvents of choice by most workers since that time and formed the basis of extraction in present work.

Conclusion

The five spectra obtained using the ¹³C-NMR spectroscopy fell within the ranges of -5 to +210 ppm, covering the aliphatic, carbohydrate, aromatic and carboxyl C groups. The largest peak was found in the carboxyl C region, while the second peak was located in the aromatic region. Even though small spectral differences may be difficult to distinguish using ¹³C-NMR spectroscopy due to high complexity of the signals/peaks present in such a spectrum; it is still a popular method among the scientific community.

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