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REGULAR ARTICLE

Fluorescence quenching as an indicator of the conformational change of humic acids

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Abstract

Humic acids as a water soluble fraction of humus play important role in the plant growth. Even though structure of humic acids is not clear, their spectroscopic properties can reveal us useful information. To investigate influence of humic acids reorganization, HPSEC fractionation at mild fractionation conditions has been carried out. Reconstruction of secondary chromatograms of separated fractions showed molecular size increase and increased response on fluorescence detector, although neither molecular size change nor significant absorbance increase was observed on UV-VIS detector. However, bigger aggregates, that didn't fluoresce in the unfractionated sample started to fluoresce. The reason for this behavior is not some change of spectral properties of a specific fraction, but a general fluorescence increase indicating humic acid reorganization. Therefore spectroscopic changes can be used as a tool for monitoring their reorganization, which might play an important role in nutrients soil plant mobility and should be studied in more details from that perspective.

Key words: Conformational change, Fluorescence, Fluorescence quenching, Humic acid, HPSEC

Introduction

In soil science humus refers to any organic matter that has reached a point of stability, where it will break down no further. Stable (or passive) humus consists of humic acids and humins (Di-Giovanni et al., 1998).

Humic acids (HA) as a water soluble fraction of humus play important role in the plant growth (Lee and Bartlett, 1976; Nardi et al., 2002). The beneficial effects of HA on plant growth may be related to their indirect (increase of fertilizer efficiency or reducing soil compaction), or direct (improvement of the overall plant biomass) effects. They play important role in ion absorption (increase the availability of micronutrients from sparingly soluble hydroxides (Stevenson, 1994)). Apart from that, HA are absorbed by plants, influencing intermediary metabolism, or plant growth and development directly, by acting as hormone-like substances (Piccolo, 1996).

Even though structure of HA is not clear, their spectroscopic properties can reveal us useful information. HA exhibit rather unusual absorption and emission characteristics: absorption decreases with increasing wavelength in an approximately exponential fashion, but extends well into visible and in some cases the near infra-red. HA spectra does not arise from a superposition of many independent chromophores (Del Vecchio and Blough, 2004), but from a continuum of coupled states formed through charge transfer interactions of a few distinct chromophores. Emission maxima shift continuously to red with increasing excitation wavelength, while fluorescence quantum yields monotonically decrease (Boyle et al., 2009).

According to available data, the fluorescence efficiency of HA is generally low. This means that a great part of absorbed energy is dissipated leading neither to fluorescence emission nor to triplet excited state production. Therefore a non-irradiative relaxation must play an important role, meaning that many aggregates do not fluoresce due to aggregation (Coelho et al., 2010). It is already reported (Engebretson and Von Wandruszka, 1999; Specht et al., 2000; Wu et al., 2003; Conte et al., 2007) that the bigger aggregates do not fluoresce. And authors generally agree that this is due to

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fluorescence quenching and consequently increased non radiative relaxation). Fluorescence quenching is strongly related to the local environment of chromophores, such as solvent or mobile phase in case of chromatographic separation or surrounding aggregates influencing the distances between fluorescence donor and fluorescence acceptor.

But with the new evidence that HA are aggregates composed mainly of fairly small subunits weakly held together by predominantly hydrophobic forces (Piccolo et al., 1996a; Sutton and Sposito, 2005; Schaumann, 2006) unusual absorption and emission characteristics make more sense: by influencing distances between donors and acceptors, aggregation of subunits does not influence only the size, but also the spectroscopic characteristics of HA.

To study the influence of the aggregation on spectroscopic characteristics of HA, mild fractionation procedure, hardly changing their natural properties is needed (Burba et al., 1998) and their characterization by physical or chemical methods should be performed under ‘in situ' or at least nature-like conditions (Buffle and Leppard, 1995). Therefore high pressure size exclusion chromatography (HPSEC) in low ionic strength mobile phase is necessary.

But even in mild, nature like condition, the fractionation changes the local environment of chromophores and therefore changes the distances between fluorescence donor and fluorescence acceptor. This should be pronounced as change in fluorescence quenching efficiency, therefore different secondary chromatograms of fractions would be expected if recorded by fluorescence or absorbance detectors. If we could confirm this hypothesis, we could use HPSEC with fluorescence detection as a tool for better understanding of aggregation behavior of HA.

Materials and Methods

Humic acid sodium salts (Aldrich) were used for the experiments, as an representative of terrestrial HA (Valencia et al., 2013). They were dissolved in ultrapure water (Barnstead NANOpure system) at a concentration of 1 g/L, to keep ionic strength as low as possible to imitate natural conditions, left on ultrasound bath for 15 minutes and then centrifuged for 30 minutes at 4000 rpm. The supernatant was filtered through a 0.45 μm PTFE filter (Chromafil).

Analyzes of HA samples were performed by aqueous high pressure size exclusion chromatography (HPSEC) using a high pressure liquid chromatograph Agilent HP 1100 Series with G1322A Degasser, G1311A QuatPump quaternary pump, G1315A DAD diode array detector, and G1321A FLD fluorescence detector.

0.001 M $NaNO_3$ (Riedel-de Haën) was used as a mobile phase at a flow rate 0.5 mL/min. The void volume was 5.5 ml.

For HPSEC 50 μL of samples were injected onto a MN Nucleogel aqua-OH 50-8 column (3000 mm x 7.7 mm, 8 μm particle size) with a MN Nucleogel aqua-OH 8P pre-column (50 mm x 7.7 mm) (Macherey-Nagel). The temperature of chromatographic column was kept constant at 25°C. The detection was performed with a diode array detector (DAD) at 280 and 230 nm and a fluorescence detector (FLD)(EX = 230 nm, EM = 440 nm). The dead volume of capillary connecting the two detectors was 0.1 mL. Data acquisition and processing were accomplished by the HP ChemStation A.07.01 software.

For the fractionation experiment, 250 μL fractions of HA, separated with HPSEC, were collected behind the second detector (FLD). 100 μL aliquots of each fraction were immediately analyzed with HPSEC again (three replicates).

Results and Discussion

From the chromatogram of fractions (Figure 1) it is obvious, that the biggest aggregates (retention times shorter than 13 minutes) do not fluoresce even after fractionation.

But before discussing the reconstructed chromatograms, we have to consider the fact that only 100 μL aliquots of collected fractions (250 μL) were analyzed with HPSEC again. Furthermore the initial sample is diluted. It has been observed in our experiments, as also reported in the literature (Swift and Posner, 1971), that decreasing concentrations of HA produced shifts of peaks from low to high molecular size ranges (shorter retention times).

Of course, the peak area decreased as well, as a result of smaller amount of HA (100 μL aliquots) and consequently smaller absorbance. On the basis of 6 replicates at 3 different concentrations we have calculated the expected retention time and peak area, as if the whole collected fraction would be injected ($R^2 > 0,90$ for retention time and $R2 > 0,99$ for peak area).

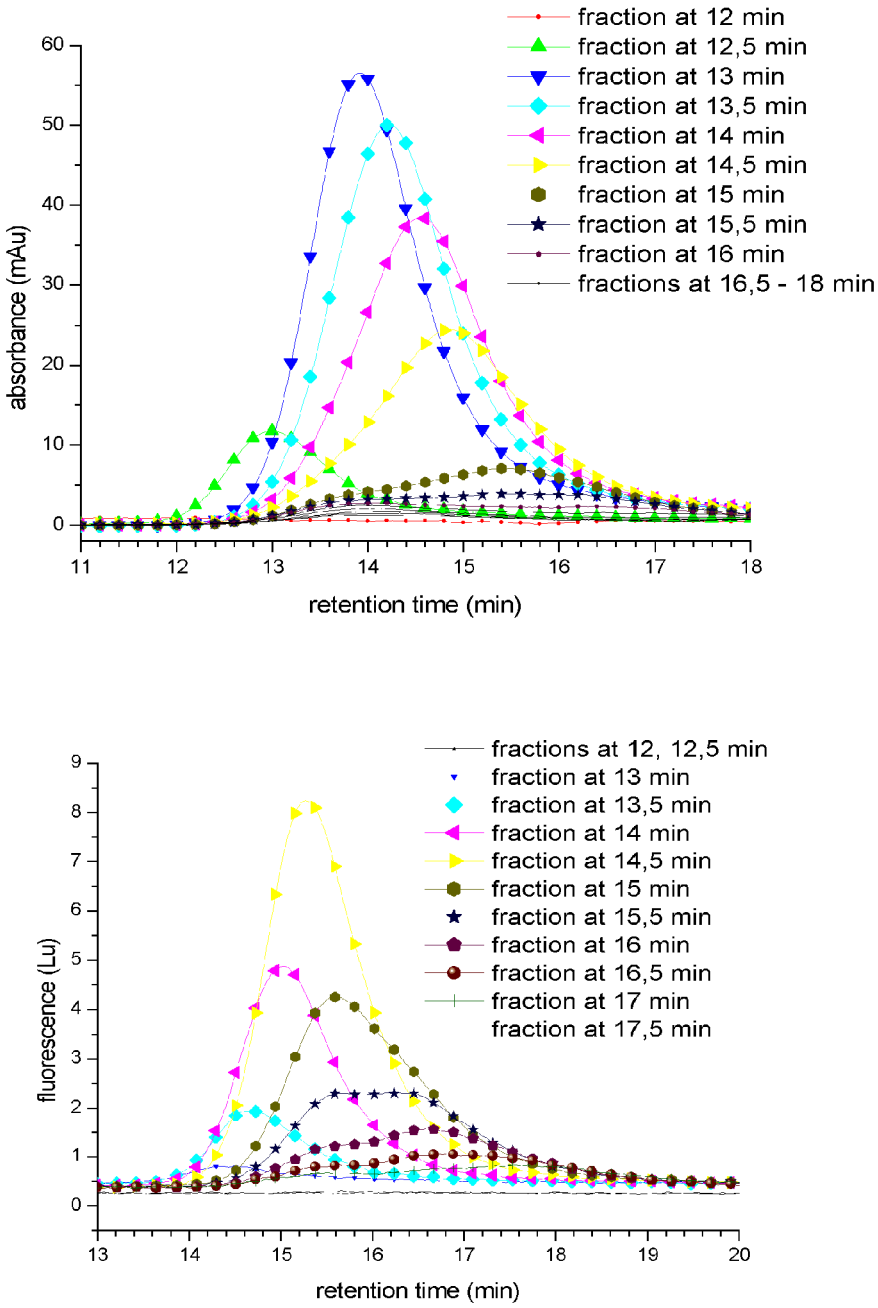

Figure 1. Chromatograms of fractions.

Therefore, when reconstructing the primary chromatogram from chromatograms of fractions (sum of signal intensities from chromatograms of fractions), 2.5 times smaller peak area and a shift of the retention time by up to 0.2 min toward shorter retention times on both detectors, compared to unfractionated sample, would be expected as a consequence of dillution (100 µL of 250 µL injected).

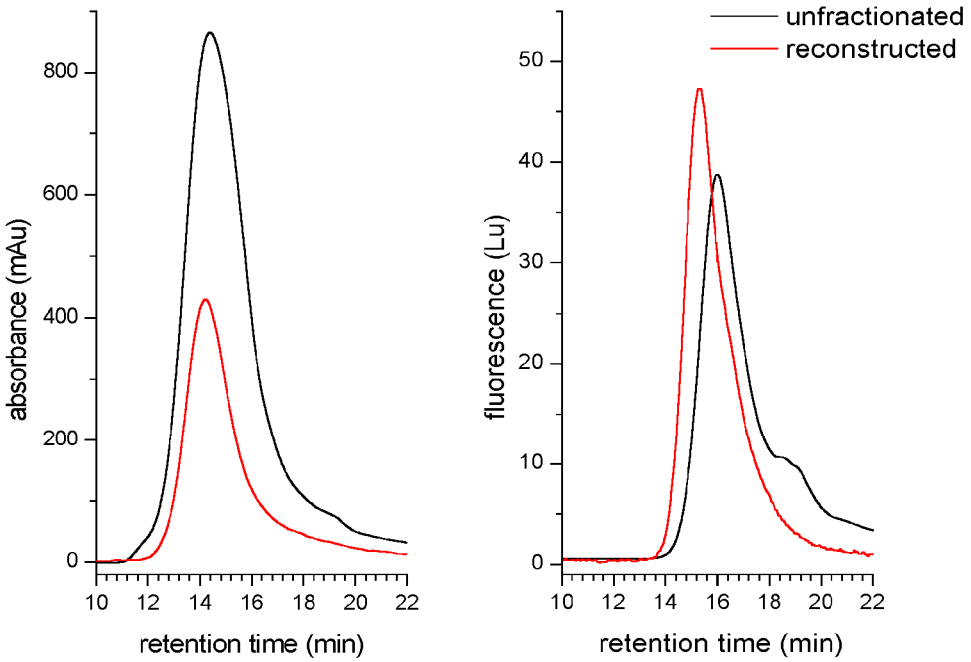

Figure 2. Reconstructed chromatogram of fractions.

However, the reconstructed DAD chromatogram exhibits a 5 times smaller peak area and 0.2 min shorter retention time as compared to the unfractionated sample. The difference in the retention time between reconstructed and original (unfractionated) chromatograms corresponds approximately to the expected retention time decrease due to dilution (0.2 min). DAD peak area of the reconstructed chromatogram, corrected by the dilution factor 2.5 is however significantly smaller (50 %) than the peak area of the unfractionated sample (Figure 2).

The decrease of absorbance in our experiment cannot be attributed to the dilution effects and neglection of fractions with retention times above 18 min, since they were estimated to represent less than 8% of the total amount of injected HA. Also material loss due to adsorption on stationary phase is not likely (a low ionic strength mobile phase) because of electrostatic repulsion between stationary phase and HA (De Nobili and Chen, 1999; Perminova, 1999). We realize that the choice of a low ionic strength mobile phase has also its drawbacks: it is not enough to eliminate all ionic interactions (Perminova, 1999), but this would be important only if we would like to interpret results with absolute values of molecular size. Which we don't.

Actually, the decrease of absorbance has already been observed in different fractionation processes (Burba et al., 1995; Aster et al., 1996; Schimpf and Petteys, 1997; Lin et al., 1999, 2000; Kitis et al., 2002; Hoque et al., 2003; Hur and Schlautman, 2003; Alberts and Takács, 2004; Zanardi-Lamardo et al., 2004; Richard et al., 2004) or changed in their aggregation through acidic treatment (Piccolo et al., 1999; Cozzolino et al., 2001; Baigorri et al., 2007) but mostly left without explanation. Piccolo explained that absorbance decrease is a consequence of the disruption of weak intermolecular bonds; holding aggregates together (Piccolo et al., 1996b). We find it as the only reasonable explanation also in our experiment. Of course fractionation under mild conditions is much less radical than organic acids treatment, therefore only partial disruption, influencing mostly spectroscopic properties has been observed.

Conformational changes of HA are even more pronounced on the fluorescence detector: the retention time at the fluorescence maximum is 0.8 min shorter than for the unfractionated sample (Figure 2). This retention time shift cannot be explained only with the dilution effect (0.2 min), and therefore indicates that bigger aggregates, which did not fluoresce in the unfractionated sample start exhibiting higher fluorescence or that smaller aggregates do not fluoresce anymore.

Table 1. Fluorescence of unfractionated sample, fraction, collected at specified retention time and sum of fractions at the retention time of maximal absorbance of each fraction (tmax). Fluorescence of fraction and sum of fractions has been corrected by the dilution factor 2.5.

Fraction, collected at (min)	13.5	14	14.5	15	15.5	16	16.5	17	17.5
Maximal fluorescence of fraction	2.5	5	12.5	20	10	5	5	2.5	2.5
Fluorescence of all fractions	10	22.5	42.5	47.5	42.5	25	20	17.5	10
Response in original chromatogram	2	5	13	20	31	35	29	25	15

An increased overall fluorescence of the fractions shown in the reconstructed chromatogram indicate that retention time shift is due to increased fluorescence of bigger aggregates: cumulative FLD peak area of individual fractions, multiplied by the dilution factor 2.5, is bigger than the peak area of unfractionated sample, regardless much smaller absorbance and therefore weaker excitation (Figure 2).

Similar observation (increase of fluorescence even if the UV absorption decreases) has been reported in the literature as a consequence of degradation: the fluorescence of the NOM samples investigated was increased upon hydrolysis (Kumke et al., 2001), after chlorination (Korshin et al., 1999), ozonation and UV irradiation of HA (Win et al., 2000).

The fact that fractionation experiment lead to the same result as photo or chemical degradation can only be explained with the decreased quenching effect, caused by change in the local environment of HA aggregates. Changes of the local environment influence the distance between fluorescent donor and acceptor and consequently the rate of energy transfer (Gilbert et al., 1991) without bond breaking.

The quenching is directly related also to rigidity, because more rigid structures decrease the possibility of fluorescence quenching (Specht et al., 2000) So the fluorescence increase could be caused by increased rigidity, such as formation of hydrogen bonds (Conte et al., 2006). But we would expect, if this is the reason, that fluorescence increase would be pronounced only on specific fractions and pronounced more like fluorescence concentration (Alberts and Takács, 2004) than general fluorescence increase.

Closer inspection of the chromatogram of fractions (Figure 1, Table 1) reveals that fluorescence increase after the process of fractionation is not characteristic for a specific fraction. Actually, the fractions collected at 14, 14.5 and 15 minutes (highest absorbance) show as high fluorescence as unfractionated samples. Lower fluorescence of fractions collected after 15.5 min (highest fluorescence of unfractionated samples) might not be a consequence of actual fluorescence decrease, but more likely a consequence of the shift of fluorescence excitation/ emission maxima (Richard et al., 2004). The shift of ex/em maxima should be taken into account also in biggest aggregates, but it is obviously less pronounced than quenching effect decrease.

Similar behavior, indicating different spectroscopic properties of fractions, collected before 15th minute and after could be observed also by comparing UV-Vis absorption ratios: absorbance at 300 nm to absorbance at 400 nm (E300/E400) of unfractionated sample and fractions.

Fractions, collected at 13 and 14 minutes, that represent major contribution to the absorbance of the unfractionated samples and practically no fluorescence, show lower E300/E400 absorbance rate compared to the unfractionated sample, indicating shift of the absorbance maxima toward shorter wavelength (blue shift). This shift can be explained with changed spectroscopic properties due to increased polarity of environment (Skoog et al., 2007) in the process of fractionation: HA aggregates, which were surrounded with bigger or smaller aggregates prior fractionation, are exposed to mobile phase, and therefore more polar environment influences their spectroscopic properties.

On the other hand, E300/E400 ratio is increased for fractions, collected at 15 and 16 minutes (major contribution to fluorescence of unfractionated sample). The smallest size-fractions, eluted at the end of the HPSEC chromatographic separation are predominantly composed of oxidized carbons (Chen et al., 2003; Conte et al., 2007). This is in agreement with the loss of absorbance due to donor-acceptor interactions that are expected to red-shift and broaden near-UV absorbances giving long wavelength absorptivity (Richard et al., 2004).

Table 2. Absorbance at 300 nm divided with absorbance at 400 nm of unfractionated sample and fractions.

fractions, collected at (min)	unfractionated	13	14	15	16
E_{300}/E_{400}	3.0	2.8	2.8	36	3.7

Obviously we observe a dual behavior of HA regarding size: bigger aggregates exhibit intensive increased fluorescence and blue shift in absorbance spectra after fractionation/treatment, however smaller aggregates exhibit smaller fluorescence and red shift in absorbance spectra after fractionation /treatment, which is in accordance also with the results of NMR analysis, revealing that structure of DOM is significantly altered with size (Conte et al., 2006; Lam and Simpson, 2009; Woods et al., 2010).

Conclusions

The biggest HA aggregates do not fluoresce, because of fluorescence quenching. The reason for this behavior is not the property of a particular HA fraction, but a consequence of the local environment (which aggregates are nearby, which functional groups participate in molecular bonding etc.). When local environment changes, as in HPSEC fractionation, different response in secondary chromatograms of fractions on UV-VIS and fluorescence detector is observed indicating that HA have reorganized.

And changes of environment happen in soil all the time. It means that humic substances reorganize all the time, which can be a simple explanation of their environmental resistance, but even more, continuous reorganization can play an important role in nutrients soil plant mobility. And should be studied in more details from that perspective.

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