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Spectroscopic study of humic acids fractionated by means of tangential ultrafiltration

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Abstract

Different chemical and spectroscopic techniques—diffuse reflectance infrared Fourier transform (DRIFT), surface-enhanced Raman spectroscopy (SERS), and ^{1}H, ^{13}C nuclear magnetic resonance (NMR) have been applied to investigate a peat humic acid (HA) separated by tangential ultrafiltration into different nominal molecular weight (NMW) fractions.

Each fraction analyzed showed a characteristic DRIFT and NMR pattern. High nominal molecular weight fractions were mainly characterized by long chains of methyl and methylene groups and poorly substituted aromatic rings, while in low nominal molecular weight fractions (L-NMW), phenolic and oxygen-containing groups were predominant. A comparative study on fractions before and after treatment with 0.5 M HCl was carried out. Purified fractions showed either an increase in the carboxylate and phenolic OH groups or an improvement in signal-to-noise ratio of their NMR spectra.

The SERS study of NMW fractions allowed significative information on structure and conformation of these fractions. In particular, L-NMW fractions showed a great structural modification, when different alkaline extractants or treatment with HCl were used.

Humic-like substances obtained by catechol and gallic acid polymerization on metal surface were investigated using SERS. The SERS spectra of these polymers were compared and discussed with those of NMW HA fractions.

Keywords: Humic acid; Diffuse reflectance infrared Fourier transform; Surface-enhanced Raman spectroscopy; Nuclear magnetic resonance; Nominal molecular weight

1. Introduction

Soil humic substances (HS) are chemically and physically heterogeneous macromolecules produced by biological and chemical degradation of organic residues in the natural environment [1]. A major goal in the study of HS is to obtain information on their chemical structure, size and molecular weight, which could be useful in clarifying the nature and formation processes of HS. The separation of HS into different nominal molecular weight (NMW) fractions to reduce their heterogeneity is a necessary step to facilitate the study of their structural features. Usually, ultrafiltration (UF) [2–5] or size exclusion chromatography (SEC) techniques [6–10] are applied

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to achieve this. These techniques (SEC and UF) are in agreement on the molecular size distribution of humic acid (HA) fractions that they present [11].

Studies performed on different NMW HA fractions have shown wide ranges in structure, conformation and binding properties for different metal ions [2,5,12]. These results showed substantial differences between high nominal molecular weight (H-NMW) and low nominal molecular weight (L-NMW) fractions suggesting the importance of size fractions in the metal complexing phenomenon.

For a better understanding of the composition, structure and physical–chemical properties of NMW fractions a multi-method spectroscopic approach has been deemed the most beneficial [2,3,5,13]. Although ^{1}H, ^{13}C nuclear magnetic resonance (NMR) [14–18] and diffuse reflectance infrared Fourier transform (DRIFT) [12,19–22] spectroscopies are considered the most powerful tools available to determine the structure of both organic and inorganic species, they cannot provide direct information on conformation.

Surface-enhanced Raman scattering (SERS) is an important tool used to obtain valuable structural and conformational information on different HS [2,23–26]. This technique is based on the enormous enhancement of the electromagnetic field occurring in the vicinity of a rough metallic surface. It has demonstrated its potential in the study of biological molecules from the analytical and structural point of view [27]. An advantage of SERS over normal Raman techniques in the characterization of HS is that no interference due to intense fluorescence emitted by the HS is observed. Besides the smaller sample size required for SERS measurements, the application of this Raman technique also produces an intense signal from the large amount of aromatic or aliphatic groups contained in HS. This is in contrast to IR techniques, which are more sensitive to more polar groups. The SERS technique also allows for the structural and dynamic study of HS (unfolding and adsorption on a charged surface, interaction with pesticides, etc.) at very low concentrations because of the short-range character of the SERS effect [2,28–30].

The objective of this paper was to study: (i) the chemical composition and structure of NMW HA fractions isolated from a *Sphagnum* peat using several spectroscopies DRIFT, SERS and ^{1}H and ^{13}C NMR; and (ii) the influence of treatment with HCl on chemical composition and structure of these fractions. Finally, a tentative structure of NMW fractions is presented based on model HS molecules produced from catechol and gallic acid.

2. Experimental

2.1. Extraction of humic acid

A representative sample of an Irish *Sphagnum* peat, total organic C = 52.3%, total N = 0.7%, total P = 0.03%, pH = 4.8, ash = 4.5%, was extracted to yield the HA. Briefly, 2 kg of air-dried peat, previously crushed and sieved to 0.5 mM sieve, were extracted under N_2 with 40 l of 0.5 M NaOH at room temperature using a vertical stirrer for 24 h. The suspension was centrifuged at $5000 \times g$ for 30 min and then filtered through a 0.22 μm filter using a Minitan S System (Millipore, Bedford, MA). The solution was acidified to pH < 2 with 5 M HCl to precipitate the HA and afterwards was centrifuged for 20 min at $5000 \times g$. The HA was dissolved with NaOH 0.5 M to achieve a Na-humate.

The Na-humate was separated into six NMW fractions using UF technique as described by Francioso et al. [2]. The following molecular weight cut-offs of HA > 300 kDa ($HA_{>300}$); HA 100–300 kDa ($HA_{100-300}$); HA 50–100 kDa (HA_{50-100}); HA 20–50 kDa (HA_{20-50}); HA 10–20 kDa (HA_{10-20}) and HA 5–10 kDa (HA_{5-10}) were obtained. H-NMW include the following three fractions: $HA_{>300}$; $HA_{100-300}$ and HA_{50-100}, while L-NMW include the other ones.

The NMW of each fraction obtained by UF was evaluated by SEC [11]. All samples were dialyzed against Millipore water, using a Spectrapore tubing with a molecular mass cut-off of 500 Da, and were then freeze-dried.

The mass balance of NMW fractions compared to unfractionated HA was as follows: $HA_{>300} = 54.0\%$; $HA_{100-300} = 19.6\%$; $HA_{50-100} = 14.0\%$; $HA_{20-50} = 12.0\%$; $HA_{10-20} = 0.5\%$ and $HA_{5-10} = 0.04\%$.

The three H-NMW ($HA_{>300}$; $HA_{100-300}$ and HA_{50-100}) and one L-NMW (HA_{20-50}) fractions were treated twice with 0.5 M HCl solution at room temperature to reduce their metal content. Insoluble residues were centrifuged, and then washed with distilled water until a neutral pH and freeze-dried.

Table 1
Total metal contents of NMW HA fractions. All data are expressed on dry weight (n.d.: concentration less than the detection limit)

Size-fraction (kDa)	Cu	Zn	Fe (mg kg^{-1})	Mn	Mg	Ca
$HA_{>300}$	92 ± 2.8[a]	157 ± 7.0	1241 ± 34	26 ± 4.2	555 ± 17	2150 ± 54
$HA_{>300}$[b]	25 ± 0.6	12 ± 0.9	462 ± 28	n.d.	95 ± 1.0	197 ± 19
$HA_{100-300}$	147 ± 7.7	218 ± 11	1062 ± 29	8.4 ± 0.6	455 ± 13	1958 ± 26
$HA_{100-300}$[b]	41 ± 0.8	11 ± 0.4	384 ± 15	n.d.	33 ± 1.0	112 ± 6.9
HA_{50-100}	396 ± 20	566 ± 19	358 ± 17	7.9 ± 0.8	352 ± 14	1793 ± 20
HA_{50-100}[b]	214 ± 10	81 ± 1.0	301 ± 6.9	n.d.	148 ± 7.8	1160 ± 23
HA_{20-50}	409 ± 21	688 ± 14	764 ± 27	5.9 ± 0.4	652 ± 15	3278 ± 30
HA_{20-50}[b]	204 ± 12	111 ± 9.0	302 ± 14	n.d.	256 ± 13	1277 ± 24

[a] (±) Standard deviation.
[b] HA treated with 0.5 M HCl.

2.2. Chemical characterization

Total metals content (Ca, Mg, Fe, Cu, Zn, Mn) were determined by an atomic absorption spectrophotometer (AAS) using a Varian Spectra AA 200 (Australia Pty. Ltd) after mineralization with HNO_3.

2.3. Diffuse reflectance infrared Fourier transform

Ten milligram of lyophilized HA were thoroughly mixed with 100 mg of KBr (FT-IR grade, Aldrich Chemical Co., Milwaukee, WI). The spectra were recorded with a Nicolet Impact 400 FT-IR Spectrophotometer (Madison, WI) fitted with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT). The spectra are the average of 200 scans and the resolution was set at 4 cm^{-1} [2].

2.4. Surface-enhanced Raman spectroscopy

The silver colloid for SERS measurements was prepared as described by Lee and Meisel [31]. An aqueous solution of each NMW fraction was obtained by dissolving 1 mg of the lyophilized material with 1 ml of water as described by Sánchez-Cortés et al. [30].

Catechol and gallic acid were purchased from Sigma (St Louis, MO). The 5×10^{-2} M catechol and gallic acid stock solutions were prepared separately in ethanol. Aliquots of these solutions were added to the silver colloid to give a final concentration of 5×10^{-3} M.

Raman spectra were recorded by using a U-1000 Jobin–Yvon spectrophotometer as previously described by Francioso et al. [2] and Sánchez-Cortés et al. [30].

FT-Raman and FT-SERS spectra were obtained using an RFS 100/S Bruker spectrophotometer. An Nd:Yag laser was used to produce excitation energy at a wavelength 1064 nm. Resolution was set at 4 cm^{-1} and a 180° geometry was employed to record the data. The laser power output was 150 mW in the SERS measurements. The final spectra were recorded after averaging 100 scans.

2.5. Nuclear magnetic resonance spectroscopy

The samples were prepared by dissolving the L-NMW and three H-NMW HA fractions (40–50 mg) in 0.6 ml of D_2O and 0.3 ml of NaOD. The HA solutions were filtered before being placed in the NMR tube.

Spectra were recorded at 300.0 and 75.45 MHz frequencies for ^{1}H and ^{13}C, respectively. In a standard experiment, the proton spectrum was acquired with a single pulse sequence using a relaxation delay of 3-s, a sweep width of 4500 Hz, a pulse angle of 45° and 256 transients. The ^{13}C spectra were acquired using a proton-decoupled single pulse sequence with a relaxation delay of 10 s, a sweep width of 20,000 Hz and a pulse angle of 70° according to Thorn et al. [15]. The number of transients ranged from 50 to 100 thousand. In order to achieve an acceptable signal-to-noise ratio, a line broadening of 40 Hz was used in the Fourier

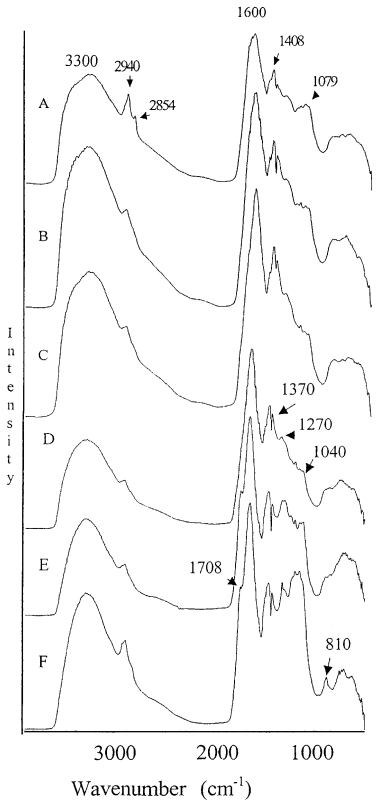

Fig. 1. DRIFT spectra of NMW HA fractions: (A) $HA_{>300}$; (B) $HA_{100-300}$; (C) HA_{50-100}; (D) HA_{20-50}; (E) HA_{10-20} and (F) HA_{5-10}.

transform. Chemical shifts are referred to 3-trimethylsilyl-tetradeutero propionate (TSPA-d^4).

3. Results and discussion

3.1. Elemental analyses

The metal concentrations of the most quantitatively significant fractions are shown in Table 1. The higher contents of Cu, Zn, Mg and Ca were found in the L-NMW fraction (HA_{20-50}), whereas Fe was much more concentrated in the H-NMW fractions in agreement with results obtained by Aster et al. [32,33]. In a previous study, Francioso et al. [12] found similar concentration of metals in HA extracted with NaOH + $Na_4P_2O_7$ from the same peat sample. However, the HA extracted with NaOH showed a higher Fe content in the H-NMW fractions, as well as a lower Mg content in the L-NMW fractions, when compared to those extracted with NaOH + $Na_4P_2O_7$ 0.1 M [12]. We deduce from different metal distributions for the NMW fractions that a different structural composition might be present in each fraction. The high Fe content in H-NMW fractions might be due to the existence of aromatic salicylate structures, whereas the high content of Cu and Zn in the L-NMW fractions may be related to the existence of phthalate and carboxyl groups in the aliphatic structures [34–37].

The metal content undergoes a significant decrease until 70–90% in the H-NMW fractions after treatment with 0.5 M HCl (Table 1). Consequently, the concentrations of paramagnetic metals decreased and the signal-to-noise ratio in the NMR spectra (shown later) increased. In the L-NMW fraction (HA_{20-50}), the decrease in the metal content was less significant.

3.2. DRIFT spectroscopy

DRIFT spectra of different NMW fractions are shown in Fig. 1. The interpretation of DRIFT spectra was in accordance with previously published spectra [1,19,20,34,38–40]. All spectra are dominated by a broad band at 3350 cm^{-1} attributed to ν(O–H) vibration of carboxylic and alcoholic groups in different environments [41]. The peaks at 2940 and 2854 cm^{-1} are assigned to asymmetric and symmetric ν(C–H) motions of aliphatic groups, respectively. The relative peak intensity (I) at 2940 cm^{-1} decreases and shifts towards lower wavenumbers in the L-NMW fractions ($I_{2940} = 0.165$ in the $HA_{>300}$ and $I_{2920} = 0.090$ in the HA_{5-10}). The factors affecting these changes might be related to the presence of elements, such as oxygen and sulfur adjacent to CH_3 and CH_2 groups, which can produce perturbations in the stretching vibrations of C–H [42]. The high concentration of S and oxygen found in L-NMW (data not shown) might support this suggestion. We also suggest that long chains of methyl and methylene groups of saturated hydrocarbons might be present in $HA_{>300}$ fraction.

The prominent band appearing at about 1600 cm^{-1} can be attributed to $\nu_{as}(COO^-)$ vibration and aromatic (C=C) vibration, together with olefinic C=C bands [12,20,34,39,42,43]. The ν(C=O) motion of carboxyl groups, appearing at 1708 cm^{-1}, is only a shoulder in

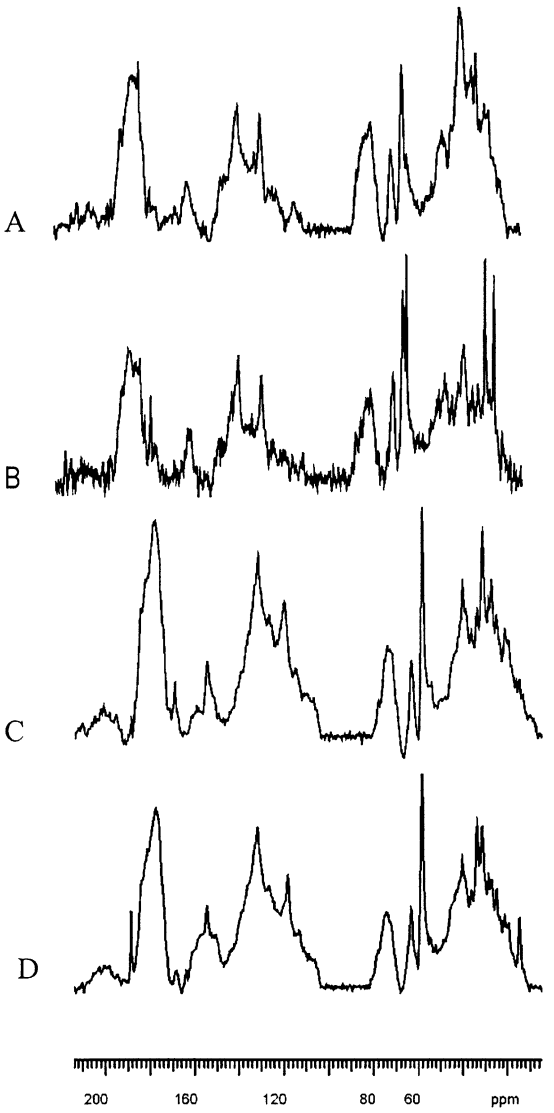

Fig. 2. ^{13}C NMR spectra of the three H-NMW HA fractions: (A) $HA_{>300}$; (B) $HA_{100-300}$; (C) HA_{50-100}; and (D) HA_{20-50} corresponds to L-NMW fraction.

the L-NMW (Fig. 1(E) and (F)). The region between 1450 and 1370 cm^{-1} is also related to the aliphatic groups: the shoulder near 1450 cm^{-1} is attributed to $\delta_{as}(CH_2)$ motions [41], while the band at 1407 cm^{-1} corresponds to $\delta(CH_2)$ and $\nu_s(COO^-)$ symmetric stretching motion [1,20,41,43]. The band appearing near 1370 cm^{-1} can be attributed to the $\delta_s(CH_3)$, and also to $\nu_s(COO^-)$ in dissociated acids. The relative enhancement of this band in L-NMW fractions could also be related to an increase in carboxylate moieties in these fractions. The bands appearing in the 1277–1260 cm^{-1} region can be attributed to ν(C–O) vibrations of alcohols, phenols and carboxyl groups. The relative intensity of these bands markedly increases in the L-NMW fraction (Fig. 1(F)). The bands appearing at 1100–1000 cm^{-1} are attributed to C–O stretching of carbohydrates and alcohols [40,41], as well as to C–C stretching motions of aliphatic groups, and in-plane C–H bending of aromatic rings. The relative intensity of these bands is progressively enhanced as the NMW of the fraction becomes lower. The band centered at 810 cm^{-1} can be attributed to the γ(CH) of aromatic rings (*p*-substituted) [20] and to ν(C–C) in aliphatic moieties [41].

3.3. ^{13}C NMR spectroscopy

The ^{13}C NMR spectra of four NMW HA fractions are shown in Fig. 2. The assignment of spectra were based on the reports of Newman and Tate [15,16,44–49]. The spectra show broad resonances in the aliphatic (0–105 ppm), aromatic (105–165 ppm), and carboxyl (165–190 ppm) regions. In particular, the intense resonances between 16 and 32 ppm are characteristic of methyl and methylene groups in linear alkyl chains, respectively [16,50]. These resonances undergo a clear intensity decrease in the L-NMW fraction (Fig. 2(D)). However, the resonance at 40 ppm that could include contributions from branched alkyl groups or amino acids [51] undergoes an intensity increase in the L-NMW fraction indicating the presence of a greater number of branched aliphatic chains in this fraction.

The region between 40 and 80 ppm is characterized by aliphatic C-substituted by N and O. The sharp peak at 58 ppm and the broad peak at 62 ppm can be assigned to O–CH_3 and O–CH_2 moieties, respectively, as well as to carbohydrates and α-C of amino acids. The O–CH_3 peak shows a marked intensity in the L-NMW.

Carbohydrate C gives rise to the broad signal between 70 and 80 ppm [51–53]. The intensity of this signal seems to decrease in the L-NMW fractions.

The aromatic region corresponds to the signals appearing in the 100–150 ppm range. It can be divided into two parts: (i) 100–130 ppm, assigned to protonated C of aromatic rings; and (ii) 130–150 ppm, due to

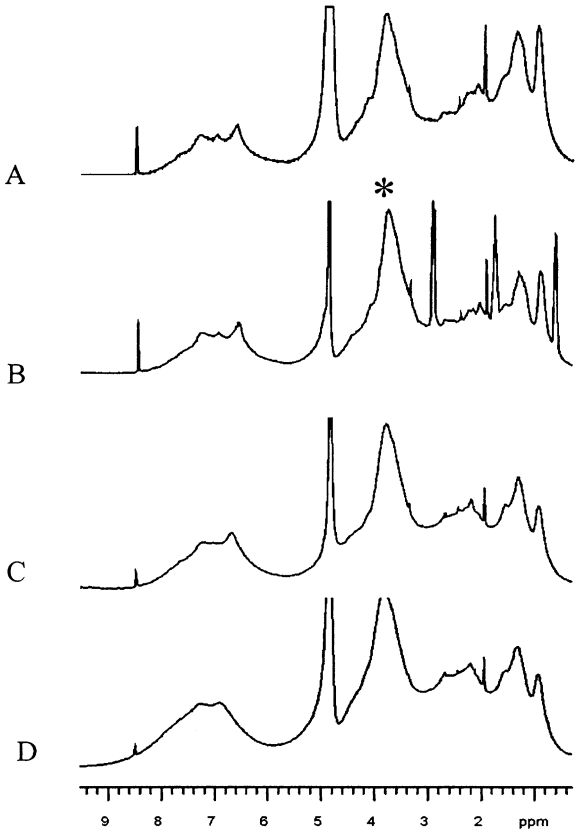

Fig. 3. ^{1}H NMR spectra of three H-NMW HA fractions: (A) $HA_{>300}$; (B) $HA_{100-300}$; (C) HA_{50-100}; and (D) HA_{20-50} corresponds to L-NMW fraction.

quaternary C of aromatic rings [16,53]. The region between 90 and 110 ppm has been also assigned to anomeric carbon of carbohydrates, C-2, C-6 of syringyl units of lignin [17]. The relative intensity of this region appears stronger in the H-NMW fractions suggesting the presence of more syringyl groups in this material. The two intense signals at 122 and 132 ppm have been assigned to protonated C in aromatic rings alkyl substituted or with N or O electron donor groups [45,51,54].

The peak between 150 and 160 ppm indicates the presence of N- or O-substituted aromatic quaternary carbons, and the relative intensity increases in the HA_{20-50} fraction. The broad signal between 170 and 185 ppm can be assigned to quaternary C in amide, ester or carboxyl groups [13,55]. However, resonances at 170, 178, 180 ppm may be assigned to phenolic C close to quinonoid groups [51]. Furthermore, the sharp resonance at 188 ppm that may be due to quinone moieties seems to be more intense in the HA_{20-50} fraction.

3.4. Effect of HCl treatment

The presence of metal ions, free or complexed in functional groups of HA, can cause a broadening of NMR resonances. Among the H-NMW fractions treated with HCl, only the $HA_{100-300}$ spectrum is discussed here, because it displays weaker signals in all of the spectra due to the high Fe(III) content. After treatment with HCl, the Fe(III) content decreased and the signal-to-noise ratio in the spectrum improved. The main effect observed on the $HA_{100-300}$ spectrum was the decrease in the resonances at 18, 22, 56 ppm assigned to aliphatic moieties and that at 172 ppm is due to the quaternary phenolic C.

We suggest that the peaks assigned to aliphatic moieties are LMW compounds, such as amino acids [51,56], monosaccharides or aliphatic acids, which could be removed with metal ions during the treatment [45]. The absence of major changes in the spectra suggests that this treatment could improve the signal-to-noise ratios of the NMR spectra.

3.5. ^{1}H NMR spectroscopy

Spectra of four NMW fractions are shown in Fig. 3. The assignment of spectra were based on the reports of Wilson et al. [57]; Thorn et al. [15]; Preston [16] and Francioso et al. [2]. On the basis of a qualitative analysis, the spectra are subdivided into three main resonance regions: (i) 0.8–3.0 ppm; (ii) 3.0–4.5 ppm; and (iii) 6.0–8.5 ppm. In the first region, the peaks are commonly assigned to aliphatic protons, and those in the range of 2.0–2.8 ppm are attributed to aliphatic groups linked to electronegative atoms (O or N). The discrete, narrow peaks emerging from broad signals at 1.93, 2.43, 3.36 and 8.40 ppm are attributed to acidic compounds, such as acetate, succinate, methanol and formate [16,57]. In particular, the resonances centered at 0.9 and 1.3 ppm can be assigned to protons of methyl groups of highly branched aliphatic structures and terminal methyl groups of methylene chains, respectively [58]. An additional difference in the region is the resonance at 0.9 ppm that decreases in the HA_{20-50} fraction. Signals at 1.3 ppm have also been attributed by Wilson et al. [57] to hydrogens,

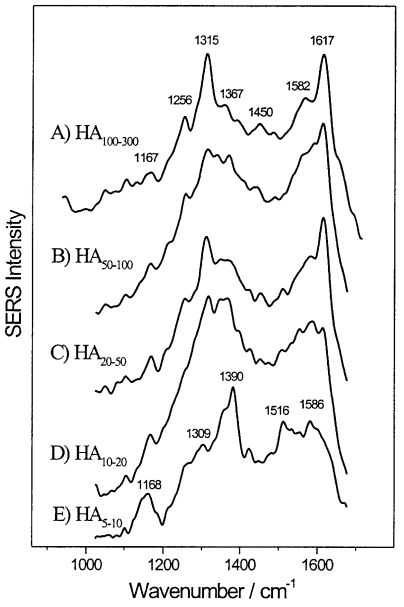

Fig. 4. SERS spectra of NMW fraction HA fractions: (A) $HA_{100-300}$; (B) HA_{50-100}; (C) HA_{20-50}; (D) HA_{10-20} and (E) HA_{5-10}.

which are β- or γ-attached to benzene rings, respectively. The peaks appearing in the 1.8–2.5 ppm range are attributed to protons on C atoms adjacent to functional groups, such as carbonyl, carboxyl, aromatic rings or unsaturated groups [47,58,59].

The second region corresponds to a broad resonance assigned to protons on the α-C relative to an O atom, which are generally assigned to sugar-like components, polyether materials or methoxy groups [58]. This region is prominent in the HA_{20-50} spectrum (Fig. 3(D)). Finally, the signals in the 6.0–8.5 ppm range can be attributed to the presence of both heterocyclic or highly substituted aromatic ring hydrogens, and seem to increase in the HA_{20-50} spectrum (Fig. 3(D)).

3.6. Surface-enhanced Raman spectroscopy

The SERS spectra of the different HA fractions are shown in Fig. 4. The existence of two strong bands at 1617 and 1315 cm^{-1}, can be attributed to benzene derivatives with few substituents [30,60]. This result indicates a high content of poorly substituted aromatic groups, mainly in the H-NMW, in agreement with the DRIFT and NMR spectra (Figs. 1 and 2). In fact, we

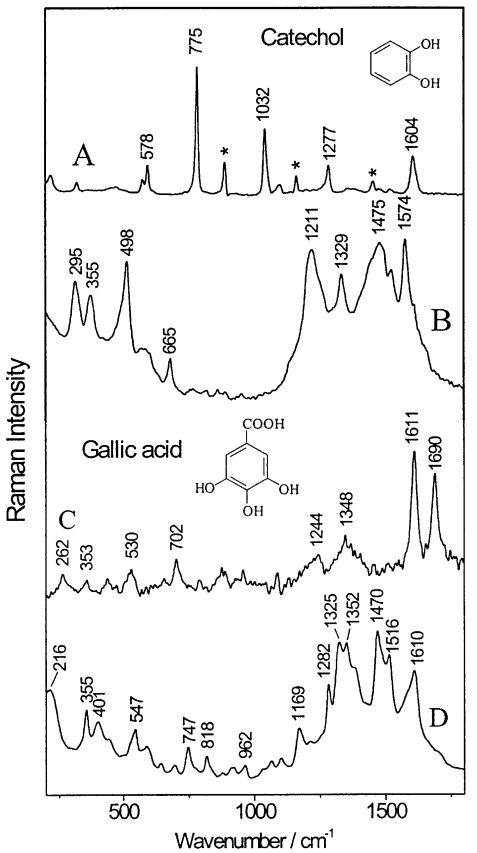

Fig. 5. FT-Raman of catechol (A) dissolved in ethanol (0.05 M), after subtracting the ethanol spectrum (asterisks represent the still ethanol bands remaining after the subtraction); (B) FT-SERS of catechol (5×10^{-3} M) adsorbed on Ag colloid (pH 6.0); (C) FT-Raman of gallic acid (0.06 M) in ethanol solution after subtracting the ethanol spectrum; and (D) FT-SERS spectrum of gallic acid (5×10^{-3} M) on Ag colloid (pH 3.9).

have found bands at 1604 and 1610 cm^{-1} in the Raman spectra of catechol and gallic acid, respectively, which can be related to that at 1615 cm^{-1} appearing in the HA SERS spectra (Fig. 5). The intensity of these two bands decreased in L-NMW fractions, thus indicating an increase in the substitution degree in the aromatic rings in L-NMW. This change is accompanied by an intensity increase in bands at 1168, 1390, 1430, 1516 and 1586 cm^{-1} that are more evident in the SERS spectrum of HA_{5-10} fraction (Fig. 4(A)). Some of these bands are attributed to highly substituted aromatic groups.

Catechol can be used as a model molecule for

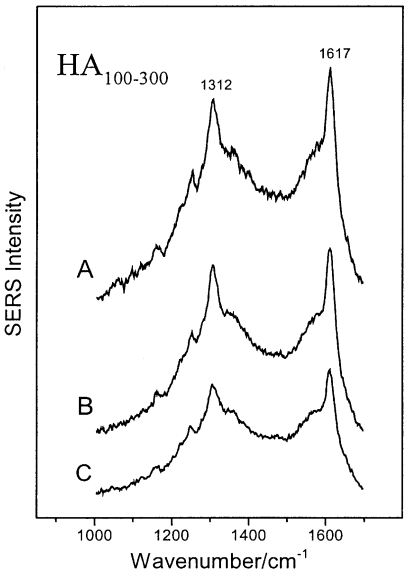

Fig. 6. SERS spectra of $HA_{100-300}$ fraction extracted with (A) 0.1 M NaOH solution and then purified with 0.5 M HCl; (B) 0.1 M NaOH and (C) 0.1 M NaOH + 0.1 M $Na_4P_2O_7$.

studying the SERS of HA. This molecule is able to polymerize, when adsorbed on metal surfaces, giving rise to polymers that may resemble the HS. The SERS spectrum of catechol (Fig. 5(B)) reveals the existence of polymeric benzene rings (corresponding to the bands at 1574, 1475 and 498 cm^{-1}), with ether and phenolic substituents, which give rise to the bands appearing at 1211 and 1329 cm^{-1}, respectively [61].

As in the case of catechol, the SERS spectrum of gallic acid (Fig. 5(D)) shows spectral changes in relation to the spectrum in aqueous solution (Fig. 5(C)), this also suggests that polymerization on the silver surface produces polyphenols similar to those produced by the catechol polymerization. The presence of a band at 1379 cm^{-1} attributed to a $\nu_s(COO^-)$ motion indicates that these polyphenols contain carboxylate groups. Many bands appearing in the HS fraction spectra can also be correlated to the SERS of catechol and gallic acid. The bands seen at 1516, 1309 and 1168 cm^{-1} in the SERS spectrum of HA_{5-10} can be correlated to bands appearing in the SERS of catechol and gallic acid at approximately the same wavenumbers. This suggests the existence of similar polyphenols in this fraction. In addition, the band at 1390 cm^{-1} can be assigned to asymmetric stretching vibrations of carboxylate moieties, which are much more frequent in the HA_{5-10} fraction, as the high intensity of this band indicates. The band at 1586 cm^{-1}, attributed to in-plane vibrations of highly substituted phenols, increases markedly on decreasing the NMW of the fraction. As in the case of catechol and gallic acid, this may indicate the existence of polyphenols with a large number of substituents.

3.7. Extraction method effect on the HA conformation

Figs. 6 and 7 show the SERS spectra of two fractions, $HA_{100-300}$ and HA_{20-50}, obtained after extraction with 0.1 M NaOH + 0.1 M $Na_4P_2O_7$ (Fig. 6(C)), NaOH (Fig. 6(B)), and NaOH after treatment with HCl (Fig. 6(A)). The extraction procedure with NaOH + $Na_4P_2O_7$ has been previously described by Francioso et al. [12]. The HA_{20-50} fraction showed a higher sensitivity to the extraction procedure (Fig. 7). In fact, the bands at 1617 and 1315 cm^{-1} are strongly enhanced either after extraction with NaOH (Fig. 7(B)) or after treatment with HCl (Fig. 7(A)). In the case of extraction with NaOH + $Na_4P_2O_7$ (Fig. 7(C)) prominent broad bands at 1385 and 1572 cm^{-1} were observed.

The spectral profile of the $HA_{100-300}$ fraction does

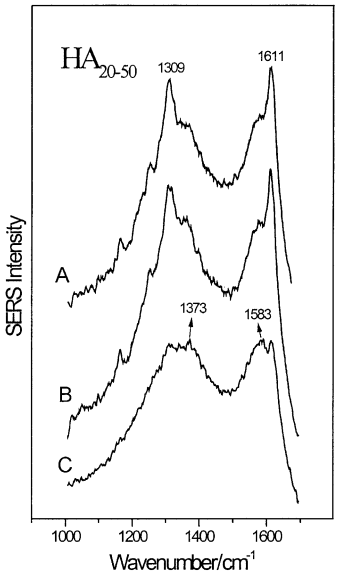

Fig. 7. SERS spectra of HA_{20-50} fraction extracted with (A) 0.1 M NaOH solution and then purified with 0.5 M HCl; (B) 0.1 M NaOH and (C) 0.1 M NaOH + 0.1 M $Na_4P_2O_7$.

not show any significant change, when different alkaline extractants are used. However, the relative intensity increases, going from NaOH + $Na_4P_2O_7$ to that treated with HCl and NaOH (Fig. 6(A)). This result must be considered with care, since the absolute SERS intensity can change because of a different aggregation pattern induced by nitrate on the metal colloid.

In order to understand the effect of alkaline extractants on the SERS spectra of the fractions discussed earlier, it is necessary to consider the conformational structure sensitivity of the short-range character of SERS [28,62]. While the alkaline extractants have a negligible influence on the conformation of the $HA_{100–300}$ fraction, the effect on the $HA_{20–50}$ fraction is more important. We suggest that extraction with NaOH or later purification with HCl might lead to a more open structure. This is demonstrated by the appearance of intense bands at 1617 and 1315 cm^{-1} that are believed to be due to internal groups [30]. This result can be related to the metal content of this fraction. In particular, the concentrations of Cu, Zn, Mn and Mg were lower in the $HA_{20–50}$ extracted with NaOH and in that fraction after treatment with HCl (<50%) compared to the same fraction extracted with NaOH + $Na_4P_2O_7$, although the Fe content is about five-times higher (Table 1). The dramatic reduction in the content of these cations leads to a more open structure.

In a previous study, Sánchez-Cortés et al. [30] demonstrated that the $HA_{20–50}$ fraction extracted with NaOH + $Na_4P_2O_7$ leads to relatively tight structures. Therefore, high alkaline pH values were necessary to open the HA structure and allow the internal aromatic groups to approach the metal surface. The bands corresponding to internal aromatic groups appeared even at neutral pH in the same fraction, extracted with NaOH (Fig. 7(A)) and treated with HCl (Fig. 7(B)). For the L-NMW fraction, the influence of metal content on the structure is very strong. This could also occur in the environment, where the higher or lower amount of metals can dramatically modify the structure of L-NMW. It is worth noting that no significant structural modification was observed in the H-NMW fractions after HCl treatment.

4. Conclusions

Chemical and spectroscopic (DRIFT, SERS and NMR) investigation of a peat HA fractionated into NMW fractions by tangential UF showed significant changes in chemical and structural composition between fractions with H-NMW and L-NMW. The aliphatic character was found to be more concentrated in H-NMW fractions, where chains of saturated hydrocarbons were prevalent. The aliphatic composition in the L-NMW fraction, however, was mainly constituted by branched chains. The aromatic component in the H-NMW fractions, which has often been considered to be one of the major 'building blocks' of HS, consisted of poorly substituted benzene rings, while electron donating substituents, such as N and O were prevalent in L-NMW fractions. In addition, a greater amount of phenolic and other oxygenated group fractions present in L-NMW might justify the presence of transition metals found in these fractions. The different concentration of metals, such as the Fe, which is higher in the H-NMW, and Cu and Zn, which are more concentrated in the L-NMW, might indicate the existence of different oxygen-containing ligands [34–37].

SERS spectroscopy has provided information regarding the conformational structure and the chemical groups, which are directly involved in humic adsorption on metal colloids and the investigation of the effects of purification treatment on fractions. Differences suggest that H-NMW fractions have more stable structural characteristics, because no significative structural modification was observed. These results were also supported by ^{13}C NMR spectra analysis. The L-NMW fraction showed structural modifications indicating that was strongly affected by the extraction method. We conclude that metals present in the L-NMW fraction are important factors that can dramatically modify the structure of these HS. On the basis of these results, we suggest that a 'milder' treatment might be used to purify the isolate humic material. However, particular care should be taken with L-NMW fractions due to their sensitivity to pH and metal ions. The SERS of catechol and gallic acid are particularly relevant to an understanding of the HA structure. These molecules are able to polymerize on metal surface yielding humic-like polymers.

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