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Capillary zone electrophoresis for the separation and characterization of humic acids

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Abstract

Capillary zone electrophoresis (CZE) was used for the separation and characterization of humic acids (HAs) of different origin. UV–Vis detection and/or fast scanning of spectra during the separation was applied for the detection. From several background electrolytes (BGEs) studied, it was found that especially those containing amino acids are the most suitable and led to the separation of HAs into several fractions. The composition and pH of buffers were optimized. Up to 30 fractions were separated using a high boric acid concentration (350 m*M*) BGE. The results can be used as real ''fingerprints'' to characterize HAs of different origin. © 1998 Elsevier Science B.V.

Keywords: Buffer composition; Humic acids

1. Introduction

Widely distributed throughout the world, humic acids (HAs) form biogenic, heterogeneous organic yellow to brown color substances with high molecular mass ([1] and references therein). One of the latest reviews is that of Shevchenko and Bailey [1]. Many techniques and methods have been used for obtaining more information about HAs. Different chromatographic techniques such as size-exclusion chromatography (SEC) followed by IR, UV, fluorescence, electrochemical detection, pulse polarography, or electrophoretic techniques using polyacrylamide gel [2] and gel permeation chromatography [3] were used. Moreover, high-performance liquid chromatography (HPLC) coupled with fluorescence or UV detection, gas chromatography (GC) with microwave emission or nitrogen-selective detection and sorption chromatography followed by ^{13}C nuclear magnetic resonance (NMR) spectrometry [1] have been applied for studying HA substances ([4] and references therein).

The ionic nature of HAs enables their study also using electrophoretic techniques. Different authors already proposed methods for the separation of HAs into some fractions using capillary zone electrophoresis (CZE) with UV–Vis, diode array or laser-induced fluorescence detection with the most commonly used background electrolytes (BGEs) in CZE, i.e., borate or phosphate [4–9].

HAs play an important role in environmental chemistry. They form chelates with metal ions ([10] and references therein) and thus influence heavy metal ion transport in nature [11–13]. Furthermore, the modeling of radionuclides transport from a depository to the biosphere as part of the nuclear

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waste deposits safety/performance assessment is of great importance [8,14–16] stressing the necessity to develop methods able to determine the different organic matter fractions [17] in soils, especially HAs.

For this purpose characterization of HAs was studied, using CZE as the method possessing the highest separation efficiency. Some results of this work have been preliminarily reported elsewhere [18].

2. Experimental

2.1. Apparatus

SpectraPhoresis 2000 (Thermo Bioanalysis, CA, USA), instrumentation was used with untreated fused-silica capillary (Avery Dennison, MA, USA), 43.5 cm (length to the detector window 35.5 cm)×75 μm I.D., filled with the corresponding buffer solution. Separation voltage from 5 to 30 kV was applied using both polarities (cathodic injection/anodic detection or vice versa). The instrument equipped with a fast scanning detector with deuterium and tungsten lamps working from 190 to 700 nm was used.

The electropherograms were recorded using PC1000 program and data were reprocessed.

Acidity of solutions was measured using 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 of Radiometer and/or Radelkis were used for the calibration. The pH was measured with precision of ±0.05.

All solutions were filtered through G4 sintered porous glass filter before use.

2.2. Reagents

Nine BGEs of different concentrations were used: HCl–DL-alanine; HCl–β-alanine; HCl–β-phenylalanine; HCl–DL-serine; HCl–DL-proline; HCl–L-cystine; HCl–glycolic acid; borate and boric acid.

All chemicals were of analytical grade purity from Merck (Darmstadt, Germany), Lachema (Brno, Czech Republic), or Loba Chemie (Vienna, Austria). For preparation of all solutions, bidistilled water obtained using a Heraeus Quartzschmelze (Hanau, Germany) quartz apparatus was used.

BGE solutions were prepared by mixing a solution of the weighted amount of the compound with a suitable volume of 0.1 mol/l HCl or NaOH solution in various proportions to obtain the appropriate pH and concentration.

2.3. Humic acids

The HA preparations used in this work were supplied by the Research Institute of Inorganic Chemistry (VÚANCH), Ústí nad Labem, Czech Republic. HAs were extracted from high-quality oxyhumolite originated from bohemian brown coal North Bohemia Coal (Colliery Bílina and Vršany) marked as 193 MAR and 317 MAR [12], one of them which was prepared from Bílina locality brown coal, ''248 MAR'', has showed the particularity to being membrane separated just after the extraction [12].

HA preparation No. 53680 (analysis number 38537/1 293) supplied by Fluka (Switzerland) was used as a reference sample.

HA characteristic parameters are collected in Table 1 [12,16].

2.3.1. Procedure of HAs dissolution

2.3.1.1. First method

A weighed amount of HAs was exposed to ammonia vapors in a dessiccator for 24 h. Ammonium salt formed was then dissolved in water. This stock solution (concentration about 2 g/l) was then diluted.

2.3.1.2. Second method

Direct dissolution of the weighed amount in a small volume of 0.1 mol/l NaOH and after dissolution applying also ultrasound bath (Branson ultrasonic cleaner, USA), the solution was transferred to corresponding measuring flask which was filled up to the mark with water.

2.4. CZE procedure

Washing solutions of 0.1 mol/l NaOH or 0.1 mol/l HCl were prepared using analytical-grade

Table 1
Characteristic parameters of HAs [12]

HA sample	Water content (%)	Ash content in dry matter (%)	Total acidity (mequiv./g)	Carboxylic acidity (mequiv./g)	Molecular mass
193 MAR	5.99	4.13	8.42	3.64	M_n = 10 189 M_w = 19 301 M_w/M_n = 1.89
Fluka	5.63	18.07	7.46	1.01	M_n = 9787 M_w = 20 032 M_w/M_n = 2.04
317 MAR	7.50	18.93	9.18	3.59	M_n = 12 676 M_w = 19 879 M_w/M_n = 1.57
248 MAR	1.72	0.74	8.77	4.61	M_n = 8299 M_w = 12 791 M_w/M_n = 1.54

M_n = Number-average molecular mass, M_w = weight-average molecular mass, M_w/M_n = degree of polydispersity.

reagents from Carlo Erba (Italy) and Merck and prewash procedure was optimized for each BGE. Detection between 190 and 600 nm was done using fast scanning detector. At wavelengths higher than 600 nm no absorbance of HAs was observed. The temperature was set at 40°C in all experiments, if not otherwise stated. All samples were injected by hydrodynamic injection in the capillary using a vacuum (10342.14 Pa).

3. Calculation

Reproducibility was estimated repeating the experiments 3- to 5-times and the average value of the residual standard deviation (R.S.D.) of different parameters (migration time, peak height and peak area) was determined using Eqs. (1) and (2):

$$s_i = \sqrt{\frac{1}{n} \cdot \sum_j^n (x_j - \bar{x})^2} \quad (1)$$

$$\text{R.S.D.} = \frac{1}{m} \cdot \sum_i^m s_i \quad (2)$$

where s_i is the residual standard deviation of parameters, x_j is the jth experimental value, n is the number of experiments, m is the ith parameter (migration time, peak height and peak area) of which the standard deviation is determined and $\bar{x}$ is the average value.

4. Results

The crucial point for efficient CZE separation of whichever compound(s) is often the selection of suitable BGE and pH. The systematic study of various BGE has been done here in order to search the most suitable electrolytes for the separation of HAs.

4.1. DL-Alanine as BGE

The DL-alanine BGE was a mixture of 60 mmol/l DL-alanine with 8 mmol/l HCl at pH = 3.20. In order to obtain reproducible results, it was necessary to wash the capillary before each use. The following procedure gave the most reproducible results. The capillary was washed first with 0.1 mol/l HCl for 1 min, redistilled water for 1 min, 0.1 mol/l NaOH for 1 min, water for 1 min and, finally, the BGE was applied for 2 to 4 min for conditioning the capillary. The sample of HAs was hydrodynamically injected 3- to 6-times for 15 to 30 s (injected volume 120–300 nl) and separation was achieved applying a 15

kV separation voltage (anodic injection). The baseline stability observed was reproduced within ±0.5 mAU (absorbance units). The electroosmotic flow (EOF) value detected using mesityl oxide was higher than 60 min. Furthermore, no changes were observed in electropherograms for HAs prepared applying ammonia vapors or when dissolving them in 0.1 mol/l NaOH.

Using Fluka HAs, the effect of separation voltage was studied in the range 5–30 kV. It was found that the highest value of the theoretical number of plates (TNPs) was obtained in the interval 12–17 kV and the peak symmetry factor was (1.00±0.15).

The stability study of HA solutions during 24 h by CZE was followed at 15 kV, 40°C, C_{HA}=0.495 g/l and hydrodynamic injection time for 30 s. Retention times for each fraction were reproducible within ±5% and absorbancy values at ±1 mAU. Greater differences were observed after more than 24 h but this was found to be mainly due to alanine deterioration by the action of fungi. No antifungal agents were added to BGEs, because it is known that HAs also react with organic compounds [7]. Thus, the stock solution of DL-alanine and other BGEs were kept in a freezer.

Results of CZE separations obtained confirmed the presence of two fractions A and B, as observed by Rigol et al. [4] when using L-alanine BGE. In addition, a third fraction C with the retention time ~3 min was observed at 200 nm (Fig. 1). The gap observed after fraction C we suggest to be a HA fraction too which is absorbing less at given wavelength than the BGE as EOF is higher than 60 min.

Relation of the peak area and/or height was also studied. The peak area (or peak height) versus HA concentration relations were linear from 5 to 500 mg/l, with squared regression factors higher than 0.99. The best linear relationships were observed for the first two fractions. Migration time was stable for $C_{HA} \geq 50$ mg/l at $\lambda < 210$ nm and for $C_{HA} \geq 20$ mg/l at $\lambda \geq 210$ nm with a R.S.D.≤±4%. For lower concentrations the relationship is represented by another linear equation with a high slope value.

For fractions A and C, higher precision is obtained when following the peak height whereas for fraction B both peak height and/or area parameters can be used. The results reproducibility evaluated on 20 days was very good [R.S.D. <3 (or 9)% for peak height (or area)].

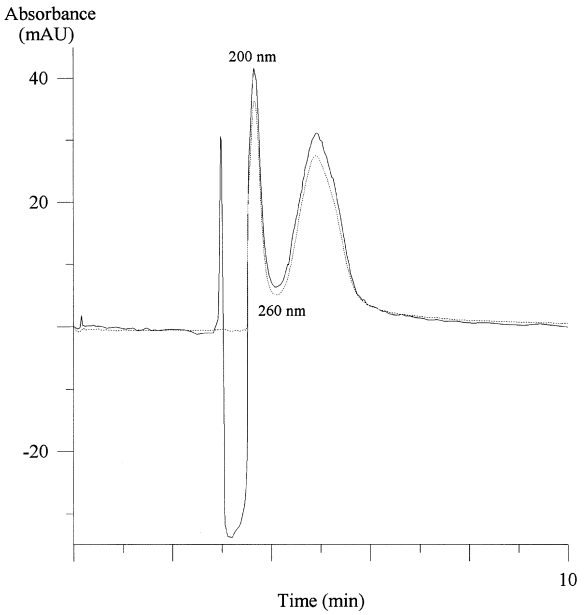


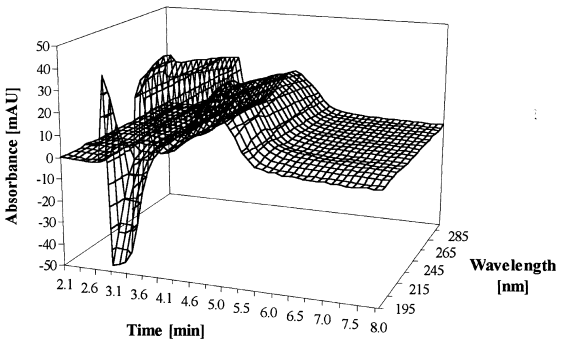
Fig. 1. Electropherograms of Fluka HAs at 200 nm and 260 nm in DL-alanine BGE. BGE: 60 mmol/l DL-alanine with 8 mmol/l HCl at pH=3.20. Conditions: 15 kV, 40°C, 20 s hydrodynamic injection and C_{HA}=250 mg/l. (Solid line 200 nm, dotted line 260 nm). Remark: EOF determined using mesityl oxide was higher than 60 min.

Using spectrophotometry in the range 190–220 nm it was proved that interaction occurs between HAs and DL-alanine as when increasing concentration of DL-alanine, the absorbancy of HAs solution decreases.

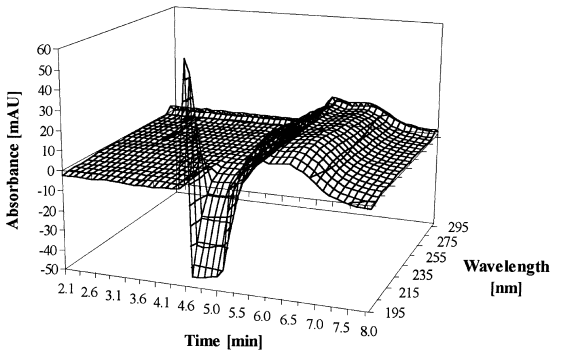
4.1.1. Effect of filtration

All solutions were filtered through G4 glass filter before use, nevertheless, the effect of additional filtration was followed. Acrodisc Nylon 0.2 μm (Elman, Germany), Chromafil 0.2 μm (Macherey–Nagel, Germany), Anotop 10 0.02 μm (Merck) (Fig. 2b) and Anotop 10 0.02 μm (Merck) followed by 15 min ultrasound treatment were used.

No differences in three dimensional electropherograms were observed, except for 0.2 μm Chromafil where two principal fractions (A and B) were not



a)



b)

Fig. 2. Effect of different filters on Fluka HAs 3D electropherograms in DL-alanine BGE. BGE and conditions as in Fig. 1. (a) Not filtrated, (b) 0.2 μm Chromafil. Remark: EOF determined using mesityl oxide was higher than 60 min.

well separated and the migration time was 2 min higher (Fig. 2).

4.1.2. Three dimensional electropherogram "fingerprints" of studied HAs

Fig. 3 presents a three dimensional electropherogram obtained in DL-alanine BGE for HAs of different origin. It is evident that fractions A and C are similar for Fluka (Fig. 2a), and to HAs originated from oxyhumolite, i.e., 193 MAR and 317 MAR (Fig. 3). Fraction B shows high differences between HAs in the migration times which are increasing in the order Fluka<317 MAR<193 MAR (Fig. 2a and Fig. 3). The order is the same as the carboxylic acidity content of these HAs (Table 1). A possible explanation of the slower migration time observed when the carboxylic acidity content increase might be possible interactions between the untreated fused-silica capillary inner surface (hydroxy groups) with the carboxylic groups of HA. For 248 MAR, which differs from 193 MAR only with respect to the way of separation on a membrane, an identical fingerprint can be observed for both preparatives but the fraction C is very small (for 248 MAR) and the global migration time of both fractions A and B present values of about 1 min lower. Furthermore, R.S.D. values obtained are in the range of 1 to 3%. In addition, the appearance of a small peak D was observed for 248 MAR between A and B fractions.

A comparison of the electropherograms for 248 MAR, 193 MAR and 317 MAR HAs with that of Fluka shows that they are similar to each other.

4.2. Application of alanine derivatives as BGEs

The use of β-alanine and β-phenylalanine as BGEs was also studied. Procedure for equilibrating and washing of the capillary was the same as mentioned above.

4.2.1. β-Alanine

Sixty mmol/l β-alanine with 8 mmol/l HCl at pH=3.20 solution was examined as a BGE. Faster separation of fractions A and B was reached here in comparison to DL-alanine, i.e., the migration time was between 1.2 to 2.5 min. However, separation quality is lower under the same conditions as used for DL-alanine BGE (15 kV, 40°C, 20 s hydrodynamic injection) and C fraction was not observed neither for Fluka nor for 193 MAR and 317 MAR HAs, while the gap at low migration time is observed for all HAs studied samples.

4.2.2. β-Phenylalanine

When using 30 mmol/l β-phenylalanine with 8 mmol/l HCl at pH=3.20 solution as a BGE, fractions A and B were not separated. Fraction C was missing and the gap with a maximum absolute value

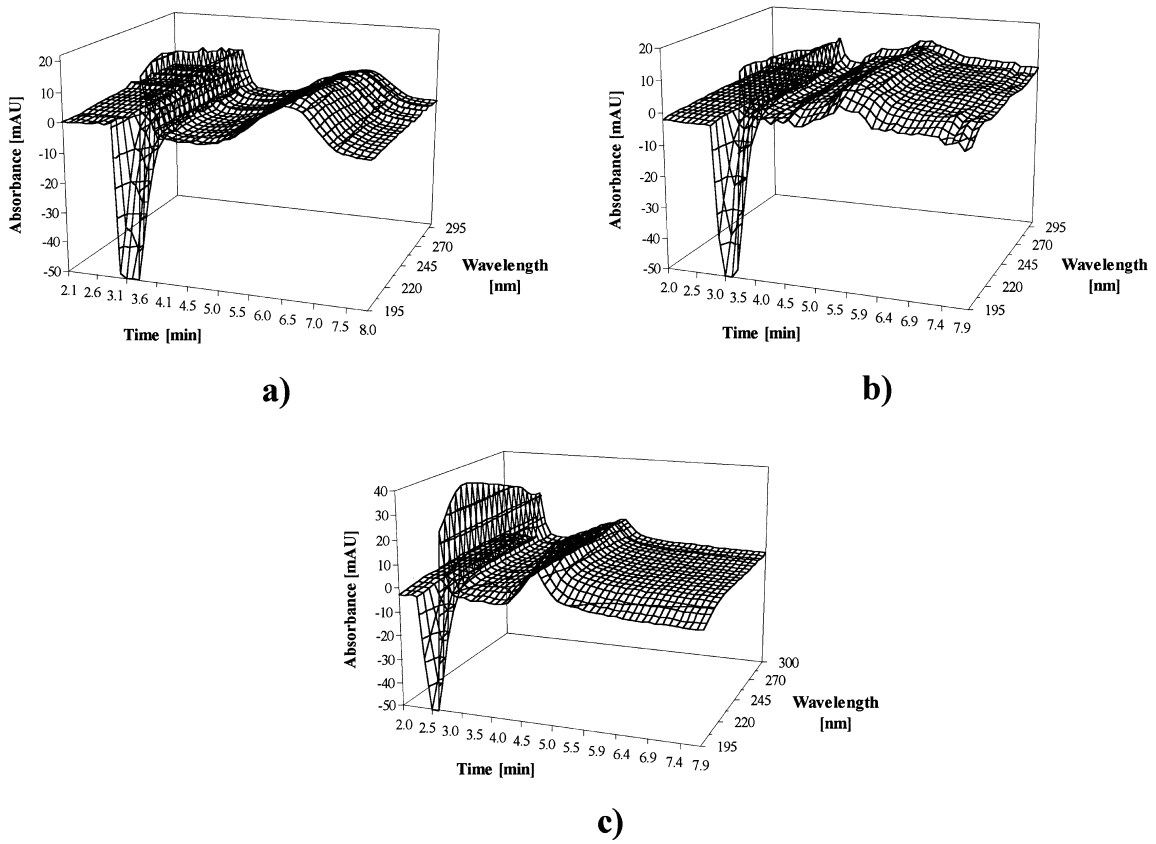


Fig. 3. Electropherograms ''fingerprints'' of HAs of different origins in DL-alanine BGE. BGE and conditions as in Fig. 1. (a) 193 MAR, C_{HA} = 101.4 mg/l, (b) 317 MAR, C_{HA} = 101.8 mg/l and (c) 248 MAR, C_{HA} = 99.8 mg/l. Remark: EOF determined using mesityl oxide was higher than 60 min.

of 250 mAU was 5-times deeper than that one we have observed for DL-alanine. It was suggested that deeper gap is caused by the fact that β-phenylalanine is absorbing more than DL-alanine BGE. This is in agreement with known correlation between molar absorptivity of BGE and the relative peak area in indirect detection in CZE [19].

4.3. Other amino acids as BGEs

We have also examined the use of the other amino acids, i.e., DL-serine, DL-proline, L-cystine, while the same equilibrating procedure as described above for DL-alanine was used here.

4.3.1. DL-Serine

Ten mmol/l DL-serine with 8 mmol/l HCl at pH = 3.20 solution was used as a BGE and similar results as for DL-alanine BGE (at 15 kV, 40°C and 20 s hydrodynamic injection) were obtained. However, the electropherograms have lower quality because peaks of A, B and C fractions are not so narrow. The gap was also observed with an absolute absorbance value near to that obtained for DL-alanine BGE. Negligible differences in the migration times for DL-serine BGE (in comparison to DL-alanine BGE) were observed.

4.3.2. DL-Proline

A BGE consisting of 15 mmol/l DL-proline with 8 mmol/l HCl at pH = 3.20 yielded identical electropherograms as those observed for DL-alanine BGE but with worse separation of A and B fractions. Furthermore, whereas the gap is still observed with a

lower absolute value of the absorbance (only 5 to 20 mAU), the fraction C is completely missing here. A quite good separation is obtained at 5 kV after 40 s hydrodynamic injection and a new fraction E between the gap position and fraction A was observed with a migration time of around 14 min. Peak quality and reproducibility are lower for fractions A and B (R.S.D.$<$6 to 12%), and higher for fraction E (R.S.D.$<$0.5 to 1.5%).

4.3.3. L-Cystine

When using a solution of 30 mmol/l L-cystine with 8 mmol/l HCl at pH=3.20 as BGE, the similar electropherograms as for DL-proline were observed with almost the same absolute value for the gap. Furthermore, no separation of fractions A and B was observed and fraction C was missing, whereas fraction E was present, as in the case of DL-proline BGE. The global migration time was 3.7-min higher in comparison to DL-alanine BGE.

4.4. The use of other BGEs

4.4.1. Glycolic acid

A 60 mmol/l glycolic acid (with 8 mmol/l HCl) of pH=3.20 solution was tried as a BGE at 40°C under the assumption that the functional group –C(O)–OH is the main factor allowing HA separation when using amino acids containing BGEs. Only one broad peak was observed and neither glycolic acid concentration nor pH changes helped to improve separation. A small gap before this single peak was observed. It seems that the presence of –C(O)–OH functional group in the BGE is not the factor improving HAs separation by CZE.

4.4.2. Borate

A BGE containing 20 mmol/l $Na_2B_4O_7$ with 100 mmol/l H_3BO_3 at pH=8.45 was used. Obtained results were similar to those for glycolic acid BGE. The gap peak was also observed and just one peak with positive absorbance was obtained without any separation into the fractions (applying 15 kV, 40°C and 20 s hydrodynamic injection).

4.4.3. Boric acid

Mostly, it is suggested that boric acids and borates are reacting with phenols, phenol carboxylic or polycarboxylic acids in neutral or alkaline medium ([18,19] and references therein). But it is known, that for example oxalic acid reacts with boric acid also in slightly acid solution [21,22]. Similarly, we can suggest that HA fragments containing salicylic acid functional group, *o*-hydroxycarboxylic, dihydroxy or perihydroxy groups in the HA skeleton, are complexed under the excess of boric acid. This lead us to examine the use of boric acid as a complexing buffer for the separation of HAs. We found that the higher was the concentration of boric acid, the better results obtained.

Boric acid BGE (0.1 mol/l H_3BO_3, 10 mmol/l HCl) of pH=3.15 was used and quite different separation for Fluka HA (15 kV, 40°C, 15 s hydrodynamic injection, prewash of 1 min 0.1 mol/l NaOH, 2 min water and 5 min BGE) in comparison to previous results was reached with this buffer. Surprisingly, about 20 fractions or peaks were observed (those with the height of three times higher than the baseline noise), see Fig. 4. We suggest that peaks numbered 1 to 12 and 16 to 19 belong to more defined compounds while peaks 13 to 15 are fractions of HAs due to their broad peak shape (Fig. 4). However, electropherograms in boric acid BGE show poorer reproducibility of the migration times, but the order and the number of the peaks remained always the same.

When using 0.35 mol/l H_3BO_3 with 10 mmol/l HCl (pH=3.15) as a BGE, the similar electropherograms to those obtained for DL-alanine BGE were observed for C_{HA} (Fluka)$<$60 mg/l with the absence of the gap between fractions C and A. For samples with higher concentrations of Fluka HAs, a quite different separation was observed, however with a low reproducibility of the migration times.

The highest number of fractions (peaks), 10 to 30, was obtained when performing the separation with 0.35 mol/l H_3BO_3, 10 mmol/l HCl and pH=3.15 BGE (20 kV, 40°C and 15 s hydrodynamic injection) if the capillary was pre-washed as follows: 3 min 0.1 mol/l HCl; 4 min water; 4 min 4 mmol/l 18-crown-6; 4 min water and, finally, 5 min with BGE.

The electropherograms reached are quite different from those obtained with the previously studied BGEs (Figs. 5 and 6). No gap was observed under these conditions.

At low concentrations of humic acid (266.8 mg/l),

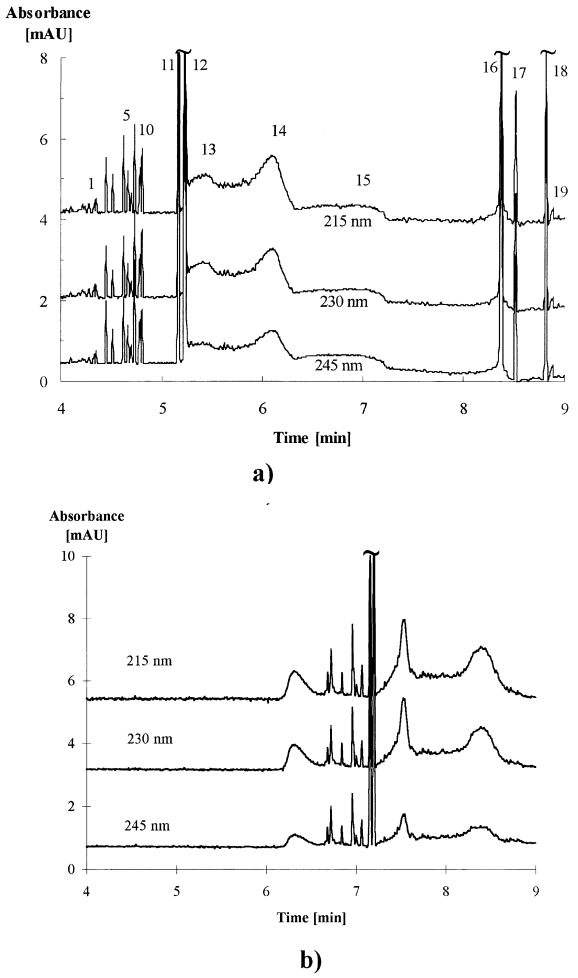


Fig. 4. Electropherograms of HAs at 215, 230 and 245 nm in boric acid BGE. Conditions as in Fig. 1; 15 s hydrodynamic injection. BGE: 100 mmol/l H_3BO_3 with 10 mmol/l HCl at pH=3.15. (a) Fluka 533.5 mg/l; (b) 193 MAR 502.4 mg/l. (For migration time 0–4 min, no significant peaks were observed).

one major fraction of HA was observed at 20.4 min (Fig. 5d) and the second one was observed at 4.2 min (Fig. 5a), whereas smaller peaks are obtained between 9.6 and 17.2 min (Fig. 5b and c).

At higher concentrations of humic acid (533.5 mg/l) (Fig. 6), between 20 to 30 fractions or peaks were obtained. The peak at 12.3 min is assumed to correspond to the fraction described as ''Mount Everest'' group of peaks (Fig. 5d) obtained at lower concentrations. Some unseparated fractions of HAs are still present as in the HA ''Cathedral'' group of peaks (Fig. 6a). The other peaks obtained represent probably either free compounds or unseparated compounds of similar molecular mass and structure [18,20]. Peak splitting phenomena described recently for β-naphthol [23] due to the interactions between boric acid polymers with β-naphthol were also suspected, but at this pH a low amount of polymeric species of borate are formed and so we can suggest that this seems not to be the case of any peak splitting phenomena.

Interaction between boric acid and Fluka HAs was proven by spectrophotometry. In the range 190 and 205 nm high decrease of the absorbance of HA solution was observed when increasing boric acid concentration [24].

5. Discussion and conclusions

At pH 3.2, for all buffers studied, we have observed that HA fractions are charged entities moving to the cathode.

Better separation of humic acids observed for α-amino acids BGEs than that one for β-amino or β-phenyl derivatives used as BGEs is probably caused by a spherical hindrance due to phenyl substituent or by a difference in stability of supramolecular complexes between amino and humic acids.

Also the effect of molecule size of the buffer molecules was observed. E.g., for β-substituted –OH functional group (DL-serine) the ''quality'' of observed peaks was better, whereas it was observed that a longer chain (L-cystine) and a cyclic form (DL-proline) molecules do not enable such efficient separation as it was reached for BGE containing DL-alanine. Furthermore, migration times are higher here.

Moreover, α-NH_2 functional group in BGE constituent seems to play an important role, as with glycolic acid poorer separation has been obtained. Alkaline borate BGE at pH=8.45 shows rather bad separation of HA fractions.

It is interesting to notice that ''best'' CZE separation was obtained for the compounds constituting BGEs possessing values of the dissociation constants (p*K* values) between 2.1 and 2.5 [21,22].

In contrast to the previous work [4], we found that

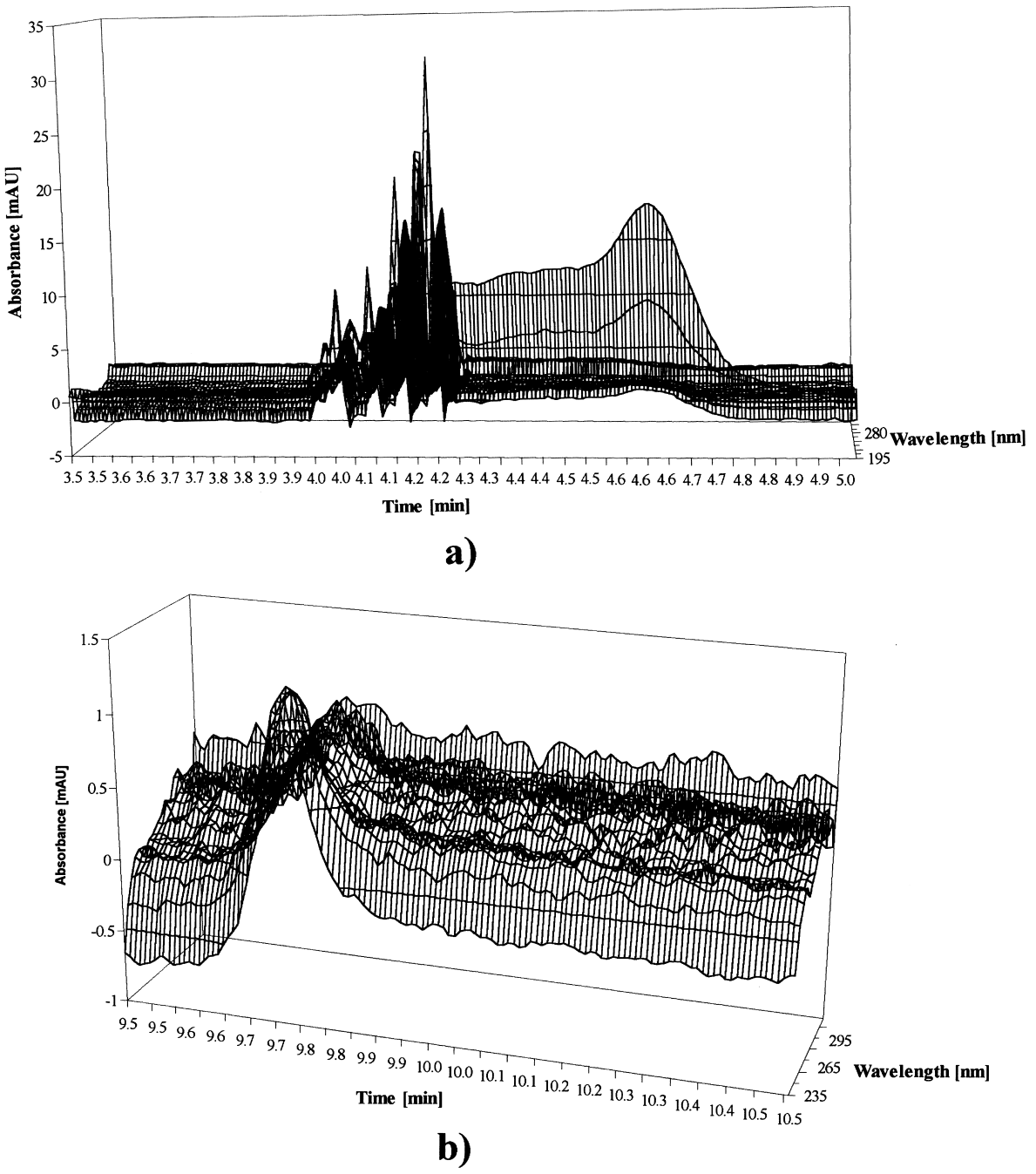
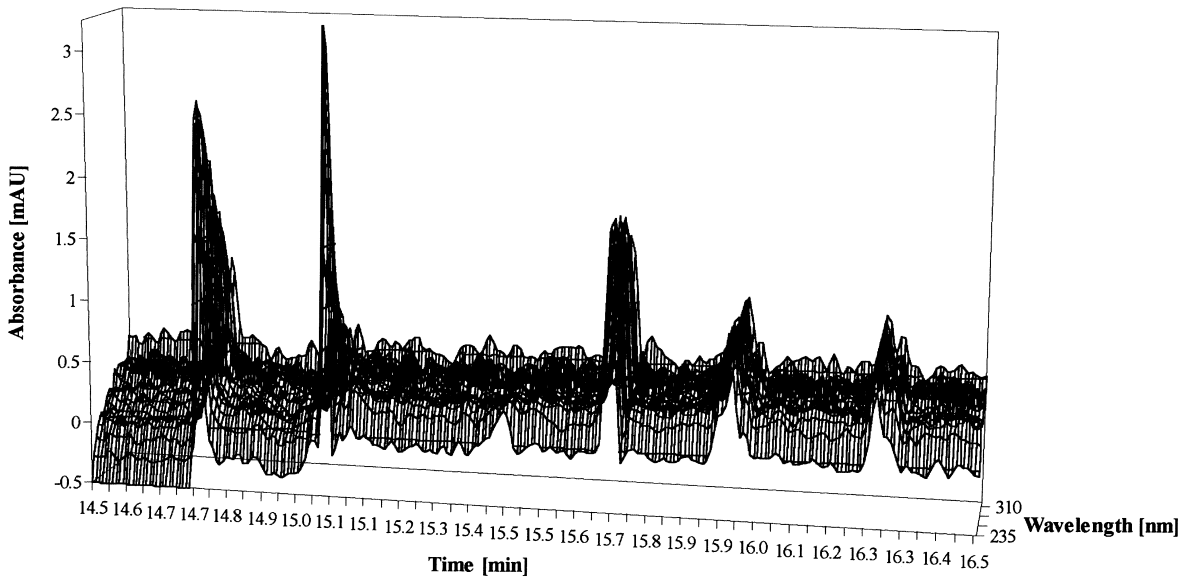


Fig. 5. Fluka HAs 3D electropherogram at lower HA concentration in boric acid BGE. BGE: 350 mmol/l H_3BO_3 with 10 mmol/l HCl at pH = 3.15. Conditions: 20 kV, 40°C, 15 s hydrodynamic injection and C_{HA} = 266.8 mg/l. (a) HA ''Cathedrale'', (b) HA ''Volcano'', (c) HA ''Alpes'' and (d) HA ''Mount Everest''.



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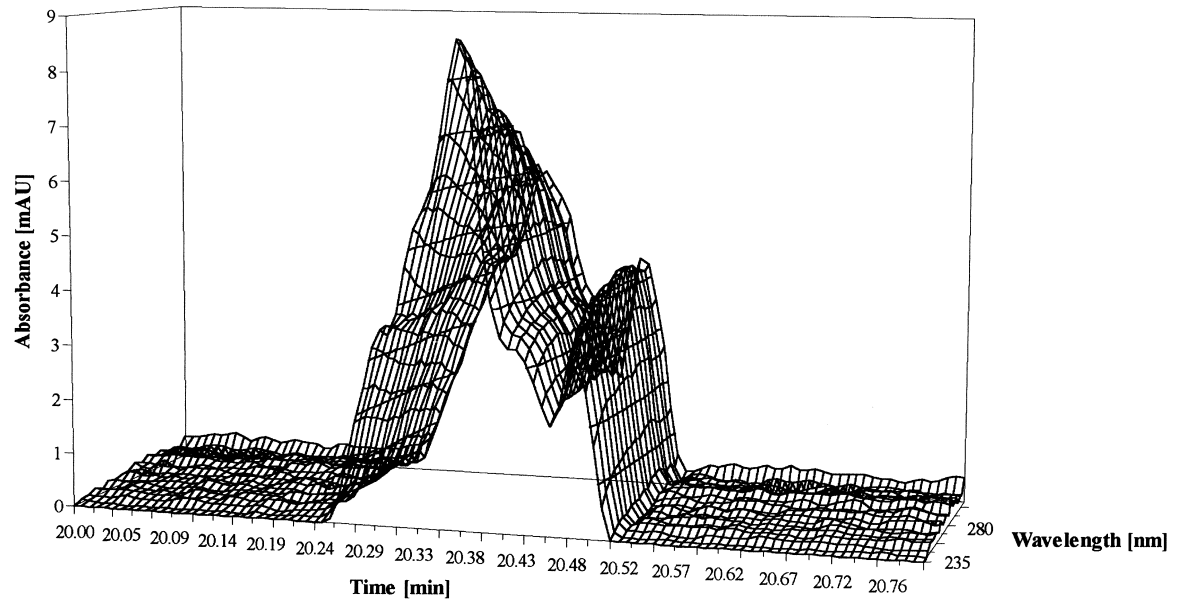


d)

Fig. 5. (*continued*)

D forms of amino acids permitted the observation of C fraction on the electropherograms, while Rigol et al. [4] working with L forms of amino acids, obtained only two fractions (denoted as A and B in this work) and a gap for a low migration time.

Concerning DL-alanine, this compound is a zwit-

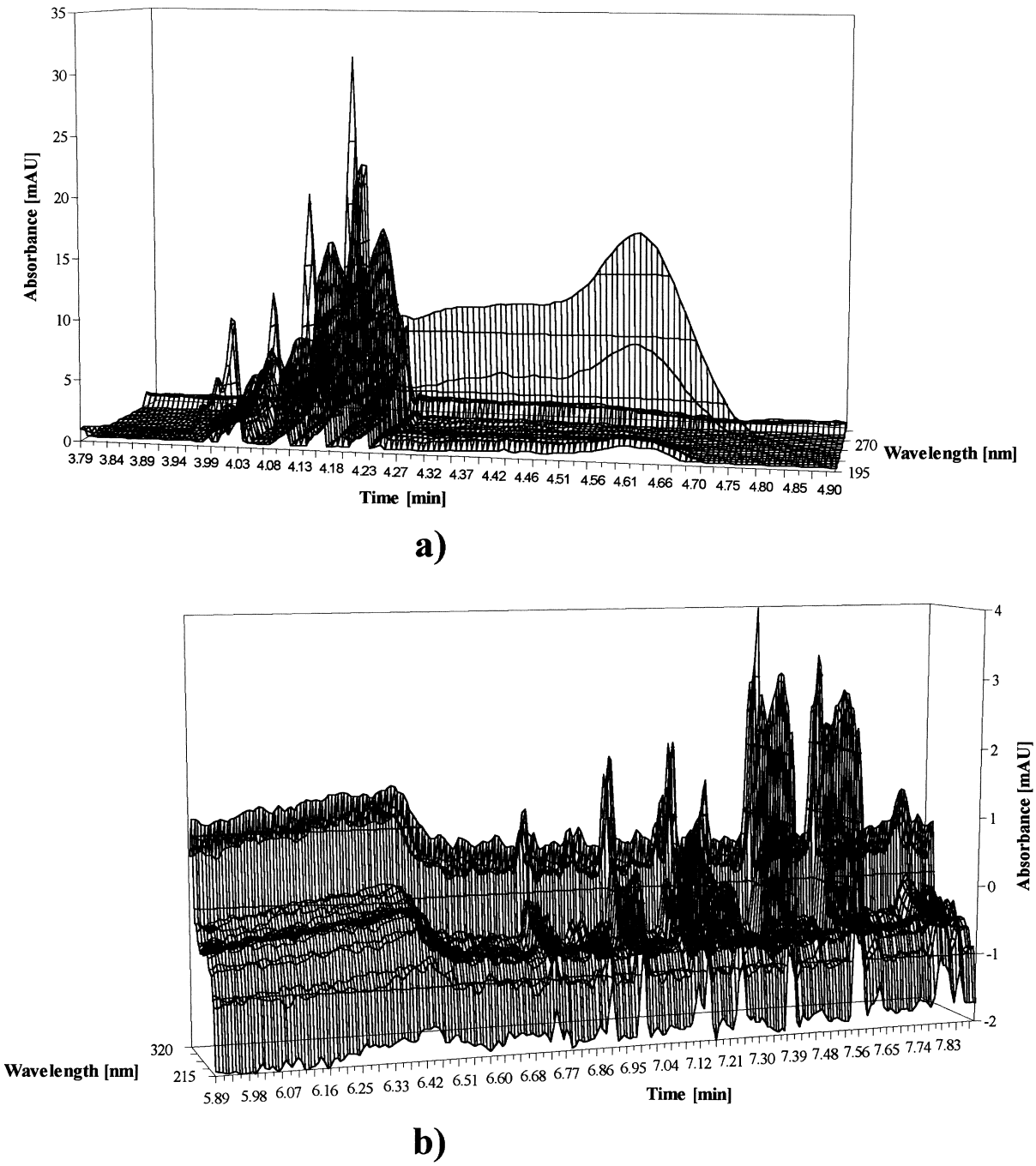
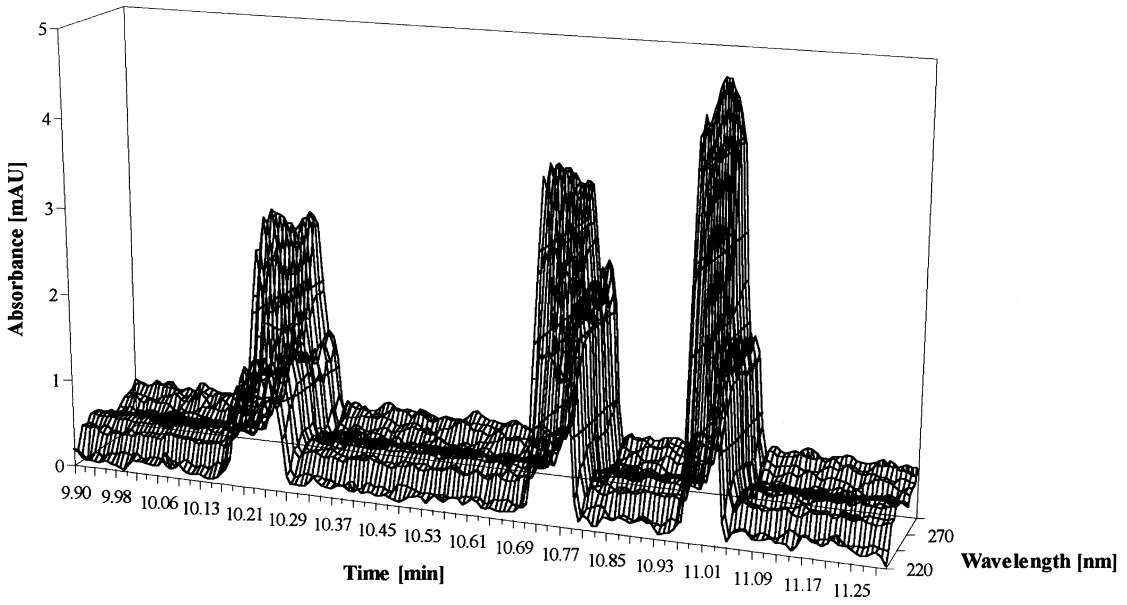


Fig. 6. Fluka HAs 3D electropherogram at higher HA concentration in boric acid BGE. BGE and conditions as in Fig. 6, C_{HA} = 533.5 mg/l. (a) HA ''Cathedrale'', (b) HA ''Tanker'', (c) HA ''Alpes'' and (d) HA ''Austrian Alpes''.



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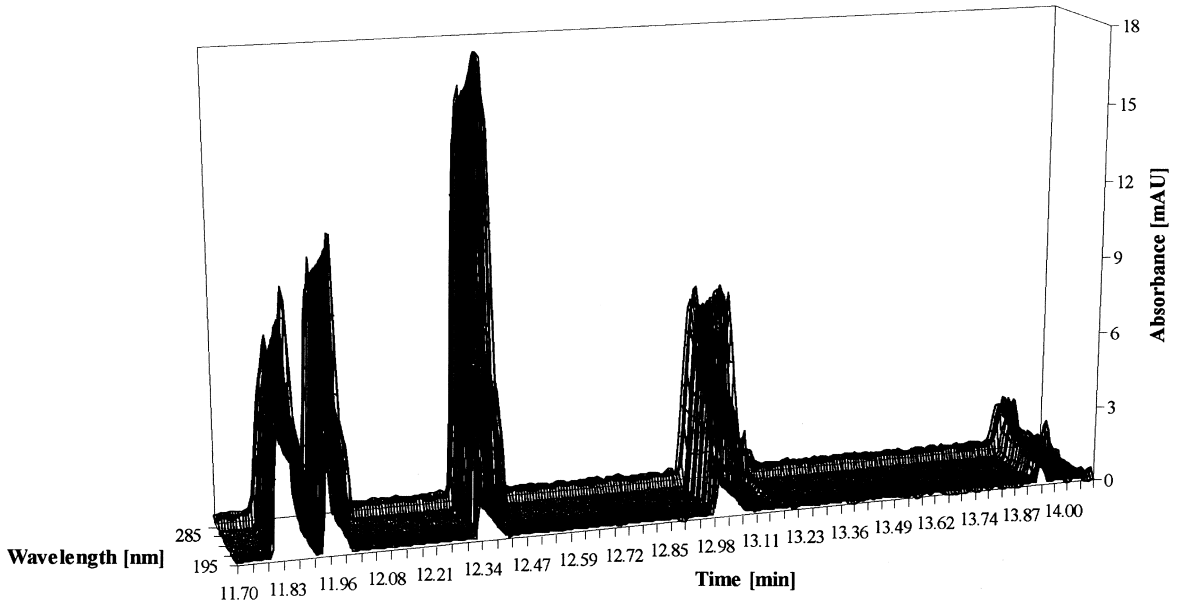

d)

Fig. 6. (*continued*)

terion at pH used and it was observed that HAs move towards cathode during separation. This observation can be explained by the interactions of DL-alanine zwitterion with HA molecules under the formation of positively charged supramolecular structures. This conclusion is supported by described sorption (inter-

action) of humic acids with various organic compounds [25,26].

It has already been noted in the literature that HA molecules, supposedly consisting of a long flexible polymers, have a tendency to coil up [27] and aggregate [1,20]. This is thought to be due to the functional groups bridging and combined to charge neutralization. We suggest that mutual repulsion among carboxyl and hydroxyl groups causes the HA polymers to adopt a stretched configuration. Thus, few association sites for nonpolar species in solution are provided. Upon the addition of DL-alanine zwitterion, this effect is minimized and HA forms a compact structure. Functional group bridging is considered to enhance this effect by drawing together various groups on the humic acid chain, as DL-alanine (BGE) zwitterion concentration is higher than HA ones. Finally, all HA solutions are polydisperse (Table 1) and the interaction of segments of different sizes might lead to a various degree of intermolecular interactions that further can increase supramolecular or aggregation formation.

Furthermore, the observed ''gap fraction'' in absorbing BGE is due to HA fraction absorbing less than BGE itself, while for boric acid (it is much less absorbing) it is not the case as only positive peaks or fractions are observed.

Concerning results obtained for boric acid BGE, a possible explanation is that at higher HA concentrations, polymers or clusters are formed (Fig. 5). Increasing HA concentrations is perhaps rearranging the different molecules present. These intramolecular interactions accompanying the conformational changes which are influencing the acidic sites of the HA seems to confirm the theoretical approach of Schulten [28] with his three dimensional modeling of HA structures. In fact, it has been found that at high boric acid concentrations at pH = 3.15, the separation of HAs into three (low HA concentration) or up to 30 (high HA concentration) fractions or peaks can be observed. How to explain the phenomena? Are the 30 fractions or peaks really the chemical entities?

We suggest that the possible explanation is that in reality the samples of HAs studied contain limited number of chemical entities. However, as it is suggested in literature, when the concentration of humic acid is increased, the HA species do polymerize [20]. In the same time, the complexation of boric acid with phenolic or carboxylic groups might proceed and so the number of species might change. We suggest that in boric acid solution the individual fragments of HAs are reacting with boric acid in the sense of equilibrium (A) or (B) and in this way the formation of oligomers of HAs is prevented. Other combinations can be obtained as for example *o*-hydroxycarboxylic functional groups. Therefore, peaks obtained in boric acid BGE represent probably mainly the individual monomeric fragments of HAs (Fig. 7).

We can conclude that perhaps we have managed for the first time to separate HAs into the real fractions [18], while in the amino acids BGE the separation of HA oligomers was performed only. The study of this problem is under further detailed investigation in our laboratory.

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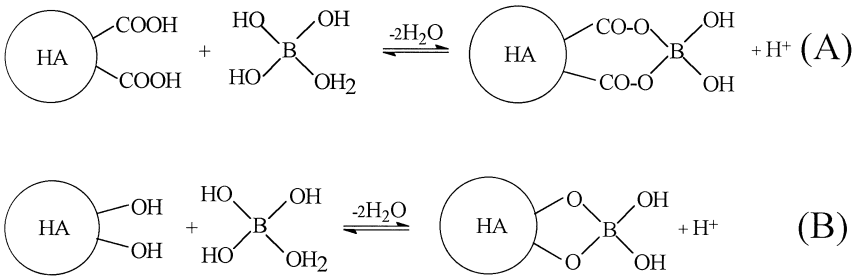

Fig. 7. Possible reaction of individual HA fragments with boric acid.

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References

[1] S.M. Shevchenko, G.W. Bailey, Crit. Rev. Environ. Sci. Technol. 26(2) (1996) 95–153.
[2] P. Kopáček, D. Kaniansky, J. Hejzlar, J. Chromatogr. 545 (1991) 461.
[3] H.K.J. Powell, J. Fenton, Anal. Chim. Acta 334 (1996) 27–38.
[4] A. Rigol, J.F. López-Sánchez, G. Rauret, J. Chromatogr. A 664 (1994) 301–305.
[5] A.W. Garisson, P. Schmitt, A. Kettrup, Water Res. 29 (1995) 2149.
[6] C. Ciavatta, M. Govi, C. Gessa, Comm. Soil Sci. Plant Anal. 26 (1995) 3305.
[7] M. Norden, E. Dabek-Zlotorzynska, Electrophoresis 18 (1997) 292.
[8] S. Pompe, M. Bubner, M.A. Denecke, T. Reich, A. Brachmann, G. Geipel, R. Nicolai, K.H. Heise, H. Nitsche, Radiochim. Acta 74 (1996) 135–140.
[9] S. Pompe, K.H. Heise, H. Nitsche, J. Chromatogr. A 723 (1996) 215.
[10] M. Samadfam, Y. Niitsu, S. Sato, H. Ohashi, Radiochim. Acta 73 (1996) 211–216.
[11] J. Havel (Editor), Research Report: Modeling and Experimental Verification of Transport Mechanism of Pollutants and Evaluation of Risks (in Czech), Grant GA 1487/93, Czech Ministry of Enviromental Protection, Prague, 1994.
[12] J. Havel (Editor), Research Report: Structural Characteristics of Humic Acids and Their Derivatives (in Czech), Grant of Research Institute of Inorganic Chemistry, Ústí nad Labem, 1994 and 1995.
[13] D. Široký, D. Fetsch, P. Lubal and J. Havel, Chemometrics, the 4th Int. Chem. Conf. of the Czech Chem. Soc., Pardubice, Czech Republic, 3–7 July, 1995, p. 121.
[14] I. Grenthe, Radiochim. Acta 52–53 (1991) 425.
[15] G.R. Choppin, J. Chem. Ed. 71 (1994) 856.
[16] D. Fetsch, P. Lubal, D. Široký, A.M. Albrecht-Gary and J. Havel, Inorganic Reaction Mechanisms Meeting 95, Société Chimique de France–Royal Society of Chemistry, Le Bischenberg, France, 4–6 January 1996, p. 121.
[17] A. Rigol, M. Vidal, G. Rauret, J. Radioanal. Nucl. Chem. 208 (1996) 617.
[18] D. Fetsch, and J. Havel, presented at the 10th International Symposium on High-Peformance Capillary Electrophoresis, Kyoto, Japan, 9–11 July 1997, p. 112.
[19] P. Jandik and G. Bonn, Capillary Electrophoresis of Small Molecules and Ions, VCH, Weinheim, 1993, p. 146.
[20] D. Fetsch, A.-M. Albrecht-Gary and J. Havel, XII Celostátní Seminář o Separační Chemii (XIIth State Seminar on Separation Chemistry), Lázně BohdaneČ, Czech Republic, 25–26 June 1997.
[21] L.G. Sillén, IUPAC Chemical Data Series, Pergamon Press, No. 21, 1982 and No. 22, 1979.
[22] J. Perrin, IUPAC Dissociation Constants of Organic Bases in Aqueous Solution, London Butherworths, 1965 and Supplement, 1972.
[23] A.L. Revilla, J. Havel, P. Jandik, J. Chromatogr. A 745 (1996) 225–232.
[24] K. Ghosh, M. Schnitzer, J. Soil Sci. 30 (1979) 735–745.
[25] J. Pörschmann, U. Stottmeister, Chromatographia 36 (1993) 207.
[26] M. Remmler, F.-D. Kopinke, J. Pörschmann, Naturwissenschaften 82 (1995) 28.
[27] R. Von Vandruszka, C. Ragle, R. Engebretson, Talanta 44 (1997) 805.
[28] H.-R. Schulten, Int. J. Environ. Anal. Chem. 64 (1996) 147–162.