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Capillary zone electrophoresis study of aggregation of humic substances

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Abstract

The process of humic acids (HAs) component agglomeration (oligomerization) can be followed by capillary zone electrophoresis (CZE). Oligomerization of HAs of different origin was found to be strong, it leads to kinetically robust entities which can be separated by CZE and their formation is one of the reasons for unsuccessful separation by previous authors. Another difficulty comes from the sorption of some of the HA fractions on the fused-silica capillary wall. Critical concentration for the formation of aggregates was found to be mainly around 30 mg/l. Results of CZE were confirmed by vapour pressure osmometry.

Keywords: Background electrolyte composition; Humic acids

1. Introduction

With more than 300 published works in the last year alone, humic substances (HSs) research is of high interest in the scientific world. Even if several different models were proposed and properties of HSs were intensively studied ([1–3], and references therein), the real structures of humic acids (HAs), fulvic acids (FAs), humin and hymatomelanic acids still remain unknown, in spite of the fact that for the characterization of HAs many different techniques and methods have already been used ([1,4,5] and references therein).

Our first studies of Czech HAs of different origin [6] (and some commercial ones) concentrated on HA interactions with metal ions, e.g., Cu^{2+}, Ba^{2+}, Ca^{2+}, Pb^{2+} and UO_2^{2+} (stability decreases in this order) [2,3,7] and a general hypothetical model of HA structure was proposed (Fig. 1) [7,8]. In the literature, an excellent work on ''tight metal binding by HAs and its role in biomineralization'' was published recently by Davies et al. [9].

The formation of HA aggregates was already described in the literature [1]. Von Wandruszka et al. [10] refer to pseudomicelles. They observed that aqueous solutions of soil HAs respond to the addition of cations by the formation of molecular microdomains of relatively low polarity. These domains were referred to as pseudomicelles in view of their similarity to detergent behaviour. Chien and Bleam [11], for their study of fluorine-19 nuclear magnetic resonance (NMR) of atrazines in humic micelles adopted Wershaw's [12,13] model of humic membrane–micelle. This model accounts for the dual nature of HAs by postulating that micellar humic aggregates with hydrophilic exterior surfaces and predominantly hydrophobic interiors. Dachs and Bayona [14] presented fractal aggregates in their

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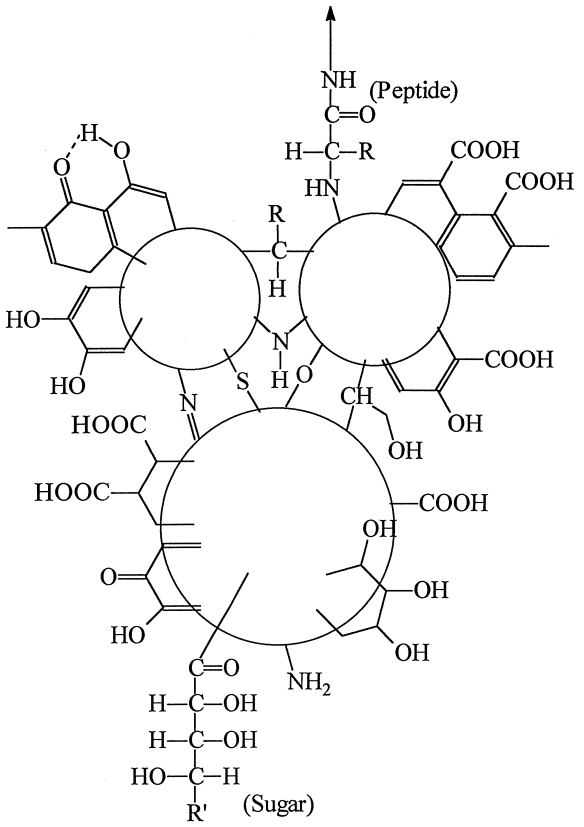

Fig. 1. Hypothetical model of HA structure.

Langmuir-derived model for diffusion- and reaction-limited adsorption of organic compounds on HAs. It is based on the property that natural colloids, aerosols and aggregates generated in wastewater treatments have fractal geometries. Thus, not all the surface sites of these aggregates possess the same collision probability. Shevchenko and Bailey [15] reexamined lignin–humic relationships and not only proposed lignin and HS structures but also presented the formation of polymers. Finally, considering that HAs were a natural mixture with a defined number of compounds, we proposed the possible formation of oligomers [7,8].

Capillary zone electrophoresis (CZE) is nowadays often used for the separation and characterization of HAs due to the ionic and/or polyelectrolyte properties of HAs. In this work, we have mainly concentrated on CZE of HAs as a tool for their aggregation study.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade purity. Mesityl oxide (MSO) used as a neutral marker for electroosmotic flow (EOF) determination was from Fluka (Buchs, Switzerland). NaOH and HCl were from Carlo Erba (Milan, Italy) and Merck (Darmstadt, Germany), respectively. Deionized water used to prepare all solutions was double-distilled from a quartz apparatus (Heraeus Quartzschmelze, Hanau, Germany).

2.2. Humic acids

The HA samples used in this work were: Fluka HA preparative No. 53680 (analysis No. 38537/1 293) (Fluka I), and Fluka HA No. 53680 (analysis No. 38537/1 594) (Fluka II), both supplied by Fluka. The other HAs were supplied by the Research Institute of Inorganic Chemistry (VÚANCH) (Ústí nad Labem, Czech Republic). HAs ''193 MAR'' and ''317 MAR'' [6] were extracted from high quality oxyhumolite originating from Bohemian brown coal of North Bohemia Coal (Colliery Bílina and Vršany, respectively). HA ''248 MAR'', prepared from Bílina locality brown coal, was membrane separated just after extraction [6].

2.2.1. Procedure for humic acids dissolution

Stock solutions of HAs were prepared by weighing a given amount, dissolving it in a small volume of 0.1 *M* NaOH and diluting to a fixed volume using double-distilled water. An ultrasound bath (Branson ultrasonic cleaner, Shelton, CT, USA) was applied for 3 to 5 min to enhance the dissolution.

2.3. Solutions

Standard stock solutions of HAs were filtered through a sintered porous glass filter (G4 porosity) before the use. For background electrolytes (BGEs) used, the value of pH was adjusted with diluted aqueous solutions of NaOH and/or HCl. BGE solutions were prepared daily, filtered through a Chromafil 0.2-μm filter (Macherey–Nagel, Düren,

Germany) and degassed in an ultrasonic bath. BGE solutions were kept in a freezer in order to prevent decomposition by the action of fungi. Because HAs can interact with organic compounds [16–18], antifungal compounds were not added either to BGE or to HA solutions.

2.4. Apparatus

A Spectraphoresis 2000 (Thermo Bioanalysis, CA, USA) unit equipped with a fast scanning detector with deuterium and tungsten lamps working from 190 to 700 nm was used. Untreated fused-silica capillaries of varying total length (length to detector)×75 μm I.D. (Avery Dennison, MA, USA) were housed in a cartridge (see corresponding figures for dimensions). A polyacrylamide (PAA)-coated capillary of 43.6 cm (length to the detector 35.6 cm)×75 μm I.D. (Watrex, Prague, Czech Republic) was used. Samples were injected hydrodynamically under a vacuum (10 342.14 Pa).

The electropherograms were recorded and data reprocessed by PC1000 software (Thermo Bioanalysis, CA, USA) to obtain peak parameters: migration time, peak height, peak area, theoretical number of plates, symmetry factor, etc.

A Knauer vapour pressure osmometer (Berlin, Germany) was used to measure the vapour pressure of aqueous HA solutions. VPO measurements were performed at similar temperature as used for CZE, i.e., at 45°C.

The pH was measured using a glass G202C electrode, standard calomel electrode K401 (Radiometer, Copenhagen, Denmark) and Precision Digital pH meter OP-208/1 (Radelkis, Budapest, Hungary) while standard buffer solutions from Radiometer and/or Radelkis were used for the calibration.

2.5. Procedure

The capillary was daily filled first with the BGE solution which was degassed for 10 min. Furthermore, it was washed 5 min with 0.1 *M* NaOH at 25°C, 10 min with deionized water at a temperature gradient from 25 to 40°C and 5 min with BGE at 40°C. The capillary inlet vials were replenished after each injection. In each analysis a pre-wash for 2 min with NaOH, 3 min with deionized water and 5 min with BGE at 40°C was applied. Separation of HAs was done at various temperatures, mostly 40°C, and hydrodynamic injection time was 18 s if not otherwise mentioned. Separation potential value was optimized for each BGE. Finally, the EOF was determined using MSO under the same conditions as for the separation of the analytes.

Concerning VPO, either saccharose (molecular mass 342) or urotropin (M_r 140) were used for the calibration. The measurement of the temperature difference between two drops, drop of the solvent and that one of the test solution, both placed in a chamber with saturated vapours of the solvent (water in the present case) was performed. Results were expressed as resistance difference ΔR. In fact, we are measuring the difference of temperature ΔT between two drops, which is proportional to the resistance difference measured by the apparatus afforded with a Wheatstone bridge. Resistance difference ΔR is the measure of the oligomerization of solutes in solution, for further details refer to the literature [19].

2.6. Calculations

Reproducibility of the CZE analysis (migration time, peak area, etc.) and of VPO measurements was determined by repeating each measurement three to five times and estimating the relative standard deviations (R.S.D.s) of the parameters using a standard formula.

3. Results and discussion

3.1. CE separation of humic substances

Several methods for the separation of HAs using CZE with UV–Vis, diode array or laser-induced fluorescence (LIF) detection were already suggested [4,5,20–32]. An overview of the BGEs used in the literature and in this work is given in Table 1. We can observe that a high variety of BGEs for HS characterization were used and different results were obtained. In fact, four main types of the separation electropherograms can be observed. The first one consists of broad shaped peaks which can be called humic ''humps'', which sometimes have shoulders

Table 1
Review of BGEs examined for HA separation by CZE

BGE	pH	Ref.	Used HS concentration (g/l)
20 m*M* 2-(*N*-Morpholino)ethanesulphonic acid (MES)	6.15	[28]	High
MES–NaOH	≈3	[20]	0.1
20 m*M* Tris(hydroxymethyl)aminomethane (Tris)	8.30	[28]	High
HCl–Tris	≈3	[20]	0.1
20 m*M* 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS)	10.4	[28]	High
20 m*M* 2-(*N*-Cyclohexylamino)ethanesulfonic acid (CHES)	9.5	[28]	High
10 m*M* Tetraborate	9.0–9.2	[22,23,28]	1 to high
20 m*M* Tetraborate	8.3	[31]	1
25, 50 and 100 m*M* Tetraborate	9.25	This work	0.01–0.5
40 m*M* Tetraborate	9.0–9.3	[29]	≥0.05
90 m*M* Tetraborate	8.3	[30]	1
200 m*M* Tetraborate	10	This work	0.15
6 m*M* Tetraborate–3 m*M* dihydrogenphosphate	8.9	This work [24,25]	0.05–0.5
10 m*M* Tetraborate–5 *M* urea	7.4	[28]	High
10 m*M* Tetraborate with 10% (v/v) acetonitrile	9.0	[22]	1
10 m*M* Tetraborate with 10% (v/v) acetone	9.0	[22]	1
10 m*M* Tetraborate with 10% (v/v) isopropanol	9.0	[23]	0.01–0.1
10 m*M* Tetraborate with 10% (v/v) 2-propanol	9.0	[22]	1
10 m*M* Tetraborate with 10% (v/v) 2-propanol and 5 m*M* urea	9.0	[22]	1
10 m*M* Tetraborate with 10% (v/v) tetrahydrofuran	9.0	[22]	1
20 m*M* Tetraborate with 5 m*M* *trans*-1,2-diaminocyclo-hexane-N,N,N′,N′-tetraacetic acid (CDTA)	8.6	[23]	0.01–0.1
20 m*M* Tetraborate–100 m*M* boric acid	8.45	This work	0.1–0.25
Citric acid–citrate	≈3	[20]	0.1
HCl–imidazole	≈3	[20]	0.1
5 m*M* Imidazole–acetic acid–20 m*M* boric acid	4.5	[23]	0.01–0.1
5 m*M* Acetate	4.75	[28]	High
50 m*M* Acetate	4.6–5.2	[29–32]	0.01–1
8 m*M* L-Alanine	3.17	[28]	High
8 m*M* HCl–59.8 m*M* L-alanine	3.17	[20]	0.1
8 m*M* HCl–30 m*M* DL-alanine	3.40	This work	0.01–0.5
8 m*M* HCl–60 m*M* DL-alanine	3.2	This work [4,5]	0.1–0.25
8 m*M* HCl–60 m*M* DL-alanine with 10% (v/v) methanol	3.2	This work	0.1–0.25
8 m*M* HCl–60 m*M* L- or D-alanine	3.2	This work	0.1–0.5
8 m*M* HCl–60 m*M* β-alanine	3.2	[5,20]	0.1–0.25
8 m*M* HCl–30 m*M* β-phenylalanine	3.2	[5]	0.1–0.25
8 m*M* HCl–30 m*M* L-cystine	3.2	[5]	0.1–0.25
HCl–L-leucine	3.17	[20]	0.1
HCl–L-lysine	≈3	[20]	0.1
HCl–L-serine	3.17	[20]	0.1
8 m*M* HCl–10 m*M* DL-serine	3.2	[5]	0.1–0.25
8 m*M* HCl–15 m*M* DL-proline	3.2	[5]	0.1–0.25
HCl–L-aspartic acid	≈3	[20]	0.1
HCl–glycylglycine	≈3	[20]	0.1
HCl–glycine	3.17	[20]	0.1
8 m*M* HCl–60 m*M* glycolic acid	3.2	[5]	0.1–0.25
10 m*M* HCl–100 or 350 m*M* boric acid	3.15	This work [5]	0.25–0.5
HCl–500 m*M* boric acid	3.38	This work	0.15
25–50 m*M* Dihydrogenphosphate	9.2	This work	0.01–0.5

Table 1
Review of BGEs examined for HA separation by CZE

BGE	pH	Ref.	Used HS concentration (g/l)
100 m*M* Dihydrogenphosphate–5 m*M* phosphate–250 m*M* boric acid	3.3	This work	0.15
67 m*M* Dihydrogenphosphate–3.3 m*M* phosphate–167 m*M* boric acid–3.3 m*M* wolframate	5.0	This work	0.15
50 m*M* Phosphate	9.2	This work	0.1
100 m*M* Phosphate	6.3	[30]	1
20 m*M* Rimantadine hydrochloride	3.40	This work [26,27]	0.01–0.5
20 m*M* Rimantadine hydrochloride with 2, 6, 9, 14 and 50 m*M* $MgCl_2$	3.40	[26,27]	0.01–0.5
50 m*M* Carbonate	9.0–11.4	[29]	≥0.05

but little definition [23,24,28–30]. For these types of electropherograms, it is generally suggested that the ''hump'' corresponds to the average electrophoretic mobility of a HA mixture.

We have examined or re-examined several other possible BGEs for HA separation. With amine and amino acids BGEs [5,20] the separation in 3–5 fractions can be observed (Fig. 2). These electropherograms show only small differences between HAs of different origin. None of the authors studied effect of the temperature on the separation patterns. Thus, we have therefore studied the effect of temperature between 25 and 60°C in rimantadine BGE (Fig. 3) using a PAA-coated capillary. It was found that the highest peak area and greatest peak height and the best separation was obtained for the measurements performed at temperatures in the range from 30 to 40°C. Moreover, increasing the temperature increased the theoretical number of plates (N) from 6160 to 16 619. For subsequent experiments, we therefore worked mainly between 30 and 40°C.

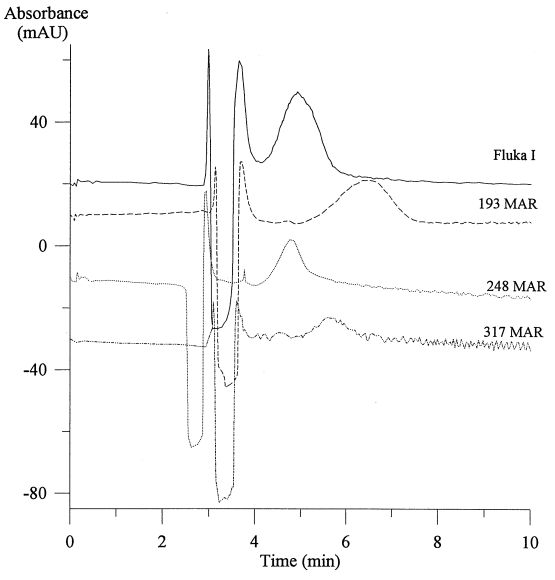

Fig. 2. Electropherogram fingerprints of different HAs in DL-alanine electrolyte. Fused-silica capillary: 43.5 cm (length to detector 35.5 cm)×75 μm I.D. BGE: 60 m*M* DL-alanine with 8 m*M* HCl, pH 3.20. Separation conditions: 15 kV, 40°C, 20 s hydrodynamic injection and C_{HA}=100 mg/l. EOF determined using mesityl oxide was higher than 6.0 min.

Furthermore, from the other buffers studied the separation into five fractions and one sharp peak was obtained when using a dihydrogenphosphate–borate mixture as BGE (Fig. 4).

Finally, the separation into 30 fractions or peaks [4,5] when using highly concentrated boric acid as BGE was obtained (Fig. 5b) even if with rather low reproducibility. In this case, some of the fractions observed were attributed to the formation of humic–boric acids complexes. Increasing boric acid concentration (Fig. 5), increases the number of fractions or peaks observed. It is known that boric acid reacts with oligoalcohols, *o*-diphenols, hydroxycarboxylic acids, oxalic acid [33], etc. Functional groups of these compounds are often present in HAs structure [1,3,7–9,12,15]. Using spectrophotometry we have already proved interactions of boric acid with HAs [5]. We have therefore suggested that the peaks observed in boric acid or borate BGE with a high concentration of boric acid in slightly acid medium

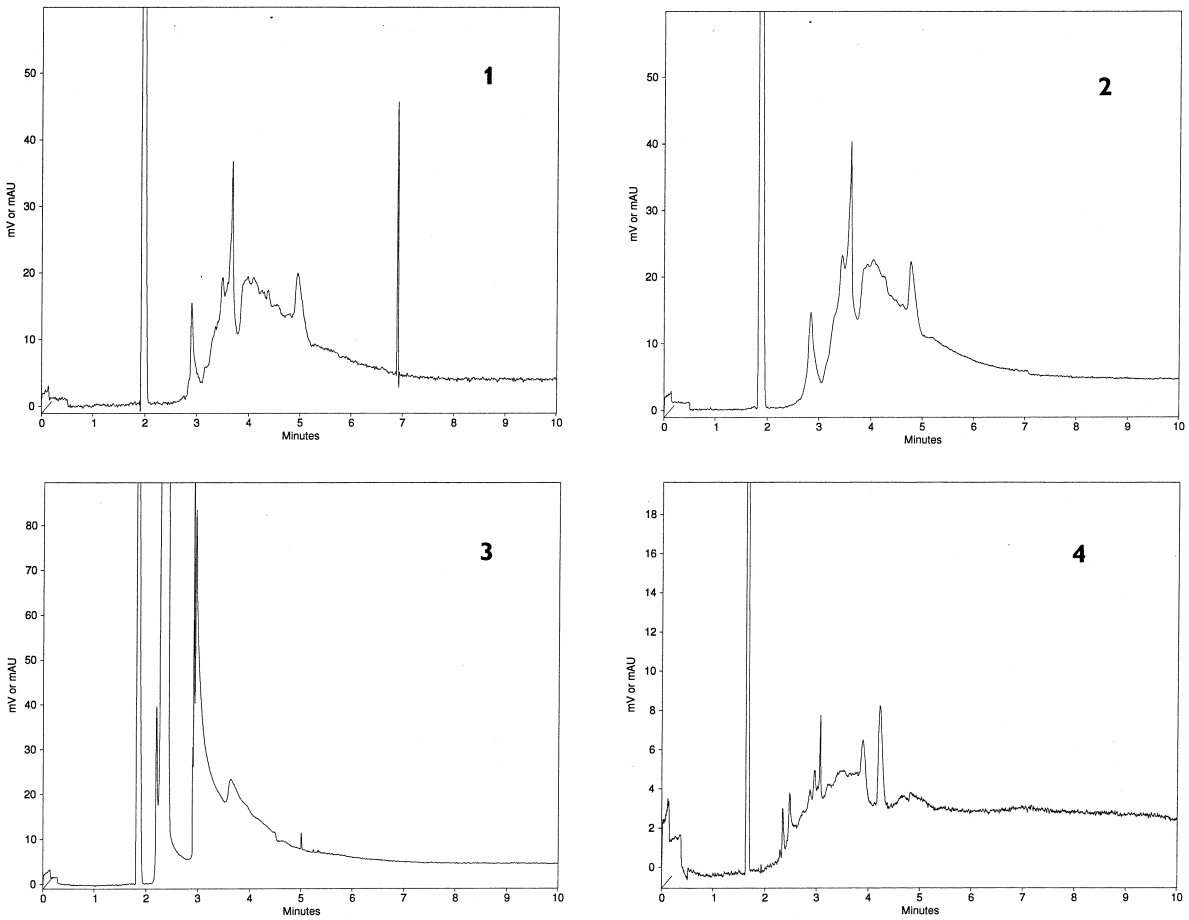

Fig. 3. Effect of the temperature on HA separation by CZE on a PAA-coated capillary. BGE: 20 m*M* rimantadine hydrochloride, pH 3.40. Separation conditions: −25 kV, 18 s hydrodynamic injection, 215 nm and HA concentration 534 mg/l. Temperature: (1) 25, (2) 30, (3) 40, (4) 50°C.

are due to the breaking of oligomers in real fractions [5].

On the other hand, when using BGEs that do not interact with HAs (for example rimantadine), high sorption of HAs on the fused-silica capillary wall was observed [26,27]. In fact, HAs are known to be adsorbed highly on silica rocks and silicates [1,34] or to induce interaction with metal ions on clay surface [35]. This explains observed high sorption of HAs when an uncoated capillary was used. When adding magnesium(II) (>14 m*M*) to the rimantadine BGE, the sorption of HAs was completely prevented. Fig. 6 presents two examples of more real separation patterns of HAs, showing that some of the fractions observed for HAs of different origin have similar migration times. However, even if more fractions are seen, still these peaks observed present a humic ''hump'' pattern. We therefore decided to study this behaviour.

3.2. Oligomerization of HSs followed by CZE

In the literature concerning studies of HS separation, the attention was mainly paid to three major points: HS separation by CZE [4,5,20–32]; study of BGE and pH effect on the separation of HSs [30,31]; interaction of HSs with organic compounds and/or metal ions [21–25,30–32]. Only a few works

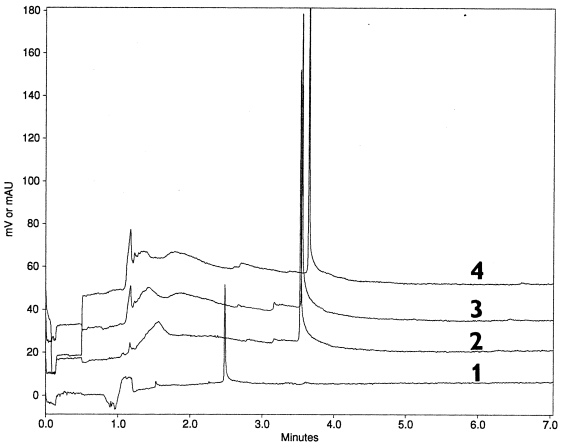

Fig. 4. Electropherogram of Fluka II HA in phosphate–borate buffer. Fused-silica capillary: 43.9 cm (length to detector 35.9 cm)×75 μm I.D. BGE: 3 m*M* KH_2PO_4 with 6 m*M* $Na_2B_4O_7 \cdot 10H_2O$, pH 8.9. Separation conditions: 30 kV, 215 nm, 30°C and 18 s hydrodynamic injection. HA concentration: 1=53, 2=387, 3=480, 4=534 mg/l.

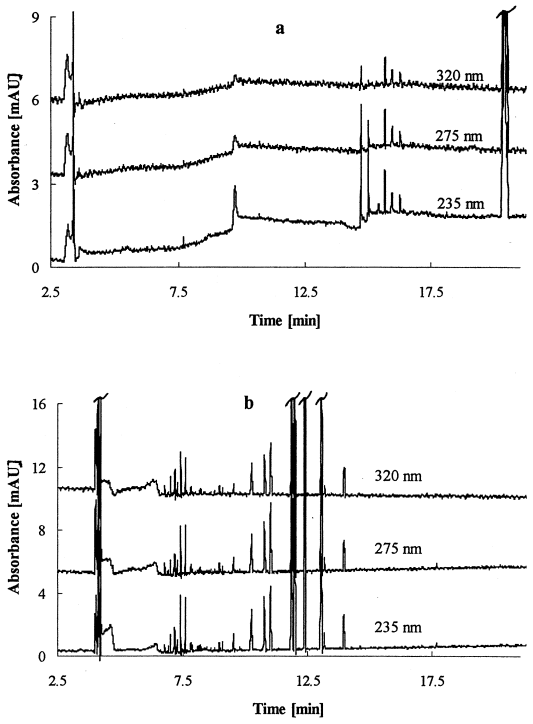

Fig. 5. Electropherograms of Fluka I HA. Fused-silica capillary: 43.5 cm (length to detector 35.5 cm)×75 μm I.D. BGE: (a) 100 m*M* H_3BO_3 with 10 m*M* HCl, pH 3.15 and C_{HA}=267 mg/l; (b) 350 m*M* H_3BO_3 with 10 m*M* HCl, pH 3.15 and C_{HA}=534 mg/l. Separation conditions: 15 kV, 40°C and 15 s hydrodynamic injection. (For migration time 0–2.5 min, no significant peaks were observed).

[26,27,32] up to now mentioned the effect of HS concentration on CZE separation patterns.

In the first part of this study, we have returned to the DL-alanine BGE as suggested by Rigol et al. [20]. These authors were working at very high HA concentrations and they did not explain why. CZE electropherograms (Fig. 7a and b) show that for obtaining any separation patterns of HAs, we must really use either rather high HA concentration or a high injection time value. In case of Fluka II HA, working with constant HA concentration, we can see that a 10 s injection time (Fig. 7a) is not sufficient to obtain any sign of a peak on the electropherogram of HAs. The separation patterns are observed only for injection times higher than 15 s. Evidently, higher injection time with electrostacking phenomena is leading to a zone with a high concentration of HAs. The observed slight decrease of the migration time is due to the increased injected volume leading to a shorter distance to the detector. On the other hand, when increasing HA concentration, at constant injection time (Fig. 7b), we can observe a sharp change of the separation patterns between curves 5 (267 mg/l) and 6 (534 mg/l). We suggest that electrophoretic patterns are obtained only after a ''critical concentration'' of HA (similar to critical micellar concentration, CMC) is overcome. This fact (and the sorption) explains why almost all authors of the works dealing with capillary electrophoresis of HSs were working with high HA concentrations (200 to 1000 mg/l) to obtain some separation patterns. Even if the sharp change of the separation patterns occurs between curves 5 and 6 (Fig. 7b), the CMC value of Fluka II HA in 60 m*M* DL-alanine BGE (pH 3.20) was estimated to be ≈50 mg/l as at this concentration a good electrophoretic pattern was obtained. In fact, due to HA sorption on the fused-silica capillary inner surface studied already in a previous work, even for this BGE [26,27], the ''effective'' humic CMC is slightly lower, about 35 mg/l. In a former work, we have shown that when using solution with a concentration 15 mg/l of HAs (at 18 s injection time and 10 min HA plug standing in the capillary) HAs were completely adsorbed onto the

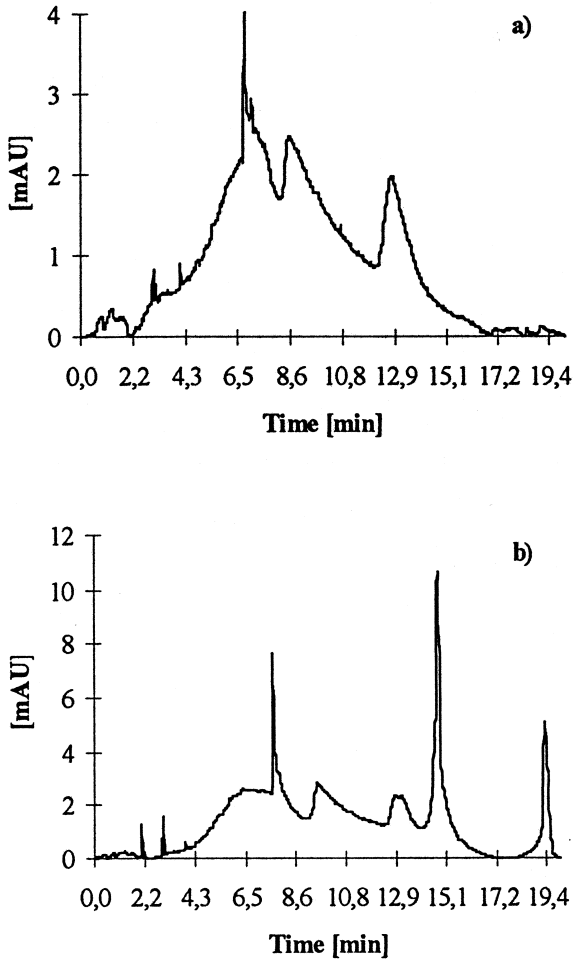

Fig. 6. Electropherogram fingerprints of two HAs of different origin in rimantadine–HCl buffer. Fused-silica capillary: 43.5 cm (length to detector 35.5 cm)×75 μm I.D. BGE: 20 m*M* rimantadine hydrochloride with 14 m*M* $MgCl_2$, pH 3.40. Separation conditions: −15 kV, 40°C and 18 s hydrodynamic injection. HAs and concentration: (a) 193 MAR 101, (b) 317 MAR 102 mg/l.

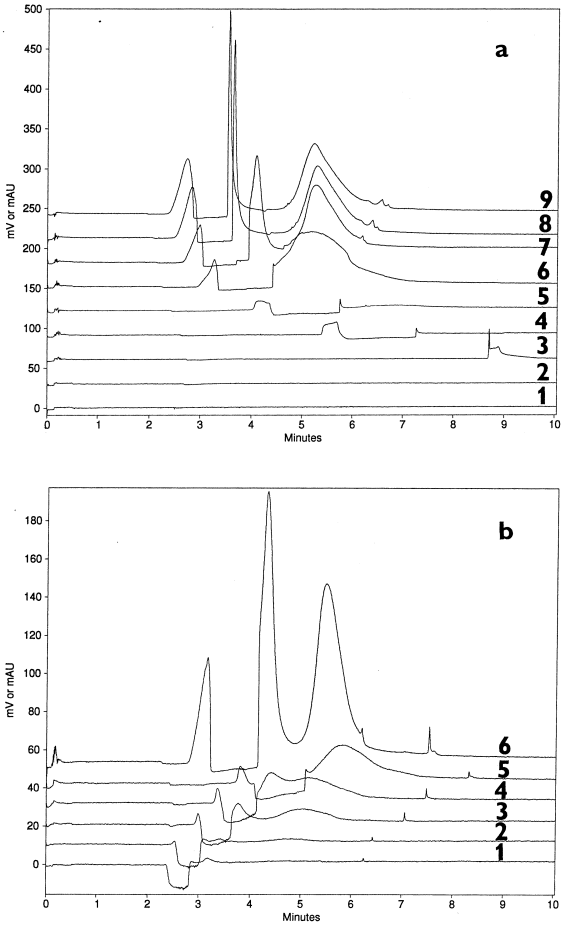

Fig. 7. Oligomerization of Fluka II HA observed when increasing (a) the injection time and (b) HA concentration. Fused-silica capillary: 43.7 cm (length to detector 35.7 cm)×75 μm I.D. BGE: 60 m*M* DL-alanine with 8 m*M* HCl, pH 3.20. Separation conditions: 15 kV, 215 nm and 40°C. (a) C_{HA}=533.5 mg/l and sample hydrodynamic injection as follows: 1=1, 2=3, 3=5, 4=8, 5=10, 6=13, 7=15, 8=18, 9=20 s; (b) 18 s hydrodynamic injection and HA concentrations as follows: 1=5, 2=11, 3=53, 4=107, 5=267, 6=534 mg/l.

fused-silica capillary surface [26,27]. It means that no humic pattern can be therefore observed and the HA plug injected in the capillary ''disappears''. Thus, when the observed humic CMC is higher than ≈50 mg/l of HAs, we must substract corresponding amount due to the sorption effect (≈15 mg/l of HAs) to get the ''effective'' humic CMC value equal to ≈35 mg/l. These results are in a good agreement with those of Schmitt et al. [32]. They were using micellar electrokinetic chromatography (MEKC) and estimated the HA CMC of both Aldrich HAs and Scheyern FAs to around 30 mg/l.

When performing similar experiments with phosphate BGE, we also observed the so-called HA ''hump'' with a number of sharp peaks on it (Fig. 8a) for a high injection time. When increasing the injection time to 36 s (Fig. 8b) the CZE pattern was distorted and just one fraction (1.3 min) and a HA ''double-hump'' (1.6 to 9.6 min) was observed.

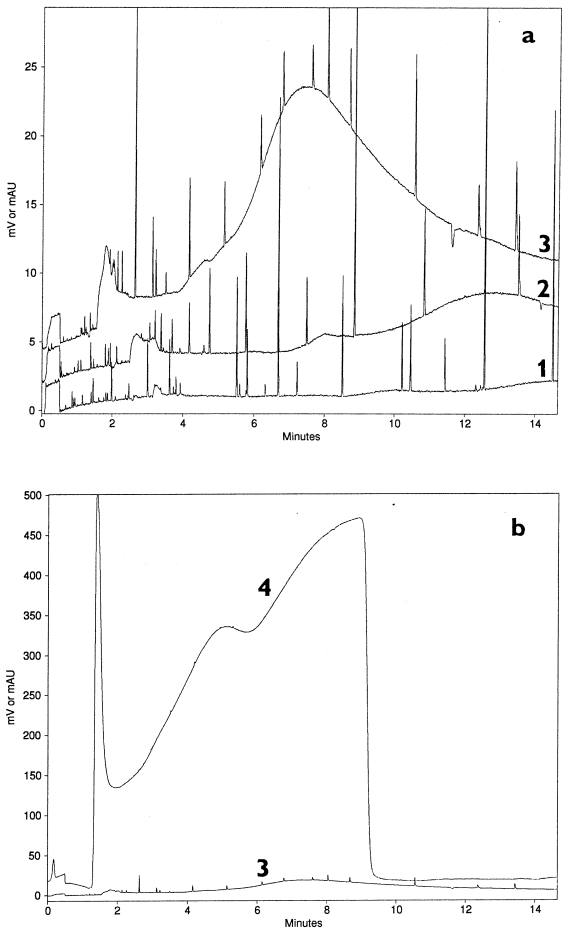

Fig. 8. Oligomerization of Fluka II HA in phosphate BGE. Fused-silica capillary: 43.9 cm (length to detector 35.9 cm)×75 μm I.D. BGE: 50 m*M* H_3PO_4, pH 9.20. Separation conditions: 20 kV, 215 nm, 40°C and C_{HA}=100 mg/l. Sample injection time (hydrodynamic): (a) 1=4, 2=9, 3=18 s; (b) 3=18, 4=36 s.

Moreover, the sharp peaks observed until 18 s injection time completely disappeared due to the ''double-hump'' of high absorbance. These electropherograms can be explained by strong interactions of phosphate with the capillary surface [36] which prevented HA sorption on the capillary wall. However, the oligomerization is observed anyway.

The oligomerization process was also observed even when using Mg(II) additive to rimantadine BGE with a fused-silica capillary as reported elsewhere [26,27]. It seems clear that the sorption process of HAs on the capillary wall is not responsible for HA oligomerization. For this BGE, HA CMC was found to be around 5 mg/l. We can suggest that this decrease of humic CMC was due to some Mg(II) interactions with HAs, the prevention of HA sorption and perhaps can also be explained by higher ionic strength used in this case. Finally, performing the same study on a PAA-coated capillary, oligomerization was also observed (Fig. 9). In this case, the HA CMC was surprisingly observed around 100–150 mg/l. The main difference to the previous experiment using similar BGE (20 m*M* rimantadine, 5 m*M* $MgCl_2$, pH 3.40) was that EOF was strongly reduced due to the PAA coating. This observation is difficult to explain and we can only suggest that this increase of humic CMC can be due to lower Mg(II) concentration used in this case. On the other hand, we have to realise that CMC for different HAs can be different. In addition as HAs are a complex mixture, CMC for different fractions of the same HA can also be different.

3.3. Oligomerization of HSs followed by vapour pressure osmometry

In order to confirm above discussed results, we have applied another method: vapour pressure osmometry (VPO). For simple compounds like urotropin which are not forming any oligomers, VPO

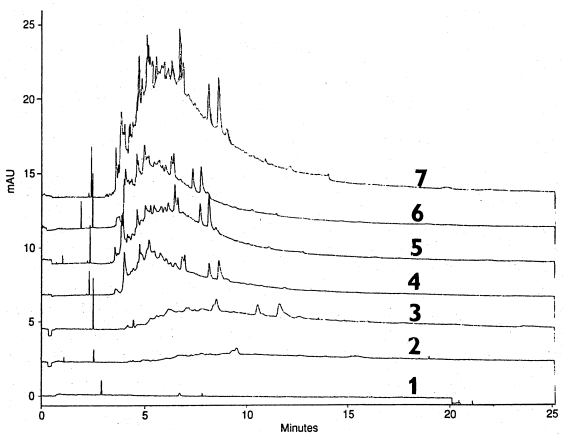

Fig. 9. Oligomerization study of Fluka II HA on a PAA-coated capillary. BGE: 20 m*M* rimantadine hydrochloride with 5 m*M* $MgCl_2$, pH 3.40. Separation conditions: −15 kV, 40°C and 18 s hydrodynamic injection. HA concentration: 1=10, 2=90, 3=150, 4=225, 5=266, 6=320, 7=534 mg/l.

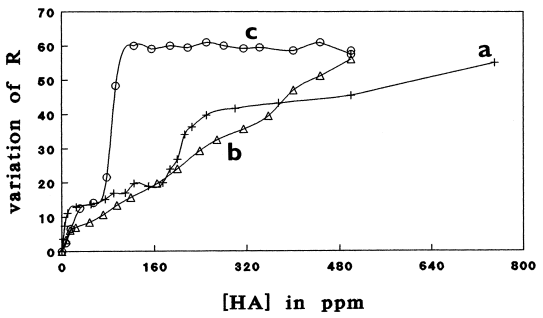

Fig. 10. VPO of aqueous solutions of different HAs. Conditions: 45.0±0.1°C, standard deviation of VPO measurement: $\sigma(\Delta R)=\pm 0.25$. HAs: (a) Fluka II, (b) 317 MAR, (c) 193 MAR. (For curve c, ΔR values have been multiplied by a factor of 10).

results show that the resistance difference (ΔR) is a linear function of the concentration. Furthermore, using a reference compound with VPO it is possible to determine an approximative value of the average molecular mass of an unknown compound with a R.S.D. value ≤5%. For studied HAs, the molecular masses between 425 and 1450 were estimated which is in a good agreement with similar work in literature [37]. Fig. 10 presents the experimental curves obtained for commercial and two Bohemian coal originated HAs. It is evident that some aggregation process occurs as no linearity is observed. Finally, we observed that the oligomerization of HAs is different for each of the HAs studied. For Fluka HA (Fig. 10a) up to four steps (waves) can be observed on ΔR=f(concentration) curves which can be explained by the formation of different oligomers. On the other hand for 317 MAR only two steps are evident (Fig. 10b). Concerning 193 MAR HA (Fig. 10c), we have observed that the response obtained by VPO presented lower ΔR values than for the other HAs. Nevertheless, it seems that four steps are present until a constant value of ΔR is reached around 150 ppm. Exact explanation is difficult but it is evident that oligomerization observed by capillary electrophoresis was also proven by VPO measurements.

4. Conclusions

In this paper various BGEs used for the separation of HSs by CZE were studied. Four different types of HS separation patterns were observed.

It was proved for several BGEs and confirmed by VPO that oligomerization or aggregation play highly important roles in the separation process of HSs. Oligomerization starts at concentrations higher than ≈50 mg/l and has been proved in DL-alanine, rimantadine and phosphate BGEs.

Another important fact is sorption of HAs on uncoated fused-silica capillaries. That is why most of the authors were working at extremely high HS concentrations and so they observed only 3–4 broad peaks which according to our results are just different aggregates or supramolecules of HAs.

Real separation of HSs can be obtained preventing oligomerization by using strongly complexing buffer, e.g., at high boric acid concentration [5].

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