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A COMPARATIVE STUDY OF SOUTH MORAVIAN LIGNITE AND STANDARD IHSS HUMIC ACIDS´ OPTICAL AND COLLOIDAL PROPERTIES

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Abstract
Optical and colloidal properties of humic acids isolated from South Moravian lignite and IHSS standards were assessed by emission, excitation and synchronous fluorescence spectroscopy, UV-VIS spectroscopy and by means of zeta potential measurement. Obtained results indicated a high aromaticity of lignite humic acids which causes a strong inner filter effect in fluorescence measurement. As a result, lignite sample showed the lowest fluorescence intensity. The comparison of obtained records as well as humication indexes calculated from UV and fluorescence spectra did not show any correlation with the elemental composition or zeta potential values of humic acids under study. The results indicate that the optical and colloidal properties of humic acids of different origin can not be explained on the base of simple molecular composition. Therefore, also the nature of secondary structure must be taken into consideration.

Key words: *lignite humic acids, synchronous fluorescence spectroscopy, humification index, zeta potential.*

1. Introduction

Humic substances (HS) are ubiquitous in almost all terrestrial and water sources. They represent a highly heterogeneous mixture of organic materials developed as a result of biotic or abiotic (or both) degradations of dead plant tissues and animal bodies. They play a principal role in many important environmental processes such as formation and stabilization of soil aggregates, in the process of binding organic chemicals and pollutants and at plants growth (drawing off nutrients from soils) [1,2]. Traditionally, HS are divided into three main groups according to their solubility: the humic acids (HAs), fulvic acids (FAs) and humins. The structure of HS has been discussed from different viewpoints including molecular conformation, molecular aggregation, macromolecularity, supramolecular characteristics, domain mobility, and many others. Nowadays, two main views of HS character are discussed: polymeric and supramolecular theory. The former describes HS as a system of polymers with a high coefficient of polydispersity [3] whereas the latter as a random assemblies of relatively small molecules holding together via weak interactions and exhibiting apparently high molecular weight [4,5].

The unique molecular structure, interactions and conformation properties of HS are and for a very long time have been studied by spectroscopic methods [6-23]. Light absorption by these substances increases exponentially with decreasing wavelength across the visible and ultraviolet spectrum and also fluorescence is a generally known phenomenon in HS [23]. The fluorescent intensity of HAs and FAs increase with decreasing apparent molecular size [24], changes with different humification degree [25] and significantly differs in HS of different origins [26]. Accordingly, fluorescence indexes, e.g. the ratio of main peaks in the fluorescence spectra or the ratio of excitation and emission peaks served for determination of degree of humification and a high correlation coefficient with elemental analysis and mainly total carbon content has been obtained [14, 21].

An attempt was also made to explain the spectroscopic behavior of HS on the base of their chemical composition. The fluorophores in HA are thought to contain substituted and condensed aromatic structures [27]. Cory and McKnight [28] stated that thirteen components (seven quinone-like, two

amino acid-like and four not yet classified groups of molecules) are responsible for the HS fluorescence. Peuravuori et al. divided fluorescence spectrum into several regions according to certain wavelengths and assumed that certain polycyclic contributors are responsible for humic fluorescence properties [19]. Further, it is thought that in the structure of HAs derivatives of coumarine are present [29] since emission peaks of such derivatives have been found in the region 400 to 550 nm for excitation at 340 nm [30]. Other possible fluorophores could be quinone, phenols and perylene [14].

In the contrary, it has been demonstrated [31] that optical properties of humic substances are the result of intramolecular charge-transfer interactions between hydroxy-aromatic donors and quinoid acceptors and therefore the absorption and emission spectra obtained by spectroscopic methods cannot result solely from a simple superposition of the spectra of numerous independent chromophores. Moreover, up to now there is no convincing evidence for condensed aromatic components in the humic structure [5,29]. Thus, despite the number of excellent works, the origin of fluorescence of humic molecules remains still not completely solved problem.

Currently, in contrast to common fluorescence techniques, the synchronous fluorescence spectroscopy (SFS) gains steadily interest in HS research [14, 19-22, 26]. In principle, a constant difference of wavelengths between the excitation (λ_{ex}) and emission (λ_{em}) monochromator is used. Thus, synchronous measurement is performed in accordance with the condition $\Delta\lambda=\lambda_{em.}-\lambda_{ex}$. For humic acids $\Delta\lambda$ is usually reported either 18 or 20 nm. It has been stated, that higher values of $\Delta\lambda$ caused the increase of relative intensity of fluorescence at lower wavelengths and vice versa. SFS has been successfully employed for characterization of mixtures giving a better resolution of spectral peaks in comparison with common excitation and emission techniques [19].

The aim of this work was to study the South Moravian lignite HAs by means of fluorescence spectroscopy and other additional methods as UV-VIS spectroscopy and zeta potential measurement. The effort was made to find the appropriate measuring conditions since literature data provide rather scattered information. Further, optical and colloidal properties of the lignite humic acids were compared with properties of humic acids supplied by the International Humic Substances Society (IHSS) and it was checked if correlations obtained in previously published papers are consistent with those obtained for the South Moravian lignite humic acids.

2. Material and methods

Humic acids

Humic acid (HA1) was isolated from the South Moravian lignite (mine Mikulčice, Czech republic) and purified by common procedures as described elsewhere [32]. In brief, original material was shaken for 24 hours under nitrogen atmosphere in 0.5 M-NaOH and 0.1 M-$Na_4P_2O_7$ (60 g lignite: 2000 mL of extraction agents) in plastic flasks overnight. The HA1 was precipitated from alkaline extract by adding 6M HCl until pH 2 and treated with a 0.5% (v/v) HCl-HF solution for 24 hours, dialyzed (Spectrapore 3, 3500 Mw cutoff) against distilled water until chloride free and freeze-dried. Details on obtained humic acid are given in[33].

Other humic acid samples were purchased from the IHSS (International Humic Substances Society): lignite (LHA), and Elliot soil humic acid standard (Elliot soil) and Suwanee River humic acid standard.

All the humic samples were suspended in distilled water and titrated by 0.5 M NaOH to pH 7 to dissolve the solid HA and to form soluble humates. Obtained solutions were freeze-dried, homogenized and stored. For the purpose of analysis water soluble humate salts were dissolved in Mili Q water.

Fluorescence, zeta potential, UV/VIS and elemental analysis

An Aminco Bowman spectrofluorimeter, Series 2 equipped with Xenon lamp was employed to acquire excitation, emission and two-dimensional SFS. Excitation spectra were measured in the interval from 300 to 480 nm with the emission at 528 nm and emission spectra from 480 to 600 nm at excitation 468 n. Synchronous fluorescence spectra were scanned from 300 to 600 nm at various $\Delta\lambda$ differences from 12 to 80 nm. Bandpass of both monochromators was set to 4 nm. Spectral resolution of Aminco spectrofluorimeter is 1 nm. Sensitivity of all samples was set to 70 %. Scan speed was set to 60 nm per min. Fluorescence measurements were recorded and assessed by AB2 program. The correction of fluorescence records were carried out using the same voltage on the detector. UV/VIS spectra were acquired by the spectrophotometer Varian 50 Probe. Samples were measured in Spectrosil cuvett (δ=1 cm). UV/VIS limiting resolution was less than 1.5 nm.

Zeta potential measurement was carried out by means of ZetaSizer 3500, Malvern in a glass cell, by the method of the laser Doppler micro electrophoresis. Standard deviation never exceeded of 2 mV.

The elemental analysis of HA was performed on Carlo Erba analyzer, Flash 1112. Carbon content was determined as CO_2, nitrogen content as NO_2, hydrogen content as H_2O and oxygen content was calculated up to 100 %. Ash content was calculated from the weight of a sample after burning at 700°C for 3 hours. Results of elemetal analysis are summarized in Table 1.

Table 1. Ash-free elemental analysis of humic samples, ash content, fluorescence and absorbance indexes, zeta potential values.

HA	C (%)	O (%)	N (%)	H (%)	C/H	Ash (%)	(I_5/I_4)	E_4/E_6	A_{265}	ζ [mV]
LHA*	63.8	32.0	1.2	3.7	17.2	2.6	0.80	4.8	3.41	- 43.3
Elliot s.*	58.1	34.1	4.1	3.7	15.8	0.9	0.84	4.7	3.19	- 42.6
Suw. R.*	52.6	41.7	1.2	4.4	11.9	3.1	0.75	4.1	2.00	- 49.7
HA1	57.2	37.2	1.0	4.6	12.4	2.3	0.83	3.99	2.41	- 35.9

** elemental analysis obtained from list of products from IHSS*

3. Results

Preliminary tests were devoted to determine the ideal concentration of humic acids for the fluorimetry measurement in order to comprehensively compare wavelength and intensities of all humic samples. Fig 1 presents emission fluorescence spectra in the concentration range from 0.001 to 0.1 $g.L^{-1}$ of HA1 with the excitation at 468 nm. As expected, the intensity of fluorescence depends on the concentration, any significant shift of the peak position has not been observed. Fluorescence emission measurement at lower concentrations gave a prominent peak at 556 nm, dissappearing at higher concentrations due to the rapid increase of intensities at lower wavelengths. Fluorescence intensity emitted at 556 nm for the concentration range from 0.001 to 0.01 $g.L^{-1}$ showed a linear increase followed by a non-linear increase at concentrations up to 0.1 $g.L^{-1}$ (results not shown). Since humic samples measured in this work showed significantly different fluorescence intensity, for their mutual comparison the concentration 7.5 $mg.L^{-1}$ was used (Fig. 2). It can be seen that the soil Elliot humate and LHA gave substantially more intensive fluorescence than the Suwanee river and the South Moravian lignite humic acids. Identification of the peak at 556 nm was in the former case hampered by the increasing intensity at lower wavelength.

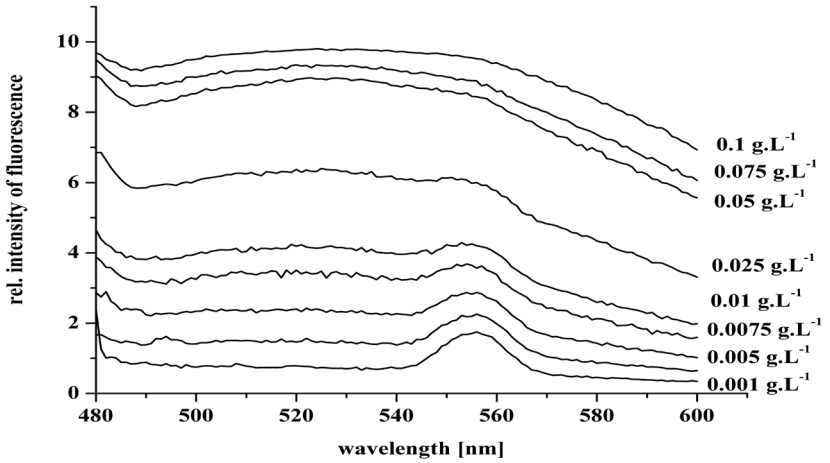

Figure 1. The influence of the sample concentration on the emission fluorescence spectra of HA1 sample (excitation at 468 nm).

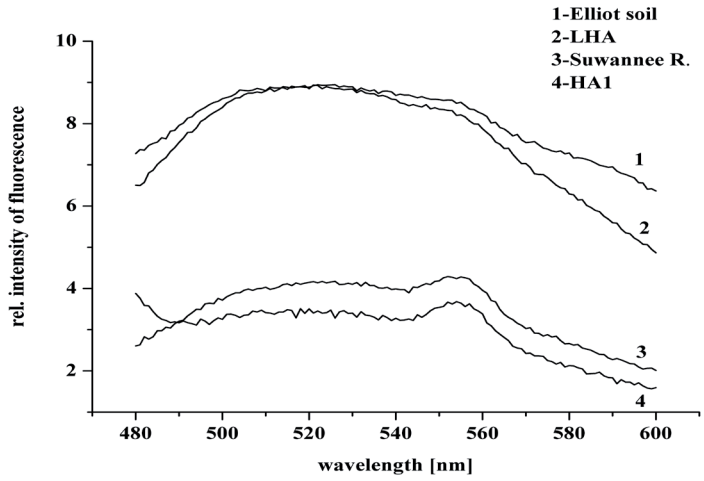

Figure 2. Emission spectra of sodium humate samples at excitation 468 nm. (7.5 mg.L^{-1})

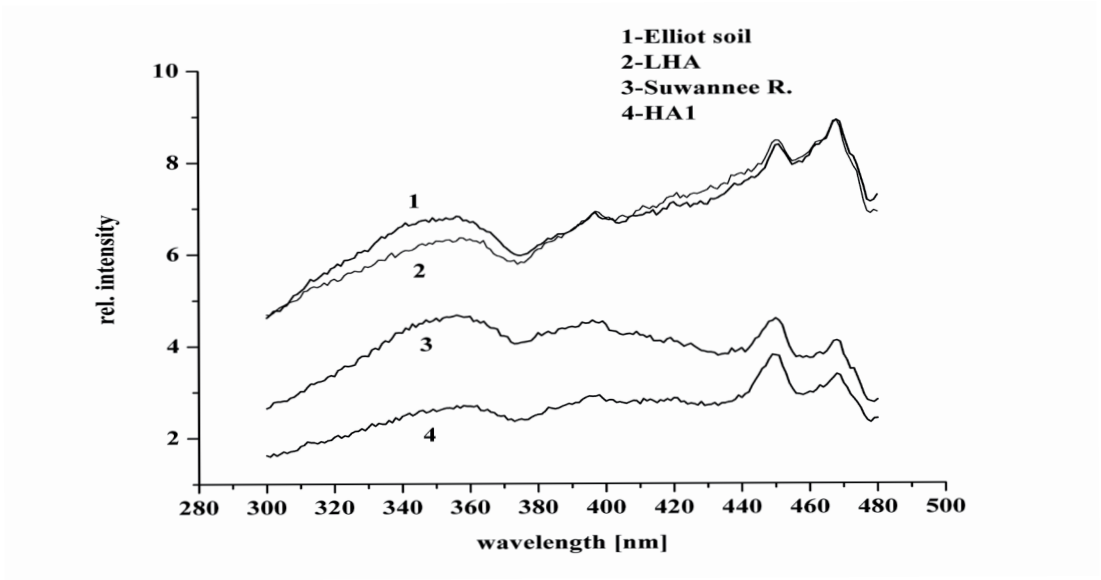

Figure 3. Excitation spectra of sodium humate samples with the emission at 528 nm. (7.5 mg.L^{-1})

Fig. 3 shows the same comparison but for the excitation fluorescence measurement with the emission at 528 nm. In this case, the resolution is significantly better than in Fig. 2. The order of intensities is the same as in the emission measurement. The HA1 sample compared with the Suwannee river humate showed even lower fluorescence intensity.

The RFI indexes were determined from the ratio of the two most intensive peaks at 487 and 501 nm from SFS (the fluorescence index). Results are reported in Table 1. The highest RFI index had the Elliot soil sample whereas the lowest one the Suwannee River sample.

The synchronous fluorescence spectra (SFS) were measured at various constant differences – 10, 20 and 80 nm (Fig. 4). SFS spectra confirmed that the optimal setting of $\Delta\lambda$ is 20 nm. As expected and in accordance with literature data, SFS spectra of humic samples resulted in fine records allowing better spectral separation. A main peak was observed at 488 nm, minor peaks at 360, 380, 420, 470, 500 and 512 nm. Fluorescence intensities obtained by SFS confirmed the previous order determined in the emission mode (Fig. 5).

UV-VIS spectroscopy is considered to be one order less sensitive technique in comparison with the fluorescence measurement. The UV profile of all humic acids showed typical record, that is, the

exponential increase of absorbance at decreasing wavelengths with a small plateau around 250 nm. Since the visible region did not give any important information, solely the UV region is reported in Fig. 6. The index of humification i.e. the ratio of specific absorbances in visible region at 465 and 665 nm ($E_{4/6}$) was also determined (Table 2). Further, absorbance at 265 nm was determined and compared since it is the wavelength frequently used for the comparison of specific humic constituents[34].

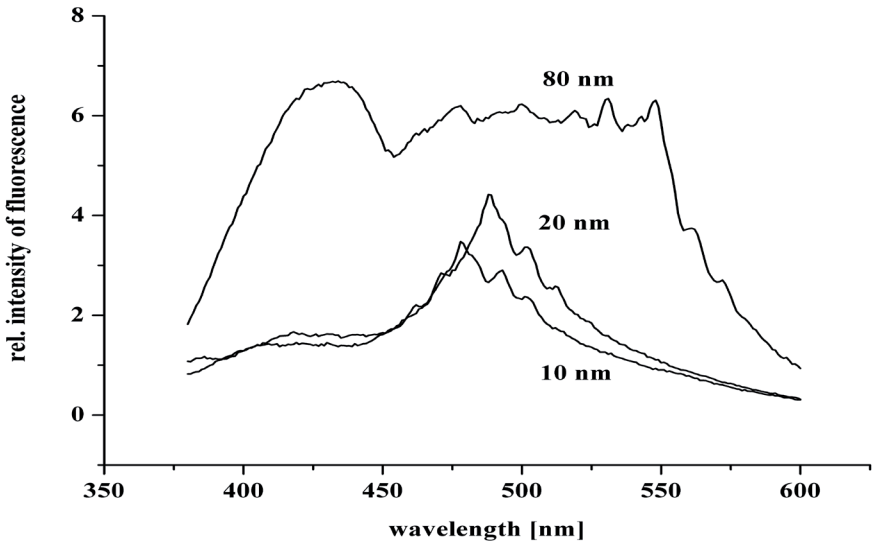

Figure 4. Synchronous scan with constant difference Δλ=10, 20 and 80 nm. (7.5 mg.L-1)

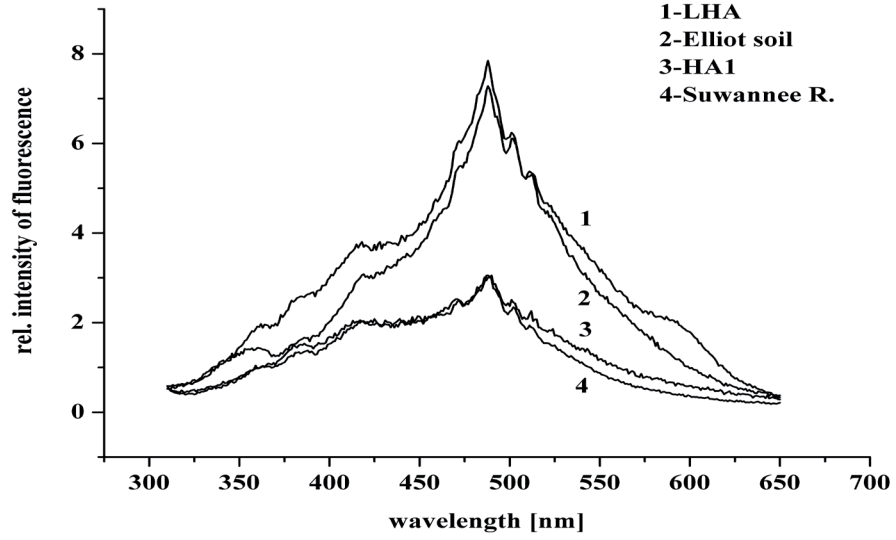

Figure 5. Synchronous fluorescence scan of sodium humate samples. (Δλ=20 nm, 7.5 mg.L^{-1}

In order to evaluate influence of concentration on the zeta (electrokinetic) potential, different concentrations of HA1 in the range from 1.10^{-4} g.L^{-1} to 1.10^{-1} g.L^{-1} of were measured as reported in Fig. 7. Values of zeta potential irregularly decreased from -5 do -46 mV and at higher concentrations slowly decreased. Since the records of other humic samples under study were similar they are not reported. Instead the averaged values of zeta potential of humate solutions at the concentration 7.5 mg.L^{-1} are given in Table 1. The lowest value indicating the most intense charge of the double layer showed the sample Suwanee River whereas the highest the sample HA1.

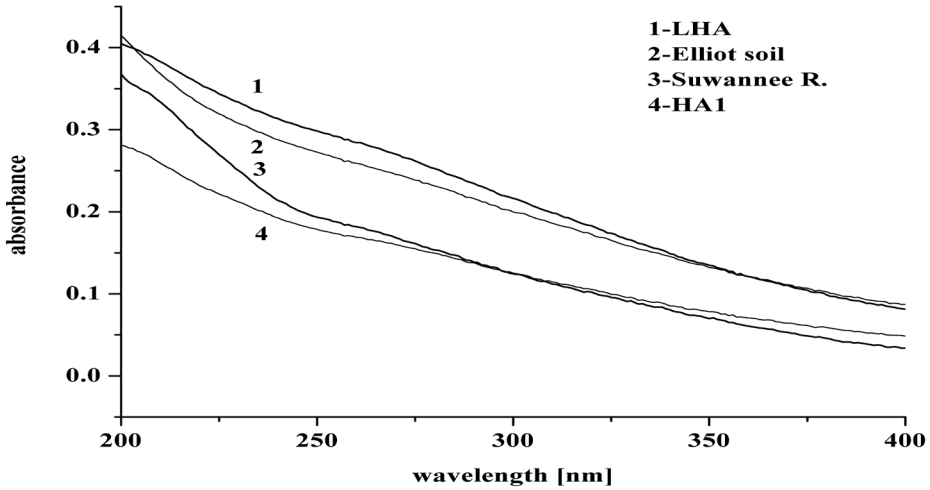

Figure 6. UV spectra of sodium salts of humic acids, concentration 7.5 mg.L^{-1}.

4. Discussion

Fluorophores represent a minor part of humic substances[29]. As a result, the fluorescence is generally weak and its intensity depends on many factors [14, 35]. One of those result has been reported the age or better maturity of humic substances [14]. In Figs 2,3 and 5 it can be seen that the lignite humic acid HA1 showed the lowest fluorescence intensity which is in agreement with literature data. Leonardite humic acids LHA, though are undoubtedly ancient, underwent their specific genesis during which they were extensively oxidized and thus it is not easy to rationalize their fluorescence properties with regard to their maturity.

As it has been demonstrated, the measurement of fluorescence of humic acids at higher concentrations (approximately above 15 mg.L^{-1}) is hampered by intrinsic fluorescence quenching whereas at lower concentrations it is difficult to be resolved from the background scattered light. Further, as stated in [7] the position of peaks can help to distinguish between humic substances of different origin. In this work, we have used river, soil and two lignite humic acids. It is noteworthy, that both lignite humic acids showed significantly different intensities, but almost the same peak positions (Figs. 2, 3 and 5). The opinion on the origin of humic fluorescence is quite scattered [14, 19, 27-30]. The common point is that humic acids are, among others, composed of substituted aromatic rings and potentially by highly unsaturated aliphatic chains. However, there is not a general agreement if the aromatic constituents are condensed or if the presence of frequently reported highly aromatic structures is not rather a result of the hydrophobic effect, i.e. a driving force sticking amphiphiles together exhibiting consequently an apparently large molecular aggregate [5]. They can be gradually created from diluted solutions and their dimension increases with increasing concentration. Individual constituents exhibit fluorescence at low wavelengths while the fluorescence of their aggregates are shifted to higher wavelengths (red shift). This can be identified in the Fig. 1, if the concentration is increased the ratio of peak intensities around 520 and 550 is changed. Undoubtedly, the role of above mentioned light scattering and quenching must be taken into account. Nevertheless, similar results as published here were obtained also by Yang and Zhang [35] who employed the polarization technique to suppress undesirable light scattering effects. Such explanation sounds reasonable since i) as a result of solubilization of hydrophobic pollutants at very low concentrations, humic acids were proposed to aggregate from significantly lower concentrations than is usually reported their critical micelle concentration [36] and ii) based on the UV/VIS fluorescence measurement, the optical properties of humic acids is not a result of the superposition of numerous independent chromophores, but rather of their mutual interactions [31]. Consequently, although the elementary analysis and humification indexes reported in Table 1 as well as literature data [32] imply the higher aromaticity of the lignite humic acids HA1 in comparison with the Suwanee river sample, their

fluorescence intensity is the same or even lower. Kononova reported that the ratio $E_{4/6}$ corresponds to the degree of condensation of aromatic substances. Low ratio corresponds to high degree of humification and indicates low number of aliphatic chains [37]. Table 1 reports the lowest $E_{4/6}$ for the HA1 but the value does not follow the order of fluorescence intensity. We hypothesize that the reason of fluorescence fluctuation in dependency on the aromaticity degree indicators can be seen in the quenching or, to call it more precisely, in the inner filter effect [38]; in principle, it can be static (e.g complex formation) and dynamic (e.g. collision quenching). Regarding the heterogeneity of humic acids, the former seems to be more probable. Further, the high $E_{4/6}$ value of the LHA indicates a higher content of aliphatic chains, but the C/H ratio indicates the high aromaticity degree. Our hypothesis is supported by data published in [34] where the authors measured emission spectra of soil humic acids and its sub-fractions. The latter exhibited more intensive fluoresce emission than the former while peak maxima of sub-fractions were shifted to lower wavelengths. Moreover, as demonstrated by [39] humic acids of different origin create similar domains exhibiting mechanistically similar behavior (glass transitions) and they are reported being composed mostly by aromatic units [40]. This can be an explanation of similar peak position of different humic acids as reported here. Thus, we assume that in line with the previous observation [31] the overall optical properties of humic acids are a function of intrinsic humic components aggregation and do not have necessarily and simply to follow the basic parameters such as the aromaticity degree, ultimate analysis or functional group content.

Humic substances generally show strong absorbance in the UV/VIS range, particularly in the UV region because of the presence of aromatic chromophores and/or other organic compounds [41]. In fact, the UV absorptivity at 265 nm (ABS_{265}) is commonly used to determine the relative abundance of aromatic C=C content of NOM (natural organic matter) because of π-π* transitions substituted benzenes or polyphenols occurs in this wavelength region [23]. Accordingly, the records given in Fig. 6 shows that both the LHA and soil Elliot HA contain relatively high amount of the above mentioned constituents. In addition, the Suwanee river sample showed a more rapid decrease in absorptivity as the wavelength increased in comparison with the HA1. However from 320 nm the absorptivity of river sample was lower than for HA1. This observation could be explained by the fact that that UV/VIS absorption of natural organic matter is affected by relative abundance of the aromatic C=C and ketonic C=O functional groups (or chromophores) and auxochormes such as C-OH, $C-NH_2$ and others. The ketonic functional groups give a weaker absorption in the visible range, whereas auxochromes do not confer color by themselves but increase the color of chromophores [23]. Elemental analysis of HA1 indicated that it contained a higher amount of carbon and lower of oxygen than Suwanee river sample (Table 1). The strong UV absorption (at < 250 nm) may result both form the aromatic C=C and ketonic C=O functional groups. However, UV absorption by the river humic sample may result primarily from the absorption of by ketonic functional groups. This is also in agreement with the genesis of river humic sample. Further, both the low amount of auxochromes and lower C/H is probably the reason of significantly lower absorptivity of HA1 in comparison with LHA and Elliot soil. In the former case it is likely a high content of N-containing auxochromes.

The determination of the zeta potential in order to assess humic acids properties has not been frequently reported [42, 43]. That method allows assessment of an apparent surface charge of colloidal particles or aggregates. Since humic acids molecules contain a number of miscellaneous polar groups (carboxyl, hydroxyl, alcoholic, carbonyl and amidic) having a strong influence on the total charge, its application for the characterization of humic substances seems to be quite reasonable. The value of zeta potential is an important measure of aggregation properties of the system and allows the determination of equilibrium dissociation constant. As reported in Table 1, the values of zeta potential do not correlate with the oxygen content in humic molecules. Instead, the lowest value have sample LHA having the lowest oxygen content. The explanation can be seen in the fact, that the significantly highest influence on the value have deprotonized functional groups such as COO^-. Leonardite, a source of LHA is known as an oxidized coal with pronounced high content of carboxylic groups. Nevertheless, the influence of sterical effects, i.e. protection of a number of groups by a hydrophobic barrier can not be excluded. This can be identified in the Fig. 7. Aggregation of humic substances as illustrated by the increasing concentration proceeds in non-linear or non-exponential decrease of zeta potential while at higher concentrations seems to increase again. Similar results have already been reached by means of size exclusion chromatography [44] and it has been stated, that in diluted solutions of humic acids (at pH 7) prevail hydrophobic interactions between molecules (π-π, *CH*-π interactions and van der Waals forces). Increasing concentration causes closer contact of aggregates and increasing importance of both H-bonds and repulsive forces of charged sites. As a result, the mean surface charge of humic aggregate is slowly reduced and humic secondary structure altered.

5. Conclusions

The optical and colloidal properties of humic substances of different origin can not be explained on the base of simple molecular composition. Instead, the nature of secondary structure must be taken into consideration as well. We do not intend to doubt about results and correlations obtained and published so far by many authors, rather, to suggest the future investigation of humic substances based on the interrelationship between the primary and secondary humic structures.

Acknowledgements

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