Spectroscopic characteristics of derivatized humic acids from peat in relation to soil properties and plant growth

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Abstract
The humic acid from a sapric peat was subjected to several treatments in order to modify its structure and functionality, or to introduce different forms of N. These treatments included methylation, sulphonation, acid and alkaline hydrolysis, nitration, ammonia fixation, amidation, acetylation, oxirnation, hydrogen peroxide treatment and fixation of stearic acid.

The preparations were characterized by routine analyses, solid-state 13C NMR spectrometry and IR derivatographic spectroscopy. In addition, the effect of the K-humates were tested in two soils, where physical and chemical properties, as well as plant growth were evaluated by greenhouse experiments.

It was found that some of the latter results were reflected in the spectroscopic patterns of the humic acids used. In this study, the positive humic acid properties in the soil correlated better with the amount of aliphatic structures than with other classical indicators.

1. INTRODUCTION
The beneficial effect of the humic acids (HA's) on most physico-chemical processes involved in soil fertility has largely been recognized in the literature. The colloidal character of such soil components, their functional groups and their tendency to form organomineral derivatives have been considered important factors regulating the dynamism of most organic and inorganic molecules in the soil.

Although some empirical approaches showing the effect of HA's on parameters of environmental relevance have been carried out [1,2], such studies are greatly limited by the complex HA composition. From the structural viewpoint, humic substances consist of a chaotic macromolecular system the properties of which cannot deterministically be expressed in terms of their various independent units. In particular, the steric impediments in polymer structures, affect the possibilities of external exposure of the functional groups. This is reflected in their colloidal properties in soil and, in most cases, led to meaningless laboratory determinations of functional groups by wet chemical methods [3].

In the present study, HA's derivatized or chemically transformed are studied by IR spectroscopy and 13C NMR spectrometry. The research focuses on two parallel aims: (i) correlating spectroscopic information with parameters of structural or environmental relevance and (ii) checking the possibility to transform native HA's into improved materials of agronomic interest.

2. MATERIALS AND METHODS
Extraction and transformation of the HA
After continuous shaking for 4 hours, the HA was extracted from the sapric horizon of peat from Vivero (Lugo, Spain) by using 0.5 mol L−1 KOH in a 50 L reactor.

Sample ACE was the acetylated preparation obtained by heating the HA with acetic anhydride at 95 °C for 24 h [4]. Sample ALK was the residue after alkaline hydrolysis: the HA was heated in 1 mol L−1 KOH for 12 h. The so called 'ammonia fixation' is a classical treatment for lignocellulosic products [1]: sample AMO was prepared with NH4OH at 95 °C for 5 h. Sample AMI was amidated with diethylamine [5]. Sample HID is the residue after boiling the HA with 6 mol L−1 HCl for 6 h
(4 fold). The retention of hydrophobic substances on the HA (LIP) is expected to modify its partition coefficient through the modification of the reactivity of some HA macromolecule surfaces, which should then be retaining the lipid added; sample LIP contains 5% of stearic acid; the mixture of K-humate and K-stearate in 0.1 mol L\(^{-1}\) KOH was precipitated with HCl. Sample MET was methylated with diazomethane [6]. Sample NIT was a nitrohumic acid prepared with 50% (v/v) HNO\(_3\) [7]; the precipitate was dialysed. Sample OXI was oxidized with hydroxylaminium chloride in pyridine [5]. Sample PER was obtained by treating the original HA in 0.1 mol L\(^{-1}\) KOH with 30% wt H\(_2\)O\(_2\) (4 h at 60 °C). Finally, sample SUL was sulphonated with 65% HSO\(_3\) in H\(_2\)SO\(_4\) at 80 °C for 1 h.

2.1. Spectroscopic methods

The IR spectra were acquired between 4000–300 cm\(^{-1}\) with a Perkin-Elmer 680B (2.00 mg sample in KBr). For digital data treatments, 740 data points were transferred to a personal computer subjected to baseline subtraction (polygons below 4000, -1850, -865 and 300 cm\(^{-1}\)) and smoothed by 10 iterations of the moving averages algorithm. When resolution enhancement was required, a method was used [8] based on subtracting a multiple (x 50) of the second derivative from the raw spectrum.

Solid-state \(^{13}\)C-NMR spectra were obtained with the CPMAS technique at 74.5 MHz in a Bruker MSL 300. The pulse repetition rate was 5s, and the cross polarization contact time was 1 ms. For each spectrum 1000 cycles were accumulated. The sweep width was 31.25 KHz, the filter width was set to 37.5 KHz and the acquisition time was 0.016 s. Magic-angle spinning was performed at 4 KHz [9].

The digital processing of the spectra included the automatic plotting of the linear correlation indexes between variables with structural and/or environmental relevance, and the arrays containing the twelve spectral intensities for the successive data points in the IR or NMR range. Such correlation spectra are useful to detect readily the spectral regions being directly (positive peaks) or inversely (valleys) correlated with the individual variable under examination [10,11]. The plots were obtained with the authors' programs, where special care was taken in displaying the regression plots in the case of the spectral peaks which correlated significantly (P < 0.05 and P < 0.01) with the dependent variable monitored. This was done to avoid any interpretation of maxima where meaningless significant indexes may be due to individual points in a very extreme position in the regression plot.

2.2. Analyses on the transformed HA's and the soils treated with the K-humates

Spectroscopic measurements in the visible and UV ranges were made from 0.02 mol L\(^{-1}\) NaHCO\(_3\) solutions of 0.1 and 0.01 mg mL\(^{-1}\), respectively.

In order to test the effect of the humid preparations on some soil properties, the samples dissolved in 0.01 mol L\(^{-1}\) KOH were added in triplicate to soil samples in 1-kg pots at the rates of 2 and 4 Mg ha\(^{-1}\). Soil material was collected from two plots of a Calcare Luvisol with a mosaic-like distribution of carbonates [12]: the plots selected were neutral (sample A) or calcic (7.9% CO\(_3\)^2\(\)) (sample C).

In a greenhouse experiment, rye-grass (Lolium rigidum) plants were grown (4 g seed per pot). To prevent deficiency in minerals Hoagland's solution was regularly applied. Rye-grass biomass was harvested every two weeks in all 4 growth stages. After the greenhouse experiment, the soil samples were air-dried and put through a 2 mm sieve. To evaluate the soil structural stability, two independent methods were used: the determination of the percentage of stable aggregates (benzene pretreatment) and that of the mean weight diameter (MWD) after dry sieving [13]. The Richards pressure-membrane apparatus was used for the determination of WHC at the 15 atm point. The cation exchange capacity of the soils [14] was also analyzed.

3. RESULTS

3.1. Spectroscopic characterization

The aliphatic region (0–110 ppm) of the \(^{13}\)C NMR spectrum of the original peat HA (REF) (Fig. 1) showed the alkyl signal ca. 33 ppm and peaks for methoxyl (56 ppm) and glucopyranoside-derived structures (73 ppm). The aromatic region (110–160 ppm) showed unsubstituted and hetero-substituted carbons (126 and 147 ppm, respectively) and the C=O region evidenced carboxyl (172 ppm) and ketone or aldehyde C's (198 ppm) [15].

The IR spectra (Fig. 1) show C=O groups (1720 cm\(^{-1}\)), aromatic ring vibrations (1510 cm\(^{-1}\)), alkyl deformations (1460 cm\(^{-1}\)) and the unspecific band around 1620 cm\(^{-1}\), to which aromatic, conjugated carboxyl and/or quinone and amide groups contribute. Other bands in the resolution-enhanced
spectrum, such as that at 1420 cm⁻¹, or those contributing to the broad band around 1230 cm⁻¹ also matched typical lignin vibrations. Carbohydrate-like structures, methoxyl groups and minerals can contribute to the 1100–1025 cm⁻¹ peaks [16].

The acetylated HA (ACE) showed the acetate signal at 21 ppm [17] as well as enhanced intensity of the 172 ppm C=O signal. The IR spectra showed a broadened 1730 cm⁻¹ band, and peaks at 1370 cm⁻¹ (CH₃-C deformation), 1230 cm⁻¹ (C-O of anhydrides and alcoholic acetates) and 1025 cm⁻¹ (v, C=O) [4,16]. As expected, sample MET gave the enhanced methoxyl signal (56 ppm) and prominent peaks at 2920, 1730 and 1460 cm⁻¹. The C=O stretching band at about 1210 cm⁻¹ also increased due to the methylation of phenolic OH groups [4]. The increased 1120–1095 cm⁻¹ peaks suggest aromatic methoxyl groups.

The oximation (OXI) caused the total disappearance of the 198 ppm signal and the decrease in that at 172 ppm. The broad peak at 147 ppm suggested increased proportion of hetero-substituted groups. The decrease in the amounts of other C=O groups (bands at 1720, 1165 and 1110 cm⁻¹) contrasted with the intense and broadened 1610 cm⁻¹ peak [5]. The sulphonation (SUL) caused selective degradation of aliphatic structures (0–110 ppm), partial demethoxylation and a relative increase in resistant aromatic units. Some decarboxylation (172 ppm, 1720 and 1270 cm⁻¹) from the acid treatment was also found as expected. The sulphone groups (1025, 1165 cm⁻¹) are evident in the IR spectrum [16].

The treatments introducing N in the HA showed some common spectral characteristics: there were minimal changes in the 13C NMR spectrum after ammonia fixation (AMF) [18]. The most external COOH groups are probably involved in the fixation of most ammonia (decrease of carboxyl vibrations and a prominent 1395 cm⁻¹ peak). Characteristic NH₃⁺ bands at 3190 and 3030 cm⁻¹ appeared superimposed on the broad OH stretching band [16].

The alkyl signals at 13 and 41 ppm after amidation (AMI) conform to the derivatization -COOH→-CONH(CH₂)₃. This is also suggested by the decrease in the 1720 cm⁻¹ band and the increase in the 1630 cm⁻¹ region, as well as in the peaks of the ethyl groups at 1460, 1380 and 787 cm⁻¹ [5].

Nitrification (NIT) is known to yield considerable amount of acid-soluble aromatic material [7] which, in the conditions used here, was lost after dialyzing. This may explain the enrichment in polyalkyl structures resistant to nitric acid treatment (33 ppm, 2920 cm⁻¹). The resistance—or incorporation—of carboxyl groups is observed. Nitration is also reflected by the 1540 and 1360 cm⁻¹ peaks (νₛ and ν of C-N=O) [18].

Other treatments causing selective degradations in the HA's (ALK, HID, PER), also showed common spectral features. Alkaline hydrolysis (ALK) caused losses of O-alkyl structures as well as of carboxyl and other oxygen-containing groups. Acid hydrolysis (HID) led to increased aromaticity due to the selective removal of carboxyl and aliphatic—mainly O-alkyl—structures. Hydrogen peroxide (PER) caused both the removal of alkyl structures (33 ppm) and the ring opening of some aromatic units [19] whereas the carboxyl content remained high.

3.2. Some soil and HA characteristics connected with the spectral intensities

The correlation spectra for the plant yield of the soils amended with humates showed significant positive peaks in the alkyl and O-alkyl NMR region, which also occurred in the C=O region near 172 ppm. On the contrary, the intensity of the peaks in the aromatic region (and often that of the 56 ppm methoxyl resonance) tends to correlate inversely to plant production. The correlation spectra were comparable either with both soils or with the different humate doses or the subsequent harvesting periods. Two representative correlation spectra are shown in Fig. 2.

In our experiments, the NMR aromatic/aliphatic ratio correlated inversely with most of the of plant yield data, as illustrated in Fig 3. In addition, the area ratio between the NMR region for (O- and/or N)-substituted aromatic C's and the protonated C's, suggests the increase of plant yield in samples treated with the materials where the former structures dominated. This bears out the results from the IR spectra. Fig 2 shows positive correlations for alkyl structures (2920, 1460 cm⁻¹) as well as in the unspecific 1620–1640 cm⁻¹ region, in part affected by amide groups (it is noted that, in these experiments, the percentage of N of the humates correlated poorly with plant yield data). As opposed to the carboxyl (1720 cm⁻¹) peak, the broad 3400 cm⁻¹ region frequently appeared correlated with the plant yield.

The extent of the alkyl moiety of the HA's is also reflected in soil aggregate stability, both when such a parameter is evaluated on the basis of the MWD (Fig. 2) or on the basis of the weight of stable aggregates (Fig. 3). Positive correlations between the above physical parameters and the intensity of the whole aliphatic NMR region and the ratio between hetero-substituted and protonated aromatic C's were also detected.

No systematic correlations were found for other soil variables examined. The intensity of the 1620 cm⁻¹ IR peak tends to be inverse to the WHC at 15 atm. Small significant changes in the cation exchange properties of the amended soils were found.

Finally, when studying the spectral patterns in terms of standard HA parameters, some more
or less trivial results were obtained: the HA solubility in ethanol paralleled the amount of aliphatic C's, whereas the contrary occurred with the aromatic ones (NMR aromatic region and 1510, 1620 cm\(^{-1}\) IR peaks). The intensity of the carboxyl NMR signal tended to be enhanced as the N percentage decreased. No significant correlation was found with the O/C atomic ratio though, as expected, the H/C atomic ratio did in the NMR aliphatic area. Both the E\(_{465}\) and E\(_{275}\) extinctions were found to correlate with the aromatic/aliphatic NMR ratios (Fig. 3) [20].

4. DISCUSSION

Complementary IR and NMR techniques are required to detect the chemical transformations of the HA's, as suggested by the fact that treatments causing no substantial changes in the NMR pattern led to characteristic ones in the IR spectra, and vice-versa.

Our study indicates that alkyl structures in the peat HA take the form of some favourable soil properties. Assuming some cause-effect relationship, the protecting role of partially hydrophobic colloids [21] should be taken into account as they make the microaggregates waterproof, as well as their presumable effect—at the colloidal level—on the stable organization of hydrophobic clay particles. In some cases, the influence of the aliphatic structures seems to be greater than those of the N-bearing material, or of the carboxyl groups. In this study, no evidence was found concerning the beneficial effects attributable to changes in the cation exchange capacity of the soil.

Apart from hydrophobic interactions, it could also be postulated that the presence of alkyl and heterocyclic constituents in the HA's has a bearing on greater structural flexibility than in the macromolecules with frequent aromatic units, typical for HA's from most fossil materials. The possible role of such flexibility on the speciation of functional groups, may be found in the importance of the HA's for soil fertility.

Further studies on the properties of the derivatized HA's are in progress.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

Fig. 1. Above: Changes in the resolution-enhanced IR spectrum (2000–700 cm⁻¹) of peat HA after chemical transformation or derivatization. In the untreated HA (REF), the resolution-enhanced spectrum is compared with the original one. Below: CPMAS ¹³C NMR spectra. In both cases, and after equal-area normalization, the spectrum REF was paired (dashed line) with those of the different treatments.

ACE= acetylation, ALK= alkaline hydrolysis, AMO= ammonia fixation, AMI= amidation, HID= acid hydrolysis, LIP= "fixation" of the n-C₁₈ fatty acid, MET= methylation, NIT= nitration, OXI= oximation, PER= H₂O₂ oxidation, SUL= sulphonation.
Fig. 2. Correlation spectra for some variables determined in soils amended with transformed humates (values for correlation coefficients between the dependent variables and spectral intensities at different wavelengths in terms of the wavelength). Left: $^1$H NMR range; right: IR range. Bars at the right indicate the significant values at $P < 0.05$ and $P < 0.01$.

MWD C x1: Aggregation index (mean weight diameter, calcic soil, low dose); PII A x1: plant yield (dry wt increase from the unamended soil; 2nd harvest, neutral soil, low dose); PT C x2: plant yield (total harvest; calcic soil, high dose); PI C x1: plant yield (1st harvest, calcic soil, low dose).

Fig. 3. Regression plots between spectroscopic data and parameters determined in derivatized or transformed HA's or in soils amended with humates (for point labels see Fig. 1).

Sagg C x1 (percentage of water-resistant aggregates: calcic soil, low dose); PT A x2: Plant production (total harvest; neutral soil, high dose); PII A x1: Plant production (2nd harvest, neutral soil, low dose); E275: UV extinction at 275 nm [0.01 mg mL$^{-1}$].