The Isolation and Characterization of Humic Substances and Humin from Grey Brown Podzolic and Gley Grassland Soils

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Abstract
Humic acids (HAs) and fulvic acids (FAs) were isolated by the exhaustive extractions at pH 7, 10.6, and 12.6 of a Grey Brown podzol and of a Gley soil, each in long term grassland. A further exhaustive extraction in base + 6 M urea isolated additional HAs and FAs of significantly different compositions. A subsequent sequential extraction with dimethylsulphoxide (DMSO) + 6 M concentrated H_2SO_4 isolated humin materials. The solvent sequence used is capable of isolating > 90% of the organic matter (OM) in some soils, and at least 70% of the OM in all soils. Solid state CPMAS ^{13}C NMR spectroscopy s showed readily observable differences between the compositions of the HAs and FAs isolated at the different pH values, and by the base + urea solvent system. Humin has been considered to be the most intractable component of soil organic matter. However, applications of solid state ^{13}C NMR and of liquid state proton NMR have shown that soil humin is composed largely of biological molecules of plant and of microbial origins and with a degree of resistance to biological degradation. That resistance is enhanced by the protection afforded by close associations with the soil mineral colloids.

Key Words
Humic substances, humin, extraction, fractionation, XAD resins, proton and ^{13}C NMR.

Introduction
In the classical definitions soil humic substances (HS) are defined as amorphous, polymeric, brown coloured substances that are differentiated on the basis of solubility properties (Hayes and Swift 1978) into humic acids (HAs, precipitated when aqueous alkaline extracts from soil are adjusted to pH 1), fulvic acids (FAs, soluble in aqueous media at all pH values) and humins (insoluble in aqueous media). Humins are considered to be the major components of HS, and to compose 50% and more (Stevenson, 1994) of the transformed or humified components (that bear no morphological resemblances to the structures from which they were derived) of organic materials in soil organic matter (SOM). The procedure used (Swift, 1996) to isolate the Standard soil HAs and FAs of the International Humic Substances Society (IHSS) is widely used. That procedure uses XAD-8 resin to recover the FAs from the FA fraction, or the materials that remain in solution when the pH of the aqueous alkaline extract is adjusted to 1. When XAD-4 resin is placed in tandem with the XAD-8 significant amounts of hydrophilic biological molecules are recovered and these do not satisfy the definition for HS (Hayes *et al.* 2008).

On the basis of the classical definitions, any humified materials that are extracted, following exhaustive extraction in aqueous basic media, in organic solvents, or even in aqueous media containing organic solutes, might be regarded as humins. The present study involves an exhaustive extraction of the classical humic components of SOM, including the isolation of humin residues by novel procedures from a well drained Grey Brown Podzol (GBP) and of the humin materials from a poorly drained Gley soil after the HAs and FAs had been isolated during exhaustive extractions in aqueous sodium hydroxide (NaOH) solutions adjusted to different pH values. ^{13}C and ^{1}H nuclear magnetic resonance (NMR) spectroscopy techniques were employed to show differences in the compositions of the different isolates.

Methods
Soil; extraction and analyses procedures
A well drained GBP soil (45% sand, 20% silt, and 12% clay) and a poorly drained Gley soil (25% sand, 35% silt and 25% clay; were H^+-exchanged and exhaustively extracted in 0.1 M NaOH adjusted to pH 7, then exhaustively at pH 10.6, and at 12.6. The residual materials were exhaustively extracted in 0.1 M NaOH + 6 M urea. After the urea was washed out the residual soil was dried and exhaustively extracted with dimethylsulphoxide (DMSO) + concentrated H_2SO_4 (6% v/v). The aqueous extracts were diluted to < 50 ppm, the pH was adjusted to 2, and the solution was passed on to XAD-8 and XAD-4 resins in tandem. The HAs and FAs were recovered, as described by Hayes *et al.* (2008), from the materials sorbed on XAD-8. The

$DMSO/H_2SO_4$ mixture was poured into water; the precipitate formed was washed with water and freeze dried (Song *et al.* 2008).

Instrumental analysis using CPMAS ^{13}C and proton nuclear NMR spectroscopy followed the procedures outlined by Simpson *et al.* (2007) and by Song *et al*. (2008).).

Results

Compositions of humic and fulvic acids from the Grey Brown Podzolic soil

Both soils used in the study are in long term grassland and are enriched in SOM (with 6.7 x 10^4 and 7.2 x 10^4 kg/ha organic C in the surface layer in the cases of the GBP and the Gley soil, respectively). The CPMAS ^{13}CNMR spectra in Figure 1 clearly show that a fractionation was obtained as the result of applications of aqueous solvents of different pH values. A comparison of the HA isolates in the aqueous media at pH 7, 10.6, and 12.6 shows that the material isolated at the highest pH value, spectrum 3, has clear indications of components of lignin origins, as demonstrated by the characteristic shape of the methoxyl resonance at 56 ppm and the distinct O-aromatic resonance (145-150 ppm). The O-aromatic resonance is distinctive also in the isolates at pH 7 and at pH 10.6, but the shapes of the 50-60 ppm resonance suggest that the structures contain peptide functionalities. There is clear evidence for carbohydrate (70-90 ppm and the anomeric C resonance, 105 ppm) in all three samples, and especially in the cases of spectra 1 and 2, and the resonance around 110 ppm may be indicative of tannins. These spectra show that the HAs contain significant hydrocarbon structures, and the resonances at ca 32 ppm can be indicative of crystalline methylene structures (e.g. those arising from interactions between long chain hydrocarbons in acid and ester functionalities). Spectrum 4, for the HA isolate in base + urea, is distinctly different from the extracts in the purely aqueous basic media. There is evidence for lignin inputs to the structures (in the O-aromatic resonance, although the 50-60 ppm resonance does not have the characteristic shape for the methoxyl of lignin). The aromaticity resonance is distinct, but it is clear that aromaticity functionality is significantly less in spectrum 4 than in spectra 1, 2, and 3. The contributions of carbohydrate structures to the compositions are significant (70-90, and the 100-105 ppm resonances), and the contours of the 50-60 ppm resonance would suggest the presence of peptide structures. The most distinctive feature of spectrum 4 is, however, the major contribution of hydrocarbon-type structures (10-40 ppm) to the composition. The components in spectrum 4 more closely represent humin structures than those of HAs (*vide infra*).

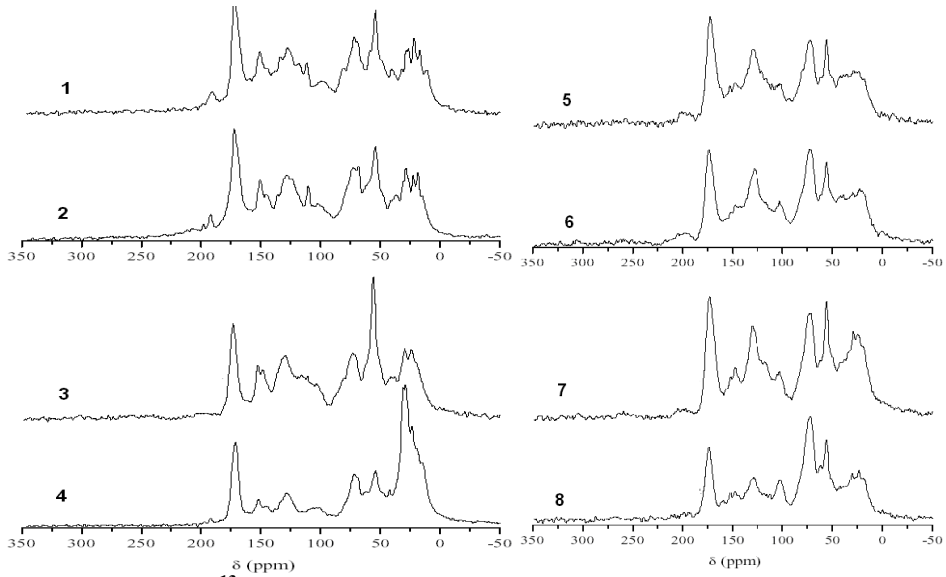

Figure 1. CPMAS ^{13}C NMR spectra of humic (HA) and fulvic (FA) acids isolated from the Grey Brown podzolic soil: 1, HAs isolated at pH 7; 2, HAs isolated at pH 10.6; 3, HAs isolated at pH 12.6; 4, HAs isolated in 0.1 M NaOH + 6 M urea ; 5, FAs isolated at pH 7; 6, FAs isolated at pH 10.6; 7, FAs isolated at pH 12.6; 8, FAs isolated in 0.1 M NaOH + 6 M urea.

Spectra 5 to 8 for the FAs also show compositional differences between the isolates at the different pH values and media. Spectra 5 and 6 are relatively similar (as is evident also for the corresponding HAs, spectra 1 and 2), and these spectra provide only weak evidence for residual lignin structures. There is, however, strong evidence for carbohydrate functionality, The resonances at 50-60 ppm are likely to have significant contributions from peptide structures (Hayes *et al*. 2008).

The evidence for contributions from lignin and for carbohydrate is strong in the case of the FA isolate at pH 12.6 (spectrum 7), and the contribution from hydrocarbon structures (which can include lipids, cutin, cutan structures, etc) is greatest for this FA isolate. Again, as was observed for the HAs, the compositions of the FAs in the base + urea isolate are different from those in the aqueous base isolates. Aromaticity is very low in spectrum 8 (compared with that in spectra 5-7), and aliphatic hydrocarbon functionality is also least. There is evidence for material with origins in lignin, and enrichment in carbohydrate is significant.

The significant enrichments of carbohydrate components in all of the FAs is surprising. Polar carbohydrate materials would not be expected to sorb to XAD-8 but to wash through and be retained by the XAD-4 in the resin sequence (Swift 1996; Hayes *et al.* 2008). The abundance of carbohydrate in all of the FAs would suggest that that the carbohydrate components were strongly associated with (perhaps even covalently linked to) the less polar components of the FAs that sorb to XAD-8.

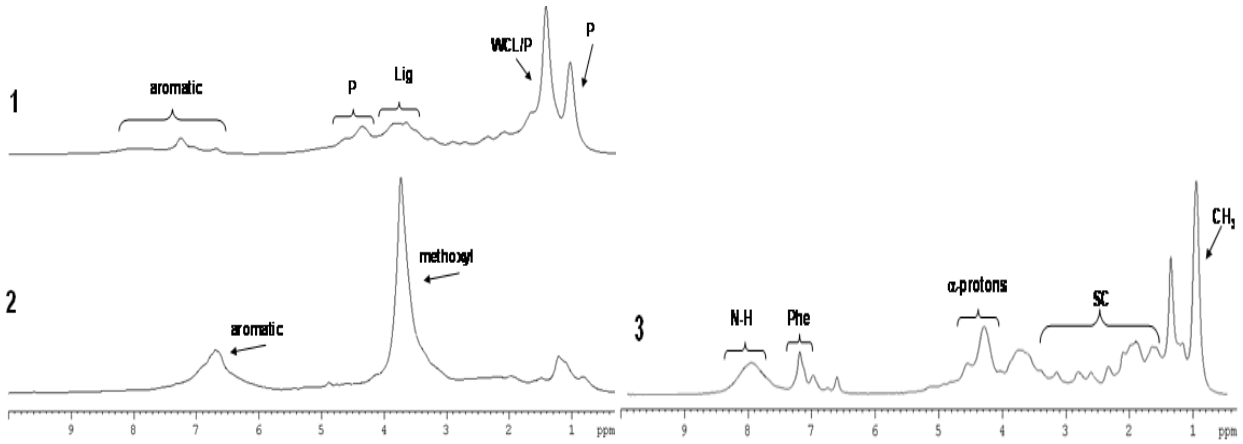

Figure 2. Diffusion edited ^{1}H NMR spectra in DMSO- d_6 for: 1, humin from the poorly drained Gley soil; 2, Organosolv Lignin; and 3, Cultured soil microbes (Simpson *et al.* 2007). P = protein/peptide; Lig = lignin-derived functionality; W = waxes; L = lipid; Phe = phenylalanine.

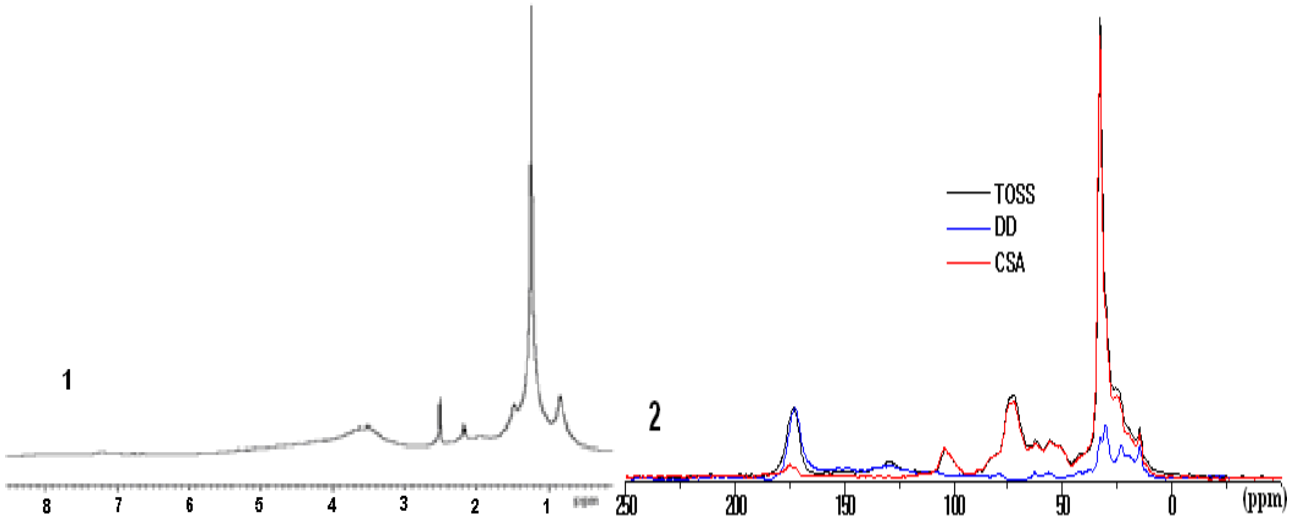

Figure 3. ^{1}H NMR spectrum in DMSO-d_6 (1) for humin from the Grey Brown Podzolic soil; and the TOSS, Dipolar Dephasing (DD) and Chemical Shift Anisotropy (CSA) ^{13}C NMR solid state spectra (2) for the humin, isolated from the same soil.

Compositions of humin materials

Figure 2(1) shows the diffusion edited proton NMR spectrum for the humin material isolated from the Gley soil. Figures 2(2) and 2(3) are markers for functionalities in organosolv lignin and identified in microbes isolated from soil (Simpson *et al.* 2007), respectively. The major contributors to the structures are aliphatic components composed mainly of lipids, waxes, and various hydrocarbon-type structures. There is evidence also for protein/peptide materials, and there is some evidence for structures with origins in lignin material. Spectrum 3(1), the proton NMR spectrum for the humin isolated from the GBP, is dominated by aliphatic hydrocarbon type structures. It has some of the features of spectrum 2(1), but with less evidence for aromaticity. The solid state spectrum, Figure 2(2), indicates a lack of significant aromaticity, but shows

significant carbohydrate, and the resonance at 50-60 ppm can be regarded as protein/peptide functionality. It is clear from the resonance in the 20-35 ppm region that aliphatic hydrocarbon type structures dominate the composition.

Discussion and Conclusions

It is clear, as was also shown by Hayes *et al.* (2008), that a fractionation of HS is achieved when exhaustive extractions are carried out at increasing pH values. Hayes (2006) has discussed how urea in aqueous base can enhance the isolation of soil humic components, and suggested mechanisms involving the breaking of hydrogen bonds binding the HS to the humin matrix, or allowing conformational changes enabling the liberation of the molecules. Although Song *et al*. (2008) found that HAs and FAs isolated from a Mollisol soil using the urea extraction method were similar to the fractions isolated at pH 12.6 in the sequential extraction, the isolates from the urea-base system in the present study resemble more strongly the humin components in the DMSO/H_2SO_4 than those in the pH 12.6 isolates.

Based on the observation that the DMSO/H_2SO_4 was able to remove substantial amounts of organic matter following exhaustive extractions of HS, Hayes (2006) recognised the potential of the DMSO/H_2SO_4 system for the isolation of humin components. Subsequently Simpson *et al.* (2007) and Song *et al*. (2008) have effectively employed this solvent system for the isolation and studies of the compositions of soil humin materials.

Humin is the major component of SOM, and its composition has baffled soil scientists in the past. The application of modern NMR spectroscopy equipment has indicated that humin is a mixture of materials of plant and of microbial origins, and the structures of the component molecules are likely to be well known. The most abundant components have significant aliphatic hydrocarbon functionalities with major contributions from components, such as waxes, lipids, long chain fatty acids and esters, cutins, cutans, suberins, with significant amounts of carbohydrate and peptide materials and with traces of lignin-derived substances and. Under normal circumstances the carbohydrates and peptides would be readily enzymatically degraded, and their presence as components of the relatively resistant humin fraction is surprising. However, the presence of these, and of lignin-derived components that are major constituents of HAs and of FAs, can be attributed to their associations with and steric protection by the largely non-polar components with a high degree of resistance to microbial degradation. Because these non-polar components have high affinities for the inorganic soil components, and especially for the clays, the protection effect is enhanced and access of enzymes to hydrolysable sites is inhibited. Thus humin should no longer be regarded as the 'mystery component' of SOM, and based on the evidence we now have it is clear that humin does not satisfy the classical definitions for humic substances. It should now be regarded as a mixture of largely identifiable biological molecules derived from plant materials composed predominantly of resistant non-polar moieties in intimate associations with, and protecting some biodegradable biomolecules, all in intimate associations with the soil mineral colloids. On the basis of this evidence it is now appropriate to reconsider the inclusion of humin in the classical definitions of soil humic substances.

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