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Characterization and analysis of soil humic acids by off-line combination of wide-pore octadecylsilica column reverse phase high performance liquid chromatography with narrow bore column size-exclusion chromatography and fluorescence detection

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ABSTRACT

Liquid chromatography method arranged around RP-HPLC using step-wise gradients of dimethylformamide (DMF) in pH 3.0 aqueous phosphate buffered mobile phase and a wide-pore (30 nm) 4 mm inner diameter (I.D.) octadecylsilica column was applied to the fractionation separation of soil and peat humic substances (HS), respectively. Combination of acceptable DMF solvating properties for HSs together with wide pore RP sorbent improved surface interactions of the analytes and suppressed influence of size-exclusion effects in RP-HPLC. Individual fractions collected from the RP-HPLC were off-line analyzed by size-exclusion chromatographic method (SEC) using 99/1 DMF/aqueous phosphate buffer pH 3.0 with Spheron HEMA 100 stationary phase filled in a 2.2 mm I.D. column. Both methods provided reproducibility of characterization profiles and robustness resulting from excellent reproducibility of HSs fraction retention times (±0.5% RSD) of peaks enforced by the step gradient shape. Obtained results indicate that the methods can be combined in a compatible, compact, automatic, 2D orthogonal separation system for fluorimetric characterization of such complicated natural substances as are examined humic acids and obtain so more information about their character.

1. Introduction

Humic substances (HS) are ubiquitous in our environment influencing numerous important processes in all compartments of the environment. They are created by a complex mixture of amorphous, yellow to black colored, hydrophilic, polyelectrolyte, poly-disperse macromolecules or supramolecular structures and maybe no two HS molecules are identical [1–4]. From the point-of-view of chemical analysis, characteristic feature of HS is their diffuse non-distinct analytical signal produced by many detection principles. This fact is a consequence of their great complexity expressed also by their chemical, structural and physical poly-dispersity, which is further expressed in a great uncertainty of their analytical signal measured by almost any analytical method. Actually, they behave like loosely defined or fuzzy chemical systems, that must be characterized not by exact numerical values, but rather by distribution functions or ranges of validity of their analytical signals induced by numerous analytical principles, or better by their combinations.

Due to extremely high complexity of HS it is evident that separation methods are inevitable part of their analysis with respect to their isolation, separation, fractionation and measurement of subfractions, respectively, e.g., by such solution analysis methods as are high performance liquid chromatography (HPLC) and electroseparation methods (ES) [5]. Gas phase analysis of HS is due to their macromolecular or supramolecular structure restricted mainly to the analysis of low molecular compounds and decomposition products resulting from HS controlled degradation.

Complexity of problems achieved in context with HS requires valuation and utilization of many possible combinations of basic operations, analytical methodologies and techniques creating so a numerous individual approaches. Structural ambiguity and typical properties of HS result in very variable effects with respect to their often unpredictable behavior under various conditions. For instance, HS have strong aggregation and disaggregation ability and they create supramolecular structures, respectively. Unfortunately, there is still lack of standardization of common procedures, methods, measurements and definition of limiting conditions of HS measurement. Interpretation of the measured data can be too general and including extrapolation out of the data scope.

Until now size-exclusion chromatography (SEC) [5] is the most utilized separation method of HS relative molar mass determination and is used also for purpose of HS macromolecules size based fractionation [6–10] in spite of the fact, that the effective size of HS macromolecule or supramolecule is influenced by many

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variables related to their quality, concentration and the composition of mobile phase (e.g., pH and ionic strength). Poor control of the separation conditions beside adsorption phenomena may strongly influence characteristic shape of SEC record [5] and its run-to-run reproducibility. Another still not solved problem is the choice of suitable molecular mass calibration standards behaving close to HS in solution. Commonly used polymeric standards (polystyrenes, polystyrene sulphonates, globular proteins, and pullulans) should be substituted by working standards of HS isolated according to officially recommended (e.g., International Humic Substances Society, IHSS) procedures from corresponding source materials and standardized by independent methods. However, additional problems could arose out of HS standard solutions changes due to, e.g., ageing.

Generally prevailing reversed-phase high performance liquid chromatography (RP-HPLC) has achieved till now only limited application in analysis and characterization of HS. Researchers used organic solvents (methanol and acetonitrile) typical for RP-HPLC separation of small molecules together with weakly pH buffered aqueous part of mobile phase. However, such mobile phases resulted in moderate satisfaction with HS samples [11–14].

Recently utilized separation and detection techniques have in comparison to the complexity of HS problem severe limitations in terms of selectivity, separation and detection range. Logical consequence of the previous statement necessitates comprehensive multi-stage separation and detection procedures. Current technological processes related to HS require rugged and reliable analytical methods giving comprehensive information about the HS raw materials, HS in process stages and HS as products. The necessity of automated complex separation procedures, minimal sample pretreatment and the use of on-line multidimensional chromatographic techniques is one of logical solutions to the requirements. Multidimensional chromatography has proven to be useful for the analysis of complex samples such as HS.

Study of literature on the topic revealed, that relatively few works solve problems of off-line combination of two or more chromatographic methods based on different principles and their application to the characterization of HS. The fact is surprising, because adoption of such approach can lead to more complex insight to the behavior of HS in different chromatographic systems, as, e.g., in the work [15], where modified method was applied for determination of HS in aluminum containing solutions from Bayer process and on-line combination SEC-RP-HPLC [16] for the characterization of HS in Bayer liquor during technology of aluminum production. HPLC using C18-bonded phase with fluorescence detection (FLD) was tested for trace analysis of HS in environmental samples, including coral skeletal matter, sea and river water, soils and plants, by Susic and Boto [17]. They also concluded that HPLC with FLD is sensitive method of choice for determination of humic acids (HA) in environmental samples at the low nanogram levels.

The aim of this work is an introductory study of off-line combination RP-HPLC and SEC with dimethylformamide (DMF) based mobile phases. With respect to the non-common approach we focused to evaluation of its potential to create orthogonal on different separation principles working two-dimensional comprehensive separation methods combined with sensitive FLD.

2. Experimental

2.1. Instrumentation

Study of the RP-HPLC retention characteristics involving fractionation and SEC characterization of the selected groups of HA was carried out by the HPLC system LaChrom (Merck-Hitachi, Darmstadt, Germany) consisting of pump L-7100 provided by quarternary low-pressure gradient, autosampler L-7200, column oven L-7300, diode-array detector L-7450A, fluorescence detector L-7480, interface D-7000, PC data station with software HSM ver.3.1 and on-line four channel solvent degasser L-7612. Measured dwell volume of the system including column was 3.80 ml and should be considered when gradient mixing profile and chromatogram appearance is to be compared.

Extractions of HS from soil were done with the aid of shaker KS 125 (IKA Labortechnik, Junke and Kunkel GmbH, Germany). Extracts of HA were desalted by repetitive batch dialysis against distilled water from dialysis tubes with relative molecular weight cut-off value 6000 that were pre-conditioned and purified according to the procedure recommended by the manufacturer (Regenerated Cellulose, Spectra/Por.1, Spectrum, Houston, USA). Additional laboratory equipment: ultrasonic bath (IKA Labortechnik, Germany); laboratory shaker type KS 125 basic (IKA Labortechnik); analytical balance Sartorius Handy (Sartorius, Göttingen, Germany); an Inolab type pH 730 pH meter provided by combined glass/AgCl electrode (WTW, Weilheim, Germany); Centrifuge K24 (Janetzski, Leipzig, Germany); Ultrafiltration Unit provided with ultrafilters Anotop 10, 0.2 μm (Merck).

2.2. Methods

RP-HPLC separation (fractionation) was carried out using a LiChroCART column 250 mm × 4 mm filled by wide pore octadecylsilica LiChrospher WP 300 RP-18, 5 μm spherical particles, guarded by LiChroCART 4 mm × 4 mm pre-column packed with LiChrospher WP 300 RP-18, 5 μm particles. Dead volume (calculated from the manufacturers data [18]) of the column was 2.55 ml and is equal to the retention volume of the first HS eluted peak. Flow-rate was maintained at 1.00 ml/min. Separation conditions for optimized gradient elution of HS were as follows. Mobile phase A composition: aqueous phosphate buffer (pH = 3.00, 50 mM) containing 1% (v/v) dimethylformamide (DMF). Mobile phase B was 100% DMF.

2.2.1. Step-wise gradient

Gradient program was set from 0.0 min to 3.6 min isocratic 0%B in A, and from 3.7 min. Every 4 min there was isocratic step added increasing content of B in A by 10% up to the last step increased by 9% ending in 99% B in A, maintained till 55.0 min isocratic 99% B in A, from 55.1 min to 60.0 min linear decrease from 99% B in A to 0%B in A and between runs 10 min re-equilibration.

Column oven temperature was maintained at 35.0 ± 0.1 °C. Injection volume 50 μl and 100 μl, alternatively, was injected by the autosampler. Wavelength range of DAD was set to 280–800 nm. Monitoring wavelength was set to 280 nm or 420 nm. Fluorescence detection parameters were set to excitation wavelength 470 nm, emission wavelength 530 nm according to the published data [19] of fluorescence spectra measured in DMF solutions. Photomultiplier gain medium was selected.

The RP-HPLC method was used to obtain elution profiles and also for fractionation of analyzed humic samples. Each fraction was collected in a time range which was calculated from the FLD response. Volume of each of these fractions was 400 μl collected around the peak maximum. All collected fractions were analyzed by the second SEC method.

SEC separation was carried out using a stainless-steel column 250 mm × 2.2 mm filled by Spheron HEMA 100 (copolymer of hydroxyethylmethacrylate with ethylenedimethacrylate) sorbent, spherical particles diameter was 25 μm. Relative molar mass exclusion limits of polymers in Spheron HEMA 100 range from 70 000 to 250 000 according to the manufacturer [20]. During the development of SEC method for the reliable characterization of HS and their fractions, several mobile phases were tested (1% DMF/99%

phosphate buffer, pH = 3.00 (v/v); 50% DMF/50% phosphate buffer (v/v); 99% DMF/1% phosphate buffer (v/v). Finally, mixture of 99% DMF/1% phosphate buffer, pH = 3.00 (v/v) was chosen. Pump operated at flow-rate 0.2 ml/min. Volumes 20 μl or 50 μl of selected HSs fractions were injected into the SEC system.

Void volume (V_0; 0.35 ml) and total permeation volume (V_i; 0.95 ml) of the column were determined using Blue Dextran 2000 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and toluene, or nitrobenzene (Lachema, Brno, Czech Republic), respectively. The column system was calibrated using polystyrene standards (Polymer Laboratories, Amherst, USA) with different nominal molar mass for estimating the averaged molar mass of injected fraction of HS.

2.2.2. SEC column calibration

Calibration plot of Spheron HEMA 100 narrow bore (2.2 mm × 250 mm) column was accomplished by injection of 5 μl of solution containing standards dissolved in DMF mobile phase at concentration level 1.0 mg/ml, determination of elution time at peak apex and plot of molar mass vs. elution tome. Calibration standards were Blue Dextran 2000 kDa; polystyrene standards 498 kDa, 175 kDa, 37 kDa, 10 kDa, 4 kDa, 2.6 kDa, 1 kDa; styrene 0.1 kDa (Polymer Laboratories, Inc., Amherst, MA, USA). Column oven temperature was set at 35.0 ± 0.1 °C.

2.3. Materials

Target group of humic substances was obtained by the procedures published by Kandráč et al. [21], Procházková et al. [22] and Góra and co-worker [23] from soil samples pedologically characterized and collected by standard procedures. Soil sampling was done by experts from Department of Pedology, Faculty of Natural sciences, Comenius University in Bratislava on sub-contract within the project APVV-0595-07.

Fractionation procedure to get humic acid fractions HA J and HA I

We applied sequentional fractionation procedure to the oven dried (3.5 h; 104 °C) soil sample from the locality Dunajská Streda, Slovakia (I horizon, 10–20 cm depth). This soil morphogenetically belongs to Calcaric Fluvisoil (FAO classification) and is intensively used for cereals production.

1st step: Soxhlet extraction

200 g of the soil was twice extracted by Soxhlet extraction with methanol/benzene (1/2) mixture for 12 h. Extracts were discarded. Soil sample after the 1st step was next air dried for 12 h to remove free solvent and then it was further treated in oven for 3.5 h at temperature 104 °C.

2nd step: decalcination

Dried soil sample after the first step was decalcinated by 350 ml of 0.1 M HCl for half an hour. Then, after removal of the acid by careful decantation the rest of soil was repeatedly washed-out with distilled water to negative reaction for chloride (tested by 2% $AgNO_3$) and pH higher than 5.0.

3rd step: dissolution of humic substances from the soil rest by sodium pyrophosphate

900 ml 0.1 M $Na_4P_2O_7$ was added to the soil sample after decalcination and this mixture was shaken for 12 h. Supernatant was separated by centrifugation and this step was repeated twice.

4th step: humic acids precipitation and purification

Merged liquid portion was used for precipitation of HA by HCl acidification to pH = 2.0. After 2 h of standing (under nitrogen gas) the HA precipitate was separated from the liquid phase by centrifugation for 30 min at 3000 rpm. The precipitate of crude HA was refined by twice repeated dissolution in NaOH, centrifugation, HCl precipitation, centrifugation phase separation and final dialysis (6000 MWCO, cleaned regenerated cellulose membrane) till the negative reaction for chlorides. After drying under nitrogen stream HA J was obtained and stored in dark vessel and dark box at ambient temperature till the use.

5th step: acid hydrolysis

300 ml of 6 M HCl was added to the rest of soil sample from the 3rd step. This mixture was heated for 12 h at 90 °C in a ventilated glass vessel. Then it was washed with distilled water to the negative reaction of chloride (tested by 2% $AgNO_3$). After centrifugation the supernatant was discarded and the soil residue was air dried for 12 h.

6th step: Soxhlet extraction II

Soxhlet extraction (methanol/chloroform, 1/5) for 12 h was applied to the rest of soil residue from 4th step. This extraction was repeated twice. Solvent extract was discarded and the soil residues after the Soxhlet extraction II were air dried for 12 h.

7th step: separation of humic substances from the soil rest by NaOH

Solution of 0.5 M NaOH (900 ml) was added to the soil residue from preceding step and this mixture was shaked for 12 h under nitrogen atmosphere. Supernatant was separated by centrifugation and decantation and this whole step was repeated twice. The procedure of the supernatant processing was identical with the description of 4th step. After drying under nitrogen stream HA I was obtained and stored in dark vessel and dark box at ambient temperature till the use.

Difference between HA I and HA J is in the succession of extraction steps of the same soil sample. Chelated HA I is released from processed (degreased, decalcinated) soil by sodium pyrophosphate and HA J is strong alkali released from the soil rest processed by vigorous acid hydrolysis and degreasing. HA J should be therefore of older origin than HA I, because it is more tightly bound to the soil constituents.

For comparison purposes we selected also commercially available humic acid sodium salt, technical grade (H 16752, Sigma–Aldrich, Inc., St. Louis, MO, USA) in spite of the fact that manufacturer data of this product are weak (this product is of natural mined origin and the only purification step is washing with deionized water). However, it is well characterized by Kim et al. [24–26] and proposed by Susic and Boto [17] as a standard for environmental samples: its elemental composition is according to Rajec et al. [27] C-55.23, H-4.48, N-0.32, 0-37.64, S-2.33%; inorganic major impurities are Al-2.95, Ca-9.93, Fe-12.21, Mg-0.70, Na-75.12 and Si-3.33 mg/g, respectively. The cation exchange capacity is 7.06 mequiv./g. The size distribution, determined by gel permeation chromatography was found to be 95.9% in range up to 8 kDa and the rest in the class above 100 kDa. This HA is favoured by radiochemists, e.g., [27] in SEC (radio)chromatography of aqueous humic acid solutions with radioisotopes to study their interactions with HAs. Another samples of commercially available free HA, technical grade (Sigma–Aldrich, Inc., St. Louis, MO, USA) and commercially available ammonium salt of peat humic acid (fertilizer, Ecohum Ltd, Senica, Slovakia) were also involved in our studies.

Solutions of HS were prepared daily fresh by dissolution of weighed HS exactly to prepare around 3 mg/ml concentration level in initial composition of mobile phase for RP-HPLC and their pH values were adjusted to around 8 by 0.05 M NaOH.

Dimethylformamide (Merck, Darmstadt, Germany; Lachema, Brno, Czech Republic) was of pro analysi purity. Water for gradient

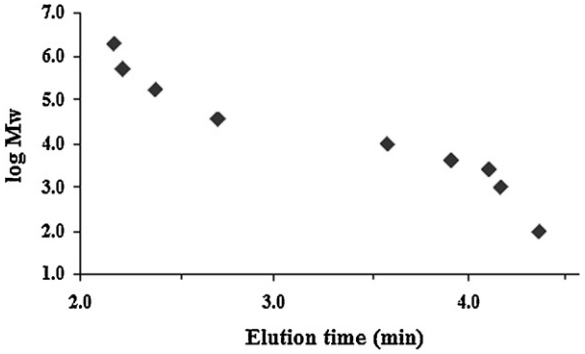

Fig. 1. Calibration plot of Spheron HEMA 100 column by polystyrene standards in DMF mobile phase. Plotted marks (from left to right) correspond to the Blue Dextran 2000 kDa (t_R = 2.18 min); polystyrene standards 498 kDa (t_R = 2.22 min), 175 kDa (t_R = 2.39 min), 37 kDa (t_R = 2.71 min), 10 kDa (t_R = 3.59 min), 4 kDa (t_R = 3.92 min), 2.6 kDa (t_R = 4.11 min), 1 kDa (t_R = 4.17 min); styrene 0.1 kDa (t_R = 4.37 min). For more details see the text.

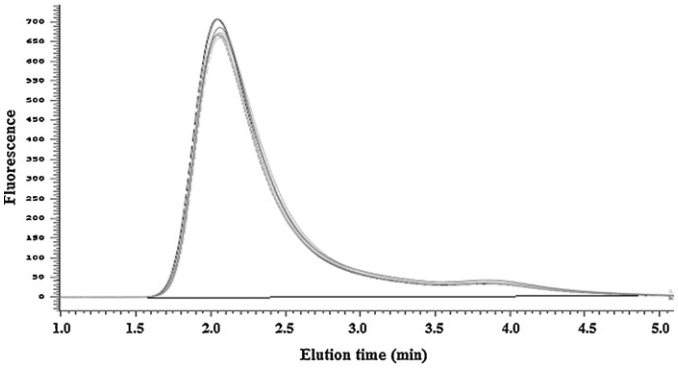

Fig. 2. Overlay of 9 SEC chromatograms of Ekohum sample (peat derived humic and fulvic acids. Column Spheron HEMA 100 (2.2 mm × 250 mm), mobile phase 99/1 DMF/phosphate buffer pH 3.0, flow-rate 0.25 ml/min, column oven temperature 35 °C. Fluorimetric detection 470 nm (Ex.) and 530 nm. (Em.)

HPLC was prepared by Labconco Pro-PS unit (Labconco, Kansas City, USA). Phosphoric acid (Lachema, Brno, Czech Republic), sodium dihydrogen phosphate and sodium hydroxide, both (Merck, Darmstadt, Germany) of pro analysis grade were used for preparation of buffered mobile phases.

3. Results and discussion

3.1. RP-HPLC method description and narrow-bore SEC column calibration and testing

The devised step-wise gradient chromatographic [28,29] method with tandem DAD and fluorimetric detection was used for characterization and fractionation of HA by their chromatographic profiles. The pore size (30 nm) of selected stationary phase allows an efficient mass transfer, very good recovery of HA and fulvic acids (FA) and also non-restricted steric distribution of HS into the pores (molar mass exclusion limit around 2 MDa [30]) for interaction with surface functional groups (specific surface area is 80 m^2/g) [28,29]. These values fit size of HS without size discrimination and result in good shaped chromatographic peaks.

The shape of HA chromatogram actually depends also on the elution technique. By the use of linear gradient elution one can get chromatographic record in a shape of a broad peak regardless the efficiency of chromatographic column. However, by use of step-wise gradient elution we can get separation of HS in shape of several well defined peak-like record.

Very good solvating power of DMF toward HAs was already utilized for their isolation from various soils [19]. In the above mentioned works [28,29] it was proved that dimethylformamide (DMF) is also acceptable solvent of macromolecular polyelectrolyte and organo-inorganic HS in gradient RP-HPLC. Another important parameter which was strictly held under control is pH of aqueous portion of the mobile phase. On the base of our previous results we continued with combination of the DMF RP-HPLC method and SEC method under the condition of high mobile phase compatibility.

At Fig. 1 is presented plot of relative molar mass calibration of narrow bore SEC column that was further used for analysis of selected RP-HPLC collected fractions.

SEC using narrow bore column was tested also for the other figures of-merit. Similarly to repeatability and reproducibility of RP-HPLC chromatographic peaks induced by step gradients [28] we studied repeatability of SEC chromatographic profiles by peat derived HA and FA of Ecohum, which was selected as the worth defined HS material. At Fig. 2 overlay of 9 repeated 1.0 μl injections of 3.0 mg/ml sample is depicted. The main peak of HA with higher molar mass eluted at the average elution time 2.06 min with RSD ± 0.64%, the second peak of lower molar mass HS eluting at 3.84 min had RDS ± 0.45%. Total area of SEC chromatogram repeated with RSD ± 4.1%. Fig. 2 shows that SEC of HS is usable for their characterization via chromatographic profiles. Reproducibility of the results is up to RSD ± 1.1% of elution time and RSD ± 5.2% of total chromatogram area.

Sample load influence to chromatographic profiles of HS analyzed at narrow bore SEC column was tested for all studied HS by change of injection volume up to unrealistic values 100 μl. At Fig. 3 we can observe changing profiles (peaks proportions) of HA Aldrich. Increase of sample load via change of injection volume favours the second eluted peak (low molecular mass HS). It is maybe due to smaller resolution obtained under the overloaded conditions. Surprisingly, even under the condition total chromatogram area fits linear regression with high correlation coefficient 0.9986 as is evident from the plot of total area vs. injected volume at Fig. 4. For further experiments injected volume 10 μl was set as optimal value for samples or fractions containing less than 2 μg of HS per injection.

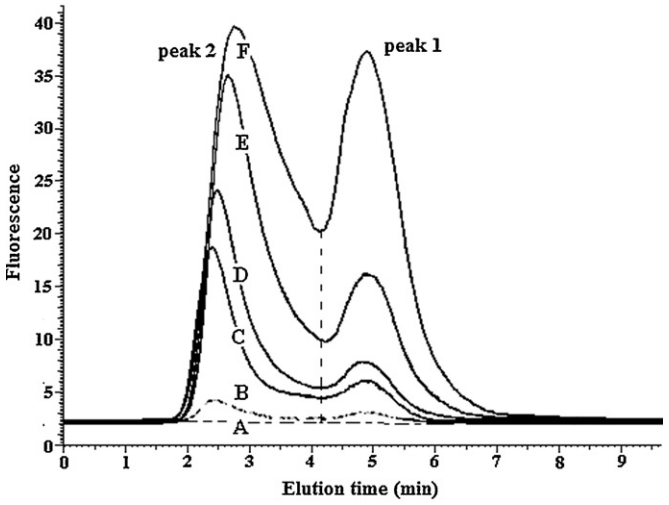

Fig. 3. Influence of sample load via change of injection volume to SEC chromatograms of HA Aldrich fraction No.6. A is the blank chromatogram after the first injection (injected volume 10 μl); B is the blank after 50 injections (overall injected volume 1000 μl); C is injection of 10 μl (1.5 μg HS); D, 20 μl (3.0 μg HS); E, 50 μl (7.5 μg HS); F, 100 μl (15 μg HS) of sample HA Aldrich fraction no 6 collected from RP-HPLC step gradient. Flow-rate was set to 0.20 ml/min (final and optimal selected flow-rate), 35 °C, 99/1 DMF/phosphate buffer pH 3.0.

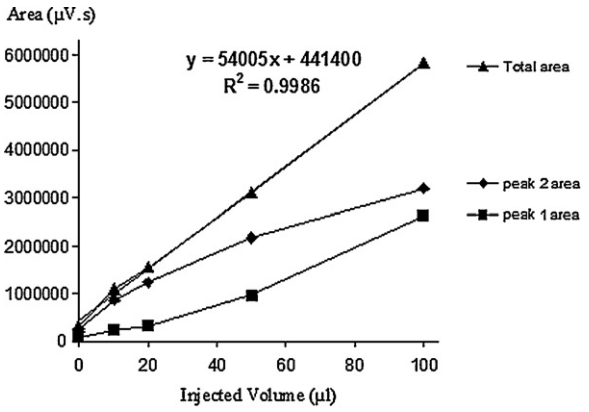

Fig. 4. Plot of peak areas (μV s) vs. injected volume (μl) of peaks obtained by SEC from HA Aldrich fraction no. 6 of Fig. 3.

3.2. RP-HPLC fractionation of HA and analysis of fractions by narrow-bore SEC

Horizontally located chromatograms of normalized SEC profiles in Fig. 5 show typical example of the profiles of sodium salt of HA Aldrich as resulted from analysis of humic acid fractions collected from RP-HPLC step-gradient run. Similarly, normalized fluorescence profiles were measured for HA I (Fig. 6) and HA J (Fig. 7). Sodium salt of HA Aldrich gives overall profile different from the other two soil derived HA, moreover total fluorescence signal of sodium salt HA Aldrich is much lower, maybe due to higher content of inorganic ballast substances or more aliphatic character of the material in comparison to HA I and HA J. The last two HA were isolated and purified from very fertile Calcaric Fluvisoil that is used for cereals production.

From the drawings it is evident that the combination of chromatographic methods is capable to distinguish among the fractions of humic acids. Repeatabilities of retention times of artificial peaks of HA were evaluated from the data obtained in 8 consecutive runs

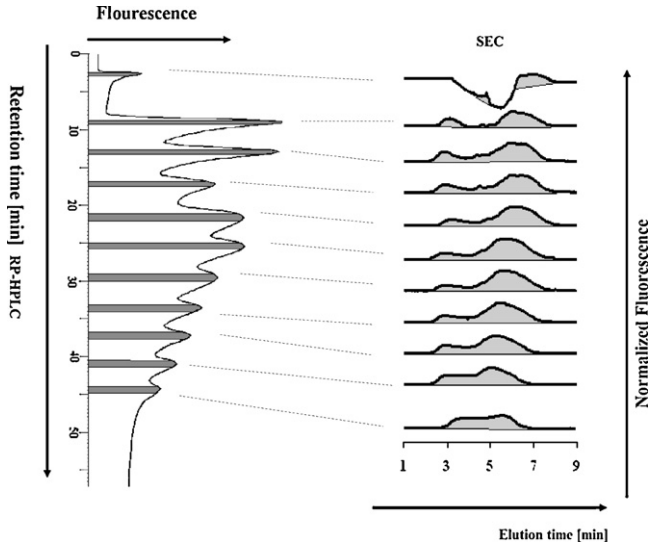

Fig. 5. Multichromatogram of sodium salt HA Aldrich. SEC chromatograms were normalized with respect to the highest peak for emphasis on qualitative differences in individual fractions taken from RP-HPLC. Vertically located record is background corrected RP-HPLC profile obtained using FLD at Ex. 470 nm/Em. 530 nm, flow-rate 1.0 ml/min, after injection of 100 μl sample volume. Horizontally located records are SEC profiles of major HA fractions (FLD at Ex. 470 nm/Em.) at flow-rate 0.2 ml/min. Injection volume was 10 μl. For the other conditions see the text of Sections 2.2 and 2.3.

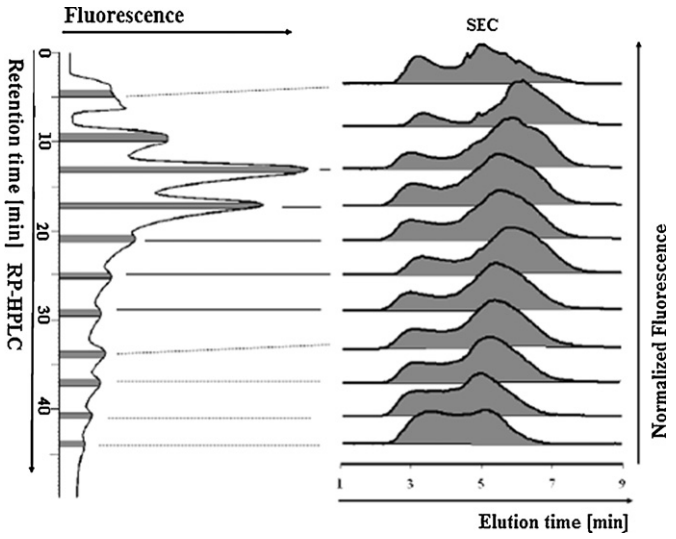

Fig. 6. Multichromatogram of HA I. SEC chromatograms were normalized with respect to the highest peak for emphasis on qualitative differences in individual fractions taken from RP-HPLC. For experimental conditions see the text of Fig. 5. For the other conditions see the text of Sections 2.2 and 2.3.

of sample HA J (HA isolated from calcaric fluvisoil soil samples taken from locality Dunajská Streda, Slovakia). Analyzed were solutions at concentration level 2.55 mg/ml (FLD Ex. 470 nm/Em. 530 nm), for the conditions see Fig. 5. Calculated reproducibility of retention time (t_R) of selected peak (t_R = 24.97 min for FLD detection) was ±0.42% RSD. Repeatability of the data within 1 week (set of 5 data) was ±1.5% RSD. These data are representative also for the other well-shaped peaks of analyzed substances.

The records show that in each of collected fractions from RP-HPLC we can find by SEC different peaks coding distribution of HA fractions with the highest signal in the region below molar masses value 5 kDa. It means that regardless the hydrophobicity and/or interaction of HA-DMF ability of humic acids—their SEC profiles are similar but not identical under the FLD detection conditions.

The recorded chromatograms data can be transformed to the plot of individual recognizable chromatographic peaks that represent two-dimensional separation space of RP-HPLC and SEC separation mechanisms, respectively. Fig. 7 (similarly to

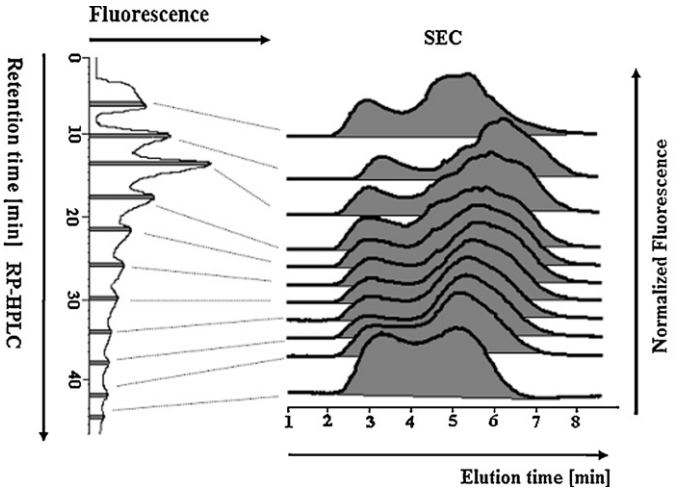

Fig. 7. Multichromatogram of HA J. SEC chromatograms were normalized with respect to the highest peak for emphasis on qualitative differences in individual fractions taken from RP-HPLC. For experimental conditions see the text of Fig. 5. For the other conditions see the text of Sections 2.2 and 2.3.

Table 1
Statistical parameters obtained by application of Spearman Rank Correlation [31] for calculation of degree of correlation for retention data obtained using application of above described combination of chromatographic methods (RP-HPLC and SEC) for selected fractions of HA I.

Spearman Rank Correlation			
Correlation rho		−0.0527	
2-Sided *p*-value		0.8929	
S		126.3245	
Number of pairs (*N*)	0.05		0.01
7	0.714		0.893

Table 2
Statistical parameters obtained by application of Pearson Product Moment Correlation [31] for comparison of degree of correlation of data obtained using application of above described combination of chromatographic methods (RP-HPLC-SEC) for selected fractions of HA I.

Pearson product moment correlation—ungrouped data				
Statistics	Variable *X*		Variable *Y*	
Mean	12.7600		3.6989	
Biased variance	10.5611		1.7804	
Biased standard deviation	3.2498		1.3343	
Covariance		−0.0003		
Correlation		−0.0001		
Determination		0.0000		
T-Test		−0.0002		
p-Value (2 sided)		0.9999		
p-Value (1 sided)		0.4999		
Degrees of freedom		7		
Number of observations		9		
Level of significance (*p*) for a two-tailed test				
df (*n* − 2)	0.1	0.05	0.02	0.01
Critical values	0.582	0.666	0.75	0.798

Figs. 6 and 5) shows wide spread and non-correlation of obtained retention times data pairs for the most pronounced fractions of HA. Actually we can accept it as a graphical evidence of certain degree of orthogonality of 2D method.

To prove the non-existence of retention times (RP-HPLC/SEC) correlation (its existence is defined as H0 hypothesis) we did statistical test by Spearman rank correlation and Pearson product–moment correlation coefficient. The treatment is given in Tables 1 and 2, respectively.

From both tables follows that by comparing calculated Spearman correlation coefficient Rho having value −0.0527 with the critical values at 95% confidence interval 0.714, or calculated Pearson correlation coefficient having value −0.0001 with the critical values at 95% confidence interval 0.666 we can refuse H0 hypothesis. It means that retention and elution data are not correlating, what means also that both methods separation mechanisms are not correlating or redundant. It creates basic condition for effective evaluation of two-dimensional separation space for unique positioning of individual constituent molecules of HA within larger separation space than is offered by the two combined methods. This gives us potential for further study of HA under investigation. Similar results were obtained also by processing RP-HPLC retention data and SEC data measured for humic acids HA J, HA I and sodium salt HA Aldrich, respectively. For comparison purposes refer to Figs. 5–7, respectively, where normalized fluorescence chromatograms are shown.

In the near future the research will be conducted to prove this conceptual strategy also for the comprehensive 2D separation of fulvic acids, lignins and humin, respectively, in combination with advanced detection (multiangle laser light scattering, evaporative laser light scattering, 2D fluorescence, etc.) and identification (mass spectrometry) detection techniques.

4. Conclusions

The results suggest that the devised off-line 2D RP-HPLC and SEC method is highly reliable for characterization and fractionation of soil HAs in a wide concentration range and also at trace concentration levels. Analysis of individual fractions obtained by the described RP-HPLC method by the method working on independent separation principles – described SEC method – provided data of even higher dimensionality needed for HSs investigation.

Obtained results indicate, that such methods could be combined in compact, automatic, orthogonal separation systems for characterization of such complicated natural substances as are examined HA and obtain so more information about their character.

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