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Post Burial Modifications of Humic Acids in a Paleosol Sequence from the South of Spain

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Abstract

Humic acids (HA) were extracted from four Chromic Luvisc paleosols belonging to the Pinos Genil formation (Granada, SPAIN). Comparison of HA extracted from the surface soil to those of paleosols showed substantial structural alterations, mainly decarboxylation and progressive degradation of aromatic structures. Changes observed in FT-IR spectra of HA after heating pellets at 105 °C suggested an increasing presence of strong metal-humate complexes with soil age. Chromatographic profiles obtained by HPLC-SEC were modified in presence of EDTA, confirming extensive formation of inter-molecular cationic bridges. Electrofocusing carried out with and without EDTA confirmed the hypothesis of metal-humate complexes formed in paleosols.

Key words: humic acids, paleosols, paleohumus, HPLC-SEC, electrofocusing, IR spectra.

Introduction

Despite the interest in buried paleosols as stratigraphic markers and as indicators of past environments, the information which can be obtained from the chemistry of their humus has often been ignored (Stevenson, 1969; Retallack, 1989). The reasons for this include (1) the very low organic carbon content of most paleosols, which commonly comprises only 10-20% of the organic C present in equivalent modern soils (Retallack, 1989), and (2) the difficulty of extracting their humic substances because of the formation of organo-mineral complexes, a problem which increases with the age of the buried horizon (Yoshida and Kumada, 1979; Sakai and Kumada, 1982).

The humic substances of buried peats formed in a predominantly wet environment have been studied more commonly (Schnitzer et al., 1990), but extrapolation of results to soils formed in drier conditions is difficult. Stevenson (1985) suggested that the

chemical properties of the organic matter of many buried soils may be intermediate between those of present soils and lignites or brown coals.

Humus is normally susceptible to change, but since microbial activity virtually ceases at very low soil water contents, dry environments can favour humus preservation (Stevenson, 1969). Under these circumstances labile organic molecules can persist for a long time and, through the diagenetic alteration of organic matter (for example by polymerisation and loss of weakly attached polysaccharides, proteins, and oxygen containing groups), can lead to the production of humic substances of unusual structure (Catt, 1990). The reaction of aminoacids with reducing sugars can, for instance, contribute to the formation of humic substances in paleosols to a much greater extent than in surface soils of high biological activity, where these easily degradable substances are quickly decomposed by microorganisms (Stevenson, 1978). The soil we studied occurs in the Pinos Genil Formation (also called "Block Formation"), located

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on the western border of Sierra Nevada Mountains (Granada, Spain) close to Durcal, a small village 15 km south of Granada. The Pinos Genil Formation is a thick deposit (>100 m) of very heterometric conglomerates (some rock fragments are more than 1 m³ in size), with few sandy and silty beds. The clasts are mainly of metamorphic rocks (mica schists and gneisses), with minor amounts of limestones and dolostones. The age of this formation is uncertain because few fossil remains (occasional pollen grains) are preserved. However, it overlies with angular discordance siltstones, sandy stones and claystones of upper Tortonian to Messinian age (between upper Miocene and lower Pliocene), but underlies the Alhambra conglomerate (Quaternary) and is therefore thought to be Pliocene, though it could be partly Messinian. The heterometry of the rock fragments indicate that the materials were deposited in a very energetic fluvial environment. The geographical distribution and the lateral wedging of the formation indicate that the materials were deposited by more than one, probably three, water streams. It probably corresponds to a period of tectonic instability with rapid uplift and erosion episodes. Most of the clasts were derived from distant parts of the Sierra Nevada; carbonate clasts are in a minority, although there are extensive dolomite formations nearby. At that time the limestones and dolostones were either under water or their relief was insufficient to allow erosion.

The aim of this work was to characterise humic acids (HA) in a time transgressive chronosequence of paleosols (Vreken, 1975) within the Pinos Genil Formation, in order to improve our insight into the effects of the different pedogenetic and post burial regimes on humic acids. To understand the effect of the time factor, HA were extracted from four red paleosols and compared with those from the surface soil. Particular attention was given to the formation of strong organo-mineral complexes or "bound-humus", whose formation seems to be related to soil age through polymerisation or de-polymerisation reactions involving changes in the molecular weight distributions of HA.

Materials and methods

Soils

The soils studied comprise a group of four red soils that occur in the upper part of the Pinos-Genil formation, within 10 m of the surface (Figure 1). The sequence is composed of 5 paleosols: counting down from the surface soil (S0), soils 1, 2, 3 and 5 are red Mediterranean soils (Chromic Luvisols), but soil 4 is a brown soil (Cambisol). Their main physico-chemical characteristics are given in Table 1. All the red soils

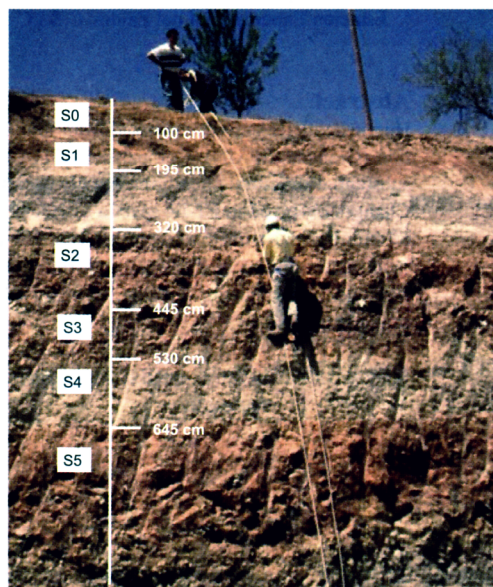


Figure 1. Profile of the paleosol chronosequence and location of the five paleosols: S1, S2, S3, S4 and S5.

Table 1. Main physico-chemical characteristics of Ap horizon of surface soil (S0) and Bt horizon of paleosols (S1 to S5).

Soil	Gravel	Sand	Silt	Clay	pH (H ₂ O)	CaCO ₃	Organic C
							%
S0	31.2	61.2	28.7	10.1	7.7	3.5	1.81
S1	35.5	43.2	24.6	32.2	7.4	0.8	0.52
S2	37.0	31.6	13.2	55.2	7.3	0.6	0.32
S3	41.0	36.5	15.9	47.6	8.3	0.5	0.14
S4	39.3	41.6	29.3	29.1	8.3	2.8	0.08
S5	38.5	55.6	22.5	21.9	8.5	0.7	0.18

Table 2. Elemental composition, on an ash free basis, percent of ash content and atomic elemental ratios of humic acids.

Soil	C	H	O	N	Ash	Atomic ratio		
						H/C	O/C	N/C
S0	55.9	4.5	35.7	3.8	1.23	0.97	0.48	0.06
S1	60.0	4.7	32.0	3.2	4.80	0.95	0.40	0.05
S2	61.2	4.9	30.9	3.1	5.67	0.95	0.38	0.04
S3	62.7	4.7	29.7	2.8	6.26	0.90	0.36	0.04
S5	63.2	4.6	29.6	2.6	7.59	0.87	0.35	0.03

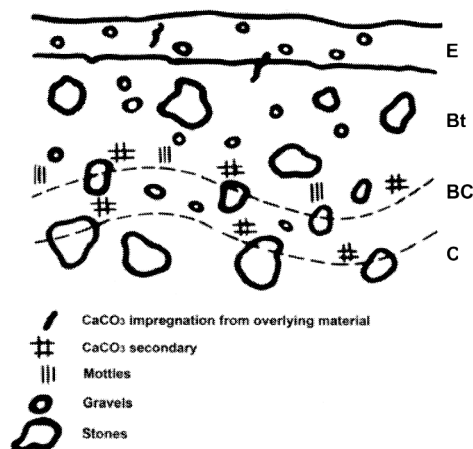


Figure 2. Profile of red paleosols S1, S2, S3, and S5.

are remarkably similar in morphology (Figure 2): they have a reddish brown to red-purple, loamy and massive E horizon 25 cm thick. Their upper boundaries are clear to abrupt. Their thicknesses are very uniform, although locally the E horizon has been thinned by erosion. The lower boundaries of the E horizons are clear and flat, but with local indentations into the underlying Bt horizons. The Bt horizons are about 75 cm thick: they are dark red (Munsell color = 2.5YR 3/6) clay, with a medium, moderately developed angular blocky structure breaking to finer peds, occasional slickensides and common stones of very weathered schists. In the lower half of the Bt horizons there are frequent but faint strong brown-olive (2.5Y 4/4) mottles increasing in abundance with depth. In these parts of the red soils there are also frequent tubular pores and root imprints on ped faces; and occasional carbonate coats and “calcitans” (calcic cutis) occur on ped faces near the lower limit of the Bt horizons. The Bt horizons pass gradually down into BC horizons which are olive brownish loam to sandy loam that is massive, approximately 25 cm thick, and contains a diffuse lower boundary to the parent material. In this horizon there are occasional carbonate segregations and concretions. Secondary carbonate is most abundant in soil 5 where there is a well formed calcic horizon with occasional indurated laminations.

Except for variations in the calcic horizons, all other morphological features are remarkably similar. They probably correspond to a stage of advanced but not strong weathering, since there are still abundant weatherable materials in the Bt horizon. These are lacking in the Bt horizon of the modern Alhambra

soils of the area.

The parent materials consist mainly of gravel-bearing schists and gneisses with occasional dolomite fragments in a sandy matrix. There are frequent lateral changes and paleochannels that locally erode the soils. There are also some silty layers which are more frequent near the surface of the formations.

Unlike the others, Soil 4 is very weakly developed. It consists only of a Bw horizon, 25 cm thick, of brown silt loam, massive, with some tubular pores. The upper limit is abrupt but there is some mixing with the burying material (a grey silt) by bioturbation (filled borrows). The lower limit of the Bw horizon is diffuse, the soil thins out and disappears laterally. Because of its pedological difference within the other soils and its low organic matter content, this soil was not considered in the present work.

Extraction and purification of humic acids

Humic substances were extracted with a 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ plus 0.1 M NaOH solution (1:5 w/w soil:solution) under nitrogen gas flow for 150 min from Ap horizon of the surface soil (S0) and Bt horizon of paleosols (S1, S2, S3, S4 and S5). The extracts were centrifuged at 2400 g for 30 min, and the supernatant was passed through 0.2 μm Whatman membrane filters, purified and concentrated by adsorption chromatography on cross-linked polyvinylpyrrolidone. The humic substances were adsorbed onto the column at pH 2 and eluted with 0.5 M NaOH after washing the column with dilute HCl. Humic acids (HA) were then precipitated twice at pH 1-2 with HCl and redissolved with diluted NaOH in order to decrease inorganic contaminants. The solution was then treated with amberlite IR 120 H^+ resin until a constant pH was reached to remove metal cations. The HA were then freeze dried in their acid form and kept at room temperature over dried silica gel. An aliquot of HA solution was kept at pH=7.0 for HPLC-SEC and electrofocusing analysis.

Analytical methods

Elementary analysis: carbon, hydrogen and nitrogen was performed using a Carlo Erba elemental analyzer (CHN 1106), whereas oxygen was calculated by subtracting the weights of C, H and N from the total ash-free weight. Ash content was determined by heating up to 550 $^\circ\text{C}$ for 24 hrs.

Infrared spectra were recorded from KBr discs of the freeze dried HA preparation in their protonated

form by a Perkin Elmer system 2000 FT-IR spectrophotometer. The discs were prepared by pressing a mixture of 1.5 mg of HA and 180 mg of anhydrous KBr to 8000 kg cm^{-2} . The pressed discs were then dried under vacuum overnight at $105 \text{ }^\circ\text{C}$ before recording a second set of spectra.

Analytical electrofocusing was performed in 5% cross-linked acrylamide gel slabs (De Nobili et al., 1985). Samples ($100 \text{ }\mu\text{l}$ of HA solution, corresponding to 0.1 mg C , at pH 7.0) were applied in precast application pockets. Ampholytes (LKB, Sweden) with a pH range of 2.5 to 8 were used to form the pH gradient. Electrofocusing assays were performed on a water cooled ($4 \text{ }^\circ\text{C}$) LKB ultrophor electrophoresis cell; the gel slabs were read with a LKB ultrascan laser densitometer before and after staining with crystal violet (De Nobili, 1988).

The molecular weight (MW) distribution of humic acids was estimated from size exclusion chromatography (HPLC-SEC) using a Waters 160 pump and UV-Vis detector at 460 nm (Tsutsuki and Kuwatsuka, 1984; Rausa et al., 1991). The separation was achieved on a Biosec-250 column (Biorad) using 0.025 M Tris buffer at pH 7.0 as eluent at 1.0 ml min^{-1} flow rate. The column was calibrated using humic fractions of reduced molecular weight dispersion obtained by ultrafiltration (De Nobili et al., 1996) and a set of polystyrene sulphonates. The separations were carried out twice, with and without addition of 0.025 M EDTA to both the HA samples (1 mg C ml^{-1}) and the eluant. The injection volume used was $20 \text{ }\mu\text{l}$.

Results and discussion

FT-IR spectra

Humic acids extracted from the eluvial horizon of the first paleosol (S1) showed FT-IR spectra (Figure 3) similar to that of HA extracted from the surface soil (S0), indicating that very little alteration has occurred in the buried S1 soil and that major climatic changes, affecting vegetation type and hydrology, are unlikely to have occurred since the soils were buried. There is a visible regular trend in the spectra from the surface soil to the deepest paleosol. In fact, the $\text{C}=\text{O}$ stretching at 1720 cm^{-1} decreases in intensity with increasing depth from S0 to S5, together with the 1620 cm^{-1} absorption band (quinonic $\text{C}=\text{O}$ and $\text{C}=\text{O}$ conjugated with aromatic $\text{C}=\text{C}$, but also $\text{C}=\text{C}$ in open chain systems). The 1510 cm^{-1} (aromatic $\text{C}=\text{C}$

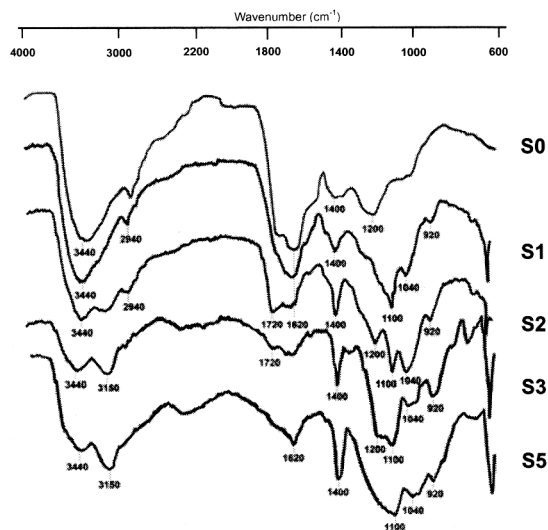


Figure 3. FT-IR spectra of humic acids extracted from the surface soil (S0) and from the four paleosols (S1, S2, S3 and S5).

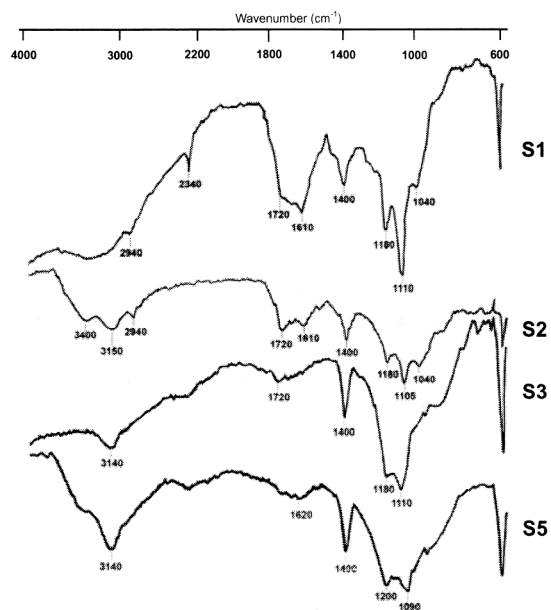


Figure 4. FT-IR spectra of humic acids extracted from the four paleosols (S1, S2, S3 and S5) after drying at $105 \text{ }^\circ\text{C}$ under vacuum.

stretching absorption shoulder super-imposed on the 1400 cm^{-1} band) decreased as well. This trend is consistent with that observed in the infrared spectra of fulvic acids extracted from buried humic horizons derived from volcanic ash and suggests a progressive degradation of aromatic structures (Yoshida and Kumada, 1979).

The spectra from all four red paleosols provide evidence for the presence of metal-humate com-

plexes as described by Piccolo and Stevenson (1982). Spectra, in fact, show a decrease in absorption at a frequency of $3420\text{--}3440\text{ cm}^{-1}$ after heating pellets at $105\text{ }^{\circ}\text{C}$ (Figure 4). This decrease is due to loss of the hydration water of metals in 1:1 complexes with humic substances. The intensity of absorption at 3420 cm^{-1} decreased with increasing depth; this unmasked the absorption of phenolic and/or alcoholic groups (bending of secondary and tertiary alcohol groups) to give a distinct band at about 3150 cm^{-1} that is not affected by heating and occurs in paleosol S2, S3 and S5.

Heating pellets at $105\text{ }^{\circ}\text{C}$ did not substantially lesser absorption at 3420 cm^{-1} , but had a pronounced broadening effect, caused by an increase in intramolecular hydrogen bonding. The 2340 cm^{-1} band, characteristic of heated samples, was present only in paleosol S1, thereby confirming its possible origin from the decarboxylation of COOH groups (Piccolo and Stevenson, 1982). Compared to organic matter extracted from Tertiary paleosols of the Canadian Arctic (Schnitzer et al., 1990), these HA seem to have a much smaller aliphatic content, as indicated by the absence of a well defined absorption band at 2940 cm^{-1} . The aromatic-aliphatic ratios of the HA under investigation were similar to those found by Calderoni and Schnitzer (1984) in other Mediterranean paleosols and decreased with depth.

As the humic acids were freeze dried in their acid form, little of the absorption around 1450 cm^{-1} can derive from COO^- bending, apart from that of carboxyl groups in metal-humate complexes. Considering also that there is no corresponding increase in absorption at 2940 cm^{-1} , the 1400 cm^{-1} band can be more attributed to the bending vibration of alcoholic groups in tertiary alcohols rather than to C-H deformation of aliphatic groups. Absorption due to the bending frequencies of alcoholic -OH ($1100\text{--}1200\text{ cm}^{-1}$) was very strong in all the four paleosols we examined and increased with depth. The broad weak band in the region around 2300 cm^{-1} , which appears in the deeper paleosols, is probably an overprint of the absorption in the $1100\text{--}1200\text{ cm}^{-1}$ region.

The band at 1110 cm^{-1} could also be attributed to Si-O-Si stretching but the absence of absorption around 900 cm^{-1} (Si-OH) in the spectra of heated pellets indicates that there were few silicate impurities. This confirms that the purification procedure was effective in removing mineral impurities not bound to HA.

During coalification COOH groups disappear first, followed in order by O-CH₃ and -OH groups (Stevenson, 1985). However, the IR spectra of all four paleosols differed greatly from those of humic acids extracted from other fossil substrates, such as peat or coal deposits (leonardite), and their extent of modification is still rather small compared to them.

Molecular weight distribution

HPLC-SEC chromatograms are given in Figure 5. Humic acids extracted from the surface soil (S0) showed no adsorption on the column since they were generally eluted with a $K_{av}<1$. They were almost evenly distributed in the apparent molecular weight (MW) range from 60 to 0.8 kDa (Figure 6a), with a relative abundance in the higher apparent molecular weight range.

The apparent MW distributions of HA from paleosols S1 and S2 were similar and contained a much larger proportion of medium size molecules. They differed greatly from those of paleosol S3 and S5, whose molecular weight distributions were shifted towards lower molecular weights and showed a small adsorbed peak in the elution profile (Figure 5).

Addition of 0.025 M EDTA, which is thought to

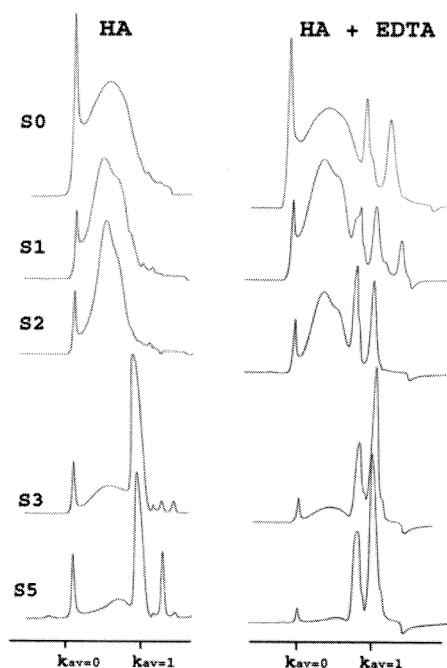


Figure 5. Size exclusion chromatography profiles of humic acids extracted from the surface soil (S0) and from the four paleosols (S1, S2, S3 and S5) before and after treatment with 0.025 M EDTA by HPLC-SEC using 0.025 M Tris buffer at pH=7.0 as eluent.

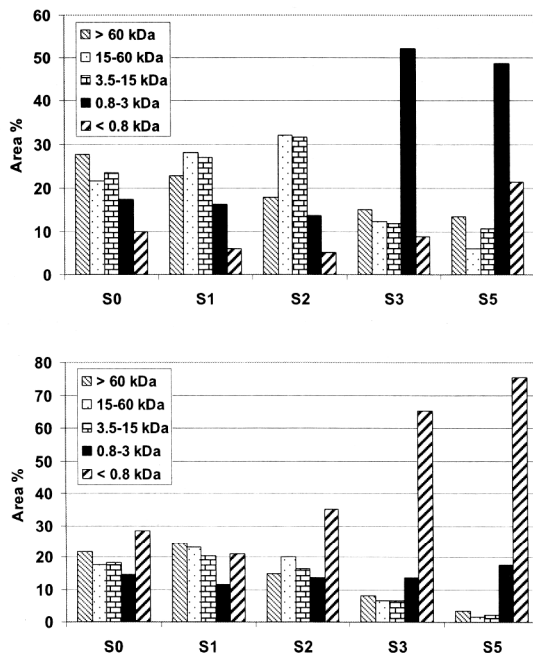


Figure 6. Apparent molecular weight distribution of humic acids extracted from the surface soil (S0) and the four paleosols (S1, S2, S3 and S5) before (a) and after (b) EDTA treatment estimated by HPLC-SEC using 0.025 M Tris buffer at pH=7.0 as eluent.

break cation bridges between HA molecules, caused a slight, but appreciable shift in the apparent molecular weight distribution of HA from surface soil (S0) (Figure 5); whereas a stronger effect was observed after addition of EDTA in the S1 and S2 HA. The effect is still low if compared to S3 and S5 (Figure 5). The fact that the excluded peak in the elution profile of S5 was almost completely destroyed by addition of EDTA suggests that the increase in the fraction of HA molecules with apparent MW>60 kDa observed in the deeper paleosol (S5) was not only due to progressive polymerisation of HA with burial time, but mainly to the formation of intermolecular cationic bridges. Addition of EDTA caused also an increase in the adsorbed fraction: this could result from an enhanced salt boundary effect caused by excess of EDTA and EDTA-Fe or -Al complexes; in this instance the shift in MW would be the result of the adsorption of HA onto the column. Unfortunately samples could not be dialysed before HPLC-SEC as this would have meant the loss of the more than 70% of the humic substances due to their low molecular size shown by the elution profiles. We must, however, keep in mind that detection of HA was performed at 400 nm and only molecules absorbing light in the

visible range were detected, whereas EDTA does not appear on the chromatograms. The results obtained by size exclusion chromatography therefore needed to be examined by another analytical technique.

Electrofocusing

Electrofocusing is an electrophoresis carried out in a pH gradient, which separates substances on the basis of their true or apparent isoelectric point. Humic substances of different molecular weight focus at different pH intervals (Ceccanti and Nannipieri, 1988). Detection of organo-mineral complexes is also possible because they focus in the pH>5.5 region and they can be easily identified by treating samples with a strong chelating agent such as EDTA (De Nobili et al., 1985).

Humic acids extracted from the surface soil (S0) showed many well-defined main bands in the 4.8-5.5 pH region and only minor bands in 5.8-6.0 pH region as is typical of soil HA; the addition of EDTA did not significantly change its electrofocusing profile and only caused an increase in intensity of all bands (Figure 7). This was due to dissolution of HA that had precipitated in the sample application pocket. Humic acids extracted from paleosols S1 and S2 showed fewer bands at both pH 4.5-5.2 and poorly resolved broad bands in the pH range between 5.2

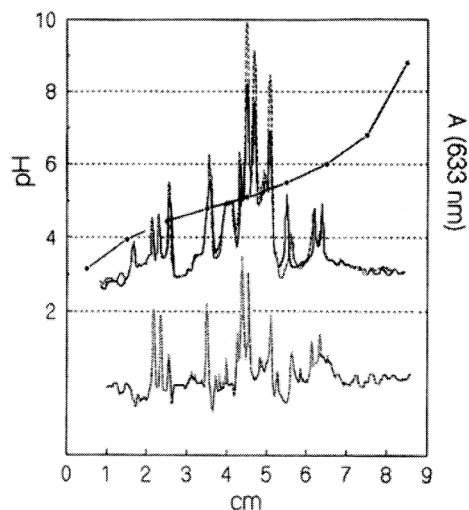


Figure 7. Electrofocusing profile of humic acids extracted from the surface soil (S0) without (continuous line) and with (dashed line) addition of EDTA. The pH gradient is reported on the left axis, while the relative absorbance of the electrofocusing profiles is reported on the right axis. The lower profile represents the difference between the EDTA treated and the untreated HA.

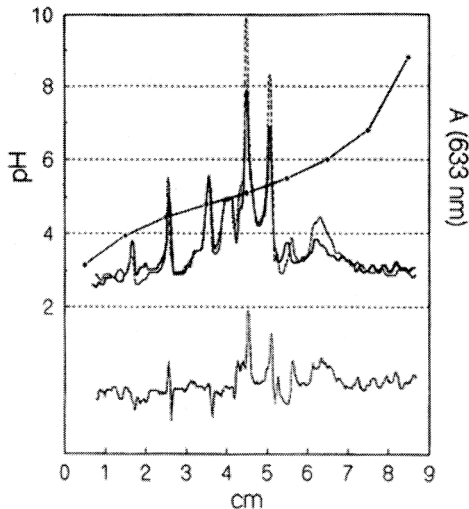


Figure 8. Profile of humic acids extracted from the surface soil (S1) without (continuous line) and with (dashed line) addition of EDTA. The pH gradient is reported on the left axis, while the relative absorbance of the electrofocusing profiles is reported on the right axis. The lower profile represents the difference between the EDTA treated and the untreated HA.

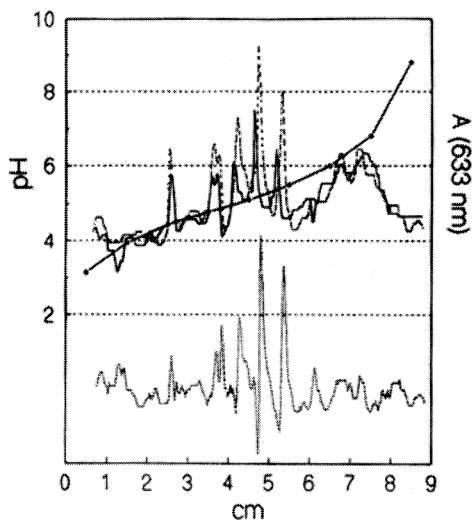


Figure 9. Profile of humic acids extracted from the surface soil (S2) without (continuous line) and with (dashed line) addition of EDTA. The pH gradient is reported on the left axis, while the relative absorbance of the electrofocusing profiles is reported on the right axis. The lower profile represents the difference between the EDTA treated and the untreated HA.

and 6.5 (Figures 8 and 9). Broad bands at $\text{pH} > 6.0$ are often due to the presence of complexes between humic substances and Fe^{3+} or Al^{3+} ions. Humic substances extracted with NaOH from the Bh horizon of spodic soils, for instance, show characteristic bands

in this range, that disappear after treatment with EDTA (De Nobili, 1988). These bands cannot be found in pyrophosphate extracts of Bh horizons indicating that pyrophosphate can effectively compete with humic substances of Bh horizons in complexing Al^{3+} and Fe^{3+} (De Nobili et al., 1985). In the paleosols of this study, interactions were not destroyed by pyrophosphate during extraction. In S1 and S2 (Figures 8 and 9) the addition of EDTA increased the intensity of the bands between $\text{pH} 6.0$ and 6.5 less that of bands between $\text{pH} 4.5$ and 5.0 , probably because EDTA dissolved HA precipitated in the application pockets. The solubilization of material precipitated in the application pockets after addition of EDTA was also observed for paleosols S3 and S5 (results not shown). This material was, however, unable to migrate to the very acid region of the pH gradient and caused the formation of broad poorly defined bands around $\text{pH} 6.5$. Humic substances extracted from the deeper paleosols (S3 and S5) produced only very weak electrofocusing profiles, which did not show any meaningful band in the pH gradient region above 4.5, even after staining with crystal violet (results not shown). The behaviour was probably caused by the much lower content of carboxylic groups in these samples: Crystal violet, in fact, is a cationic dye and binds to either hydrophobic groups or negatively charged groups. The progressive decrease in absorption at 1720 cm^{-1} frequency shown in the IR spectra of the paleosol sequence confirms this hypothesis.

Conclusions

Comparison of HA extracted from the surface soil to those extracted from four buried paleosols showed that substantial alterations occurred in the structure of humic substances after the soils were buried. The changes include decarboxylation and progressive degradation of aromatic structures with an increase in the olefinic character. On the base of our results we can only reach these conclusions: FT-IT spectra indicate a strong decrease in quinonic-type structures and an increase in OH-groups linked to aliphatic chains. The fact that the HAs are colored implies that they must still be a system consisting of conjugated double bonds and therefore the back bone must be olefinic.

Humic substances extracted from the buried soils of the Pinos-Genil formation seemed to be strongly

associated with mineral soil components: a substantial fraction of extractable organic carbon, which increased with the age of the paleosol, consists of strong HA-metal complexes. Metal ions interacted with functional groups belonging to more than one molecule, so thus act as cationic bridges and increase the apparent molecular weight of HA. Chemical characteristics of humic substances in this chronosequence changed evenly with depth and therefore seemed strongly related to the age of the soil.

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