HUMIC SUBSTANCES FROM TYPICAL CZECH FOREST SOIL HUMIC PODZOL: CHEMICAL AND SPECTROSCOPIC CHARACTERISATION

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ABSTRACT

The aim of this work was to study chemical composition, chemical properties and humification degree of different soil humic substances (HS). Five various samples of humic substances were the object of our study. Humic acids (HAs) and fulvic acid (FA) were isolated from typical Czech forest soil Humic Podzol (locality Krkonoše, Czech Republic). Isolation of soil humic substances was performed according to the procedure recommended by the International Humic Substances Society (IHSS). Samples of soil HS were compared with Elliott Soil humic acid (1S102H) and fulvic acid (2S102F) as standards of soil humic substances. All samples of soil HS were characterized by elemental analysis (EA), total organic carbon analysis (TOC), ultraviolet-visible spectroscopy (UV/Vis), Fourier transform infrared spectroscopy (FTIR), steady-state fluorescence spectroscopy and nuclear magnetic resonance (^{13}C NMR). The elemental composition was determined by a CHNS/O Microanalyser Flash 1112 Carlo Erba. Absorption coefficients (E_{ET}/E_{Bz}, E_{250}/E_{365} and E_{465}/E_{665}) of soil HS were calculated from the absorbance values. Infrared spectroscopy is a useful technique in characterization of structure, functional groups and formation modes of HS. For the fluorescence experiments, the final concentration of the HS was adjusted to 10 mg·L^{-1}. The pH-value of the samples was adjusted to seven using a standard phosphate buffer. Fluorescence mono-dimensional spectra and total luminescence spectra (TLS) of soil HS were obtained using steady-state fluorescence spectroscopy. All fluorescence spectra were performed on a Horiba Scientific Fluorolog. Total luminescence spectra (TLS) were obtained in the form of excitation/emission matrix (EEM) by scanning of emission wavelengths over the range of 300–600 nm and the excitation wavelengths were in 5 nm steps from 240 to 550 nm. Fluorescence index (Milori index and HIX) of HS was calculated from the area of the emission spectra. The fluorescence intensity (I_F) values (in CPS) of samples were corrected using method of Lakowicz. ^{13}C NMR spectra of soil HS were obtained with a Bruker Avance III NMR spectrometer at an observation frequency of 125.8 MHz for ^{13}C. The approximate number of scans was 25.000. Aromaticity (f_a), hydrophilicity and hydrophobicity ratio (Hfi/Hfo) and biological activity (BiA) of HS were calculated from the area of the NMR spectra. Soil A55 HA was characterized high molecular weight, low molecular heterogeneity, high degree of aromatic polycondensation, high level of conjugated fluorophores, and high humification degree. HS isolated from organic horizon were characterized by lower molecular mass, simple structural components of wide molecular heterogeneity, lower degree of aromatic polycondensation, and lower humification degree.

Keywords: soil humic and fulvic acids, fluorescence, ^{13}C NMR spectroscopy, absorption and fluorescence indexes, chemical composition and structure, humification degree, oxygen-containing functional groups

1. Introduction

Humic substances (HS) are a major component of natural organic matter (NOM) and are the dominant products of plant and animal degradation by microbial activity. HS as the main organic constituents of soil and sediments are widely distributed over the Earth's surface, occurring in

almost all terrestrial and aquatic environments. Humic substances are complex mixtures of high to low molecular weight species, so they are polydisperse systems with a specific distribution of molecular weights. From a theoretical viewpoint, a better knowledge of the chemical structure of HS is fundamental in order to more fully understand a great number of natural processes occurring in natural ecosystems, such as the dynamics of different elements, principally micronutrients, the transport of xenobiotics or the development of plants and microorganisms; as well as those question related to the chemical features of HS. According to their solubility, HS are classified into three main fractions: [i] fulvic acids (FAs) are soluble in aqueous media, [ii] humic acids (HAs) are insoluble under acidic conditions and [iii] humins (HU) stay insoluble throughout the whole range of pH-values (Doskočil et al., 2015). The main sources of HAs and FAs are soil and peat, from which they can be extracted by well-known chemical methods. The following spectroscopic techniques are prominent among those used for characterization of HS: UV/Vis spectroscopy, Fourier-transform infrared spectroscopy (FTIR), fluorescence spectroscopy and ^{13}C nuclear magnetic resonance spectroscopy. In addition to those techniques, fluorescence spectroscopy has also been used in the study of HS.

Fluorescence techniques provide important information on the chemical nature of the HS: the position, shift and intensity of fluorescence peaks can be correlated to structural information such as functional groups (electron-donating/withdrawing groups), polycondensation, aromaticity, heterogeneity and dynamic properties related to their intramolecular and intermolecular interactions (Rodríguez et al., 2014).

The object of our study was to investigate the chemical properties and humification degree of HS. For this purpose, elemental analysis (EA), UV/Vis spectroscopy, FTIR spectroscopy, steady-state fluorescence spectroscopy and ^{13}C nuclear magnetic resonance spectroscopy were used.

2. Materials and methods

Five various samples of HS were the object of our study. HA and FA were isolated from typical Czech forest soil Humic Podzol (locality Krkonoše, Czech Republic), by conventional procedure recommended by the International Humic Substances Society (IHSS).

UV/Vis spectra were measured by Hitachi U-3900 in the wavelength range of 200–900 nm. Absorption coefficients (E_{ET}/E_{Bz}, E_{250}/E_{365} and E_{465}/E_{665}) of HS were calculated from the absorbance of HS in UV/Vis spectral range. The FTIR of HS were recorded over the range of 4000–400 cm^{-1} on pellets obtained by pressing under reduced pressure a mixture of 1 mg of samples and 400 mg of dried KBr, spectrometry grade. Fluorescence spectra were recorded in aqueous solutions of 10 $mg \cdot L^{-1}$ HS using FluoroLog luminescence spectrophotometer after overnight equilibration at room temperature. The pH-value of the samples was adjusted to seven using standard phosphate buffer. 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 300–500 nm at a fixed emission wavelength of 520 nm. Total luminescence spectra (TLS) were obtained in the form of excitation/emission matrix (EEM) by scanning of emission wavelengths over the range of 300–600 nm and the excitation wavelengths were in 5 nm steps from 240 to 550 nm (Enev et al., 2014). The following fluorescence coefficients were obtained: [i] *fluorescence index* (FI): ratio of the emission intensity at λ_{Em} 450 nm to that at λ_{Em} 500 nm, following excitation at λ_{Ex} 370 nm. [ii] *Milori index*: Emission spectra were collected over the range of 460–650 nm using an excitation wavelength of 440 nm, and the total area under these spectra was calculated. [iii] *biological/autochthonous index* (BIX): BIX is calculated from the ratio of emission intensities at a shorter (λ_{Em} 380 nm) and longer (λ_{Em} 430 nm) wavelength using a fixed excitation (λ_{Ex} 310 nm). [iv] *Zsolnay index* (HIX): HIX is calculated from the ratio of two integrated regions of an emission scan (sum from λ_{Em} 435–480 nm divided by the sum from λ_{Em} 300–345 nm) using a fixed excitation (λ_{Ex} 254 nm) (Birdwell and Engel, 2010).

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.

There are primary and secondary inner filter effects. Primary inner filter effects originate in absorbance of light with excitation wavelength. Secondary inner filter effects on the other hand originate in absorbance of light with the emission wavelength of the fluorophore.
The fluorescence intensity (I_F) values (in CPS) of samples were corrected using 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} and F_{obs} are the corrected and uncorrected fluorescence intensities and A_{ex} and A_{em} are the absorbance values at the current excitation and emission wavelengths. Primary inner filter effects are corrected as well as secondary inner filter effects.

^{13}C NMR spectra of soil HS were obtained with a Bruker Avance III NMR spectrometer. The approximate number of scans was 25.000. Aromaticity (f_a), hydrophilicity and hydrophobicity ratio (Hfi/Hfo) and biological activity (BiA) of HS were calculated from the area of the NMR spectra.

3. Results and discussion

3.1. Elemental analysis, UV/Vis and FTIR spectroscopy

The values of the different absorption indexes were calculated from the UV/Vis spectra (E_{ET}/E_{Bz}, E_2/E_3 and E_4/E_6) of soil HS and standards HS which they are presented in Table 1 (including elemental composition). The higher value of E_{ET}/E_{Bz} ratio of HAs may be indicative of the presence of O-containing functional groups (hydroxyl, carbonyl, carboxyl, ester and ether groups). Thus, lower E_{ET}/E_{Bz} ratio of FAs can be associated with scarce substitution on the aromatic ring or with the substitution of aliphatic functional groups.

Absorption coefficients E_2/E_3 and E_4/E_6 give information about aromaticity of HS. The lower value of E_2/E_3 ratio of HAs may be indicative of the presence of structures with higher molecular weight, aromaticity and humification degree. E_4/E_6 is the ratio of absorbance at 465 nm to at 665 nm. 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 lower value of humification index for HAs confirmed the presence of HS with higher molecular weight and humification degree. The higher value of E_4/E_6 ratio of FAs may be indicative of the presence simple aromatic structures with higher degree of substitution with oxygen containing functional groups. All absorption coefficients of HS are in good agreement with results of FTIR, steady-state fluorescence spectroscopy, ^{13}C NMR.

Table 1. Elemental composition (weight %) and absorption indexes (E_{ET}/E_{Bz}, E_2/E_3 and E_4/E_6) of HS

sample	C	H	N	S	O	E_{ET}/E_{Bz}	E_2/E_3	E_4/E_6
A55 HA	42.91	4.30	4.00	–	30.52	0.76	3.27	5.72
A15w HA	55.80	5.36	1.93	–	35.55	0.48	3.61	8.86
A15w FA	50.49	4.71	0.89	–	37.96	0.55	3.75	12.22
IHSS HA	58.13	3.68	4.14	0.44	34.08	0.83	2.33	3.49
IHSS FA	50.12	4.28	3.75	0.89	42.61	0.67	4.96	12.83

All spectra feature common and distinctive absorption bands, with some differences in their relative intensity. The main characteristics of these spectra are the following: about 3400–3300 cm^{-1} (O–H stretching and, secondarily, N–H stretching of various functional groups); about 2935–2925 cm^{-1} (asymmetric C–H stretching or of CH_2 groups); about 1720–1710 cm^{-1} (C=O stretching of COOH), whose higher relative intensity was determined for FAs; 1620–1600 cm^{-1} (aromatic C=C skeletal vibrations, C=O of strongly H-bonded conjugated ketones, whose higher intensity was determined for HAs; about ≈1510 cm^{-1} (preferentially ascribed to simple aromatic C=C vibrations, N–H deformation and, C=N stretching of amides); about 1420 cm^{-1} (O–H deformation and C–O stretching of phenolic OH); about ≈1380 cm^{-1} (C–H deformation of CH_2

and CH_3 groups, and/or asymmetric stretching of COO^- groups); about 1270–1260 cm^{-1} (C=O stretching of aryl esters), whose higher intensity was detected for HAs; about 1220 cm^{-1} (C–O stretching of aryl ethers and phenols); 1130–1080 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).

3.2. Steady-state fluorescence spectroscopy and ^{13}C NMR

The values of the fluorescence intensity and excitation-emission wavelength pair of the main peaks in the EEM spectra, including fluorescence coefficients of HS, are presented in Table 2. The fluorescence EEM spectrum of A15w HA is characteristic with two unique fluorophores centered at an excitation/emission wavelength pair (EEWP) of 275/425 nm (*peak A*) and 380/450 nm (*peak C*). The fluorescence EEM spectrum of A55 HA is characteristic with three unique fluorophores centered at an excitation/emission wavelength pair (EEWP) of 270/500 nm (*peak A*), 360/500 nm (*peak C_1*) and 445/510 nm (*peak C_2*). The long wavelength and less fluorescence intensity of the major peaks of HAs 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". The fluorescence EEM spectrum of A15w FA is located by three fluorescence maxima at an excitation/emission wavelength pair of 250/430 nm (*fulvic-like*), 310/430 nm (*humic-like*) and 270/310 nm (*tyrosine-like*) which are typical for terrestrial origin. On the contrary, the prevalence of fluorescence bands and peaks with high relative intensity at short wavelengths, such as those measured for the peaks of FAs, 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.

The values of the different indexes calculated from the emission spectra (FI, BIX, Milori index and HIX) are presented in Table 2. The values of FI and BIX of soil HS are typical for terrestrial origin and autochthonous sources. The higher values of Milori index and HIX of HAs may be indicative of greater humification degree. Thus, values of FI, BIX, Milori index and HIX of HS are in agreement with previous results.

Table 2. Position of excitation-emission wavelength pair of the main peaks in the EEM spectra and values of fluorescence intensity ($\times 10^6$) these fluorescence peaks of HS and fluorescence coefficients (FI, BIX, Milori index and HIX)

sample	peak fulvic-like		peak humic-like		peak tyrosine-like		FI	BIX	Milori index ($\times 10^8$)	HIX
	EEWP (nm)	I_F (CPS)	EEWP (nm)	I_F (CPS)	EEWP (nm)	I_F (CPS)				
A55 HA	270/500	6.4	360/500 445/510	2.2 1.6	–	–	0.57	0.40	3.21	11.35
A15w HA	275/425	3.9	380/450	1.0	–	–	0.90	0.75	0.58	1.94
A15w FA	250/430	7.0	310/430	4.8	270/310	4.6	1.06	0.46	0.88	2.23
IHSS HA	270/520	7.0	440/530	2.4	–	–	0.65	0.35	3.38	21.48
IHSS FA	250/430	18.0	310/430	10.0	–	–	1.03	0.49	1.99	16.42

The values of f_a, Hfi/Hfo and BiA of forest soil HS are typical for terrestrial origin and autochthonous sources. The higher values of f_a and BiA of all HAs and IHSS FA may be indicative of greater humification degree and biological activity.

4. Conclusions

Our results showed that the chemical properties of the different HS used in these experiments were well described using seven complementary indexes derived from the ultraviolet-visible and fluorescence spectra (E_{ET}/E_{Bz} ratio, E_{250}/E_{365} ratio, E_{465}/E_{665} ratio, FI, BIX, Zsolnay, Milori index, f_a, Hfi/Hfo and BiA). Soil A55 HA was characterized high molecular weight, low molecular heterogeneity, high degree of aromatic polycondensation, high level of conjugated fluorophores, and high humification degree. HS isolated from organic horizon were characterized lower molecular mass, simple structural components of wide molecular heterogeneity, lower degree of aromatic polycondensation, and lower humification degree. The fluorescence EEM spectrum of A15w FA was located by unique one fluorescence maximum at an excitation/emission wavelength pair of 270/310 nm (*tyrosine-like*) which are typical biological or microbial origin.

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