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Article

Influence of Extractant and Soil Type on Molecular Characteristics of Humic Substances From Two Brazilian Soils

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Num estudo anterior, observou-se que substâncias húmicas (HS) extraídas com solução de NaOH e com solução de $Na_4P_2O_7$ apresentaram diferentes pesos moleculares, e que o rendimento de HS extraídas por cada método variou entre um Oxisol e um Mollisol do Sul do Brazil. No presente trabalho, o estudo da matéria orgânica nestes solos foi continuado, através da caracterização das HS extraídas com solução de NaOH 0.5 mol L^{-1} e com solução neutra de $Na_4P_2O_7$ 0.15 mol L^{-1} das amostras acima mencionadas, empregando-se análise elementar e espectroscopia de ressonância magnética nuclear (^{1}H- and ^{13}C-NMR no estado líquido). As diferenças moleculares foram relacionadas com o método extrator e com o tipo de solo. HS extraídas com pirofosfato foram mais humificadas, apresentando caráter aromático e proporção de grupos carboxílicos superiores. As HS extraídas com NaOH foram mais alifáticas e continham uma maior proporção de grupos O-alquila, o que é indicativo de um natureza menos humificada do que as HS extraídas com pirofosfato.

In a previous study it was observed that humic substances (HS) extracted with NaOH solution and with $Na_4P_2O_7$ solution presented different molecular weights, and also that the extracted HS yield by each method varied between an Oxisol and a Mollisol from South Brazil. In the present study, we further investigated the organic matter in these soils by characterizing HS extracted with 0.5 mol L^{-1} NaOH and with neutral 0.15 mol L^{-1} $Na_4P_2O_7$ solutions from the above mentioned samples, using elemental analysis and nuclear magnetic ressonance spectroscopy (liquid state ^{1}H- and ^{13}C-NMR), and by relating the molecular differences to the extraction method and soil type. HS extracted with pyrophosphate were more humified, showing a higher aromaticity and higher carboxylic content. The NaOH-extracted HS were more aliphatic and contained a higher O-alkyl proportion, which is indicative of a less humified nature than the pyrophosphate-extracted HS.

Keywords: *humic substances, oxisol, mollisol, aromaticity, ligand exchange*

Introduction

Humic substances (HS) from environmental compartments can strongly differ in their chemical and physical characteristics, as a result of the diversified humification conditions. In soils, the structure and composition of HS seem to be influenced, among other parameters, by parent material, soil pH, vegetation, soil management system and cultivation[1,2,3]. Also the soil type, including its mineralogy, which in turn, is related to soil age and climate, can affect the quality of HS[4,5,6].

The extracting agents most commonly employed for the analytical separation of HS from soils are NaOH (0.1 or 0.5 mol L^{-1}) and 0.1 mol L^{-1} $Na_4P_2O_7$ solutions (pH 9 to pH 7), or a combination of both[2,7,8,9]. In the first part of this work[10], we verified that in a Brazilian Oxisol, a greater yield of extracted HS was obtained with NaOH solution when compared to pyrophosphate solution, while in a Mollisol, an inverse behaviour was observed. We related this fact to the soil mineralogy and the type of bonding mechanism of HS, and proposed that in the Mollisol, where 2:1 clay minerals predominated, HS were preferentially aggregated among themselves and bonded to clay minerals through cationic bridges (pyrophosphate extracted HS). In the Oxisol, HS molecules were bonded mainly via H-bridges and through surface complexation to the oxide surfaces (NaOH extracted HS). HS solubilized by the two methods also

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exhibited different molecular weight distributions and E_{350}/E_{550} ratios.

Aiming to complement the verifications obtained earlier [10], the main objective of this study was to characterize HS conventionally extracted by 0.5 mol L^{-1} NaOH and 0.15 mol L^{-1} $Na_4P_2O_7$ solutions from the mentioned soils, using elemental analysis and molecular spectroscopy methods (^{1}H-NMR and ^{13}C-NMR) and to reveal potential molecular differences in the HS extracts.

Materials and Methods

Soils

The investigated HS were obtained from the A-horizon of two soil samples, an Oxisol and a Mollisol from Rio Grande do Sul state, Brazil. The Oxisol (Santo Angelo region) was under crop rotation oat/soja, contained 27 g kg^{-1} soil of organic Carbon, and its clay mineralogy consisted mainly of Fe-oxides and kaolinite [11]. The Mollisol (Rio Pardo region), was under native vegetation, contained 21g kg^{-1} soil of organic Carbon, and smectite and kaolinite dominated its clay mineralogy[12].

Extraction and isolation of humic acids (HA) and fulvic acids (FA)

The HS were extracted either with 0.5 mol L^{-1} NaOH or with 0.15 mol L^{-1} Na-pyrophosphate (pH 7) solutions (250 mL /11 g soil) during 3 hours under continuous shaking and the procedure is described in detail in our previous study[10]. The HS extract was acidified to pH 2 by diluted HCl, and the precipitated HA was separated by centrifugation from the remaining FA solution. The latter was separated by means of a XAD-8 column.

Elemental analysis

The determination of C, H and N contents were performed in duplicate with an elemental analyser (Perkin Elmer 4000) on FA and HA samples. Prior to the elemental analysis, the samples were dried in a vacuum oven at 60 °C to a constant weight. The ash content was determined by the weight loss after ignition at 750 °C for 4 h. The experimental values of C, H and N contents were recalculated for an ash free basis and the oxygen content was calculated by the difference method.

^{1}H- and ^{13}C-NMR spectroscopy

The samples were dissolved in dilute NaOD-D_2O, with solution volume of 0.55 mL and pD = 8.5. The NMR spectra were recorded on a JEOL GX 400 spectrometer. All measurements were performed on a 5 mm dual (C,H) probehead. The following experimental conditions were employed: -^{1}H-NMR measurements: frequency for ^{1}H of 400 MHz, frequency range of 25 ppm, pulse repetition time 1.1 s, number of scans 1200 - 6000. -^{13}C-NMR measurements: frequency for ^{13}C of 100 MHz, frequency range of 480 ppm, pulse repetition time 1.2 s, number of scans 100,000 - 215,000.

Results and Discussion

Elemental composition

The NaOH-extracted HS (HA-N and FA-N) from the Oxisol contained similar amounts of C, but O was greater in the FA (Table 1). In the Mollisol, HA extracted either with pyrophosphate as well as with NaOH, exhibited a higher C and lower O content than the corresponding FA, in agreement with data cited in the literature[2]. Furthermore, the HA-N, that is, HA extracted by breaking up hydrogen-bridges (H-bridges) and/or surface complexation interactions, contained less O and more C than the HA extracted via breaking up of cationic bridges (HA-P). This result is not surprising, since oxygenated groups play the main role in the coordination with metals[2,13]. The same trend was observed with HA's and FA's from organic soils[14].

The sample OX-FA-P presented a lower value for C content when compared to OX-FA-N (Table 1), while the O content was similar in the two FA. Regardless of the sample and extracting solution, the FA always showed smaller contents of H and N and larger O content, when compared to the respective HA counterparts.

Table 1. Elemental composition of the HS samples.

Sample	C * (g kg^{-1})	H* (g kg^{-1})	N*(g kg^{-1})	O** (g kg^{-1})	Ash (g kg^{-1})
OX-HA-N	482	40	39	439	430
OX-FA-N	486	23	10	481	230
OX-FA-P	413	65	28	494	195
MO-HA-N	513	50	45	392	370
MO-FA-N	424	48	35	493	350
MO-HA-P	450	28	21	501	530
MO-FA-P	402	20	4	574	509

*: normalized for an ash-free basis; **: calculated from difference of 1000 g kg^{-1} ash-free; Legend: OX: Oxisol; MO: Mollisol; HA: humic acid; FA: fulvic acid; N: NaOH extract; P: Na-Pyrophosphate extract.

The fulvic acids in general exhibited a higher value for the C/N ratio than the humic acids (Table 2), caused by the low N relative to C content in the FA fraction[15,16,17]. C/N values similar to those observed for HA's in Table 2 had been reported earlier for different soil HA[2,15,16,17].

In the Mollisol, the NaOH extracted HS (HA-N and FA-N) showed lower C/N values than the pyrophosphate counterparts (Table 2), suggesting a selective extraction of a less nitrogenated HS by the latter. Considering the extraction mechanism, it follows that in the HA interactions, amino-groups may have been more important in H-bonding than in cationic bridges. On the other hand, the contribution of nitrogenated groups to the total bonding forces should not have been very significant, in view of the low N abundance in the HS molecule.

In the Mollisol the values for the H/C atomic ratio were lower in the HA-P and FA-P than those values obtained for the corresponding HA-N and FA-N (Table 2). These results suggest that in this soil, the pyrophosphate extracted HS had a more condensed structure/unsaturation than the NaOH-extracted ones. This behaviour was not observed with the Oxisol HS, where FA-P showed a higher H/C ratio than FA-N.

All FA showed a higher O/C ratio than the respective HA (Table 2), confirming the trend observed by other authors[2]. The NaOH extracted HS showed lower values for the ratio O/C when compared to their pyrophosphate counterparts. Consequently, an oxidative effect of the alkaline treatment was not noticed in the first 3 h extraction as had been noticed with soil HA[16].

The HA extracted with alkaline solution in both soils not only showed similar values for the ratios C/N, H/C and O/C, but also showed similar values of $<M_w>$ (Table 2). The pyrophosphate extracted HA in the Mollisol presented larger molecules which were less nitrogenated, more unsaturated and more oxygenated than the ones extracted with NaOH solution, indicative of a more humified HS.

Table 2. C/N ratio, H/C and O/C atomic ratio, and weight-average molecular weight $<M_w>$ of HS.

Sample	C/N	H/C	O/C	$<M_w>^{(*)}$ (kDa)
OX-HA-N	12	1.00	0.68	≤ 100
OX-FA-N	49	0.57	0.74	≤ 1
OX-FA-P	15	1.89	0.90	≤ 10
MO-HA-N	11	1.17	0.57	≤ 100
MO-FA-N	12	1.36	0.87	≤ 1
MO-HA-P	22	0.75	0.84	> 100
MO-FA-P	91	0.60	1.07	≤ 10

$^{(*)}$ data from Dick & Burba[10]. Legend: see Table 1.

^{1}H- and ^{13}C-NMR spectroscopy

The ^{1}H-NMR spectra for HA's and FA's showed the same general features, and differed only in the peak intensities (Table 3). Two representative ^{1}H-NMR spectra are shown in Figs. 1 and 2. The relative abundances of the H-groups were calculated by relating the respective area to the total spectrum's area, excluding the peak at approximately 4.8 ppm due to H_2O. The identified H-groups agreed with spectral data obtained by other authors[18,19,20].

The data from ^{1}H-NMR showed that the NaOH-extracted HA (HA-N) were structurally very alike in the two soils, regardless of the mineralogy, agreeing with the results obtained with elemental composition and molecular weight determinations (see former item).

In the Mollisol, the pyrophosphate extracted HS revealed a higher proton aromaticity and lower H-carbohydrate (CH_n-O) (Table 3) than the corresponding NaOH

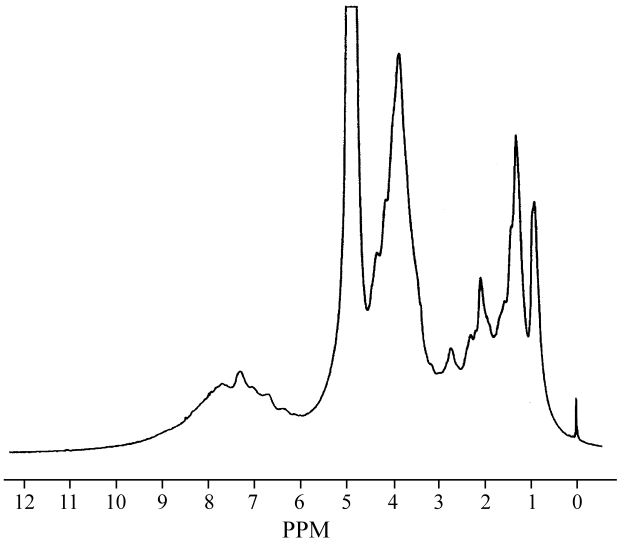

Figure 1. ^{1}H-NMR spectrum of the sample OX-HA-N.

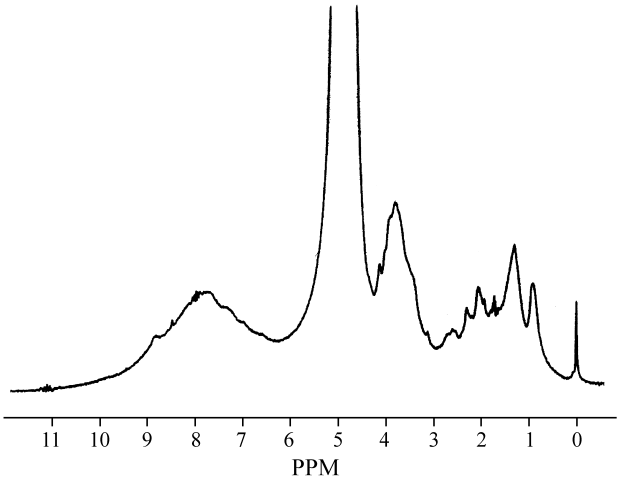

Figure 2. ^{1}H-NMR spectrum of the sample MO-HA-P.

Table 3. Chemical shift assignments for the main resonance regions in ^{1}H- NMR spectra and their relative abundance.

Sample / Assignments[6,19]	Chemical shift (ppm)			
	9.0 - 5.5 H-aromatic	4.5 - 3.3 CH_n-O-	3 - 1.6 CH_n-arom. CH_n-CO-	1.6 – 0 CH_n-C
OX-HA-N	20%	37%	19%	24%
OX-FA-N	19%	29%	24%	28%
OX-FA-P	12%	40%	28%	20%
MO-HA-N	20%	37%	19%	24%
MO-FA-N	17%	34%	25%	24%
MO-HA-P	41%	25%	16%	18%
MO-FA-P	21%	33%	26%	20%

Legend: see Table 1.

extract, agreeing with other authors[21]. The higher proton aromaticity is not a conclusive result itself because it may yield an indication of a higher aromaticity or less substituted aromatic rings. The ^{1}H-NMR results together with the elemental analysis and $<M_w>$ measurements (Table 2), indicated that HS of higher humification degree and with larger molecules were preferentially solubilized by the $Na_4P_2O_7$ solution. Recently, the stabilization of a less humified HS, determined by means of electron paramagnetic resonance (EPR) in the clay fraction of an Alfisol was related with the organo-mineral interaction on the Fe-oxides surface via ligand exchange[11].

Additionally, the NaOH extracted HS from the Mollisol showed higher H/C ratios than the pyrophosphate ones (Table 2), suggesting the solubilization of a less condensed HS by the alkali treatment. An inverse realtionship between H/C ratio and aromatic-H (determined by ^{1}H-NMR) was verified with fourty HA from various types of soils, and it was concluded that along with the humification, aliphatic side chains are split off and condensed aromatic rings are introduced into the structure[6].

The ^{13}C-NMR analysis was performed only on the NaOH extracted HA of the Oxisol and on the pyrophosphate extracted HA of the Mollisol (Figs. 3 and 4). Since the HA extracted by the alkaline treatment were chemically and structurally similar in both soils and the amount of MO-HA-N was minor, the NaOH-extracted HA of Mollisol was not analysed. Furthermore, considering that the samples MO-HA-P and OX-HA-N were the most abundant HA in the respective soils [10] they would be regarded as the representative HA for each soil.

Unlike ^{1}H-NMR, the integration of the peak area in the ^{13}C-NMR spectrum cannot be taken strictly quantitative, due to the different relaxation times of the various functional groups. Recently, a comparative study for the quantification of carboxylic groups in eight samples of soil HAs employing wet chemical analysis, FT-IR spectrophotometry and ^{13}C-NMR showed that the three mentioned methods correlated significantly[25]. Though, the COOH quantities determined by ^{13}C-NMR were higher than the values obtained by the other two methods, probably due to the presence of small amounts of esters, amides and lactones, that absorb in the same spectral region.

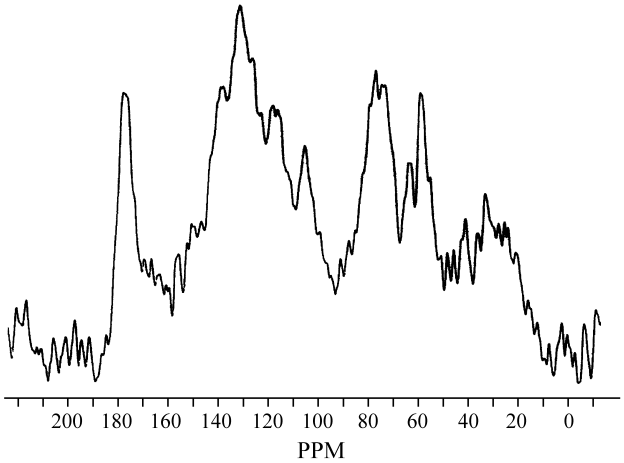

Figure 3. ^{13}C-NMR spectrum of the sample OX-HA-N.

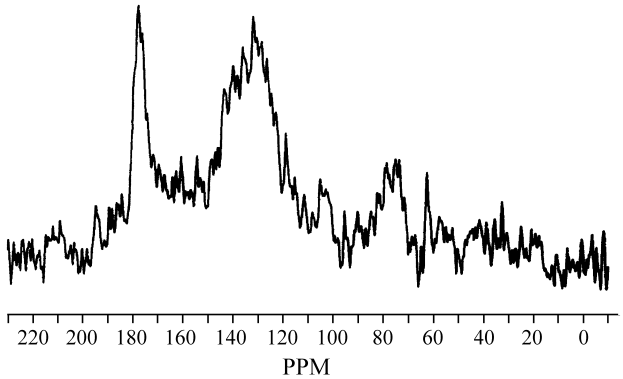

Figure 4. ^{13}C-NMR spectrum of the sample MO-HA-P.

Table 4. Chemical shift assignments for the main resonance regions in ^{13}C- NMR spectra and their relative abundance.

Assignments[9,24] / Sample	Chemical shift (ppm)				
	230 –190 C-ketone	190 – 165 C-carboxyl	165 – 100 C-aromatic	100 – 65 CH_n-O-R	65 – 0 CH_n-C
OX-HA-1A	n.i	5%	57%	20%	18%
MO-HA-P1A	n.i.	25%	64%	9%	2%

n.i.: not identified.

Since in the present study, the obtained data were very different between the two samples (Table 4), a discussion based on the ^{13}C-NMR integration area yield a relative comparison.

The ^{13}C-NMR spectra of the HA'S of the Mollisol and of the Oxisol differed basically by the peak intensities (Table 4) and their pattern agreed with the data obtained for other soil HS[5,7,22,23,24]. The pyrophosphate extracted HA from the Mollisol showed a higher content of carboxylic-C and aromatic-C than the NaOH-extracted HA from the Oxisol, indicating a more advanced humification in the first.

These observed differences could be in this case related to the nature of the soil. It was reported that HA isolated with pyrophosphate at pH 7 were more aromatic and showed a more acidic character, while HA isolated at pH 12.6 from the same soil were more aliphatic and richer in C-carbohydrates[9]. In a Gleysol and in a Chernozem, it was verified that HA extracted with 0.1 mol L^{-1} $Na_4P_2O_7$ solution at pH 9 tended to be more aromatic, and the HA extracted with 0.5 mol L^{-1} NaOH tended to be more aliphatic[7].

Conclusions

In view of our results, we may conclude that:

1. HS extracted with pyrophosphate were more humified, showing a higher aromaticity and higher oxygenation degree. Except for the sample OX-FA, the NaOH-extracted HS were more aliphatic and contained a higher carbohydrate proportion, and thus were less humified.

2. Considering the extraction mechanism of the two tested methods, it may be inferred that HS that interacted with the oxide surfaces and with other HS molecules mainly through H-bonds and ligand exchange reactions were less humified than the HS that were aggregated between themselves or bonded to clay minerals through cationic bridges. Since the present study employed only one oxidic sample, no conclusion about a stabilizing effect of iron oxides on the HS can be drawn.

3. HS extracted from soil samples showed different structural, chemical and molecular characteristics depending on the extractant chosen, and its abundance was probably influenced by the type of soil.

4. For a better understanding of the HS bonding mechanisms in soils and of the relationship between mineralogy and HS characteristics, further studies employing soil samples with more diversified mineralogical characteristics should be carry out.

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