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Chemical and spectroscopic characterization of insoluble and soluble humic acid fractions at different pH values

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Abstract

Background: Humic acids (HA) are organic molecules with complex structure and function and variable properties. They are insoluble in strongly acid pH conditions. At present, it is not clear how much is the amount of HA in solution at the pH of natural soils nor are known the characteristics of the different soluble fractions and their possible association with the inorganic phase of soil. The scope of this work was to characterize the soluble and insoluble fractions obtained by acidifying Na humate solution at pH values 3, 5, and 7 and to compare these fractions with the HA obtained at pH 1. At each pH, the precipitate and the soluble fractions were separated and characterized by elemental analysis, total acidity and carboxylic group content, infrared, and ¹³C NMR and ¹H NMR spectroscopy.

Results: The HA fraction insoluble at pH 1 had a high acid group content and aromaticity but a low content of O- and N-containing groups. At pH 3, a fraction with nearly the same characteristics was obtained. At pH 1 and pH 3, the inorganic phase bound to the insoluble humic material was largely constituted by clay minerals and some Al and Fe hydroxides. The soluble fractions at pH values 1 and 3 were very poor and they were composed of a silica gel-like phase associated with polar organic material rich in carboxylic and metal-carboxylate groups. At pH values 5 and 7, only a small fraction of the Na humates precipitated. The fractions remaining in the solution were mainly composed of organic material particularly rich in aromatic and aliphatic groups, while the inorganic phase contained phyllosilicates. The fractions insoluble at pH values 5 and 7 contained a large amount of inorganic material that consisted mainly of phyllosilicates.

Conclusions: The soluble fractions obtained at pH values 5 and 7 represent the humic component that in environmental situations would be dissolved in the soil solution. Our findings could be very useful for a more detailed investigation into the way HA influence plant metabolism under environmental-like conditions, both as regards pH conditions and interaction with the mineral fraction.

Keywords: Acid; pH; Mineral component; ¹³C NMR; FT-IR; Humic fraction

Background

Soil organic matter can be divided into two major components: organic substances of an individual nature and humic substances (HS). Traditionally, the soil HS have been subdivided into three fractions: fulvic acids, humic acids (HA), and humin.

It is widely recognized that HS are important for the sustainability of terrestrial life. The heterogeneous

chemical nature of HS exerts a multifunctional role in the environment by controlling the biogeochemical carbon cycle [1,2], providing nutrients and bio-stimulants for plant growth [3,4], and interacting with inorganic [5] and organic pollutants [6,7].

It is common knowledge that the characteristics of HS depend on the extraction procedure adopted. This fact has been attributed to the complex nature of soil, to the wide range of physical and chemical properties inherent in humic material, and in particular to the intimate association of organic and inorganic soil constituents [8]. Most of the studies reporting the effect of extraction procedures

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on the chemical structure of HS have concerned the nature of the extraction solvent. These studies pointed out that both the type of soil and the extraction pH played a role in the chemical structure and molecular weight of the humic materials. The procedure recommended by the International Humic Substances Society (IHSS) for preparing HA [9] includes 0.1 M sodium hydroxide extraction followed by 0.1 M hydrochloric acid precipitation. In accordance with the new concept of supramolecular association, the HA obtained by this procedure is composed of many relatively small, chemically diverse organic molecules which form clusters linked by hydrogen bonds (H-bonds) and hydrophobic interactions [10]. A corollary to this model is the concept of micellar structure, i.e., an arrangement of organic molecules in aqueous solution which form hydrophilic exterior regions that shield the hydrophobic interiors from contact with vicinal water molecules [11].

It is assumed that humic acids are completely soluble at pH 13 and completely insoluble at pH 1, but the solubility of HA at intermediate pH values has not been fully investigated. Brigante et al. [12] measured the dissolution kinetics of HA prepared according to the IHSS procedure at different pH values between 10 and 4. HA were completely soluble within less than an hour at pH > 10. At pH between 10 and 9, the dissolution was slower but nearly complete. At pH 8.5 and 8, more than 60% and 40% of the HA respectively were soluble. The separation of HA in a soluble and an insoluble phase at these pH values attests for the heterogeneity of the material defined as humic acid on the basis of its insolubility at pH 1. At our knowledge, the chemical differences between the soluble and insoluble fractions at a given pH have not been investigated.

A drawback of the IHSS procedure is that the HA may contain quite large amounts (e.g., 30%) of inorganic components. Although some attempts have been made to destroy the organo-mineral associations before HS extraction [13,14], a purification procedure aimed at obtaining ash-free organic fractions is commonly carried out on the extracted humic materials.

Such purification procedure, based on hydrochloric-hydrofluoric acid solubilization of the inorganic fraction, generally decreases the ash content to less than 2%. This is considered a good result as far as the purity of the humic material is concerned. However, little attention has been given to the nature of the inorganic fraction brought in solution by the alkaline extraction and so strongly bound to the humic material to require a drastic treatment to be released.

In this study, a Na humate solution was prepared following the IHSS procedure but without treatment to eliminate the inorganic phase. Aliquots of the humate solution were brought to pH 7, 5, 3, and 1, and the

corresponding soluble and insoluble fractions were collected and characterized. The scope was to determine the chemical structure of the organic and inorganic materials precipitated or left in solution at each pH and to obtain some elucidation on the nature of their interactions.

Experimental

Materials

Soil

The HA was extracted from a histosol (Trana, Turin, Italy), studied in another article [15], having the following properties: pH, 6.9; clay, 5.7%; loam, 30.5%; sand, 63.8%; organic carbon, 8.51%; cation exchange capacity, 52.9 meq/100 g; Na oxalate extractable Fe, 0.68%; Na dithionite-citrate-bicarbonate extractable Fe, 0.86%; and Na pyrophosphate extractable Fe, 0.22%.

Methods

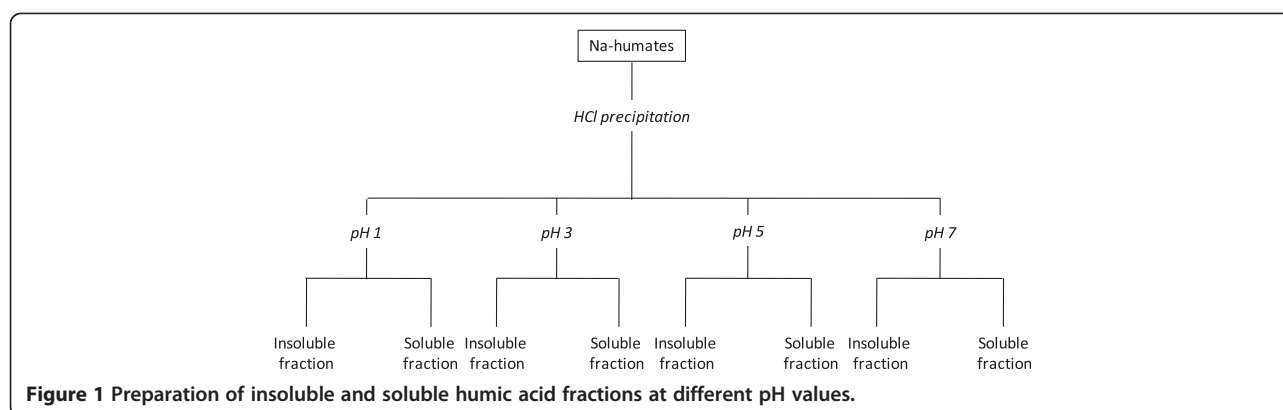
Preparation of the HA fractions

The soil (100 g subsamples in 500 ml PE flasks) was equilibrated to a pH value of between 1 and 2 with 1 M HCl; the suspension was brought to 500 ml with 0.1 M HCl, shaken for 1 h, centrifuged, and the supernatant eliminated. The solid phase was neutralized to pH 7 with 1 M NaOH, then 0.1 M NaOH was added to provide a final volume of 500 ml. The suspension was shaken under N₂ for 20 h, allowed to settle overnight, then centrifuged (1,500 g, 30 min) to separate the supernatant. A series of three alternate treatments with 0.1 M HCl and 0.1 M NaOH was performed to solubilize and remove the fulvic acids. After the final treatment, the precipitate resulting from acidification was dissolved in 0.1 M NaOH, and the whole was treated as illustrated in Figure 1. Equal parts of Na humate were acidified with 0.1 M HCl at different pH values, 1, 3, 5, and 7. At each pH, the soluble and insoluble fractions were separated by centrifugation. Subsequently, the fractions were dialyzed (MW cut-off 3,500 Da) and then freeze-dried.

Characterization of the HA fractions

The HA fractions were characterized for total acidity and carboxylic group content in accordance with the procedure proposed by Swift [9]; measurement of the phenol OH was obtained by difference; the C, H, N content was determined using a C, H, and N LECO Analyzer model 600 (LECO Corp., St. Joseph, MI, USA); O content was calculated by difference. Ash content was determined by incineration at 650°C for 4 h.

The FT-IR spectra were obtained with a Perkin-Elmer FT-IR 2000 spectrometer (Perkin Elmer Italia, S.p.A, Milan, Italy), equipped with an IR source, KBr beam splitter and DTGS KBr detector. For each sample, 64 scans were recorded with a 4-cm⁻¹ resolution over a 4,000



to 400 cm^{-1} range on pellets obtained by pressing a mixture of 1 mg organic sample and 400 mg dried KBr.

Solid-state ^{13}C MAS NMR spectra were recorded, fully proton-decoupled, on a Bruker MSL 200 NMR (Bruker Corp., The Woodlands, TX, USA) instrument operating at 50.3 MHz. Rotors with a 7-mm diameter were filled with about 50 mg of the sample; the spinning rate was 4,000 Hz s^{-1} . The following experimental parameters were adopted: spectral width 20,000 Hz, data points 2 K, 100,000 scans, 4 μs , 90 degrees of excitation pulse, and 4 s of relaxation delay. The HPDEC pulse sequence was used with a decoupling power of 300 W (9H). The FID were zero-filled and processed with 5 Hz line broadening. Chemical shifts refer to ^{13}CO of glycine, set at 176.03 ppm.

The ^1H NMR spectra were recorded using a JEOL EX-400 spectrometer (JEOL, Peabody, MA, USA) equipped with a 5 mL probe operating at 399.78 MHz. Samples were prepared by dissolving 20 mg of the sample in 0.6 mL of 0.1 M NaOD/D₂O. After filtration through a glass wool filter, the samples were placed in NMR tubes for analysis. All the samples were adjusted to pH 12. The ^1H NMR spectra were recorded using the presaturation sequence for selective saturation of the HDO resonance [16]. The main NMR parameters were 45° pulse width, pre-irradiation

10 s, acquisition time 5.46 s, and 64 scans for a total acquisition time of about 16 min.

The iron and aluminum oxide contents were evaluated by Na dithionite-citrate-bicarbonate extraction [17] at 25°C (Fe DCB and Al DCB, respectively). Aqua regia was used to attack the metals, and then the total iron and aluminum in the humic fractions were analyzed.

All chemicals were of analytical grade, and three replicates per analytical determination were carried out.

Analytical data were evaluated by one-way ANOVA ($P < 0.005$) followed by the Tukey's test for multiple comparison procedures.

Results

Yield and ash content

The yield, ash content, and elemental composition of the humic fractions at each pH are reported in Table 1.

As expected, the yield of the insoluble fractions decreased as pH increased, while the contrary occurred for the soluble fractions. The total humic material (soluble + insoluble) extracted at each pH was not about the same (86.2, 95.2, 82.5, and 74.7 g kg^{-1} soil at pH 1, 3, 5, and 7, respectively). The observed differences, in particular the low total recovery at pH 7, suggest some loss of material, probably inorganic and

Table 1 Yield, ash content, and elemental composition of the humic fractions

Fraction	Yield (g kg^{-1} soil)	Ash ^a (g kg^{-1} humic fraction)	Elemental composition ^b (g kg^{-1} humic fraction)				Molar ratio	
			C	H	N	O		
pH 1	Insoluble	83.9	247	541 ^a	42 ^a	43 ^a	374 ^a	0.5
	Soluble	2.3	196	478 ^{bd}	44 ^a	43 ^a	434 ^{bc}	0.7
pH 3	Insoluble	88.0	267	525 ^a	45 ^a	42 ^a	388 ^{ab}	0.5
	Soluble	7.2	127	493 ^{bc}	41 ^a	45 ^a	421 ^{abc}	0.6
pH 5	Insoluble	22.2	542	478 ^{bd}	55 ^b	40 ^a	427 ^{bc}	0.7
	Soluble	60.3	225	518 ^{ac}	49 ^b	41 ^a	392 ^{ab}	0.6
pH 7	Insoluble	7.4	609	451 ^d	52 ^b	39 ^a	459 ^c	0.8
	Soluble	67.3	283	534 ^a	49 ^b	42 ^a	375 ^a	0.5

^aValues are the means of three replicates, and the standard error values are < 10%; ^bon a moisture and ash-free basis. Within columns, values followed by the same letter are not significantly different ($P < 0.05$).

organic molecules smaller than the dialysis membrane MW cut-off.

The sum of the ash content of the soluble and insoluble fractions is not the same at each pH. However, this difference is only apparent when considering the value corrected by the yield (21.2, 24.5, 25.6, and 23.6 g at pH 1, 3, 5, and 7 respectively, for extracts from 1 kg of soil). This is demonstrated by the following calculation:

$$\text{Gram of ash at each pH} = \left(\text{Yield IF} \times \frac{\% \text{ IF ash}}{100} \right) + \left(\text{Yield SF} \times \frac{\% \text{ SF ash}}{100} \right)$$

where IF is the insoluble fraction and SF is the soluble fraction.

The high ash content in all fractions emphasizes the importance of employing a purification step if ash-free humic fractions are to be obtained. The effectiveness of the purification step in HA prepared from a histosol was demonstrated by Piccolo [18] who reported a decrease in the ash content from 500 to 10 g kg⁻¹ following HF-HCl washing. The ash contents of more than 500 g kg⁻¹ in the insoluble fractions at pH 5 and pH 7 attest that there is more inorganic than organic material and is the likely reason for the lack of solubility of these fractions.

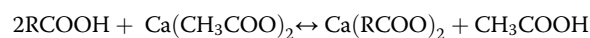
Elemental composition

The elemental composition of the humic fractions is reported in Table 1 on a moisture- and ash-free basis. Therefore, the following considerations concern the organic component of each fraction. While the insoluble fractions at pH 1 and 3 were richer in C and poorer in O than the soluble fractions, the O increased at pH 5 and 7 indicating that the precipitation pH of the HA affected the distribution of the functional groups between the precipitated and the liquid phase. The C content was lower at pH 5 and 7 than at pH 1 and 3 in the insoluble fractions, while the opposite was true for the soluble fractions. The O content and O/C ratio were lower in the insoluble fractions at pH 1 and 3 than at pH 5 and 7 indicating that polarity increased along with pH, while the opposite occurred in the soluble fractions. In the case of the N content, no clear trend could be identified indicating that pH did not affect the distribution of the N-containing moieties in the solid or liquid phase. The H content was higher at pH 5 and pH 7 than at pH 1 and pH 3 in both the insoluble and soluble fractions. This would appear to be an artifact, since the sum of each element present in the soluble and insoluble fractions deriving from the same alkali solution should be the same at any pH. The highest H contents were found in the fractions exhibiting the highest ash contents. This suggests that the presence of inorganic components could induce an overestimation of the H content. One explanation for this result is that the elemental analysis includes a high-temperature incineration step (up to 1,000°C) which could provoke the release of some

hydration water and lead to the partial decomposition of some inorganic components, such as kaolinite, thereby releasing OH groups and leading to an overestimation of the hydrogen content.

Acidic groups

The carboxylic, phenolic, and total acidity distribution in each fraction are reported in Table 2. The wet method used to determine the carboxylic group content is based on titration with Ca acetate. The aim of this technique is to convert the complex mixture of acids in HS into an equivalent amount of acetic acid by the reaction [19]:



This method is reliable only if the carboxylic groups are completely combined. This is why the carboxylic group content, measured using the wet chemical method, decreased at decreasing pH. The total acidity was not measurable at pH 5 and 7. This was most likely due to the carbonation of the barium hydroxide solution used in titration, as reported by Stevenson [20]. Consequently, the content of phenolic groups could not be calculated.

¹³C NMR

The distribution of the different forms of carbon calculated according to the area of the different NMR spectra regions (alkyl, 0 to 45 ppm; N- and O-alkyl, 45 to 95 ppm; aromatic, 95 to 160 ppm; carboxyl, 160 to 195 ppm) are reported in Table 2.

The amount of carboxyl carbon calculated from the integration of the signal in the 160 to 195 ppm region was much higher than expected considering the chemical titration. This discrepancy has already been reported and discussed in the previous papers [21,22].

The insoluble fractions at pH values 1 and 3 are largely aromatic (33.1% and 33.9% of the total carbon respectively) with a medium amount of aliphatic carbon (23.0% and 21.0% of the total carbon respectively) and a low content of substituted aliphatic groups (16.0% and 18.3% of the total carbon respectively). Similar concentrations of aromatic carbon were found in HA from histosols by ¹³C NMR investigation [15,23,24] and reflect the high maturity of the HA expected in such soils. By contrast, the soluble fractions at pH 1 and pH 3 contain a very low amount of aromatic C (2.9% and 7.5% of the total carbon) but are very rich in aliphatic substituted carbons (58.8% and 42.1% of the total carbon). These results, together with the high O content of these fractions (Table 1), suggest the presence of polar aliphatic compounds (sugars, amino acids, proteins, carboxylic acids) which are most likely responsible for the solubility at low pH values.

Similar results are obtained evaluating the degree of hydrophobicity (hydrophobic/hydrophilic carbons, HB/HI)

Table 2 Acidic groups' concentration and distribution of C intensity of ^{13}C NMR spectra of humic fractions

Fraction		Acidic groups ^a (meq g ⁻¹)			^{13}C NMR distribution (%)				HB/HI
		Carboxyl	Phenol	Total acidity	0 to 45 ppm	45 to 95 ppm	95 to 160 ppm	160 to 195 ppm	
pH 1	Insoluble	3.8	7.3	11.1	23.0	16.0	33.1	28.0	1.3
	Soluble	4.0	6.7	10.7	14.2	58.8	2.9	24.2	0.2
pH 3	Insoluble	3.1	5.9	8.9	21.0	18.3	33.9	26.9	1.2
	Soluble	2.8	6.7	9.4	14.5	42.1	7.5	35.9	0.3
pH 5	Insoluble	nd ^b	nd ^b		nd ^c	nd ^c	nd ^c	nd ^c	-
	Soluble	nd ^b	nd ^b		20.8	6.0	38.7	34.5	1.5
pH 7	Insoluble	nd ^b	nd ^b		nd ^c	nd ^c	nd ^c	nd ^c	-
	Soluble	nd ^b	nd ^b		19.1	1.3	35.2	44.3	1.2

^aValues are the means of three replicates. The standard error values are < 10%; ^banalytical method not applicable at these pH; ^cnot determinable because of the poor signal-to-noise ratio of the spectra.

as proposed by several authors [25-28]. This is given by the areas relative to resonance intervals, as follows:

$$\frac{\text{HB}}{\text{HI}} = \frac{[(0-45) + (95-160)]}{[(45-95) + (160-195)]}$$

The fractions insoluble at pH 1 and 3 have a degree of hydrophobicity (Table 2) greater than the soluble fractions, with values (> 1.0) that are consistent with those observed for HA (between 0.61 and 2.87) by Piccolo et al. [26]. On the other hand, the values of the soluble fractions at pH 1 and 3 of HB/HI are more similar to the same authors' findings for fulvic acids (< 0.5).

The ^{13}C NMR spectra of the insoluble fractions at pH 5 and 7 with a high ash content exhibit a low signal-to-noise ratio probably due to the presence of paramagnetic compounds as already observed in soils [29] and humic samples [30]. Consequently, no reliable evaluation of the carbon distribution could be made.

The soluble fractions at pH 5 and pH 7 exhibited a high content of aromatic carbon (38.7% and 35.2% of the total carbon respectively) and a low content of N- and O-substituted aliphatic groups. This is in agreement with the decrease in soluble fraction polarity as pH increases, which is assumed on the basis of the elemental analysis and the degree of hydrophobicity.

The organic component of the fractions obtained at pH 5 and 7 was also studied with ^1H NMR in order to obtain more information regarding their chemical characteristics. This technique proved easier to use with samples having a higher ash content than did ^{13}C NMR. The ^1H NMR data agree well with the data obtained from the ^{13}C NMR but tend to underestimate the aromaticity, above all when the aromatic molecules are substituted with atoms other than hydrogen [31].

The distribution of the different forms of hydrogen was calculated by integration in three regions of the spectrum (0.5 to 3 ppm, 3 to 4.2 ppm, and 6 to 8 ppm) and expressed as a percentage of the total area of each

spectrum. The allocation of the three regions was based on the work of Francioso et al. [32]. The region from 0.5 to 3 ppm corresponds to the H forms linked to aliphatic groups, the region from 3 to 4.2 ppm is assigned to the H forms linked to carbohydrate structures, and finally the region between 6 and 8 ppm is allocated to the H forms linked to aromatic molecules.

From Table 3, which relates to the distribution of the ^1H forms, it can be seen that the aliphatic region is similar for all the fractions obtained at pH 5 and 7, while the region related to carbohydrates (between 3 and 4.2 ppm) is higher in insoluble fractions than soluble. Moreover, the latter is more aromatic than the insoluble one. In agreement with the elemental analysis, these components are more polar than the soluble fractions (Table 1).

FT-IR

The FT-IR spectra indicate some large differences between the humic fractions, attesting to the different structures of both the inorganic and organic material (Figure 2). The spectra of the insoluble fractions at pH 5 and 7 (containing more than 50% inorganic component) were identical and dominated by the intense absorption of the Si-O vibration centered at 1,030 cm⁻¹ typical of montmorillonite [33]. The other bands attributable to the inorganic components have the same frequencies as those of montmorillonite defiled with kaolinite [33,34] as follows: 3,696 cm⁻¹, OH stretching of inner-surface hydroxyl groups of kaolinite; 3,621 cm⁻¹, OH stretching

Table 3 Distribution of H intensity of the ^1H NMR spectra of the humic fractions

Fraction		^1H NMR distribution (%)		
		0.5 to 3.0 ppm	3.0 to 4.2 ppm	6.0 to 8.0 ppm
pH 5	Insoluble	45.6	36.7	17.7
	Soluble	45.2	23.2	31.6
pH 7	Insoluble	47.6	40.3	12.1
	Soluble	46.9	20.5	32.6

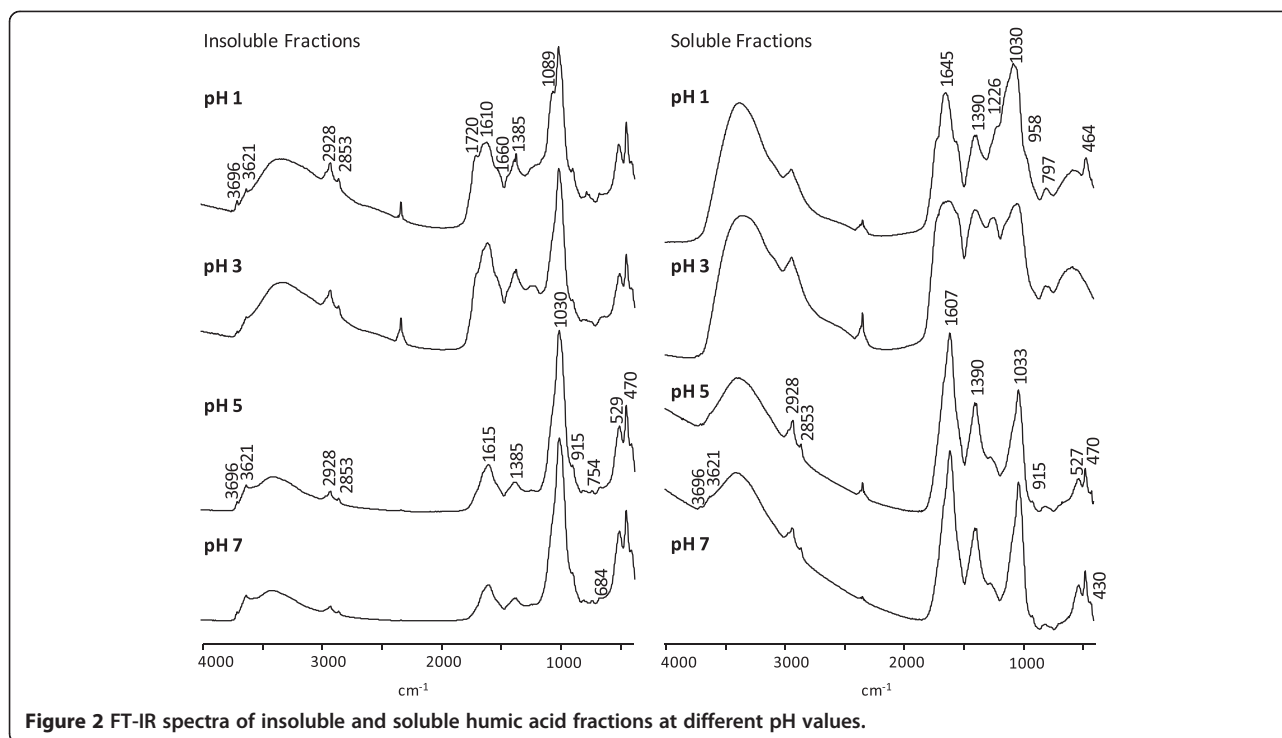


Figure 2 FT-IR spectra of insoluble and soluble humic acid fractions at different pH values.

of hydroxyl groups; 915 cm^{-1} , AlAlOH deformation; 754 cm^{-1} , Si-O perpendicular (kaolinite); 470 cm^{-1} , Si-O-Si deformation; 430 cm^{-1} , Si-O deformation (kaolinite). The band at 529 cm^{-1} could be the Al-O-Si deformation of montmorillonite, although it is generally reported at lower frequencies (521 to 523 cm^{-1}). Some of the typical bands of montmorillonite are missing (790 , 623 , and 578 cm^{-1}). The lack of such bands has already been observed in soil montmorillonites and attributed to a larger disorder which provokes the broadening and vanishing of these bands [33]. Besides the bands of the inorganic compounds, the main organic bands are the aliphatic absorptions at $2,928$ and $2,853\text{ cm}^{-1}$ (CH_2 stretching). Also, the two bands centered at $1,615$ and $1,385\text{ cm}^{-1}$, typical of the asymmetric and symmetric vibrations of carboxylates, reflect the presence of metal carboxylates.

The spectra of the soluble fractions at pH 5 and pH 7 show the same bands as those attributed to the vibrations of kaolinite and montmorillonite for the insoluble fractions ($3,696$, $3,620$, $1,033$, 915 , 527 , 470 , and 430 cm^{-1}) but with lower intensity due to their lower ash content. The COOH vibration is missing, while very strong bands are present at $1,607$ ($\nu_{\text{as}}\text{COO}^-$) and $1,390\text{ cm}^{-1}$ ($\nu_{\text{s}}\text{COO}^-$). The frequency of the $\nu_{\text{as}}\text{COO}^-$ has been seen to decrease in parallel to an increase in the ionic radii of the metallic ion [35]. According to these authors, the $\nu_{\text{as}}\text{COO}^-$ band shift toward higher frequencies corresponds to the transition from ionic to covalent bond and can be used to establish the ionic-covalent nature of metal carboxylates. This

statement was contested by Piccolo and Stevenson [36], given the possibility that the metal-complexed ketonic group could give a band in the $1,620$ to $1,610\text{ cm}^{-1}$ region. However, the $\nu_{\text{as}}\text{COO}^-$ on the spectra of the soluble fractions at pH 5 and 7 ($1,607\text{ cm}^{-1}$) does not fall in the ketonic region and is between those reported by Vinkler et al. [35] for Na humate ($1,590\text{ cm}^{-1}$) and by Piccolo and Stevenson [36] for K humate at pH 5 ($1,610\text{ cm}^{-1}$). This suggests the formation of ionic humates, probably with a great presence of Na ions in the solution at these pH values.

The spectra of the insoluble fraction at pH 1 and pH 3 exhibit the same, but weaker, bands as those attributed to the inorganic component in the insoluble fractions at pH 5 and 7. In contrast, the HA bands are stronger and some new peaks are present, in particular, the COOH vibration at $1,720\text{ cm}^{-1}$; the band in the $1,610$ to $1,660\text{ cm}^{-1}$ region is broader with a shoulder at $1,660\text{ cm}^{-1}$ and is likely due to an overlap between $\nu_{\text{as}}\text{COO}^-$ vibration, aromatic $-\text{C}=\text{C}-$ vibration and N-containing groups. A partial influence on this band can also come from the OH stretching of water bonded to the clay minerals which has been seen to absorb in the $1,630$ to $1,660\text{ cm}^{-1}$ range [33].

The spectrum of the insoluble fraction at pH 1 exhibits a strong band at $1,089\text{ cm}^{-1}$ similar to that shown on the spectrum of a montmorillonite and attributed to the Si-O stretching of a cristobalite-like phase impurity [34].

The spectra of the soluble fractions at pH 1 and 3, having the lowest ash content, exhibit the typical features of the HS spectra eliminating part of the diagnostic

bands of the inorganic components. The silicates O-Si-O vibration band centered at $1,030\text{ cm}^{-1}$ on the other spectra is broad and shifted at higher frequencies (up to $1,075\text{ cm}^{-1}$ at pH 1). A Si-O vibration at $1,075\text{ cm}^{-1}$, besides weaker bands at $1,200$, 958 , 797 , and 464 cm^{-1} , has been attributed to the silica gel [37]. The same bands are present on the spectrum of the soluble fraction at pH 1 which also exhibits the two carboxylate group bands ($1,645$ and $1,390\text{ cm}^{-1}$). Very similar spectra were reported for the soluble products formed between hydroxy-Al ions and orthosilicic acid in the presence of citric acid [37] and HS [38]. This suggests that the fractions which do not precipitate at low pH are organo-mineral colloids in which the inorganic phase is similar to the silica gel. However, the frequency of the broad band centered at $1,645\text{ cm}^{-1}$ is higher than those reported by Vinkler et al. [35] also in the case of covalent humates with Fe and Al ($1,625\text{ cm}^{-1}$) and should be overlapped by other absorptions, for example, the OH vibration of water molecules bonded to silica gel which occurs at $1,630\text{ cm}^{-1}$ [33].

Although the FT-IR spectra of these fractions provided evidence of the presence of phyllosilicates and silica gel, we cannot exclude that the inorganic fractions also contain iron and aluminum oxides. The FeOH bending and the Fe-O stretching of a Fe(III) synthetic polycation absorb at 550 and 680 cm^{-1} respectively [39,40]. Other authors report that the main absorption of ferrihydrite took place at 587 cm^{-1} while hematite gave sharp bands at 460 and 580 cm^{-1} [41]. On the spectra for those insoluble fractions at pH 5 and pH 7, a weak band is present at 684 cm^{-1} which could be attributed to Fe-O stretching, while the other bands typical of Fe vibrations could be overlapped or dwarfed by the strong absorption of silicates. The hydroxides are generally identified by several diagnostic bands in the OH stretching region, their number and frequency depending on the grade of crystallinity of the minerals [42]. However, in the presence of silicates with water in the interlayer, these bands have been seen to mix in a single broad absorption band in the $3,000$ to $3,630\text{ cm}^{-1}$ range [42]. Therefore, the lack of such bands in our samples does not mean that they do not contain aluminum hydroxides. The other typical Al hydroxide bands are at $1,025$, 970 , and 531 cm^{-1} [43]. The first two bands are overlapped by Si-O absorption at $1,030\text{ cm}^{-1}$, but a strong band at 530 cm^{-1} cannot be attributed to silicates and could indicate the presence of aluminum hydroxides.

Metal concentrations in the inorganic components

The total metal content and the DCB extractable concentration of Al and Fe in the humic fractions are reported in Tables 4 and 5. The highest concentrations of Si and Al (as total Al) were found in the insoluble fractions, which indicate the presence of phyllosilicates (Table 4). In the

insoluble fractions at pH 5 and pH 7, less than 5% Al was DCB extractable showing that this metal was mainly present in clay mineral structures. In the insoluble fractions at pH 1 and pH 3, 28% and 32% of the total aluminum were DCB extractable indicating that part of the Al was present as oxyhydroxides or HA bound. In contrast, the DCB extractable Fe was more abundant in the insoluble fractions at pH 1 and 3 (64%) than at pH 5 and 7 (34 and 46% respectively). The high proportion of DCB extractable Fe in the HA-rich fractions at pH 1 and 3 suggests the presence of organo-metallic compounds in which the Fe atom could act as a bridge between the humic and inorganic phases.

In the soluble fraction at pH 3, all the Al and Fe were DCB extractable attesting to a lack of clay minerals, which agrees with the silica-gel structure deduced from the FT-IR spectra. In the soluble fractions at pH 5 and 7, more than 70% of the total amount of Fe and more than 40% of the total amount of Al were DCB extractable indicating the presence of oxyhydroxides and HA bound forms.

The presence of Na humates and phyllosilicates in the soluble and insoluble fractions, respectively, affected the Na concentration with the increasing of pH value (Table 5).

K was much less abundant than Na. The highest concentrations were found in the insoluble fractions suggesting that besides Na^+ , K^+ also neutralized the negative surface charges of the clay minerals. Mg followed more or less the same trend as K. As far as Ca was concerned, no trend could be identified suggesting that this cation is exchangeable on both the phyllosilicate and Ca humates. It can also act as a bridge between the organic and inorganic phases.

Discussion

The HA fraction insoluble at pH 1 might correspond to the HA extracted following the IHSS procedure, except for the purification step. It has the typical composition of a mature HA formed in organic soils in a temperate climate: a high acidic group content and high aromaticity, but a low content of O- and N-containing groups [44]. A compound with nearly the same characteristics is obtained at pH 3. The inorganic phase in the fraction insoluble at pH 1 and pH 3 accounts for about 25% of the total. It is largely constituted by clay minerals (montmorillonite-kaolinite) which only in drastic treatments, including HF-HCl, are able to dissolve. We cannot establish whether these organo-mineral complexes were present in the soil and have been extracted or whether they are formed between two dissolved separated phases. On the other hand, the binding of HA to kaolinite and montmorillonite at $\text{pH} \leq 3$ has been assessed by several adsorption studies [45-49]. The interaction between humic substances and clay minerals can include specific

Table 4 Total and DCB extractable Al and Fe in the humic fractions

Fraction		Si		Fe		Al		
		Total (mol kg ⁻¹)	Total (mol kg ⁻¹)	DCB extractable (mol kg ⁻¹)	(%) ^a	Total (mol kg ⁻¹)	DCB extractable (mol kg ⁻¹)	(%) ^a
pH 1	Insoluble	1.81 ^a	0.47 ^{abe}	0.30 ^{ab}	64	1.02 ^a	0.29 ^{ac}	28
	Soluble	nd	nd	nd	nd	nd	nd	nd
pH 3	Insoluble	1.67 ^{ae}	0.60 ^{ace}	0.35 ^{ab}	58	1.03 ^a	0.33 ^a	32
	Soluble	0.87 ^{be}	0.24 ^{bde}	0.24 ^a	100	0.08 ^b	0.10 ^b	125
pH 5	Insoluble	3.67 ^c	0.73 ^c	0.25 ^a	34	2.33 ^{cd}	0.09 ^b	4
	Soluble	0.84 ^{be}	0.37 ^{de}	0.28 ^{ab}	76	0.46 ^a	0.22 ^c	48
pH 7	Insoluble	4.22 ^d	0.83 ^c	0.37 ^b	46	2.74 ^d	0.10 ^b	4
	Soluble	1.26 ^e	0.38 ^e	0.27 ^{ab}	71	0.60 ^a	0.26 ^c	43

^aPercentage of the total. Within columns, values followed by the same letter are not significantly different ($P < 0.05$). nd, not determined.

and coulombic interactions, but Schutless et al. [46] recommend being careful in interpreting adsorption studies since at low pH values, adsorption and precipitation mechanisms are difficult to differentiate. The organo-mineral aggregates could also have been stabilized by the presence of Al and Fe, not included in phyllosilicate structures, and low amounts of alkaline and alkaline earth metals. Polyvalent cations are known to promote the interaction of HA with phyllosilicates through the formation of cation bridges [47,50].

The soluble fractions at pH 1 and pH 3 account for less than 10% of the total material but this data is underestimated because part of this fraction has been lost during the dialysis step. The organic phase contains less than 10% aromatic carbon and is rich in O- and N-containing groups and carboxylic groups. Therefore, it should be composed of soluble polar molecules such as polysaccharides, organic acids, and amino acids. The inorganic fraction is mainly composed of a silica gel-like phase whose formation could be an artifact due to the polymerization of silicic acid by freeze-drying [34]. On the other hand, orthosilicic acid occurs in solutions and on the surface of

solid particles in soil [51]. The FT-IR spectra suggest the presence of Al and Fe oxides whose solubility is consistent with the low pH and a possible complexation by small organic molecules.

The fractions insoluble at pH 5 and pH 7 contain a large amount of inorganic material (54% and 61% at pH 5 and 7 respectively). The inorganic phase mainly consists in phyllosilicates (kaolinite and montmorillonite).

Less than 5% Al is DCB extractable indicating that all the Al atoms are included in phyllosilicate structures.

These results suggest that the material precipitated at pH 5 and 7 does not exhibit a typical humic structure. It is more likely an organo-mineral association of clay minerals bound to polar molecules. This result is in agreement with the study by Yong and Mourato [52], performed on the interaction of kaolinite and dextran, which demonstrated the capability of polysaccharides to act as flocculants of clay minerals.

The soluble fractions at pH 5 and pH 7 account for 73% and 90% of the total humic material, respectively. These fractions are particularly rich in aromatic and aliphatic groups and exhibit a very low polar group content as indicated by both the NMR results and the low O/C ratio. The fact that most of the polar molecules were no longer present in this typical humic material, but precipitated as organo-mineral associations (see the above paragraph), suggests that contrary to the usual assumption, they were not strongly bound to the humic acid.

There is evidence of the presence of kaolinite and montmorillonite whose solubility, probably as a colloidal solution, is promoted by the presence of large amounts of Na⁺. On the other hand, the phyllosilicates account for only part of the inorganic phase since more than 40% Al and 70% Fe are DCB extractable, indicating the presence of oxihydroxides and/or Al and Fe humates. The increased solubility of the humic fraction at increasing pH is expected due to the deprotonation of the carboxylic and phenolic groups. The carboxyl groups are

Table 5 Total metal concentration of the humic acid fractions

Fraction		Ca (mol kg ⁻¹)	K (mol kg ⁻¹)	Mg (mol kg ⁻¹)	Na (mol kg ⁻¹)
pH 1	Insoluble	0.07 ^{ad}	0.13 ^{ad}	0.17 ^{ad}	0.31 ^a
	Soluble	nd	nd	nd	nd
pH 3	Insoluble	0.11 ^{abcd}	0.18 ^a	0.22 ^a	0.50 ^a
	Soluble	0.14 ^{abcd}	0.06 ^b	0.03 ^b	1.69 ^b
pH 5	Insoluble	0.12 ^{abcd}	0.28 ^c	0.55 ^c	0.97 ^c
	Soluble	0.16 ^{bcd}	0.07 ^b	0.11 ^d	2.32 ^d
pH 7	Insoluble	0.09 ^{cd}	0.33 ^c	0.67 ^e	1.00 ^c
	Soluble	0.14 ^d	0.10 ^{bd}	0.17 ^{ad}	2.21 ^d

Within columns, values followed by the same letter are not significantly different ($P < 0.05$). nd, not determined because of the low amount of sample.

known to be completely undissociated at $\text{pH} < 3$ and mostly ionized at $\text{pH} > 7$, while the dissociation of the hydroxyl phenolic groups occurs at higher pH values. It is surprising that this fraction, whose organic phase has the typical features of mature humic acid, rich in aromatic and aliphatic moieties and therefore largely hydrophobic, was soluble at pH 5.

Our results are in contrast with the reported data concerning the solubility of HA which are usually found to be completely soluble at much higher pH (at least > 8) [53]. One of the reasons for such a discrepancy could be the solubilization procedure which several studies report as affecting the behavior of the humic substances. Klucakova et al. [54] have observed that the fresh, wet HA precipitate is very easily peptized upon washing with water which is accompanied by a gradual increase in pH. Solubility of dried, solid preparation in water is much more difficult, especially when not freeze-dried. This last observation is consistent with the experiments by Brigante et al. [12] which observed a complete solubilization only at $\text{pH} \geq 9$ (*eliminato un pezzo perché usato nell'introduzione*). The discrepancy between our results and those of Brigante et al. [12] could be explained by the different procedure for modifying the pH. In our experiment, we started from a humate solution to which amounts of HCl were added, while Brigante et al. [12] started from a solid humic acid brought to higher pH by NaOH addition. This suggests that it is less easy to form aggregates by acidifying a humate solution than to disrupt them by bringing solid HA to higher pH. Therefore, the solubilization-precipitation process of HS should behave hysteretically.

Our study points out that a decrease in the pH of a humate solution from 5 to 3 provokes a drastic change in the aggregation of the humic molecules. This pH range has not yet been fully investigated. The average $\text{p}K_a$ of the carboxylic groups of the HA is 4.5 [55,56]. More recent studies [57], based on the Anderson-Hasselbach interpretation, have established that the apparent $\text{p}K_a$ of a peat humic acid was 4.93. Alvarez-Puebla and Garrido [58] measured the zeta potential of a grey humic acid against pH and observed a drop in the pH intervals 3.6 to 4.2 and 5.9 to 6.2, with a section of null slope between the two. The null slope section between these drops seems to indicate that the electrokinetic properties of the colloids do not change in this interval. The plot of the size variation against pH showed great similarity. The porosity measured through CO_2 adsorption isotherms indicated a decrease in the pore size and surface area between pH 4 and pH 5, while no relevant difference was observed between pH 2 and pH 4. The results of Alvarez-Puebla and Garrido [58] indicate that the decrease in colloidal size appears to be more influenced by the ionization of phenolic groups than by the ionization of carboxylic groups. This is because the phenolic groups and the undissociated

carboxylic groups can participate in forming hydrogen bonds thus promoting the coiling of the molecules and the formation of aggregates.

Another factor affecting the solubility of HS is the amount and composition of the inorganic phase. Extracted HA are usually treated with HCl-HF solution until a low ash content is reached, usually $< 2\%$. Little attention is given to the residual inorganic phase because it is assumed that it does not affect the behavior of the organic material. Klucakova et al. [59] reported the equilibrium constants concerning lignitic HA treated or not treated with HCl-HF. From these results, it appears that the ratio between HA(aq) and HA(s) decreased as ash content increased.

To better understand the behavior of the HA at pH 5, especially the possibility of forming metallic humates, it would be useful to have more information on the nature of the carboxylic groups. Powell and Fenton [53] performed a titration of the carboxylic groups of a peat HA. They found that up to 20% of the COOH groups were weak acids, with titration constants above $\log K = 5.0$, indicative of polyprotic moieties which contribute significantly to the metal binding properties of HS. This suggests that the dissociated COOH groups below pH 5 are monoprotic moieties unable to bind bivalent and trivalent cations. The soluble fractions at pH 5 and 7 contain more than 20% of inorganic material probably present as a colloidal solution whose stability is promoted by the sodium ions. According to the results of Powell and Fenton [53], the formation of metal humates with bivalent and trivalent ions could take place at pH 7. On the other hand, the complete ionization of the carboxylic groups at this pH could explain the solubility of the corresponding fraction.

Conclusions

Our results indicate a notable change in the behavior and composition of the humic acid we studied in the pH range from 3 to 5. In this pH range, the humic acid changes from insoluble to a colloidal solution, notwithstanding the hydrogen bonds and weak molecular forces. On the other hand, it has also been demonstrated [54,59] that solubilization of HA is not only dependent on the deprotonation of COOH and that undissociated molecules can be water soluble.

Most of the fraction consisting in O-containing groups, notwithstanding its high polarity, does not remain in solution at pH 5 and pH 7 but precipitates with the phyllosilicate-rich inorganic phase. This suggests that it is not strongly bound to the humic fraction.

At pH 1 and pH 3, the inorganic phase bound to the insoluble humic material was largely constituted by clay minerals and some Al and Fe hydroxides. Moreover, at lower pH values, the soluble fractions isolated were

very poor and composed of a silica gel-like associated with polar organic material.

The results emphasize the effect that the preparation procedure can have on the behavior of the humic substances.

Abbreviations

HS: humic substances; HA: humic acids; IHSS: International Humic Substances Society; NMR: nuclear magnetic resonance.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The work presented here was carried out in collaboration between all authors. MN defined the research theme; MN and AB designed methods and experiments; DV carried out the laboratory experiments, AB, MG and MN analyzed the data, interpreted the results, AB and MN wrote the paper. All authors have contributed to, seen, and approved the final manuscript.

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