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Article

Criterious Preparation and Characterization of Earthworm-composts in View of Animal Waste Recycling. Part II. A Synergistic Utilization of EPR and ^{1}H NMR Spectroscopies on the Characterization of Humic Acids from Vermicomposts

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Ácidos húmicos (HA) extraídos de vermicompostos (VC) de estercos de ovelhas (SHHA), bovinos (COHA), caprinos (GOHA) e coelhos (RAHA) foram analisados com o auxílio das espectroscopias de ressonância paramagnética eletrônica e ressonância magnética nuclear de hidrogênio. Estas substâncias húmicas (HS) recentes são compostas por ácidos carboxílicos, fenóis, funções amina, amida, éster, éter, estruturas carbônicas alifáticas saturadas, alifáticas com ligações conjugadas, duplas e simples, ao lado de cadeias aromáticas. Foram caracterizados complexos de Mn^{2+} de esfera externa (SHHA, COHA), complexos de Fe^{3+} em estruturas axiais (GOHA, RAHA) e rômbicas (SHHA, COHA, GOHA e RAHA) e complexos de Cu^{2+} de campo fraco (COHA, GOHA, RAHA) e de campo forte (SHAHA, COHA, GOHA, RAHA).

Humic acids (HA) extracted from sheep (SHHA), cow (COHA), goat (GOHA) and rabbit (RAHA) vermicomposted manure were analyzed by electron paramagnetic resonance and hydrogen nuclear magnetic resonance spectroscopies. Carboxylic acids, amine, amide, ester, ether and phenol functions bonded to saturated aliphatic, unsaturated aliphatic conjugated double and single bonds, and aromatic chains constitute the backbone structure of these fresh humic substances (HS). Mn^{2+} outer sphere complexes (SHHA, COHA), Fe^{3+} axial (COHA, RAHA) or rhombic (SHAHA, COHA, GOHA, RAHA) complexes and Cu^{2+} as weak field (COHA, GOHA, RAHA) and strong field (SHAHA, COHA, GOHA, RAHA) complexes were characterized.

Keywords: vermicomposts, humic acids, EPR and ^{1}H-NMR spectroscopies

Introduction

A research area of increasing interest consists in the recycling of pollutant animal manure accumulated in the environment. Throughout the world, farmers spread cow and other animal manure on fields to improve soil fertility. As animal waste contains bacteria such as *Escherichia coli*, a normal inhabitant of the gastrointestinal tract, this practice is dangerous to human health. Coliform bacteria can survive for long periods in cattle manure and would be mixed with the soil matrix and migrate towards the ground water after the manure was spread on fields[1,2]. The environmental contamination with animal manure in some places of southern Brazil, mainly of ground water used as municipal undertakings to supply the population needs without previous treatment, is a potential problem. It occurs as a result of the growing food industry activities in that region. Very recently, we proposed to study the criterious preparation and characterization of sheep (SH), cow (CO), goat (GO) and rabbit (RA) vermicompost manures, using the earthworm *Eisenia foetida*, forming organic fertilizers for soil amendment[3]. Each corresponding vermicompost (VC), characterized as much as possible, would be recommended for amending specific type of degraded soils, already existing in southern Brazil.

In a previous study[3], thermal analysis, chemical and spectroscopic (FTIR and visible) results indicate a high nitrogen content and low carboxylic acidity, as well as a high degree of conjugated aliphatic and/or substituted or condensed

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aromatic structures for the humic acids (HA) extracted from these VC. The existence of peptide chains and N-heterocycles in the samples was proposed, but it could not be confirmed, and a differentiation between aliphatic conjugation and aromaticity degree was not possible.

The maintenance of soil fertility is intimately related with the ability of the organic matter to form complexes with trace metals[4,5]. The interaction of humic substances (HS) with metal ions is of great importance for the environment, and its study has always been of considerable interest. The transport of soil micronutrients to the plants, as well as the immobilization of toxic elements in soils and waters, is greatly influenced by metal ion complexation capacity of the existing HS[6,7].

In this work, we adopted the strategy of using thee HA native (indigenous) paramagnetic metal ions Mn^{2+}, Fe^{3+}, Co^{2+} and Cu^{2+} as EPR probes, together with ^{1}H NMR spectroscopy to deduce the functional groups and structures present in these HS. As we are interested in the use of these VC to improve the quality of degraded soils, the study of the concentration and binding of metal ions in VC and its HA is of concern. EPR spectroscopy is a suitable tool for the study of HS complexes with paramagnetic metal ions and may provide useful information about the oxidation states, modes of coordination and the geometry and the type of ligand sites[8-10]. The low concentrations of the paramagnetic metal ions will not interfere with the ^{1}H NMR studies. The use of the two magnetic resonance techniques will give more insight in both the organic structure characterization and metal ion interactions in this complex material.

Experimental

VC preparations, isolation of HA, chemical analysis and Mn, Fe, Co, and Cu determinations using atomic absorption spectroscopy were previously described[3]. Extraction of HA using aqueous alkaline solution and acid precipitation was carried out only once, in order to prevent loss or modification of important organic constituents of the samples, or excessive hydrolysis of metal ions. Reprecipitated humic acids (HAr) were also used, in order to obtain more information from EPR studies. HAr samples were obtained by alkaline redissolution and acidic reprecipitation of the HA sample. EPR spectra of the powdered HA or HAr samples were registered at room temperature (300 K) in quartz tubes. A Bruker ESP 300E spectrophotometer (LABEPR/DQ/UFPR) was used, operating at a frequency of 9.7 GHz (X-band), with a 100 kHz modulation frequency, 2.024 G modulation amplitude and ~20 mW microwave power. Simulations of EPR spectra were carried out using Win-EPR® and SimFonia® computer programs. ^{1}H NMR spectra of the HA were recorded at room temperature in an AC-400 Bruker spectrophotometer (D/Bioq/UFPR). Briefly, 50 mg of each HA sample were dissolved in 1 mL of $NaOD/D_2O$ 0.3 mol L^{-1}. Chemical shifts were recorded in ppm using the solvent resonance as internal standard (deuterated water: δ 4.70). Coupling constant (*J*) are given in Hz.

Results and Discussion

EPR spectroscopy

Mn, Fe, Co and Cu concentrations, determined by atomic absorption spectroscopy, in VC and HA, were in the order: Fe > Mn > Cu > Co (Table 1). This sequence is in agreement with the metallic contents of other vermicomposted materials[11] and contrasts with sorption capacity assays of these metals in soil HA, where the metals were sorbed in the order Fe > Cu > Mn > Co[12]. As found for HA from soil, Fe and Mn were detected in higher amounts than Co and Cu. In the HA extraction process from CV, Mn and Fe were partially lost, Cu was concentrated while Co had its concentration increased in SH and CO samples and decreased in GO and RA samples.

EPR spectra of the HA and HAr samples, at a sweep range of 5000 G, are shown in Figures 1 and 2, respectively. All spectra show a sharp line at $g = 2.00$ region typical of organic free radical absorption. Spectra for the COHA and SHHAr samples showed typical absorption lines of outer sphere Mn^{2+} ion complex, $[Mn(OH_2)_6]^{2+}$-HA, (inserts in Figures 1 and 2) with hyperfine interaction, $A = 74.7 \times 10^{-4}$ cm $^{-1}$, and $g = 2.00$, for the COHA sample, and $A = 68.2 \times 10^{-4}$ cm^{-1}, and $g = 2.00$, for the SHHAr sample (Table 2). The magnitude of the EPR hyperfine *A* parameter is inversely related to the degree of covalent bonds of Mn^{2+} ion in its complexes[13]. Those values indicated a more covalent interaction of Mn^{2+} in the SHHAr, than in the COHA sample.

Table 1. Concentrations (mg kg^{-1}) of Mn, Fe, Co and Cu in the vermicomposts (VC) and humic acids (HA) samples obtained by atomic absorption spectroscopy.

Animal manure**	Mn		Fe		Co		Cu	
	VC	HA*	VC	HA*	VC	HA*	VC	HA*
SH	2700	700	5300	2800	8.75	9.51	42.12	66.19
CO	2300	400	15700	14400	7.52	9.25	24.81	153.46
GO	3700	1800	3700	2900	4.42	3.07	28.30	62.14
RA	700	700	8900	4300	11.63	7.18	69.29	225.97

* Data from reference 3; ** SH = Sheep; CO = Cow; GO = Goat; RA = Rabbit

Table 2. EPR parameters (g, $g_{\|}$, and $g_{\perp}$ values, hyperfine coupling constants, A and $A_{\|}$, and super hyperfine coupling constant, $A_{\perp N}$) for Mn^{2+}, Fe^{3+} and Cu^{2+}-HA complexes.

	Cu^{2+}				Mn^{2+}		Fe^{3+}		
	$g_{\|}$	$g_{\perp}$	$A_{\|}$ ($10^{-4}cm^{-1}$)	$A_{\perp N}$ ($10^{-4}cm^{-1}$)	g	A ($10^{-4}cm^{-1}$)	g_1	g_2	g_3
SHHA*	-	-	-	15	-	-	4.3		
COHA*	-	2.0061	-	15	2.00	74.7	4.3,		8.9
GOHA*	-	2.0532	-	14	-	-	4.3		
RAHA*	-	2.0547	-	16	-	-	4.3		
SHHAr*	-	2.0609	-	15	2.00	68.2	4.3,		8.9
COHAr*	2.3200	2.0897	153.8	15	-	-	4.3,		8.9
GOHAr*	2.2942	2.0574	167.1	14	-	-	4.3,	6.0,	8.9
RAHAr*	2.2856	2.0554	154.6		-	-	4.3,	6.0,	8.9
	2.1005	2.0580	200.0	14					

* SHHA = sheep HA; COHA = cow HA; GOHA = goat HA; RAHA = rabbit HA; HAr = reprecipitated humic acids.

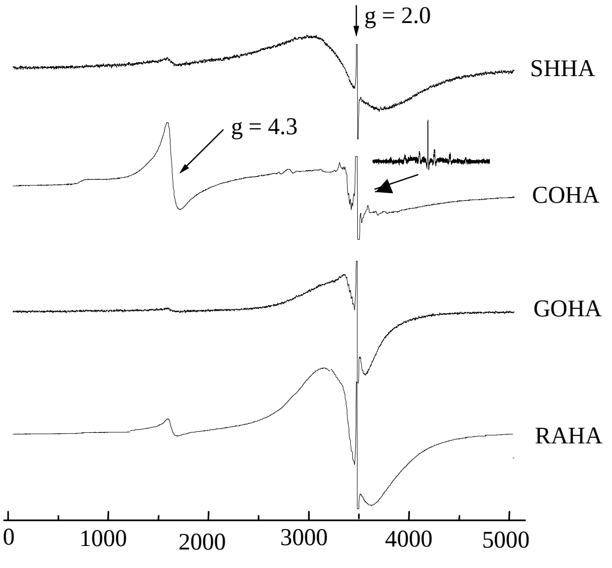


Figure 1. EPR spectra (300 K) for humic acid (HA) samples extracted from sheep (SHHA), cow (COHA), goat (GOHA) and rabbit (RAHA) vermicomposted manures. For the COHA sample detail of the second derivative mode of Mn^{2+} is also presented.

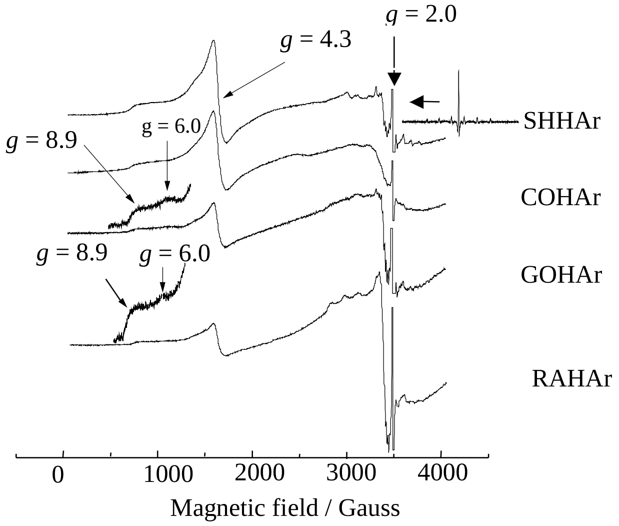


Figure 2. EPR spectra (300 K) for lower ash tenor humic acid (HAr) samples (see text) extracted from sheep (SHHAr), cow (COHAr), goat (GOHAr) and rabbit (RAHAr) vermicomposted manures. For the SHHAr sample detail of the second derivative mode of Mn^{2+} spectrum is also presented.

All samples showed evidence of high spin Fe^{3+} ($S = 5/2$) complexed in distorted octahedral and/or tetrahedral, rhombic coordination structure ($g_1 = 4.3$, $g_3 = 8.9$)[8,14,15]. For the SHHA, GOHA and RAHA samples the absorptions at $g = 8.9$ were not clearly determined from the spectra. All HA samples showed a wide line of absorption centered at $g \approx 2.2$, (Figure 1) corresponding to the superimposed lines of the spin-spin coupling (dipolar interaction), proportional to r^{-3}, where r is the distance between two of the magnetic dipoles. These wide lines occur due to the presence of paramagnetic metals and iron oxides that absorb in the same field region[9,13,16]. EPR spectra for HA suggest that the origin of these wide lines is due to a large concentration of Fe^{3+} ions forming complexes with constituents of the inorganic matter, besides iron oxides[7]. This fact is in agreement with the higher concentration of iron in view of the other analyzed metals in all HA samples (Table 1). All EPR spectra of the VC (not shown) presented similar patterns in the same g region, but with line width variation between 800 and 1100 G. The GOHAr and RAHAr EPR spectra exhibited resonance at $g = 6.0$ (inserts in Figure 2), which corresponds to a $g_{\perp}$ value due to high spin Fe^{3+} ions in sites with axial symmetry, possibly due to porphyrin type iron complexes[9,17,18]. They suggest that N-heterocyclic compounds, as pirrol rings from porphyrin, are present in these HA structures.

VC and HA presented very low cobalt contents (Table 1) and no EPR lines for Co^{2+} ions were apparent. Divalent cobalt, a d^7 ion, has ground states $^4T_1$ (weak field) and ^2E (strong field) in octahedral structures. Those states have short lifetimes, producing large linewith at room temperature. These systems normally require low temperatures (liquid helium) for EPR detection. Only in tetrahedral structures ($^4A_2$ ground state) Co^{2+} is EPR easily detected at room temperature[19].

Each HA showed a higher copper content than the corresponding VC (Table 1) and it was verified that the copper concentration rises when the extraction cycle number is increased. These facts suggest that the major quantity of copper may be contained in the bulk organic structure of the VC and that in the successive extractions, inorganic and organic materials weakly bonded to the bulk structures are lost. The EPR spectra of the samples COHA, GOHA, RAHA, SHHAr, COHAr, GOHAr and RAHAr (Figures 3 and 4 and insert) show a common line multiple pattern in the perpendicular spectrum region. This is typical of super-hyperfine interaction of the copper ion unpaired electron with four magnetically equivalent ^{14}N nucleus (I = 1). The values of the super-hyperfine interactions determined, between 14 and 16 x 10^{-4} cm^{-1} for $A_{\perp N}$, are consistent with values found in the literature for copper-porphyrin complexes[19, 20].

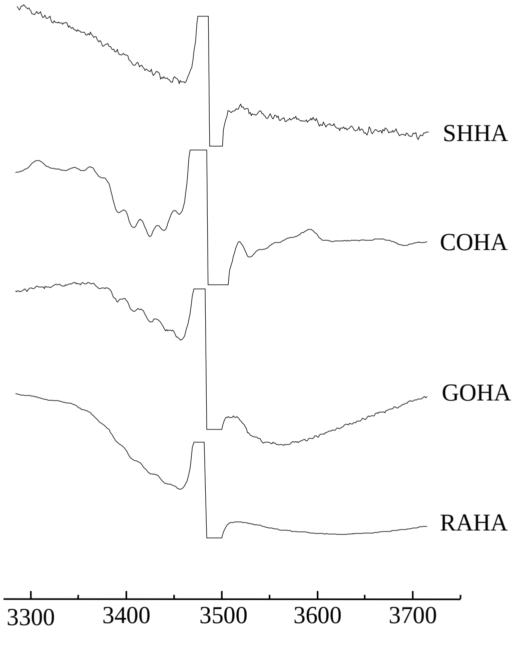


Figure 3. Perpendicular EPR spectra region (300 K) of humic acid (HA) samples extracted from sheep (SHHA), cow (COHA), goat (GOHA) and rabbit (RAHA) vermicomposted manures showing lines from super hyperfine interactions of a nitrogen containing ligand complexed to Cu^{2+} ion.

Figure 5 shows the experimental and simulated spectra for the RAHAr sample on an 1100 G field sweep range and with the 3100G as central field. The simulated spectrum (Figure 5 C) is, in fact, the sum result of two other simulated spectra: one considering the Cu^{2+} ions in axial symmetry bonded to four magnetically equivalent ^{14}N atoms (Figure 5 A), and another with Cu^{2+} inside a structure of four oxygen atoms (Figure 5 B). Through interative process, the intensity of the two added simulated spectra was varied to resemble the experimental spectrum. The parameters for the two Cu^{2+} EPR spectra of the RAHAr sample, obtained throughout the simulation, (Table 2) are consistent with the literature data for Cu-4O and Cu-4N complexes[21-23].

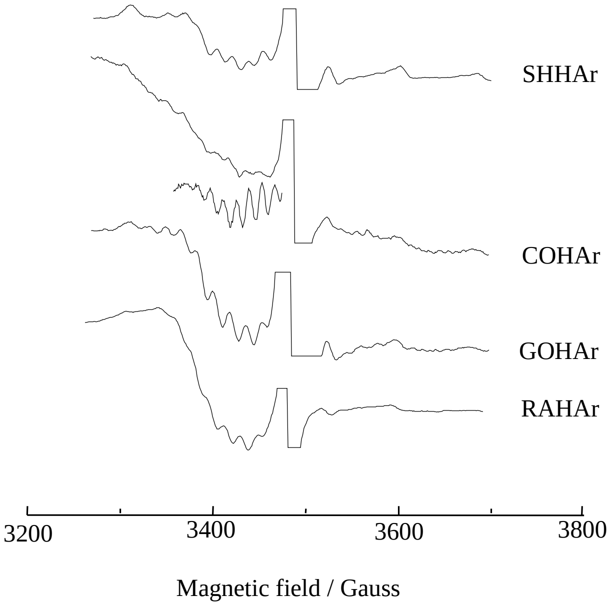


Figure 4. Perpendicular EPR spectra region (300 K) for lower ash contents humic acid (HAr) samples (see text) extracted from sheep (SHHAr), cow (COHAr), goat (GOHAr) and rabbit (RAHAr) vermicomposted manures showing lines from super hyperfine interactions of a nitrogen containing ligand complexed to the Cu^{2+} ion. For the COHAr sample detail of the second derivative mode spectrum is also presented.

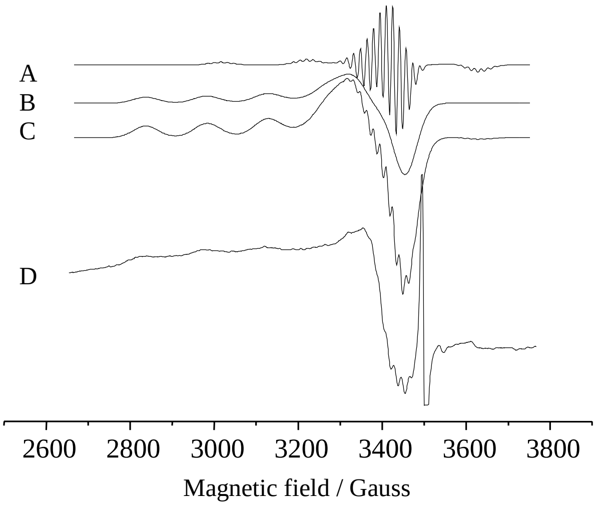


Figure 5. Simulated spectra for Cu-4N (A), Cu-4O (B) and Cu-4O + Cu-4N (C) and experimental spectrum (D) spectra of the lower ash contents humic acid sample extracted from the rabbit vermicomposted manure (RAHAr).

The RAHAr sample spectrum was simulated starting from the assumption that the experimental spectrum is a sum of the spectra of Cu-4O and Cu-4N complexes, where the Cu-4O complex is present in a larger concentration than the Cu-4N complex (Figure 5). This hypothesis is corroborated by the attributions of ν_{as} (OCO) bands of carboxylate groups interacting with metals at ~ 1645 cm^{-1} in the FTIR spectra presented in part I of this study[3]. Furthermore, ^1H RMN data (see bellow) show signals corresponding to nitrogen containing aromatic heterocycles. Spectra of Cu-4N complexes were simulated with typical $A_{||}$ values varying from 180 to 200 $\times 10^{-4}$ cm^{-1} [24]. The parallel EPR parameters, $A_{||}$ and $g_{||}$, typical for coordination of Cu^{2+} ions with four oxygen[10] atoms in the equatorial plane of an axial symmetry were more evident in the experimental spectra than the same parameters values for Cu-4N complexes. This fact can be indicative of the higher concentration of Cu-4O than Cu-4N complexes in the samples as considered above.

The assortment $g_{||} > g_{\perp} > 2.0$ (Table 2) indicated a d^9 configuration in which the unpaired electron is occupying the $d(x^2\text{-}y^2)$ orbital in the equatorial plane of Cu(II) complexes with elongated tetragonal symmetry[25,26]. It was not possible to determine the EPR parallel parameter values, $g_{||}$ and $A_{||}$, for the Cu^{2+} ion from the SHHA, COHA, GOHA, RAHA and SHHAr sample spectra.

A sharp line of organic free radicals with $g = 2.00$ and linewith varying between 6.0 and 7.0 G are apparent in all EPR spectra. These signals often found in EPR spectra of humic substances are due to semiquinone-like radicals. The presence of semiquinone functions indicates the existence of *ortho*- and *para*- biphenol functions on these organic structures. *Ortho*-biphenol are good metal ion chelating groups**.** They are also indicators of the VC humification level[27-30].

^1H NMR spectroscopy

^1H NMR spectroscopy of HS in solution has been scarcely studied because, in general, the spectra show broad signals, probably due to the presence of polymeric materials that prevent a simple structural elucidation[31]. However, the signals of these HA were reasonably well resolved using 400 MHz ^1H NMR instrumentation (Figures 6 and 7). Another approach here is the idea of the complementarity of the EPR and ^1H NMR data, which makes some high-resolution details of the spectrum nonessential.

The four HA ^1H NMR spectra showed basically the same signals, although changes in their relative intensities were observed, indicating the presence of the same species in different concentrations in the studied HA (Figures 6 and 7, Table 3).

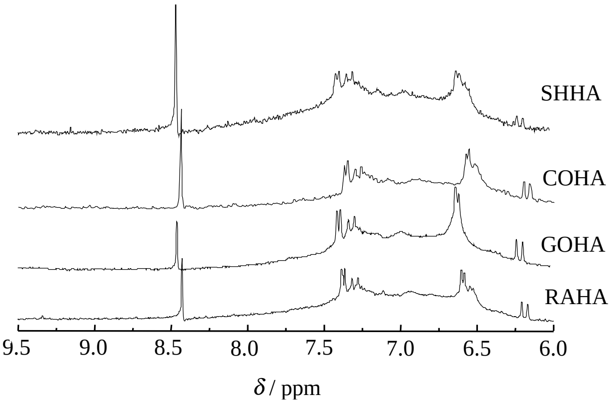


Figure 6. High frequency ^1H NMR spectra of humic acid (HA) samples extracted from sheep (SHHA), cow (COHA), goat (GOHA) and rabbit (RAHA) vermicomposted manures.

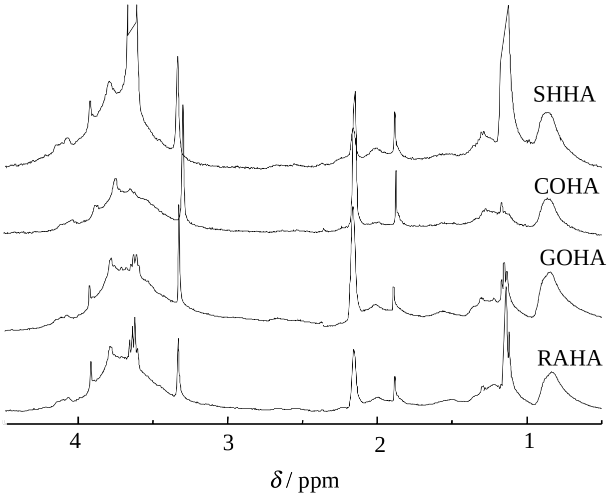


Figure 7. Low frequency ^1H NMR spectra of humic acid (HA) samples extracted from sheep (SHHA), cow (COHA), goat (GOHA) and rabbit (RAHA) vermicomposted manures.

The δ 9.5-6.0 region (Figure 6) of the COHA, GOHA and RAHA spectra contains approximately 20% of all hydrogens. This value indicates high contents of highly substituted and/or condensed aromatic structures[32], as well as a high degree of double and single aliphatic bonding conjugation in these HA. This composition was previously evidenced by the H/C atomic ratios and E_4/E_6 values[3]. Although this character was also postulated for SHHA, the percentage of protons in the δ 9.5-6.0 region of its ^1H NMR spectrum was lower ($\approx$ 13%). This was due to the great intensity of the signals at δ 3.62 (quartet) and 1.15 (triplet) (Figure 7), indicating the presence of a large number of hydrogenated substituents in this sample (see below).

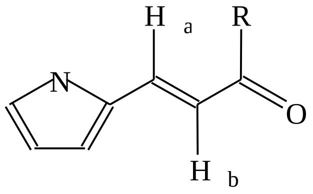
All spectra showed one sharp singlet at δ 8.43, which can be attributed to the resonance of phenolic -OH protons, probably intramolecularly H-bonded with an *ortho* carbonyl group[33]. This suggestion is in agreement with

Table 3. ^{1}H NMR data for HA in NaOD/D_2O solutions. Chemical shifts (coupling constants) in ppm and Hz.

	HA			
Assignment	SH	CO	GO	RA
Ph-OH	8.43 (s)	8.42 (s)	8.43 (s)	8.43 (s)
Aromatic H	7.38 (d, 8.0) 6.60 (d, 8.0)	7.36 (d, 8.0) 6.58 (d, 8.0)	7.38 (d, 8.0) 6.60 (d, 8.0)	7.37 (d, 8.0) 6.59 (d, 8.0)
Pyrrolic or indolic H	7.10 (br, s) 6.93 (br, s) 6.53 (m)	7.09 (br, s), 6.92 (br, s) 6.52 (bd), 6.33 (s) 6.31 (s)	7.12 (br, s) 6.96 (br, s)	7.08 (s) 6.92 (br, s) 6.53 (bd)
Double bond *trans* H	7.30 (d, 16.0) 6.20 (d, 16.0)	7.28 (d, 16.0) 6.18 (d, 16.0)	7.30 (d, 16.0) 6.20 (d, 16.0)	7.29 (d, 16.0) 6.19 (d, 16.0)
$-CH_2-X$ (X= ,-OCOR, -OCOPh, -OPh)	4.2 - 4.03 (m)	4.18 - 4.02 (m)	4.18 - 4.02 (m)	4.18 - 4.02 (m)
CH_3-X (X=-OPh, -OCOPh)	3.91 (s), 3.78 (s)	3.90 (s), 3.76 (s)	3.91 (s), 3.77 (s)	3.91 (s), 3.77 (s)
CH_3-CH_2-OR	3.62 (q), 1.15 (t)	3.67 (br, s), 1.19 (br, s)	3.62 (q), 1.16 (t)	3.61 (q), 1.14 (t)
CH_3-X or $-CH_2-X$ (X= -OR, -NHCOR)	3.33 (s)	3.31 (s)	3.32 (s)	3.32 (s)
$-CH_2-X$, (X= $-CONR_2$, -COOR, -COR)	2.16 (s)	2.15 (s)	2.16 (s)	2.15 (s)
CH_3-X (X= $-CONR_2$, -COOR, -COR)	2.01 (br, s)	2.01 (br, s)	2.00 (br, s)	2.00 (br, s)
$-CH_2-C=C-$	1.89 (s)	1.87 (s)	1.89 (s)	1.88 (s)
Aliphatic $-CH_2-$	1.29 (m)	1.28 (m)	1.29 (m)	1.22 (m)
Aliphatic CH_3-	0.87 (br, s)	0.87 (br, s)	0.84 (br, s)	0.84 (br, s)

the relatively high phenolic contents and with the absorption at 1645 cm^{-1} in the IR spectra of these HA, observed previously[3]. Formate ions[34], central protons of anthranilic structures[33] and substituted aromatic N-heterocycle like pyridines, isoquinolines, pyrimidines or pyrazines[35] can also contribute to this resonance.

In the δ 7.4 to 6.1 region, all spectra exhibit a broad signal with some well-defined peaks. Thus, the 8.0 Hz coupling constant values of the doublets at δ 7.37 and 6.59 are typical of aromatic protons. The signal at δ 7.37 can be attributed to the resonance of aromatic hydrogens of substituted anthracene type structures or of rings with -C=C, *orto*-O-COR, *meta*-COOH or -COOR substituent groups. The doublet at δ 6.59 was tentatively assigned to aromatic hydrogens or biphenolic groups, although resonance of aromatic hydrogens of rings substituted with *ortho* or *para* amine groups can also appear in this region. Other weaker signals in the δ 7.1 to 6.2 range, more abundant in the COHA ^{1}H-NMR spectrum, can be due to the resonance of pyrrolic or indolic type heterocyclic hydrogens, in agreement with the high nitrogen content of the samples[3]. The presence of pyrrolic hydrogens is in agreement with the existence of porphyrin structures, as suggested by the EPR parameters for the Fe^{3+} and Cu^{2+} complexes. The two doublets centered at δ 7.29 and 6.19, respectively hydrogen (a) and (b) of the structure below, seem to be associated. From their δ and coupling constant values ($J_{(H-H)}$ 16.0 Hz), these signals can be assigned to the resonance of *trans* hydrogens of conjugated double bonds of groups like C(=O)R (R = alkyl, ether), present in the pyrrolic structure[36].



From the study of the δ 9.5 - 6.0 region of the ^{1}H NMR spectra (Table 3 and Figure 6), the presence in these HA of substituted and condensed aromatic structures, phenolic groups, N-heterocycles and conjugated double bonds can be deduced.

Thermal analysis data suggested a partial protein loss in the HA extraction process from the VC[3]. Considering that the nitrogen contents were higher in HA compared to VC[3], the presence of N-heterocycles in these humic substance cores was proposed. Present ^{1}H NMR analysis for HA samples seem to confirm this suggestion. In this way, it is important to note that the existence of N-heterocycles in soil HA had been previously detected by pyrolysis-gas chromatography/mass spectrometry analysis[37].

All ^{1}H NMR spectra showed five signals in the δ 4.2 - 3.3 range (Figure 7), that can be attributed to methyl or methylene groups linked to nitrogen or oxygen atoms. The broad signal at δ 4.2 - 4.02 can be assigned to the resonance of methylene groups (-CH_2-) bound to -OCOR (R = alkyl), -OCOPh or -OPh groups. The existence of analogous CH_3-OPh and CH_3-OCOPh groups seems to be demonstrated by the two singlets at δ 3.91 and 3.77. The sharp singlet at δ 3.32 was attributed to CH_3- or -CH_2- linked to -OR or -NHCOR groups.

The quartet at δ 3.62 is associated with the triplet at δ 1.15, corresponding to ethyl groups linked to -NHCOR or -OR groups. These signals are extremely intense in the SHHA ^{1}H-NMR spectrum, and their intensities decrease in the order SHHA > RAHA > GOHA, being practically imperceptible in the COHA ^{1}H-NMR spectrum (Figure 7). As SHHA corresponds to the sample with the lowest nitrogen contents[3], the assignment of these signals to CH_3-CH_2-OR groups appears more adequate. In addition, these signals seem to be related with the intense IR absorption at 1384 cm^{-1}, previously assigned to an aliphatic δ (CH_2) band, which was absent in the COHA spectrum[3].

The two singlets at δ 2.15 and 2.0 were tentatively attributed to -CH_2- and CH_3- groups respectively linked to -$CONR_2$, -COOR or -COR groups, although CH_3-Ph can also contribute to the former signal. The singlet at δ 1.88 was tentatively assigned to -CH_2-C=C groups. The broad signal at δ 1.28 is mainly attributed to the aliphatic -CH_2-, although CH_3- bonded to -C-O-R groups (R = H, alkyl, Ph, COR, COPh) or -CH_2- β to amine groups also resonate in this region of spectra[33]. The broad signal centered at δ 0.85 is due to the aliphatic CH_3 groups.

A clear predominance of ether, amide and ester groups is deduced from the study of the δ 4.5 - 0.5 region of the ^{1}H NMR spectra of HA samples (Table 3 and Figure 7), in agreement to the previous FTIR study[3].

Conclusions

The ^{1}H NMR spectroscopic study of HA samples showed that both types of structures, conjugated double bonds and substituted and condensed aromatic groups are constituents of the organic matter of these fresh HS, as previously proposed[3]. This is not common for aged HS where, in consequence of the aromatic stabilization energy, conjugated double bond are present only in condensed aromatic structures. The presence of nitrogen as a constituent of amide and N-heterocycle groups, in agreement with the high nitrogen content of the samples[3], was also confirmed by ^{1}H NMR spectroscopy, which also indicated the existence of phenolic, ether and ester groups.

EPR spectroscopy was useful to determine the presence of transition metal ions such as Mn^{2+}, Fe^{3+} and Cu^{2+} in the HAs and VCs, as well as in the study of their interactions with the constituents of the organic matter.

We verified the presence of Mn^{2+}, mainly in the hydrated form, in agreement with the low capacity of chelation of this ion with soil HA organic matter. Inasmuch as manganese can be absorbed as soluble chelate or Mn^{2+} soluble ion by the roots, the VC samples here studied seem adequate to supply useful manganese to the plants[5].

EPR data provided information about the presence of inorganic iron as oxide, hydroxide, and in rhombic and axial complexes like Fe^{3+}-porphyrin. It has been shown that chelated iron complexes, in the organic matter of humic substances, are more available for plants than iron in the form of soluble salts[5].

EPR data also provided information about the complexation of Cu(II) with the organic matter of these HS. There are two types of chelates with axial symmetry, nitrogenous (Cu-4N) and oxygenated (Cu-4O) complexes. The latter is the most abundant copper species, in accordance with EPR spectroscopic parameters. It has been reported that the plants absorb chelate complexes of copper with oxygen-donor ligands faster than those with nitrogen-donor ligands[38]. Thus, the HS Cu-4N complexes would be a copper reserve during the continuing humification process on the soil.

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