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Characterization of humic substances by capillary isotachophoresis

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ABSTRACT

The use of capillary isotachophoresis (ITP) for the characterization of humic substances was studied. ITP separations of these complex mixtures were considerably improved on the addition of polyvinylpyrrolidone (PVP) to the leading electrolyte. It was preferred to work in the spike mode to improve the interpretability of the isotachopherograms obtained using a photometric detector (405 nm). Interaction of PVP with humic substances was found to differentiate humic and fulvic acids. ITP was useful also for characterizing differences in the molecular weights and hydrophobicities of humic substances fractionated by chromatography on Sephadex G-50 and on Phenyl-Sepharose CL-4B.

INTRODUCTION

Humic substances (HS) are complex mixtures of organic compounds formed in soil and water by chemical and biological degradation of plant and animal residues and by synthetic activities of microorganisms. From the physico-chemical point of view they are dark-coloured polymeric organic acids with high numbers of carboxylic and phenolic groups [1]. At present, separation methods fails to separate these complex mixtures into individual chemical. Nevertheless, their partial separations, based on well defined physical and/or chemical principles, are useful in understanding the nature and properties of HS.

The ionogenic nature of water-soluble HS enables electrophoretic separation methods to be employed for their characterization. Zone electrophoresis of HS in polyacrylamide gel [2,3], isoelectric focusing [4] and isotachophoresis (ITP) in polyacrylamide gel [5,6] are examples of the use of various electrophoretic methods for these purposes. Of these, the highest resolution has been reported for isoelectric

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focusing [6], and the resolving power of the ITP separation could be improved by adding ampholytic mixtures (continuous spacing constituents) to the samples [5,6].

In this work we studied potential of capillary ITP for characterizing HS and results concerning the optimization of the ITP working conditions are presented.

EXPERIMENTAL

Instrumentation

A CS isotachophoretic analyzer (VVZ PJT, Spišská Nová Ves, Czechoslovakia) was assembled with the column-coupling configuration of the separation unit [7,8] using modules provided by the manufacturer. The analytical column was equipped with a UVD 01 photometric detector (VVZ PJT).

The signals from the detectors were registered by a two-channel line recorder and that from the photometric detector (405 nm) was registered in parallel by an SP 4270 integrator (Spectra-Physics, Darmstadt, Germany).

The driving currents were 200 and 45 μA in the preseparation and analytical columns, respectively. Mixtures of discrete spacers (27 constituents) were chosen from the constituents suitable for this purpose [9]. The spacers were injected with the aid of a 30-μl sample loop and the sample was injected with a microsyringe (Hamilton, Bonaduz, Switzerland). The analysis was completed in *ca.* 28 min. For other experimental data, see Table I.

Chemicals for ITP experiments

Solutions of the leading and terminating electrolytes were prepared from chemicals of the highest available purity provided by Sigma (St. Louis, MO, U.S.A.), Fluka (Buchs, Switzerland) and Pierce (Rockford, IL, U.S.A.). Hydroxyethylcellulose (HEC) was used as an anticonvective additive in the leading electrolyte solutions. The preparation obtained (Serva, Heidelberg, Germany) was purified on a mixed-bed ion exchanger (Duolite MB; Duolite, France). Polyvinylpyrrolidone 360 000 (PVP) (Lachema, Brno, Czechoslovakia) was deionized in the same way as HEC.

Distilled water, further deionized on a mixed-bed ion exchanger (Duolite, MB), was used for the preparation of the solutions.

TABLE I

OPERATIONAL SYSTEMS

Solvent	Water
Leading anion	Cl^-
Concentration (m*M*)	10
Counter ion	β-Alanine
Additive to the leading electrolyte	0.1% (w/v) HEC
Co-additive to the leading electrolyte	0–2.5% (w/v) PVP[a]
pH of the leading electrolyte	3.50
Terminating anion	$(Caproate)^-$
Concentration (m*M*)	5

[a] For actual concentrations of PVP, see the legends to the figures and the text.

Humic substances and their preparations

HA-Fluka. Humic acid (HA) with molecular weight in the range 600–1000 dalton was obtained from Fluka (Cat. No. 53 680). A stock solution (0.2%, w/v) was prepared by dissolving the acid in a 10^{-3} mol/l aqueous solution of sodium hydroxide. The pH of the solution was adjusted to 5.0 on addition of morpholinoethanesulphonic acid (MES). Five-fold diluted stock solution was taken for ITP experiments.

HA-1, FA-1 and HyA-1. Humic, fulvic and hydrophilic acids were isolated from peatbog water sampled at Borkovické Blato, a bog area in South Bohemia. Humic (HA-1) and fulvic (FA-1) acids were obtained on an Amberlite XAD-8 (Serva) packed column by using the procedure described by Thurman and Malcolm [10]. In our modification of the procedure, dialysis in a Spectra/Por 6 tube (molecular weigth cut-off 1000; Spectrum, Los Angeles, CA, U.S.A.) was used instead of gel chromatography to remove low-molecular-weight compounds.

The effluent from the XAD-8 column was collected, its pH was adjusted to 4.5 and it was percolated through a column packed with a weak anion exchanger in the Cl^- form (Spheron-DEAE; Lachema). Hydrophilic acids (HyA-1) trapped on the column were eluted with a 10^{-1} mol/l aqueous solution of sodium hydroxide. The eluate was treated with Dowex 50W (H^+) cation exchanger and was finally lyophilized.

HA-2, FA-2 and HS-2. Nordic humic and fulvic acids, and lyophilized water from Hellerundmyra, a bog area near Oslo (Norway), were used [11]. HA-2 and FA-2 were isolated using the same method as for International Fulvic and Humic Acids [10]. HS-2 was prepared by 200-fold concentration of the filtered water (0.45 μm) by vacuum evaporation and lyophilization.

X1, I, II, IIIa and IIIb. Fractions of humic substances isolated from water from the Mirochovské blato peatbog, near Třeboň (South Bohemia) were used. The procedure of Mantoura and Riley [12] employing Amberlite XAD-2 resin was used to isolate the humic substances. They were neutralized with Dowex 50W (H^+) cation exchanger, freed from low-molecular-weight constituents by dialysis with a Spectra/Por 6 tube (see above) and lyophilized (sample X1). This sample was fractionated by gel chromatography on Sephadex using the procedures of Wershaw and Pinckney [13] and Hejzlar [14].

(a) A 1% solution of the sample X1 dissolved in distilled water at pH 12 (adjusted with sodium hydroxide) was applied on a Sephadex G-50 column. Water was used as the eluent (see Fig. 4a, separation 1). Three main fractions were collected (I, II and III).

(b) Fraction III was mixed with sodium chloride (15.5 g/l), its pH was adjusted to 7.0 and it was further separated on the same Sephadex G-50 column. On elution with water (Fig. 4a, separation 2) two fractions, IIIa and IIIb, were obtained.

The acids present in the fractions obtained from X1 were converted into the H^+ forms and lyophilized. These samples were reconstituted in water before the analysis (0.4 mg/ml). A detailed description of the isolation and characterization of X1 and related fractions can be found elsewhere [15].

1P, 2P, 3P and 4P. Fractions of HA-Fluka (see above) were obtained by hydrophobic chromatography on a Phenyl-Sepharose Cl-4B (Pharmacia, Uppsala, Sweden) column with a 4-ml bed volume.

A 1-ml sample volume (2 mg/ml) placed on the column was eluted with 3 ml of

distilled water and the eluate was trapped into three fractions (1P, 2P and 3P). Fraction 4P remained retained at the top of the sorbent bed (Fig. 5a). It was eluted with 8 ml of a 2% (w/v) solution of Triton X-100. No further preparation of the fractions was needed before their ITP analyses (differences in the concentrations of HS in the fractions were compensated for by sample injection volumes).

RESULTS AND DISCUSSION

ITP separation of humic substances

Humic substances migrate anionically and with high effective mobilities within the pH range currently employed in ITP. A large number of the constituents present in such samples are responsible for the appearance of the isotachopherograms (features typical of complex ionic mixtures). Their spectral properties [16] are favourable for achieving a high detection selectivity in ITP and, therefore, photometric detection at 405 nm was chosen. To improve the interpretability of the isotachopherograms obtained with the photometric detector, we preferred the work in the spike mode [17] with discrete spacing constituents added to the sample (Fig. 1b).

From the isotachopherogram in Fig. 1b it is apparent that also at a low pH of the leading electrolyte the main part of the acids migrated with effective mobilites close to that of the leading ion. Such a limited mobility span of HS indicates a poor capability of ITP from the point of view of differentiation of this group of constituents. A lower pH of the leading electrolyte (pH < 3.5) did not provide any improvement in this respect [18] and, in addition, it could be critical from the point of view of precipitation of some sample constituents [16]. A considerable improvement in the separation conditions was possible [18] when the counter-ionic constituent of an

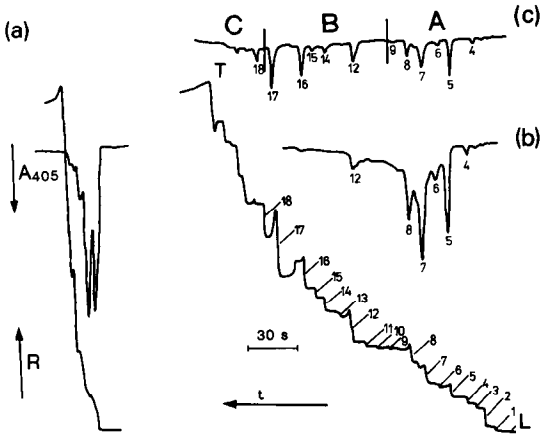

Fig. 1. Isotachopherogram from the separation of HA-Fluka. (a) Conductivity and photometric detector records as obtained for a 5-μl injection volume (for the sample description, see Experimental) in the electrolyte system (Table I) without PVP; (b) same as (a) except that a 30-μl volume of the spacers (each at a 0.05 mM concentration) was also injected; (c) same as (b) (the sample volume was 10 μl) except that the leading electrolyte contained 0.025% of PVP. The conductivity detector records were nearly identical for (b) and (c). A, B and C = symbols for the mobility regions in the span leading (L)–terminating (T) ions. 1–18 = Numbers of boundaries between discrete spacers [9].

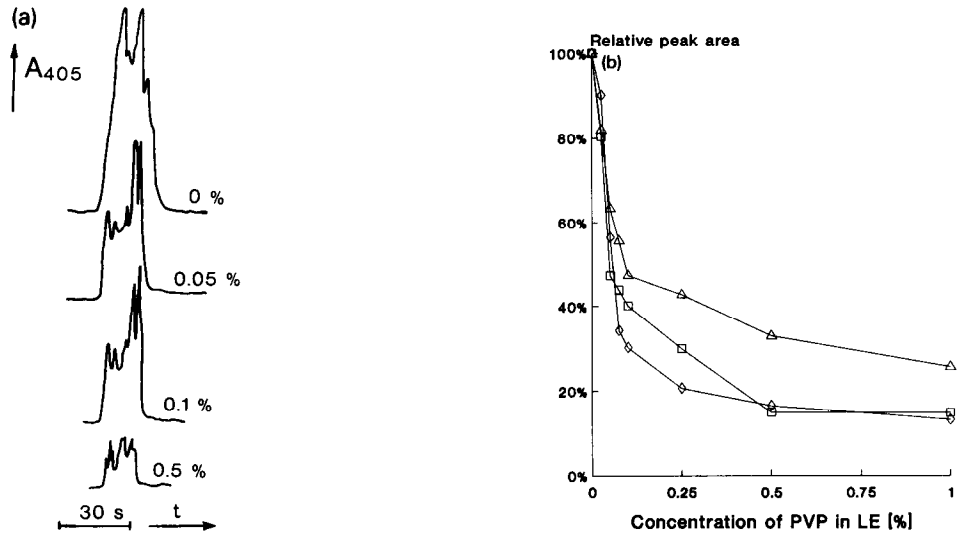

Fig. 2. Influence of PVP concentration on the migration of humic substances. (a) Profiles obtained from the photometric detector for various concentrations of PVP in the leading electrolyte (15-μl injection volumes); (b) graphical plots of dependence of the peak area on PVP concentration in the leading electrolyte. The peak areas are related to the area obtained in the run without PVP. Samples: □, HA-Fluka; ◇, HA-1; △, FA-1.

ITP profiles of some of the studied HA and FA (the acids were obtained as described under Experimental) are shown in Fig. 3a. The profiles, split into three regions (see above), show that the acids differ mainly in the occupancies of the C regions. Here, large parts of HS are present in HA samples whereas in FA this

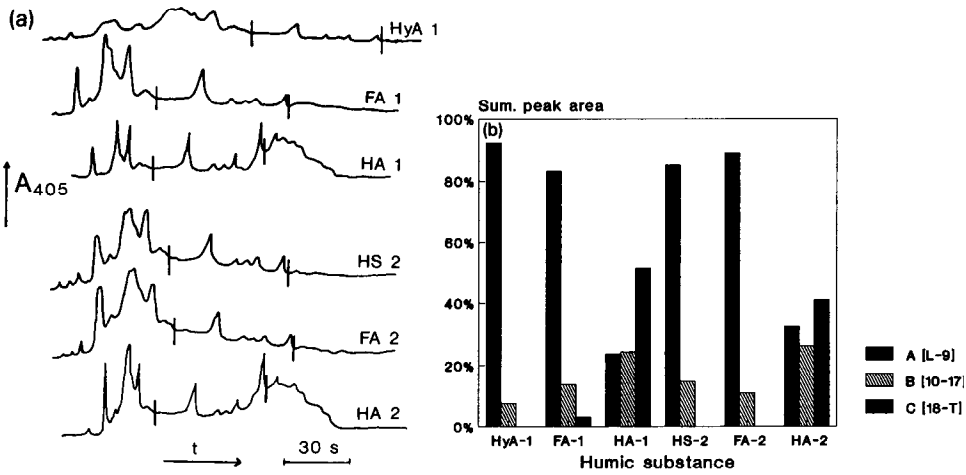

Fig. 3. Profiles of HS fractionated on an XAD resin. (a) Isotachopherograms obtained for 10-μl volumes of the XAD fractions of HS. The leading electrolyte contained 0.025% of PVP. The vertical bars divide the profiles into three mobility regions, A, B and C, from left to right. (b) Graphical representation of the profiles as evaluated on integration (sums of the relative peak areas within the mobility intervals, A, B and C are presented).

migration region is almost free of the analytes. It should be noted that without the use of PVP such a differentiation was impossible. This differentiation also did not exist when the concentration of the PVP in the leading electrolyte was higher than 0.1% (HS migrating in the C regions of the HA samples in Fig. 3 were retarded into the

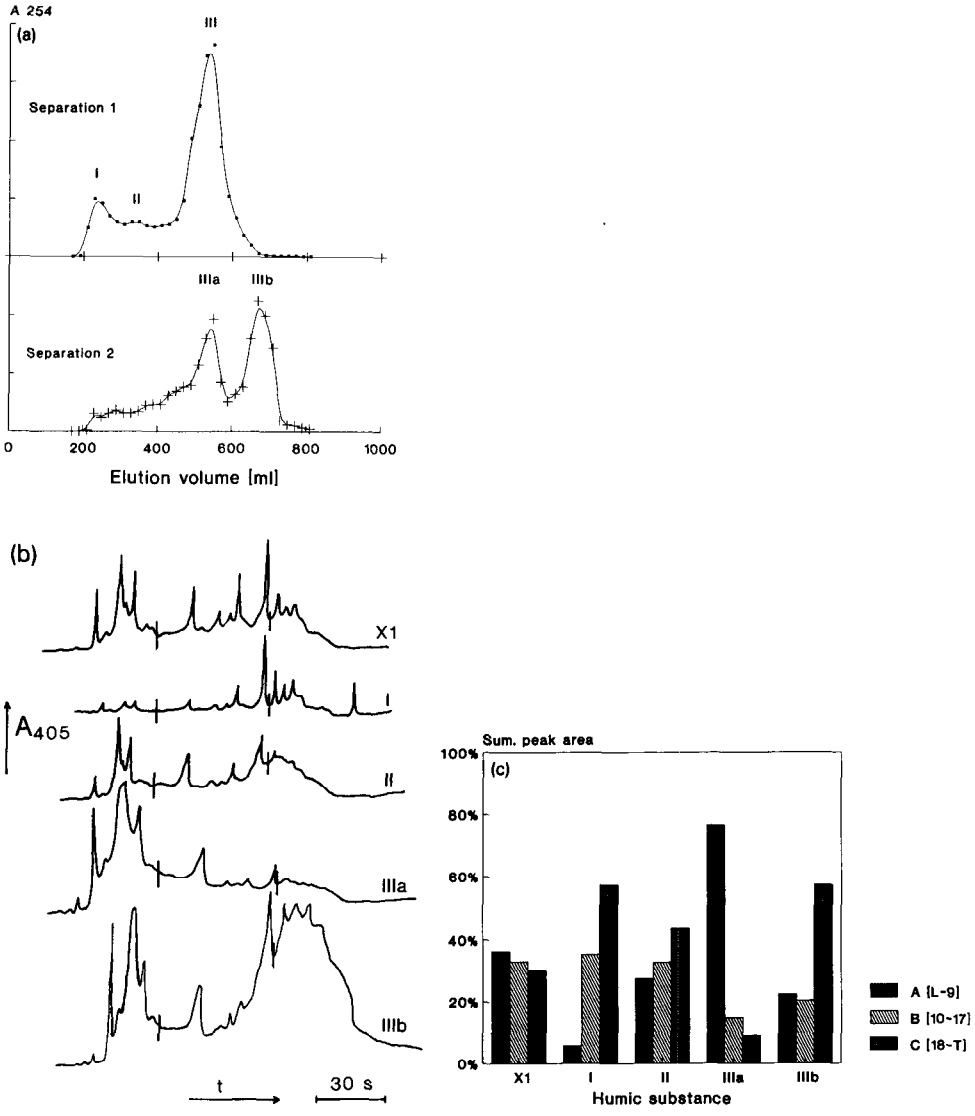

Fig. 4. Profiles of HS after fractionation on Sephadex G-50. (a) Chromatograms (254 nm) for gel chromatographic separation of X1 (separation 1) and for adsorption chromatography of fraction III (separation 2; see Experimental for details). (b) Isotachophoretic profiles of the original sample (X1) and the fractions. The leading electrolyte contained 0.025% PVP (10-μl injection volume). The vertical bars divide the profiles into three mobility regions, A, B and C, from left to right. (c) Graphical representation of the profiles of the sample and fractions (sums of the relative peak areas within the mobility intervals A, B and C are presented).

terminating zone). The effective mobilities of the constituents present in HyA-1 were influenced only negligibly by the use of PVP. It also apparent that the sample of water (HS-2) had a profile very similar to those of FA. This is in agreement with previous findings [22] that natural waters contain mainly FA.

It is known that HA have higher molecular weights and a higher content of aromatic structures than FA [1]. To decide which of these differences was responsible for the concentration of a large part of HA in the mobility region C, we carried out the experiments discussed below.

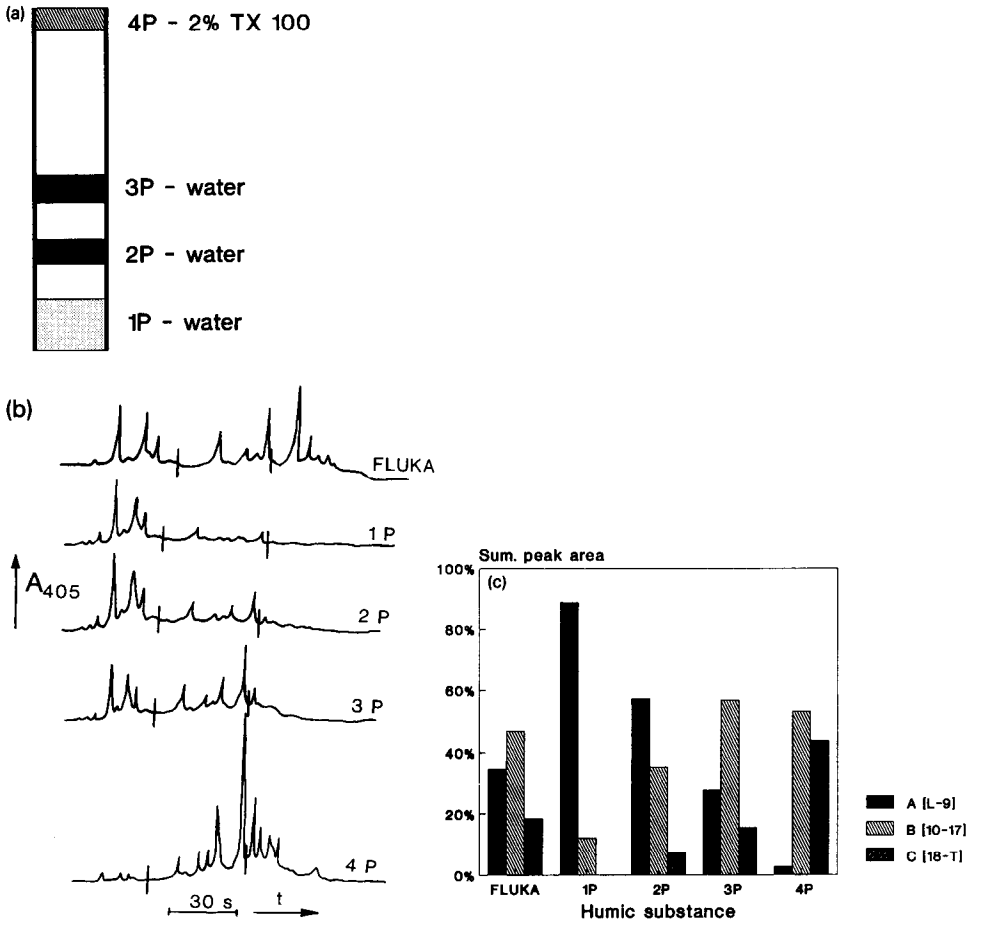

Fig. 5. Profiles of HS present in HA-Fluka after fractionation on Phenyl-Sepharose Cl-4B. (a) Schematic illustration of the fractionation of the sample on the column packed with Phenyl-Sepharose Cl-4B (for further details, see Experimental). (b) ITP profiles of the sample and fractions. The leading electrolyte contained 0.025% of PVP. Injection volumes were 5 μl for HA-Fluka, 1P, 2P and 3P and 50 μl for 4P. The vertical bars divide the profiles into three mobility regions A, B and C, from left to right. (c) Graphical representations of the profiles as in Figs. 3 and 4.

Separation on Sephadex G-50

The course of a two-step separation of X1 on a Sephadex G-50 column is shown in Fig. 4a. The first separation step was in fact gel chromatography, whereas during separation of fraction III (the second step) the experimental conditions (presence of sodium chloride) were such that adsorptive and hydrophobic effects also played a role [13]. Profiles of the original sample and fractions obtained by this two-step separation are shown in Fig. 4b. A graphical evaluation is shown in Fig. 4c. It is apparent that fraction I (containing the constituents with molecular weights > 50 000 dalton) hardly contains any of the most mobile constituents (poor A region in the profile). It is also clear that the presence of this group of constituents gradually increases in the fractions of lower molecular weight (Fig. 4c). The profile of fraction II is very close to that characteristic of the original sample (X1 in Fig. 4).

The elution volume of fraction IIIa coincided with the elution volume of fraction III (1000–5000 dalton). From its ITP profile (Fig. 4b), it can be seen that it consisted mainly of constituents with high effective mobilities. In contrast to other fractions these differences could be also detected in ITP experiments without PVP (not shown). The results suggest that HS fractions with higher molecular weights have lower effective mobilities than those with lower molecular weights. However, when PVP was present in the leading electrolyte, the behaviour of fraction IIIb (containing low-molecular-weight constituents when the elution data are considered) differed. It was found by ^{13}C NMR spectroscopy that this fraction contains a much higher ratio of aromatic structures [15], which are known to interact with PVP [20,21].

Separation on Phenyl-Sepharose Cl-4B

An HA-Fluka sample was fractionated on a Phenyl-Sepharose Cl-4B packed column (Fig. 5a). A comparison of the ITP profiles of the fractions and their graphical evaluations (Fig. 5b and c) with their elution orders (an increase with increasing hydrophobicity of the solute) shows that for the higher hydrophobicities of the fraction constituents a larger number of the constituents with low effective mobilities are present in it. However, in this instance low effective mobilities can be ascribed to the interaction of the separands with electroneutral PVP.

CONCLUSIONS

The results suggest that the presence of PVP in the leading electrolyte provides a means of determining the degree of hydrophobicity and thus the presence of aromatic structures of HA. In addition, the profiles also demonstrate differences in the molecular weights (or, better, molecular weight/charge number ratios).

Differences in hydrophobicities and molecular weights and associated solubilities of the constituents serve as a basis for classical differentiation schemes for HA and FA. The results indicate that the method employed here has potential to serve for this purpose with some obvious advantages (speed of analysis, less labour requirements, minimum sample preparation).

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