Spectral Characterization of Selected Humic Substances

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Abstract

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Current concern for soil quality has stimulated research on soil organic matter (OM). Humic substances (HS) of different origin were compared applying ultraviolet-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), "steady-state" fluorescence spectroscopy, and ^{13}C nuclear magnetic resonance (^{13}C NMR). Sodium humates samples were isolated from soil (Gleyic Luvisol), compost, and South-Moravian lignite from the mine Mír in Mikulčice. Sodium humates (SH) were extracted by a conventional procedure recommended by the International Humic Substances Society (IHSS). Results showed that the presence of *O*-containing functional groups (carbonyl in aldehydes and ketones, carboxyl in carboxylic acids, ester and ether groups) are in the order of compost > soil > lignohumate > lignite. Further, results of FTIR, fluorescence spectroscopy, and ^{13}C NMR suggested that samples of sodium humates isolated from soil, compost, and lignite were a more polycondensed, oxidized, unsaturated, humified, and aromatic structure. On the other hand, commercial lignohumate (LH) had very simple structural components and wide molecular heterogeneity. Furthermore, a small molecular size and weight, low degree of aromatic polycondensation, low level of conjugated chromophores and fluorophores, and low humification degree were characteristic for commercial LH. It should be noted that the sample of commercial LH was characterized by ^{13}C NMR analysis with a slightly higher value of aromaticity α in comparison with the sample of compost. The application of non-destructive analytical methods such as UV-VIS, FTIR, ^{13}C NMR, and fluorescence spectroscopy help us to provide main characteristics of selected humic substances.

Keywords: ^{13}C NMR spectroscopy; Fourier transform infrared spectroscopy (FTIR); humates; lignohumate; steady-state fluorescence spectroscopy; UV-VIS

Humic substances (HS) are present in many natural materials, such as soil, water, peat, compost, and brown and brown-black coal. They are intimately associated with soil fertility and quality, dynamics of nutrients, pollutants and contaminants, and the global carbon sequestration in soil. They have high complexation potential and are precursors of many carcinogenic compounds, causing their elimination or immobilization in the environment (Plaza *et al*. 2006). Transformation processes of anthropogenic chemicals are not still clearly understood because of wide heterogeneity, spatial arrangement, and chemical composition of soil HS. Humic acids (HAs), the main component of HS, are regarded as highly functionalized carbon-rich biopolymers, micellar or supramolecular substances, and nanotube membrane substances (Hedges 1988; Wershaw 1999; Picollo 2002; Sutton & Sposito 2005; Aristilde & Sposito 2013). According to the biopolymer concept of Hedges (1988) plant residues are subjected to condensation and bio-polymerization reactions producing the HS. Wershaw (1999) and Suton and

Sposito (2005) pay attention to the great amount of small broken decomposed fragments of plant residues which aggregate in solution to form micelles. Tan (2013) distinguished several types of carbon nanotubes, nanofibres, nanotube micelles, and membranes. His scanning electron microscopic investigations are explaining the principles of nanochemistry. He also showed that plant biopolymers are de-polymerized in the process and a lot of nanoparticles are capable of self-assembling and another part in a presence of peptides and other amphiphilic form nanofibres. Nano-size materials are hydrated and have large surface areas, and appear to control soil chemical properties, dissolved inorganic and organic species, moisture retention, pesticide retention, and availability for nutrients (Theng & Yuan 2008; Tan 2013). Observation made by Monreal *et al.* (2010) indicated that HS consist of the carbonaceous network of single strains linking cluster of humic material and minerals. They are in assemblies of peptide amphiphiles, carbohydrates, *N*-heterocyclics, and alkyl-aromatics. Clay fraction contains mostly phenols, lignin, lipids, and fatty acids. Their stability depends strongly on their origin and age. The main functional groups in HAs molecule are carboxylic, phenolic, and alcoholic as well as some other minor groups such as hydroxy, methoxy, and thiol, etc.

Novák *et al.* (2001) and Madronová (2011) gave characterization of alkali humates and HAs isolated from raw materials of the Czech Republic (coal, oxyhumolite, lignite, and peat), which are frequently used in agriculture for soil improvement and remediation. Coal-derived HAs could differ significantly in the mineral components (aluminosilicate) content, water content, and amount of functional groups. Data also showed differences in stability, reactivity, and affinity to water. A higher content of phenolic groups was observed in lignite and peat to compare with coal-derived HA.

The present work focused mainly on the structural characteristics and functional groups content in soil humates. Comparison on the basis of their chemical and optical properties is given between soil humates and humates isolated from the other natural sources. The application of non-destructive analytical spectroscopy methods helps us to provide synergetic data explaining main characteristics of selected terrestrial HS.

MATERIAL AND METHODS

The objects of our study were three different samples of sodium humate (SH) and one sample of commercial lignohumate (LH). Humates were isolated from brown soil (from Ap horizon) of Gleyic Luvisol type (locality Lesonice, Czech Republic), compost from ZERA s.r.o., and South-Moravian lignite from the mine Mír in Mikulčice, Czech Republic by a conventional procedure recommended by the International Humic Substances Society (IHSS) (Schnitzer 1982; Nobili *et al.* 1990). All sodium humates (SH) were prepared from humic acids samples by titration to pH = 7.

UV-VIS spectra were measured by Hitachi U-3900 (Hitachi, Tokyo, Japan) in the wavelength range of 200–900 nm. Absorption coefficients (E_2/E_3, E_4/E_6, and $\Delta \log K$) of humates and lignohumate were calculated from the absorbances of SH and LH in UV-VIS spectral range (Chen *et al.* 1977; Kumada 1987; Peuravuori & Pihlaja 1997).

FTIR spectra of SH and LH were recorded over the range of 4000–400 cm^{-1} on pellets. FTIR spectrophotometer operating with a peak resolution of 4 cm^{-1}, and 128 scans were performed on each acquisition (MacCarthy & Rice 1985).

Fluorescence spectra were recorded in aqueous solutions (Mili-Q water) of 60 mg/l SH and LH after overnight equilibration at room temperature, using Aminco Bowman Series 2 (AB2) luminescence spectrophotometer (Thermo Spectronic, Rochester, USA). Basic (one-dimensional) emission spectra were recorded over the range of 380–600 nm at a constant excitation wavelength of 360 nm. Excitation spectra were recorded over the range of 300–480 nm at a fixed emission wavelength of 520 nm. Synchronous-scan excitation spectra were measured by simultaneously scanning both the excitation and the emission wavelength (from 200 to 600 nm), while maintaining a constant, optimized wavelength difference $\Delta\lambda = \lambda_{em} - \lambda_{ex} = 20$ nm, and for LH $\Delta\lambda = 60$ (Senesi *et al.* 1996; Plaza *et al.* 2006; Pedra *et al.* 2008). The Total Luminescence (TL) spectra were obtained in the form of excitation/emission matrix (EEM) by scanning the wavelength emission over the range of 300–600 nm, also the excitation wavelength was in 5 nm steps from 300 to 600 nm (Alberts & Takács 2004; Palazzo *et al.* 2008; Fernández *et al.* 2009).

Inner filter effect correction method. Inner filter effects need to be corrected since they deplete the fluorescence signal affecting the desired linear relationship between concentration of fluorophore and fluorescence intensity (Larsson *et al.* 2007; Goletz *et al.* 2011).

The fluorescence intensity (I_F) values (in arbitrary units – a.u.) of samples were corrected using the method of Lakowicz (2006). The correction method of Lakowicz uses:

$$F_{corr} = F_{obs} \times 10^{\left[\frac{(A_{em}+A_{ex})}{2}\right]} \quad (1)$$

where:

F_{corr}, F_{obs} – corrected and uncorrected fluorescence intensities

A_{ex}, A_{em} – absorbance values at the current excitation and emission wavelengths

The path of the exciting light is assumed to be equal to the path of the emitted light. Primary inner filter effects are corrected as well as secondary inner filter effects.

^{13}C NMR spectroscopy was performed by Varian INOVA 600 (Varian, Inc., Palo Alto, USA). For experiments 100 mg of isolated HS samples were dissolved in 2.5 ml of 0.5 mol/l NaOH in deuterated water. After 24 h of intensive stirring 0.5 ml of HS sample was put in 5 mm NMR cell. All ^{13}C NMR experiments were run at 23°C on a Varian Unity-INOVA 600 MHz spectrometer using basic one-pulse experiment with the following set of the acquisition parameters: spectrometer frequency 242.803 MHz, relaxation delay 1 s, acquisition time 1.6 s, excitation pulse flip angle 45°, spectral width 50 000 Hz, and a continuous broadband decoupling of the protons. Prior Fourier transformation accumulated data were fitted with exponential function (line broadening 10 Hz). Subdivision of the spectrum was made by the commonly used scheme of Malcolm (1990). The degree of aromaticity of HS (α) was calculated by the procedure of Hatcher *et al.* (1981). Aggregability of HS was assessed according to Beyer *et al.* 1993.

RESULTS AND DISCUSSION

UV-VIS spectroscopy. The values of the different indexes calculated from the UV-VIS spectra (E_2/E_3, E_4/E_6, and Δlog K) of SH and LH samples and elemental composition are presented in Table 1. All these absorption indexes give information about aromaticity of humic substances. E_2/E_3 is the ratio of absorbance at 250 nm to at 365 nm and often used as an indicator for humification and molecular weight of humic substances (Peuravuori & Pihlaja 1997; Duarte *et al.* 2003; Uyguner & Bekbolet 2005; Li *et al.* 2009). The lower values of E_2/E_3 ratio of SH, which were isolated from brown soil, compost, and lignite may be indicative of the presence of structures with higher molecular weight, aromaticity, and humification degree. Calculated value of E_2/E_3 ratio was higher for LH, which shows on "light brown" HA with lower molecular mass and humification degree. E_4/E_6 is the ratio of absorbance at 465 nm to at 665 nm (Chen *et al.* 1977). The value of the E_4/E_6 ratio, the so called index of humification, correlates also with the average molecular weight and size and with the oxygen content of humic materials. The low value of humification index for SH isolated from lignite confirmed the presence of HS with higher molecular weight and humification degree. The higher values of E_4/E_6 ratio of SH (isolated from brown soil and compost) and LH may be indicative of the presence of *O*-containing functional groups (hydroxyl, carbonyl, carboxyl, and ester groups). Optical parameter (Δlog K) recommended by Kumada, which includes the absorbance coefficient E for 1% HS solution at 600 nm ($E_6^{1\%}$) and at 400 nm ($E_4^{1\%}$) and Δlog K = ($\log E_4^{1\%} - \log E_6^{1\%}$) are adopted in this work (Kumada 1987 ; Barančíková *et al.* 1997). The Δlog K of samples shows that the degree of humification decreases significantly in the order: SH_lignite > LH > SH_compost > SH_brown soil. Sodium humate isolated from lignite appears to be characterized by high condensation of the aromatic structure and low in aliphatic chain content. All absorption indexes of SH and LH are in good agreement with results of FTIR, ^{13}C NMR, and fluorescence spectroscopy.

FTIR spectroscopy. The FTIR spectra of SH isolated from the brown soil, compost, and lignite and commercial LH are shown in Figure 1. The main absorption bands and corresponding assignments are summarized in Table 2. All spectra feature common and distinctive absorption bands, with some differences in their relative intensity. The main characteristics

Table 1. Elemental composition (weight %) and absorption coefficients (E_2/E_3, E_4/E_6, and Δlog K) of sodium humates (SH) and commercial lignohumate (LH)

Samples	C	H	N	Ash	E_2/E_3	E_4/E_6	Δlog K
Brown soil	44.57 ± 0.06	5.39 ± 0.04	4.28 ± 0.01	4.6 ± 0.1	2.80 ± 0.02	6.75 ± 0.39	0.94 ± 0.02
Compost	44.71 ± 0.04	4.16 ± 0.07	5.09 ± 0.02	10.31 ± 0.1	2.33 ± 0.02	6.46 ± 0.07	0.85 ± 0
Lignite	55.22	4.75	1.25	22.6	2.32 ± 0.03	4.03 ± 0.39	0.67 ± 0.03
Lignohumate	30.82 ± 0.04	3.08 ± 0.16	0.15 ± 0.01	39.15 ± 0.13	3.38 ± 0.02	5.05 ± 0.11	0.82 ± 0.02

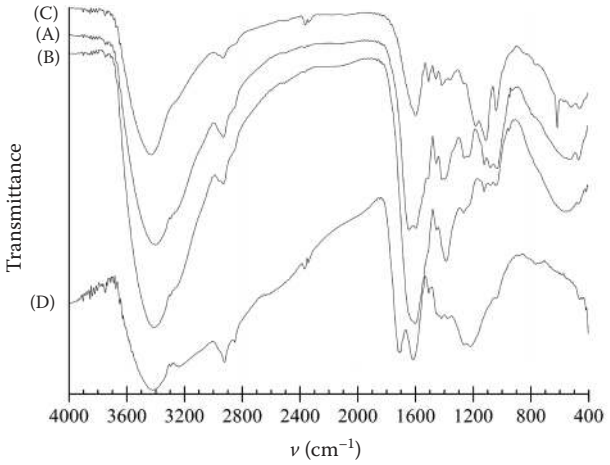

Figure 1. Fourier transform infrared spectroscopy (FTIR) spectra of sodium humates (SH) and commercial lignohumate (LH): (A) SH – brown soil, (B) SH – compost, (C) commercial lignohumate, (D) SH – lignite

of these spectra are the following: about 3400 cm^{-1} (OH stretching and, secondarily, N–H stretching of various functional groups); about 2935–2925 and 2850 cm^{-1} (asymmetric and symmetric C–H stretching or of CH_2 groups); about 1716 cm^{-1} (C=O stretching of COOH and other carbonyl groups), whose relative intensity was determined only for SH from lignite; about 1640–1600 cm^{-1} (aromatic C=C skeletal vibrations, C=O stretching of quinone and amide groups (amide I band), C=O of H-bonded conjugated ketones; about 1512–1508 cm^{-1} (preferentially ascribed to N–H deformation and C=N stretching of amides (amide II band)); about 1458–1454 cm^{-1} (C–H bending of CH_3 groups); about 1419–1416 cm^{-1} (O–H deformation and C–O stretching of phenolic OH); about 1388–1376 cm^{-1} (C–H deformation of CH_2 and CH_3 groups, and/or antisymmetric stretching of COO^- groups); about 1269–1261 cm^{-1} (C=O stretching of aryl esteres); about 1219 cm^{-1} (C–O stretching of aryl ethers and phenols), whose relative intensity was only for SH from lignite; about 1126 cm^{-1} (C–O stretching of secondary alcohols and/or ethers); and, finally, about 1045–1041 cm^{-1} (C–O stretching of polysaccharides or polysaccharide-like substances, and/or Si–O of silicate impurities) (Stevenson 1994; Senesi *et al*. 2003).

The additional band at 1184 cm^{-1} (C–O–C stretching skeletal vibration) might suggest the existence of cellulose residues in the sample of LH (Ertani *et al*. 2011). Further, the band appearing at 660–620 cm^{-1} is assigned to the sulfonic groups (S–O stretching vibration) formed from the reaction of sodium sulfite with the secondary OH of the aliphatic side chain of lignins. In FTIR spectrum of SH from brown soil band at 1032 cm^{-1} was localized which suggests the presence of hardwood lignin residues (Rodríguez-Lucena *et al*. 2009).

Fluorescence spectroscopy. Fluorescence spectroscopy has been used as a technique for classifying and

Table 2. Major Fourier transform infrared spectroscopy (FTIR) absorption bands and assignments for sodium humates (SH) and commercial lignohumate (LH)

Wavenumber (cm^{-1})	Assignment
3400–3300	O–H stretching, N–H stretching (minor), hydrogen-bonded OH
2935–2925, 2850	asymmetric and symmetric C–H stretching of CH_2 group
1725–1710	C=O stretching of COOH
1640–1600	aromatic C=C skeletal vibrations, C=O stretching of amide groups (amide I band), C=O of quinone and/or H-bonded conjugated ketones
1512–1506	N–H deformation and C=N stretching (amide II band), aromatic C=C stretching
1460–1450	C–H asymmetric bending of CH_3 groups
1420–1415	O–H deformation and C–O stretching of phenolic OH
1380	C–H bending of CH_2 and CH_3 groups, COO^- anti-symmetric stretching
1270–1260	C–O stretching of aryl esters
1220	C–O stretching of aryl ethers and phenols
1184	C–O–C stretching (skeletal vibration) of cellulose residues
1130–1110	C–O stretching of secondary alcohols and/or ethers
1045–1035	C–O stretching of polysaccharides or polysaccharides-like substances and/or Si-O of silicate impurities
660–620	S–O stretching vibration sulfonic groups

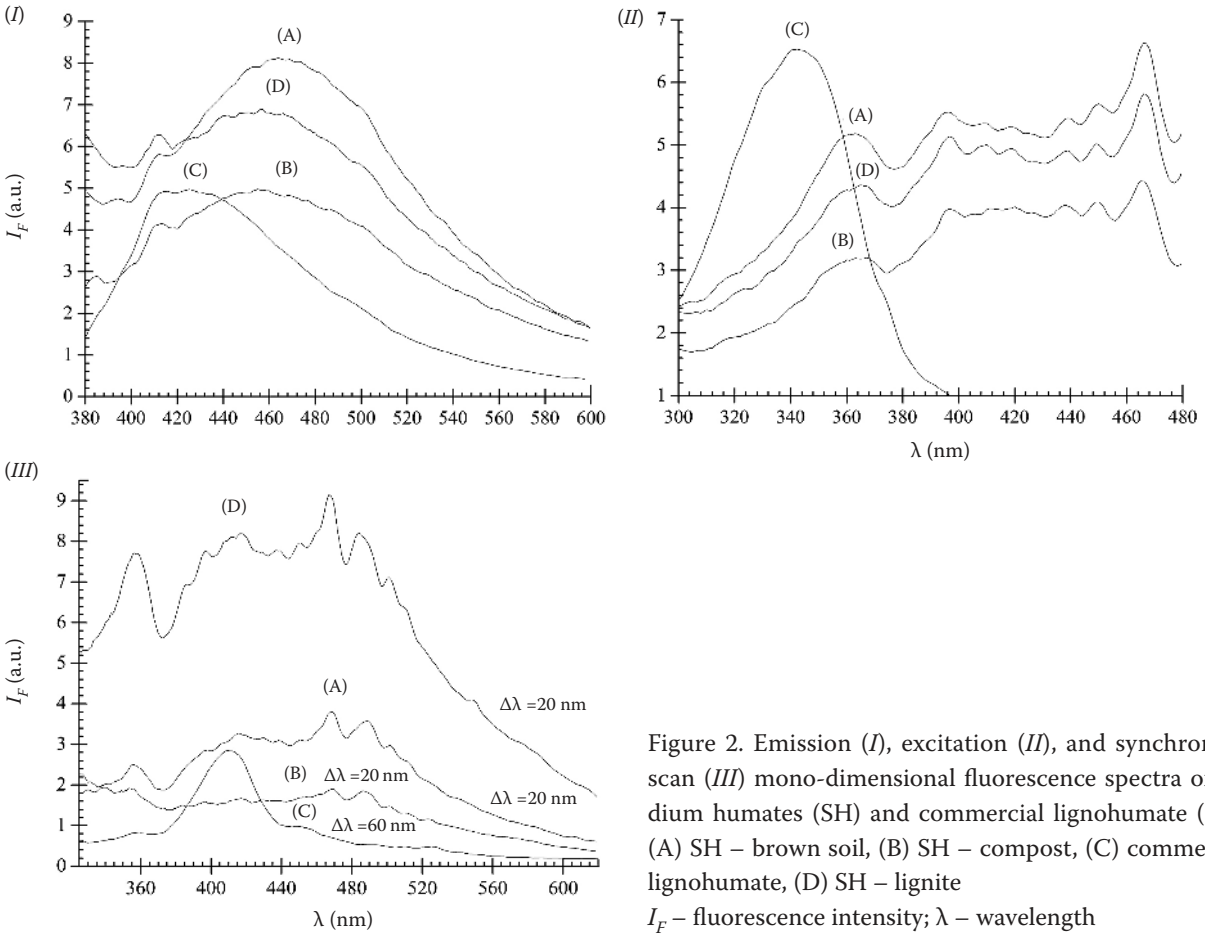

Figure 2. Emission (*I*), excitation (*II*), and synchronous scan (*III*) mono-dimensional fluorescence spectra of sodium humates (SH) and commercial lignohumate (LH): (A) SH – brown soil, (B) SH – compost, (C) commercial lignohumate, (D) SH – lignite
I_F – fluorescence intensity; λ – wavelength

distinguishing between humic substances of different origins and natures (Chen *et al.* 2002). The fluorescence emission spectra of SH and LH are shown in Figure 2. The values of the fluorescence intensity (in arbitrary units – a.u.) and fluorescence maxima are presented in Table 3. The long wavelength for the fluorescence peaks of SH indicate the presence of condensed aromatic ring and other unsaturated bond systems, a high degree of conjugation, and electron-withdrawing groups such carbonyl and carboxyl groups. The short wavelength of the main fluorescence peak of the sample LH suggest the presence of simple structural components of wide molecular heterogeneity and small molecular size, low degree of aromatic polycondensation, low level of conjugated fluorophores, and low humification degree (Senesi 1990; Pedra *et al.* 2008).

The fluorescence excitation spectra of SH and LH are shown in Figure 2. The excitation spectra of SH were characterized by two major peaks at 466–467 and 449–450 nm and two less intense peaks at 395–397 and 360–366 nm. The excitation spectrum of LH featured one intense peak at 341 nm.

The synchronous spectra of SH and LH are shown in Figure 2. The synchronous scan spectra of SH were characterized by two prominent peaks at 467–469 and 487–488 nm and two less intense peaks at 418–419 and 501–502 nm. The LH spectrum exhibited sharp peak at a shorter wavelength around 410 nm and one minor fluorescence maximum at 451 nm. The spectra of SH showed higher intensity and longer wavelength maxima that the LH spectrum, suggesting that SH contains higher amounts of conjugated aromatic π-electron systems with electron-withdrawing functional groups, which are responsible for the fluorescence shift to lower energy levels

Table 3. Emission fluorescence maxima and fluorescence intensity of sodium humates (SH) and commercial lignohumate (LH)

Samples	Main fluorescence maximum (peak)	
	λ_{em} (nm)	I_F (a.u.)
Brown soil	461	8.23
Compost	457	5.11
Lignite	457	6.99
Lignohumate	424	5.07

I_F – fluorescence intensity (arbitrary units); λ_{em} – emission wavelength

or longer wavelengths. Synchronous fluorescence can provide better sensitivity and improved peak resolution compared to the conventional emission fluorescence technique, and possibly allows differentiation of the fluorescence spectra of samples of different origins (Senesi *et al*. 1996; Chen *et al*. 2002, 2003; Fernández *et al*. 2009).

The fluorescence EEM spectra of SH and LH are shown as a contour map in Figure 3. The values of the fluorescence intensity and excitation-emission wavelength pair of the main peaks in the EEM spectra of SH and LH are presented in Table 4. The long wavelength and large fluorescence intensity of the major peak of SH may be ascribed to the presence of an extended, linearly-condensed aromatic ring network, and other unsaturated bond systems capable of a great degree of conjugation in large molecular size and extensively humified "macromolecules". On the contrary, the prevalence of fluorescence bands and peaks with high relative intensity at short wavelengths, such as those measured for the peak of LH, is associated with the presence of simple structural components of wide molecular heterogeneity and small molecular weight, small degree of aromatic condensation, small level of conjugated fluorophores, and small humification degree (Mobed *et al*. 1996; Chen *et al*. 2003; Pedra *et al*. 2008; Fernández *et al*. 2009). The main molecular components that contribute to fluorescence in this range of excitation/emission wavelength may be chromone derivates (excitation/emission wavelength pair 320–346/409–490 nm) and flavones and isoflavones (excitation/emission wavelength pair 313–365/415–475 nm) (Senesi *et al*. 1991; Pedra *et al*. 2008).

^{13}C NMR spectroscopy. Carbon type relative abundance was calculated from signal intensity in given region and results showed that it was connected with samples origin. The values of the carbon distribution and aromaticity degree (α) are presented in Table 5. ^{13}C NMR analysis showed that SH from compost contained the lowest amount of aromatic carbon (106–157 ppm) and had the lowest aromaticity degree (α = 36.6%). Soil humates (locality Lesonice) had higher aromaticity degree (α = 38.8%), more aromatic compounds, middle oxidation ability, and high aggregability (Beyer *et al*. 1993). Also higher content of sp^3 carbon (C–O and C–H) at 87–43 ppm and olephinic groups at 106–143 ppm is evident. Preston (1991,

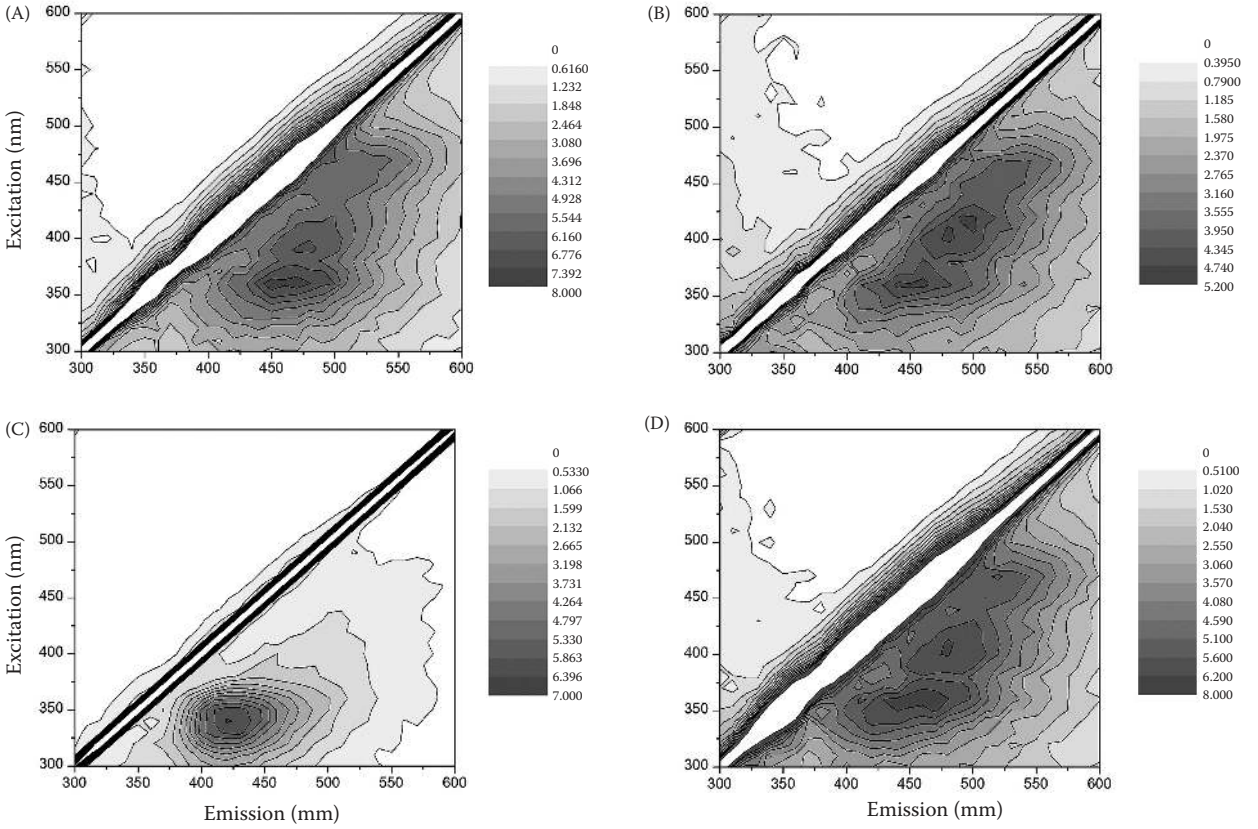

Figure 3. Fluorescence EEM spectra of sodium humates (SH) and commercial lignohumate (LH) isolated from brown soil (A), compost (B), commercial lignohumate (C), lignite (D)

Table 4. Excitation/Emission Wavelength Pair (EEWP) and fluorescence intensity (I_F, arbitrary units) of the main peaks in the excitation/emission matrix (EEM) spectra of sodium humates (SH) and commercial lignohumate (LH)

Samples	1st maximum (peak)		2nd maximum (peak)		3rd maximum (peak)	
	EEWP (nm)	I_F (a.u.)	EEWP (nm)	I_F (a.u.)	EEWP (nm)	I_F (a.u.)
Brown soil	360/450	7.86	390/480	6.99		
Compost	360/460	4.94	410/500	4.88		
Lignite	360/453	6.95	410/480	6.37	465/520	5.98
Lignohumate	340/421	6.69				

Table 5. Aromaticity degree (α) and carbon distribution (^{13}C NMR spectroscopy) of sodium humates (SH) and commercial lignohumate (LH)

Samples	sp^3 C	$C_{alif.}$	$C_{arom.}$	α (%)
Brown soil	38.0	47.0	33.0	38.8
Compost	37.0	44.9	30.0	36.6
Lignite	36.0	48.0	39.0	46.4
Lignohumate	40.4	44.0	34.2	40.0

1996) identified at 15–43 ppm presence of long alkyl groups (–CH_2–). LH had the lowest content of long alkyl groups. The last was also confirmed by FTIR spectroscopy. Aromatic carbon content at 43–106 ppm was in LH similar to soil humates, but lower than in lignite–SH. From this reason high aromaticity degree (α = 40.0%) in LH was determined. Lignite–SH contained the highest amount of aromatic and olephinic carbon. Content of aliphatic carbon was lower and aromaticity degree was high (α = 46.4%). Similar results were published by Lawson and Stewart (1989), Highasi *et al.* (1998), and Barančíková *et al.* (2003). They confirmed that lignite–SH contained more aromatic C=C and C=O groups.

CONCLUSION

Structural and functional properties of three samples of SH (isolated from brown soil, compost, and lignite) and LH were systematically characterized by a range of spectroscopic techniques. Results suggest that the presence of O-containing functional groups (carbonyl in aldehydes and ketones, carboxyl, ester, and ether groups) are in the order of compost–SH > brown soil–SH > lignohumate > lignite–SH. Further, results of the fluorescence and ^{13}C NMR suggest that samples of SH isolated from brown soil, compost, and lignite are a more poly-condensed, oxidized, unsaturated, humified, and aromatic structure. On the contrary, the sample of LH was characterized by the presence of simple structural components of wide molecular heterogeneity and small molecular size and weight, low degree of aromatic poly-condensation, low level of conjugated chromophores and fluorophores, and low humification degree.

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