Petroleum & Coal
ISSN 1337-7027

Available online at www.vurup.sk/pc
Petroleum & Coal 51 (1), 33-44, 2009

SOME REMARKS ON THE ORIGIN OF LIGNITE HUMIC ACIDS OPTICAL PROPERTIES

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Received September 3, 2008, accepted January 7, 2009

Abstract

Humic acids (HA) isolated from lignite of South Moravian and North Czech region (leonardite) were characterized by elemental analysis and fluorescence spectroscopy analysis. From both humic acids there were sequentially extracted water soluble fractions, free and bond fatty acids which were consequently characterized by CPMAS ^{13}C-NMR spectroscopy and GC-MS analysis. The respective rests were dissolved in sodium hydroxide and the synchronous fluorescence spectroscopy (SFS) was used to analyze the character of present fluorophores and assess the influence of extracted molecules on the fluorescence of humic acids. The results has revealed a number of essential outcomes for the elaboration of fluorescence records of humic matter: i) based on our and other measurements, the optical properties of humic acids could not be a result of the superposition of numerous independent chromophores, but rather of their mutual interactions; it seems, that SFS evaluates predominantly hydrophobic core of humic aggregates which has apparently highly aromatic character, ii) the inner core of a humic aggregate do not necessarily consists of condensed structures, the high aromaticity degree can be a consequence of weak interactions of simple aromatic moieties such as π-π stacking and CH-π; iii) the crucial factor in SFS measurement is quenching which can be caused both by molecules present outside and inside of a humic aggregate; iv) due to the unique and complicated secondary structure of humic acids the molecules which are not primarily fluorophores can significantly affect the fluorescence due to their interaction with fluorophores.

Key words: synchronous fluorescence spectroscopy, lignite humic acids, extractions, supramolecular structure

1. Introduction

Humic substances (HS) are complex organic materials found ubiquitously in nature where they play an essential role in numerous environmentally important processes. They are the product of biotic or abiotic (or both) degradations of dead plant tissues and animal bodies. Because light absorption by these substances increases exponentially with decreasing wavelength across visible and ultraviolet spectrum, they can provide to aquatic organisms protection from damaging ultraviolet radiation [1]. They are very active in binding ions, organic molecules and mineral surfaces and are thereby potentially important to soil structure, soil fertility and transport of pollutants in natural waters. The conformational behavior of HS in solution depends on pH and ionic strength of the

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solvent and on the concentration of HS. According their solubility under acidic or alkaline conditions they are operationally divided into: humic acids (HA), which are soluble under alkaline conditions but not acidic conditions (pH<2), fulvic acids, which are the fractions soluble under all pH conditions and humins, which are the insoluble fractions of humic substances [2].

From the chemical point of view HS are commonly believed to consist of high molecular weight and highly polydisperse heterogeneous molecules [3]. The physical-chemical behavior of HS is reconciled to their commonly accepted macromolecular structure by the random coil model [4]. By this interpretation, HS are polymers coiled-down in globular conformations at high concentrations, low pH, and high ionic strength, whereas they behave like flexible linear colloids at neutral pH, low ionic strength, and low concentration. The amphiphilic character of HS was invoked to introduce a micellar or membrane-like model [5] by which humic macromolecules arrange the hydrophobic components in the inner voids of a humic micelle, whereas hydrophilic constituents are at the interphase with the solvent. Despite its general acceptance, the polymeric nature of humic substances had never been unambiguously proved [6]. Results obtained by size exclusion chromatography suggested an alternative model in which relatively small and heterogeneous humic molecules self-assemble in supramolecular conformations stabilized only by weak forces such as dispersive interactions (van der Waals, π-π, CH-π bonds) and hydrogen bonding. Such humic associations show only apparent high molecular dimensions which could be attributed to the humic molecules aggregation. Ultraviolet-visible spectra of organic acid treated humic substances show hypochromism (decreased absorbance due to increased distance between the absorbing chromophores). Evidently, the apparent sizes of humic materials do not change due to tight coiling or uncoiling, as suggested by the polymer model, but instead change due to disaggregation or aggreagation of clusters of smaller molecules. The effects of additions of carboxylic and mineral acids on humic fractions with different hydrophobicities indicate that aggregate disruption is greatest when more hydrophobic humic materials are combined with organic molecules containing both hydrophobic and hydrophilic segments. This result suggests that humic materials are held together by hydrophobic interactions, which are easily disrupted when simple organic molecules penetrate large hydrophobically bonded associations and separate them into smaller, higher-energy H-bonded associations [7]. That view was later supported by data obtained from High Resolution Ultrasonic Spectroscopy [8] and Diffusion Ordered Nuclear Magnetic Resonance Spectroscopy [9].

Over more than three decades fluorescence spectroscopy has been applied extensively to characterization, differentiation and classification of natural organic matter (NOM) such as humic matter (HM). Likewise, attempts to identify certain structural and functional constituents (fluorophores) in natural HM have been carried out. Fluorescence is sensitive and many environmental factors (type of solution, pH, ionic strength, temperature, redox potential of the medium and interactions with metal ions and organic substances) affect it [10]. Recently, workers have begun to test whether the optical properties of HS can be attributed to a simple sum of the spectra of a series of independent chromophores or whether a more complex situation is occurring. The observed optical properties of HS could arise in two distinct fashions. The first postulates that the absorption (and emission) spectrum results from a simple linear superposition of the absorption (emission) spectra of an ensemble of independent (noninteracting or electronically isolated) chromophores (superposition model). In contrast, the second postulates the presence of only a few distinct chromophores or closely related classes of chromophores, which through electronic interaction produce new optical transitions that are responsible for the exponentially decreasing, long wavelength absorption tail (interaction model). Optical charge-transfer bands, produced via a ground-state interaction between an electron donor, D (e.g., polyhydroxylated aromatics, phenols, or indoles), and an electron acceptor, A (e.g., quinones), in close proximity represent a possible explanation for this long wavelength absorption. These donor-acceptor complexes are known to exhibit new broad absorption bands (at lower energies) that are not shown by either donor or acceptor molecule independently [1].

Single-wavelength fluorescence measurement is limited in its ability to analyze complicated multi-component samples when they have severely overlapping emission and/or excitation spectra. The general consensus is that this can be overcome by using synchronous fluorescence spectroscopy (SFS) [11]. It is a method wherein simultaneous scanning of both excitation and emission spectra is performed at a constant offset value or difference between the emission and excitation wavelengths, Δλ (Δλ = λ_{em} – λ_{ex}). SFS also provides better structured and resolved peaks compared to other conventional fluorescence methods. However synchronous fluorescence spectroscopy has also the same limitations inherent to the basic fluorescence technique, such as spectral distortions caused by intermolecular interactions and by static and dynamic quenching processes [12].

The aim of this work was to investigate the influence of specific sequentially extracted molecular fractions (water-soluble components, free and bond fatty acids) on the humic acids secondary structure (domains) and to assess consequent changes using synchronous fluorescence spectroscopy. Obtained fractions were analyzed either by CPMAS ^{13}C-NMR spectroscopy or by GC-MS. Conventional interpretation attributes individual peaks to the superposition of particular fluorophores [13,14]; in line with our results that view can be replaced by the notion that the character of fluorescence spectrum is a function of conformation arrangement of humic molecules.

2. Experimental

Humic acids

Isolation of humic acids

Humic acids were extracted from South Moravian lignite (mine Mikulčice, Czech Republic) and North Czech oxyhumolite ~ lignite (mine Sokolov, Czech Republic) by means of alkaline extraction (modified procedure of International Humic Substances Society). About 200 grams of lignite were mixed with 2 litres of 0.5 M NaOH and 0.1 M $Na_4P_2O_7$. 10 H_2O solution and shaken for about 3 hours. After centrifugation the supernatant was purified with precipitation of HCl and HF solution for 24 hours. This was again centrifuged and the residue was dialyzed (Spectrapor dialysis membranes, 3500 Mw cutoff) against distilled water until chloride free and freeze-dried.

The sample of sodium humate was prepared using following procedure. Isolated HA was mixed with distilled water and the pH value has been adjusted with 0.1 M NaOH to 7. The sodium humate solution was freeze-dried.

Extraction methods

Hot water extraction (separation of polar substances)

About 1 g of South Moravian humic acid (SMHA) and North Czech humic acid (NCHA) were overfilled with 200 ml of distilled water and heated on 60 – 80°C during about 4 hours. Then the liquid part was centrifuged and freeze-dried.

Extraction and derivatization of the free and bound Fatty Acids

200 mg of solid rest of HA (after hot water extraction) was initially oven dried at 40°C and the free lipids extracted by shaking for 2 hours at room temperature with 40 ml of a (2:1, v/v) solution of dichloromethane (DCM) and methanol (MeOH). The extract was separated from residue through centrifugation (25 min, 7000 rpm) and the supernatant removed. The residue was further extracted with 40 ml of the DCM/MeOH (2:1, v/v) solution over night at room temperature, and again separated from the supernatant by centrifugation. By this step free lipids were removed. The free Fatty Acids (FAs) present in the extract were then methylated into Fatty Acid Methyl Esters (FAMEs) with tetramethylsilyl-diazomethane [15]. The residue remaining from the lipids extraction was air-dried, added with 10 ml of 12% BF_3-CH_3OH solution and heated at 90°C over night. This treatment was repeated twice. The supernatants were recovered by centrifugation (15 min, 7000 rpm), combined, treated with an excess of water in order to destroy the remaining BF_3, and then liquid–liquid extracted with chloroform. The total extract was dehydrated with anhydrous Na_2SO_4 and was dried by rotoevaporation yielding the methylated bound FAs [16].

The procedure is summarized in following scheme (abbreviations in bold are in solid state, extracts are liquid):

SMHA, NCHA
hot water extraction
↓
SMHA1, NCHA1
free lipids extraction (extraction by dichloromethane and methanol)
↓
SMHA3, NCHA3 (yield of free lipids up to 5%)
bond lipids extraction (transesterification by boron trifluoride and methanol solution)
↓
SMHA4, NCHA4 (yield of bond lipids up to 5%)

extracted water fraction → **WSMHA2, WNCHA2 (yield ~20%)**

Fluorescence measurement

Emission and synchronous fluorescence spectra of samples at 25°C were measured by an Aminco Bowman Series 2 spectrofluorimeter equipped with a xenon lamp and a thermostated cell holder. Emission spectra were measured in the range from 460 to 600 nm at excitation wavelength 450 nm at 20±2°C. SF spectra were collected in the 250-600 nm excitation wavelength range using the bandwidth of Δλ = 20 nm between the excitation and emission monochromators. All spectra were recorded with a 4 nm slit width on both monochromators. Spectral resolution of Aminco spectrofluorimeter is 1 nm. Scan speed of spectra was set to 60 nm per min. Sensitivity of all samples was set to 60 %. Fluorescence measurements were recorded and assessed by AB2 program. The correction of fluorescence records were carried out using the same voltage on the detector.

Although no further corrections for fluctuation of instrumental factors and for scattering effects (e.g. primary and secondary inner filter effects) were applied to experimental spectra, a comparative discussion on the spectra is acceptable, at least on a qualitative basis, since all of them were recorded on the same instrument using the same experimental conditions [13].

CPMAS ^{13}C-NMR Spectroscopy

Cross polarization magic angle spinning (CPMAS) ^{13}C-NMR spectra of WSMHA2 and WNCHA2 were acquired with a Bruker AVANCE™ 300, equipped with a 4 mm Wide Bore MAS probe, operating at a ^{13}C resonating frequency of 75.475 MHz, and a rotor spin rate of 13±1 kHz. Samples were packed in 4 mm zirconia rotors with Kel-F caps. 1000 scans (NS) with 3782 data points (TD) were collected over an acquisition time (AQ) of 25 ms, a recycle delay (RD) of 2.0 s, a contact time of 1 ms. A ^{1}H Ramp sequence was used to account for possible inhomogeneity of the Hartmann-Hahn condition [17]. The Bruker Topspin 1.3 software was used to collect spectra, while spectral elaboration was conducted by Mestre-C software 4.9.9.9 version. All the free induction decays (FID) were transformed by applying, first, a 8 k zero filling, and then an exponential filter function with a line broadening (LB) of 100 Hz. A 3rd order polynomial with Bernstein algorithm was used for baseline correction of the NMR spectra [18].

Spectra were integrated over five chemical shift regions corresponding to different carbon compounds: 200–160 ppm (COOH), 160–113 ppm (aromatic C), 113–93 ppm (anomeric C), 93–44 ppm (oxidized and/or carbohydrate C), 44–0 ppm (Alkyl C). The area of each spectral region (R_i^{abs}) was divided by the sum of all spectral areas, in order to obtain a relative percentage $(R_i^{\%})$: $R_i^{\%} = \left(\frac{R_i^{abs}}{\sum_i R_i^{abs}} \right) \times 100$

Gas chromatography-mass spectrometry (GC-MS)

FAMEs were analyzed by capillary GC and GC-MS using a Hewlett-Packard 6890 GC (split injector, 250°C; flame ionization Detector (FID), 300°C) with a fused silica capillary column (SGE BPX 5%, 30 m length, 0.25 mm id., 0.25 μm film thickness) and helium as

carrier gas. The GC was temperature programmed from 60 to 300°C at 5°C min^{-1} (isothermal for 20 min final time). The GC-MS analyses were performed on a Trace GC Thermo Finnigan coupled to a Thermo Finnigan Automass (with the same GC conditions). The MS was operated in the electron impact mode with a 70 eV ion source energy and the ion separation was operated in a quadripolar filter. The distributions of FAMEs were determined by integration of gas chromatography peak areas using the *m/z* 74 ion fragmentograms.

3. Results

Elemental and NMR analysis

The elemental composition of humic acids extracted from South Moravian lignite has already been reported and analyzed in many papers [8,9,20]. The properties of humic acids extracted from North Czech oxyhumolites have been investigated in ref. [21-23]. As can be seen from Table 1, NCHA contains slightly more carbon and less oxygen content in comparison with SMHA. C/H ratio indicates the aromaticity degree and the C/O ratio shows the amount of oxygen proportional to carbon content, i.e. indicates amount of COOH and OH groups. NCHA is more aromatic and exhibits higher C/O ratio in comparison with SMHA.

Table 1. Elemental analysis of South Moravian and North Czech humic acids, values are in weight %.

Sample	C	H	N	O	C/H	C/O
SMHA	57.2	4.6	1.0	37.2	12.4	1.5
NCHA	57.9	4.3	1.5	31.3	13.6	1.9

Supramolecular arrangement of humic molecules in the mixture allowed the sequential extraction of specific molecules. Fig. 1 reports the results of CPMAS ^{13}C-NMR technique which was used to assess the composition of fraction obtained after extraction by hot water. In Table 2 there is listed the percentage of C distribution obtained as described in Experimental part. The spectrum of the HAs water fractions was divided into five regions, which are assigned to alkyl carbons (0–64 ppm), etheric/alcoholic carbons (64–102 ppm), aromatic carbons (102–150 ppm), carboxylic carbons (150–188 ppm) and carbons arise from ketones, aldehydes and quinones (188–216 ppm). Results reported in Table 2 indicate that the fraction extracted from sample NCHA by hot water has high amount of aromatic structures as well as carboxyls, ketones, aldehydes and quinones. Fraction extracted from SMHA showed similar composition, however, with higher content of etheric, alcoholic and aliphatic moieties.

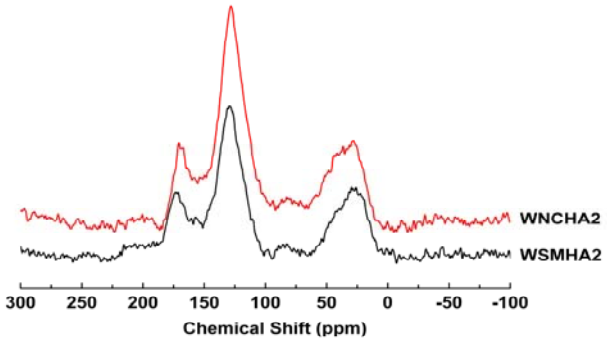


Fig. 1. CPMAS ^{13}C-NMR spectrum of the SMHA and NCHA hot water extracts.

Table 2. Distribution of C intensity in different regions of CPMAS ^{13}C-NMR spectrum of hot water extracts, for explanation of regions see text.

Sample	216-188 ppm	188-150 ppm	150-102 ppm	102-64 ppm	64-0 ppm
WSMHA2	0.50	15.76	51.14	4.05	28.55
WNCHA2	0.67	15.97	55.49	1.82	26.05

Free and bound Fatty Acids

In both cases, the distribution of FAs was dominated by the short chain range (C_{10}–C_{18}) dominated by the ubiquitous palmitic (C_{16}) and stearic (C_{18}) acids (as methyl esters) and included the *iso*- and *anteiso*-C_{15} and C_{17} members (Fig. 2). Unsaturated fatty acids, oleic ($C_{18:1}$) acids, were also present in the SMHA samples. The C_{11} component is observed in the distributions of free FAs from NCHA and SMHA samples.

Fatty acids have been used extensively as markers of the plant versus microbial origin of SOM, and the contributions of different organisms to the lipid pool [24]. While even-numbered short and linear chained fatty acids methyl esters (FAMEs) are ubiquitous, the longer mode (C_{22}+) originates from higher plants [25]. Short chain FAs (< C_{20}) have been found to be mainly of microbial origin [26,27], although C_{16}, C_{18}, $C_{18:1}$ and $C_{16:1}$ FAs have also been identified in arbuscular fungi [28,29]. Straight-chain FAME components of fungal origin typically range from C_{10} to C_{24} [30,31]. *Iso*- and *anteiso*-C_{15} and C_{17} members arise from bacteria [32,33].

The presence of a C_{11} fatty acid in the free lipids extracts is remarkable considering that these compounds are unusual constituents of extractable lipids.

Overall, the distributions clearly indicate that the origin of the extracted fatty acids is primarily from microbial sources, with only a weak contribution from higher plants, because of the absence of the longer mode (C_{20}+). While the palmitic acid (C_{16}) that dominates our chromatograms can be attributed to plant or microbial sources [32], the lack of longer chain FAMEs suggest that the shorter chain FAME were of microbial origin.

The C_{11} compounds are present only as free FAs and not covalently linked to the HAs organic network. The short chain length suggests microbial oxidation of unsaturated alkanoic acids and/or mid-chain hydroxy acids. Upon such oxidation, unsaturated bound FAs and/or mid-chain hydroxyl FAs can produce free short-chain FAs.

In addition, because unsaturated fatty acids are preferentially lost during diagenesis [34], their absence in the NCHA samples may be a sign of more evolved OM in NCHA than in SMHA.

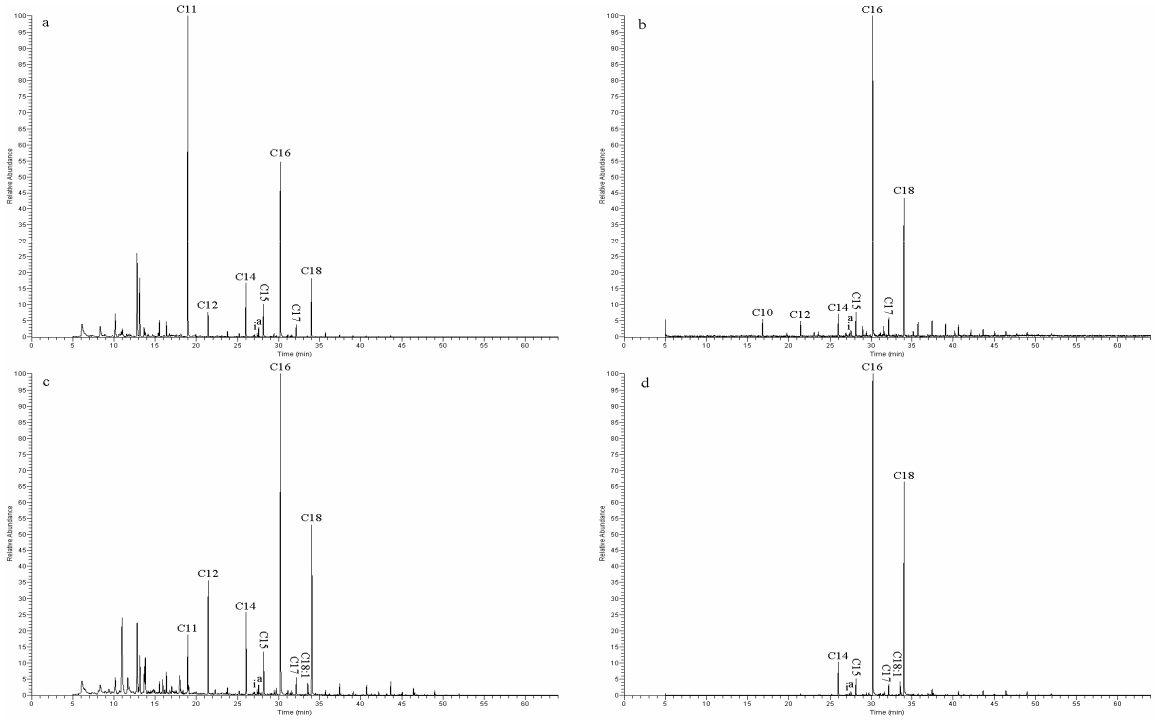


Fig. 2. Ion chromatograms (m/z 74) showing the distribution of the released fatty acids (as methyl esters) from NCHA a: free, b: bound and SMHA c: free, d: bound.

Fluorescent spectroscopy analysis

Emission fluorescence spectra (excitation at 450 nm) gave a record typical for humic substances with a broad maximum within the range 500-535 nm [19] (records not shown).

Synchronous fluorescence spectra of SMHA, NCHA and their respective hot water fractions are reported in the Fig. 3 and 4. All samples show the presence of six main spectral peaks at $\lambda_{ex.}/\lambda_{em.}$: 468/488, 481/501, 491/511, 450/470, 400/420 and 339/359 at constant difference of $\Delta\lambda$ = 20 nm. As can be seen from Fig. 3, removing of water-soluble components causes a decrease of fluorescence intensities by all peaks in comparison with original humic acid. Larger decrease is observed for the peaks in lowest wavelength region. On the contrary extracted water fraction shows higher fluorescence intensities by the peaks at 468/488, 481/501 and 491/511 nm. Separation of free and bond lipids tend to the increase of fluorescence intensity in the region around 339/359 nm and to decrease of fluorescence intensity by the peaks at larger wavelengths.

In case of NCHA sample (Fig. 4), extraction by hot water causes an increase of fluorescence intensities by all peaks. After extraction of water fraction one can observe a decrease of fluorescence intensities of peaks in the range of λ_{em} = 359–488 nm. Peaks at 481/501 and 491/511 nm have almost same intensities of fluorescence like original NCHA. Removing of free lipids shows the same feature in the lowest wavelength region and a decrease of fluorescence intensities of peaks from 400/420 nm. Separation of bond lipids causes a decrease of fluorescence intensities by all peaks in comparison with original NCHA.

Fig. 5 presents synchronous fluorescence spectra in the concentration range from 0.001 to 0.01 g/l of SMHA 3. As expected, the intensity of fluorescence depends on the concentration, any significant shift of the peak position has not been observed. Concentration lowering showed an increase of fluorescence intensity of the peak at 240/260 nm and to decreasing of fluorescence intensities by the peaks from 400/420 nm.

4. Discussion

SFS spectra were measured using the bandwidth of $\Delta\lambda$= 20 nm between the excitation and emission monochromators. This value is thought to be optimal for SFS measurement of humic acids and it has been recommended in scientific literature for a long time. SFS has also the same limitations inherent to the basic fluorescence technique, such as spectral distortions caused by intermolecular interactions and by static and dynamic quenching processes. SFS offers, however, a potentiality to reduce overlapping interferences and a possibility for each fluorescent component to be identified in a specific spectral range. Since lignin and further plant material is considered as precursors of humic acids, it is reasonable to attribute the observed fluorescence to moieties such as quinones and aromatic hydroxy carboxylic acids [35-38]. It is well-known that for this class of compounds often a large Stokes' shift is found in their steady-state emission spectra caused by a large change in dipole moment or by a proton exchange process. Moreover, humic acids are considered as fairly large polyelectrolytes or polyelectrolyte-like substances, and, therefore, inter and intra- molecular motions due to electronic excitation are likely to occur as well. Strong indications that the processes described above occur and contribute to the observed fluorescence emission were also found in time-resolved fluorescence experiments with humic substances and their metal complexes [39-40].

Table 3 summarizes the most probable individual fluorophores found in HS and though to be responsible for their fluorescence. It is noteworthy that at standard conditions all of them have the difference $\Delta\lambda$ between excitation and emission wavelength maxima about 80–100 nm. In contrast, records reported in Fig. 3, 4, 5 showed that use of $\Delta\lambda$ = 20 nm resulted in the most intensive emission peaks around 500 nm. Data listed in Table 3 indicates that no fluorophores have emission wavelength in this region as well as the difference between emission and excitation is far away from 20 nm.

Therefore, there are many conceivable possibilities for chromophores which might contribute to the fluorescence of HS. Further, to make it more complicated the interactions among different molecules must be taken into consideration as well. In SFS

records, the intensities of spectral region 280/300, typical more for aquatic humic matter, can justifiably be assigned mainly to aromatic amino acids and same other volatile acids containing highly conjugated aliphatic structures [13]. Potential structural descriptor for the intensities within the spectral region around 330/350 nm, has been attributed to naphthalene with its derivatives [41]. Potential contributors to the fluorescence behaviour within the spectral range 355/375 nm, are though the polycyclic aromatics with three to four fused benzene rings. Accordingly, the significant range centered around 400/420 nm, can correspond to polycyclic aromatic with approximately five fused benzene rings. The next significant range around 460/480 nm, is attributed to the influence of polycyclic aromatics consisting of about seven fused benzene rings. The most common lignin descriptors fluorescent within this region as well [13].

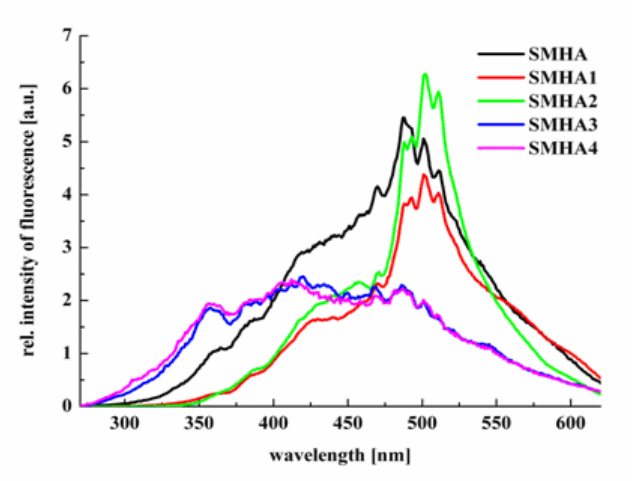


Fig. 3. Synchronous fluorescence spectra of South Moravian humic acid (SMHA), humic acid without water-soluble components (SMHA1), extracted water fraction (SMHA2), humic acid without free lipids (SMHA3) and humic acid without bond lipids (SMHA4).

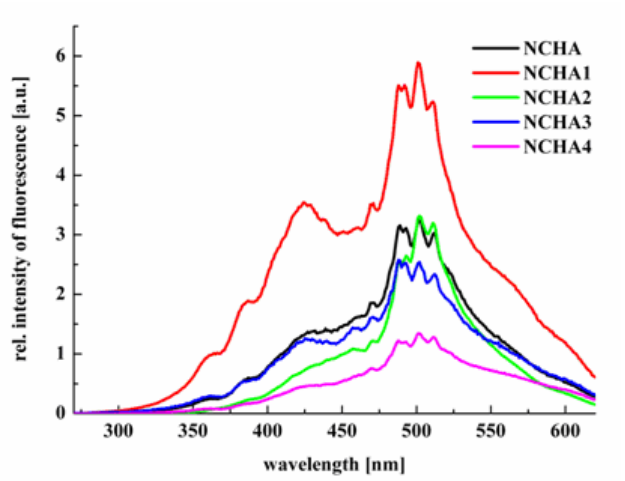


Fig. 4. Synchronous fluorescence spectra of North Czech humic acid (NCHA), humic acid without water-soluble components (NCHA1), extracted water fraction (NCHA2), humic acid without free lipids (NCHA3) and humic acid without bond lipids (NCHA4).

The spectra were recalculated with regard to the C content.

In our recent paper [19] we have published the emission spectra of South Moravian lignite humic acids which were confirmed also in this work. The excitation at 468 nm brought the emission peak at around 558 nm which fits well to the values reported in Table 3. However, in the interval up to 558 nm and above there is still evident a broad significant signal which can be attributed to the fluorescence resulted from a number of potential interactions within present fluorophores.

In light of above discussion emission spectra seem to indicate that except those fluorophores which are listed in Table 3, the weak fluorescence "background" originates also from fluorophores which optical properties are affected by their environment. Essentially, chemically identical molecules are surrounded by different solvent environments (water or other molecules) and the spectral broadening in solutions is caused by a solvent inhomogeneity. As a result, fluorophores adopt different conformations, resulting in a Gaussian distribution of energy transitions.

We assume that there are two principal groups of fluorophores present in humic acids which can be called "free" while the second "bound". While the first group is perfectly solvated and follows the Δλ excitation and emission conditions given in Table 3, the bound molecules are in close proximity and their Δλ is largely reduced. Accordingly, it seems that the former are better visible using conventional emission fluorescence spectroscopy, the latter can be better identified using SFS. This view is partially in agreement with a theory in which the physical structure of humic substances is described as the aggregates with several layers; the outer layer consists of aliphatic and simple aromatic molecules rich in polar substituents, the inner layer or core consist predominantly of aromatic, condensed structures [3].

Our hypothesis on SFS is also in line with the original application of SFS which was introduced by Lloyd [42] and primarily served for identification of condensed aromatic compounds in automobile engine oils, petrols and exhaust soots [43].

Moreover, beside the "real" fluorescence quenching, the observed steady-state fluorescence spectra are also altered due to inner filter effects and the intrinsic fluorescence of the humic substances. Thus we assume that the SFS with $\Delta\lambda$ = 20 nm reveals preferably the presence of fluorophores which consist of condensed rings or simple aromatics reported in Table 3 which are in mutual intermolecular contact affecting their spectral fluorescence characteristics. The hypothesis on apparent condensed aromatic core of humic acids is supported by our recent data [8] which revealed that humic molecules tend to recombine at very low concentrations via hydrophobic interactions (1 mg L^{-1} at least) while increasing of concentration is associated with employment of increasing number of H-bonds. Thus the optical properties are dependent on the nature of physical structure with the same importance, if not higher, as primary structure. The statement is also supported by results reported in Fig. 5 where progressive dilution of humic fraction solution resulted in an increase of fluorescence intensity at 260 nm and a dramatic decrease at 500 nm. The emission intensity is considerably shifted to lower wavelength due to the separation of individual fluorophores (originally forming aggregates with apparently high degree of aromaticity) which seem to be simple aromatic compounds in their chemical nature. Consequently, such results revealed also the nature of interaction within humic molecules forming an aromatic "core" which could be attributed predominantly to the π-π stacking or CH-π interactions of aromatic moieties.

Table 3 Nature of fluorophores in humic substances [44]

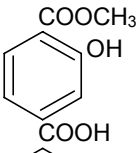
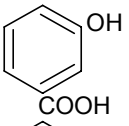
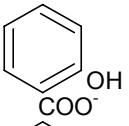
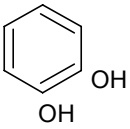
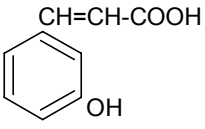
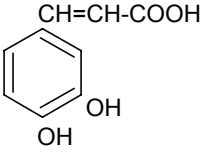
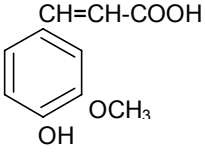
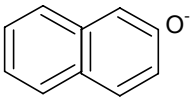
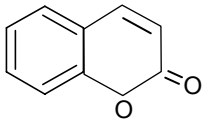
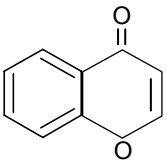
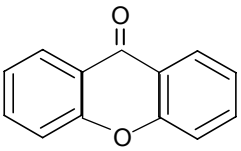
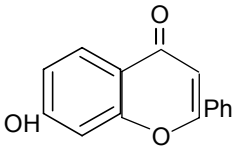
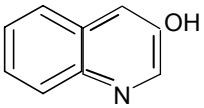
Fluorescing molecule	Structural formula	λ_{em} (nm)	Λ_{exc} (nm)
Methyl salicylate		448	302,366
Salicylic acid		410	314
3-Hydroxybenzoic acid		423	314
Protocatechuic acid (ionized)		455	340-370
3-Hydroxycinnamic acid		407	310
Caffeic acid		450	365
Ferulic acid		440	350

Table 3 Nature of fluorophores in humic substances, contd. [44]

Fluorescing molecule	Structural formula	λ_{em} (nm)	Λ_{exc} (nm)
β-Naphtol (ionized)		460	350
Coumarins unsubstituted		454	376
Hydroxy- and methoxy-		400-475	320-343
Esculetin		475	390
Scopoletin		460	390
Others disubstituted		430-462	350-419
Chromone derivatives		409-490	320-346
Xanthone and hydroxanthone		456 465	410 343,365
Flavones and isoflavones		415-475	313-365
Hydroxyquinolines		450	350
Schiff-base derivatives	-N=C-C=C-N-	470	360-390

Results obtained from CPMAS ^{13}C-NMR spectroscopy show, that extraction by hot water used in this work separated various compounds which are suppose to have fluorescent properties. That fraction shows higher fluorescence intensities by the peaks at 468/488, 481/501 and 491/511 nm. In an ideal case the sum of spectra SMHA1 and SMHA2 should give the same spectrum as the original SMHA. It can be seen that it may be possible at lowest wavelengths but definitely not at larger wavelength region. Therefore, the inner filter effect is stronger at higher wavelength in comparison with lower values which is an indirect proof that SFS is sensitive to analyze predominantly the character of inner, apparently condensed, humic core. Further, it is clear that quenching caused by secondary humic structure is a crucial parameter influencing the feature of SFS spectra of humic acids. This can be also identified in case of NCHA and its water fraction where situation is completely different. The rest after extraction (NCHA1) gave dramatically more intensive fluorescence than the original NCHA. In this case it is probable that the quenching of the inner core was largely caused also by the outer, water extractable layer.

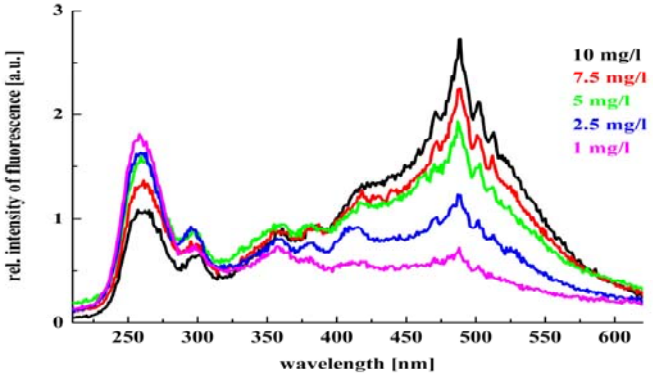


Fig. 5. Synchronous fluorescence spectra of SMHA sample after separation of free lipids (SMHA3) depending on concentration.

The extraction of free and bound lipids by organic solvents brought about molecules which are not primarily fluorescing, some significant changes in the fluorescence can be seen. This could be promoted by opening of the humic conformation structure stabilized by weak forces. It is in contrast with extraction by hot water which separated some compounds just from the surface of humic assemblies, since water due to its high polarity was not able to penetrate inside the hydrophobic interior of aggregates (core). Consequently, apolar solvents could "unblock" particular fluorescing molecules which promoted an increase in fluorescence intensities by the peaks at lower wavelengths values and vice versa to decrease of fluorescence intensity by the peaks at larger wavelengths (Fig. 3 and 5).

5. Conclusions

This study has revealed a number of essential outcomes for the elaboration of fluorescence records of humic matter:

i) based on our and other measurements, the optical properties of humic acids could not be a result of the superposition of numerous independent chromophores, but rather of their mutual interactions, which reduces the difference between excitation and emission maxima to lower values. Hydrophobic environment decreases the Δλ value while hydrophilic environment tend to increase of the Δλ value which implies that SFS evaluates predominantly hydrophobic core of humic molecules,

ii) the inner core of humic molecules do not necessarily consist of condensed structures, the high aromaticity degree can be a consequence of weak interactions of simple aromatic moieties such as π-π stacking and CH-π,

iii) the crucial factor in SFS measurement is quenching which can be caused both by inner and outer molecules,

iv) due to the unique and complicated secondary structure of humic acids the molecules which are not primarily fluorophores can affect the fluorescence due to their interaction with fluorophores.

Acknowledgements

The financial support of Ministry of Education, project MSM 0021630501 is acknowledged. Authors also thank to Daniela Šmejkalová (Centro Interdipartimentale per la Spettroscopia di Risonanza Magnetica Nucleare – CERMANU, Portici (NA), Italy) for caring out the NMR analysis and elaboration of data. Last but not least, the cooperation with Filip Mravec, MSc. (Brno University of Technology, Faculty of chemistry) is appreciated.

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