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Capillary zone electrophoresis of soil humic acid fractions obtained by coupling size-exclusion chromatography and polyacrylamide gel electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) was used for characterisation of soil humic acid (HA) fractions obtained by coupling size-exclusion chromatography with polyacrylamide gel electrophoresis, on the basis of their molecular size and electrophoretic mobility. CZE was conducted using several low alkaline buffers as background electrolyte (BGE): 50 m*M* carbonate, pH 9.0; 50 m*M* phosphate, pH 8.5; 50 m*M* borate, pH 8.3; 50 m*M* Tris–borate+1 m*M* EDTA+7 *M* urea+0.1% sodium dodecyl sulphate (SDS), pH 8.3. Independently of BGE conditions, the effective electrophoretic mobility of HA fractions were in good agreement with their molecular size. The better resolution of HA were obtained in Tris–borate–EDTA buffer with urea and SDS. This results indicated that CZE, mostly with BGE-contained disaggregating agents, is useful for separating HAs in fractions with different molecular sizes.

Keywords: Soil; Environmental analysis; Humic acids

1. Introduction

Humic substances (HSs) are the most representative part of stable organic carbon in the biosphere, comprising approximately 60–70% of the total organic carbon in soils and 60–90% of dissolved organic carbon in natural waters. Humic acids (HAs) are a fraction of the HSs and are the most abundant in the upper 30–60 cm of the Earth's crust, where they interact with water and minerals. HAs possess a variety of physical and chemical properties that make them unique from other types of environmental substances [1]. Despite of the different origins responsible for their structural characteristics, they all constitute refractory products of chemical and biological degradation and condensation reactions of plant and animal residues, and play a crucial role in many biogeochemical processes. However, due to the complexity of these macromolecules, which molecular sizes ranging from M_r values of several

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hundreds to several hundreds of thousands, it still remains difficult to elucidate the exact mechanisms of formation, their physico–chemical properties and clear up which integral components are responsible for those. One way to get better insight into these properties is to fractionate HAs into molecular size fractions having different physico–chemical properties and/or functional activity.

Much progress has been made in the characterisation and differentiation of various whole HAs with the help of different electrophoretic techniques {e.g. polyacrylamide gel electrophoresis (PAGE) [2–7], isoelectrofocusing [8–10], capillary zone electrophoresis (CZE) [11–19]}. However, attempts to find out any relationship between HA electrophoretic zones obtained by different electrophoretic fractionation methods have not been done. It can be explained, at least partially, by the fact that for many years HS electrophoretic fractions have not been obtained in preparative quantities, and electrophoretic techniques have been used only as fingerprint HS characterisation.

Recently Trubetskoj et al. [2,3] developed PAGE in the presence of disaggregating agents to fractionate different soil HAs into three fractions with exactly defined electrophoretic mobilities (EMs) and molecular sizes. A combination of PAGE with size-exclusion chromatography (SEC) allowed to obtain preparative quantities of fractions with different MS and EMs from HAs of different genesis [20,21]. These fractions shown distinct extinction coefficients [21], photochemical and fluorescence properties [22,23], and different (plant or microbial) origin [24]. The paper aim is to investigate by CZE the soil HA fractions obtained by tandem SEC–PAGE to find out the relationship between PAGE and CZE, and the feasibility of CZE for fractionation of soil HAs on the basis of molecular size.

2. Experimental

2.1. Extraction and purification

The sample used in this study was taken from the A horizon (10–20 cm) of typical Chernozem soil (Kursk region, Russia). The soil characteristics are reported elsewhere [21]. The sample was air-dried and powdered to pass a 2 mm sieve. Before extraction, plant debris were removed by flotation. Batches of 100 g of the sieved dry sample were extracted with a 1:10 cold solution of 0.1 *M* sodium pyrophosphate and 0.1 *M* NaOH under a nitrogen atmosphere. The extraction was repeated with fresh extractant until no appreciable amounts of HAs could be removed (about six treatments). The extracts were centrifuged at 45 000 *g* to remove mineral residues, acidified to pH 2 with HCl, and the precipitated HAs were removed by centrifugation at 10 000 *g*. The precipitated HAs was redissolved in 0.1 *M* NaOH, centrifuged at 10 000 *g* for 30 min, then acidified and washed with 0.1 *M* HCl until colourless, dialysed against distilled water and then lyophilised. Analytical characteristics of Chernozem HAs obtained were as follows: C 62.6%; N 3.2%; H 2.8%; ash 2.2%.

2.2. Electrophoresis

The method used for fractionation of HAs has been previously reported [2,3]. Briefly: 9.7% acrylamide and 0.3% bisacrylamide were dissolved in 89 m*M* Tris–borate, pH 8.3, with 1 m*M* EDTA and 7 *M* urea. The apparatus was a vertical electrophoresis device (LKB 2001 Vertical Electrophoresis, Sweden) with gel slab (20×20 cm). Electrophoresis was carried out for 1 h at a current intensity of 25 mA. For analytical electrophoresis we have applied on the gel 0.05–0.25 mg of each sample completely dissolved in 0.05 ml of sample buffer containing 89 m*M* Tris–borate, pH 8.3, 7 *M* urea, 1% sodium dodecyl sulphate (SDS) and 1 m*M* EDTA.

2.3. SEC–PAGE

The fractionation of soil HAs by SEC–PAGE has been previously reported [20]. Briefly: Chernozem HA sample (5 or 10 mg) were dissolved completely in 1 ml 7 *M* urea and loaded onto a Sephadex G-75 (Pharmacia, Sweden) column (1.5×100 cm) with the same solution as eluent. The flow-rate was 20 ml/h. The void column volume (V_0=47 ml) was determined using Dextran Blue 2000. The total volume (V_t) was 160 ml. The elution curves were determined by measurement of absorbance at 280 nm with an

UA-5 detector (ISCO, USA) and recorded with an automatic recorder. Effluent of the column was collected as 2 ml aliquots and each third aliquot was assayed by 10% PAGE in the presence of denaturing agents according to [2,3]. The aliquots, forming individual electrophoretic zones in the polyacrylamide gel matrix with a similar EM in full, were combined into pools, dialysed against distilled water, lyophilised, and used for further physico–chemical analysis. In order to obtain preparative quantities of HA fractions the chromatographic procedure with subsequent electrophoretic analysis was repeated several times.

2.4. Capillary zone electrophoresis

The CZE separation were made with a BioFocus 3000 capillary electrophoresis system (Bio-Rad, Hercules, CA, USA) using a 50 cm (total length, 45.4 cm at the detector window)×50 μm I.D.×375 μm O.D. uncoated fuse silica capillary (CElect-FS50 columns, Supelco, Bellefonte, PA, USA). Typical CZE conditions for separations of the HA fractions were: voltage 15 kV, polarity from the anode to the cathode, capillary temperature 20 °C, detector wavelength 254 nm, pressure injection 5–20 p.s.i.×s (1 p.s.i.=6894.76 Pa) at the anode. The electrophoretic buffer, 50 m*M* sodium carbonate (pH 9.0), 50 m*M* sodium phosphate (pH 8.5), 50 m*M* sodium borate (pH 8.3), 50 m*M* Tris–borate+1 m*M* EDTA+7 *M* urea+0.1% SDS (pH 8.3), was obtained by dissolving the respective salts (Fluka, Buchs, Switzerland), adjusted at the wished pH and filtered through a Millipore 0.2 μm filter (Bedford, USA). The lyophilised HA fraction were dissolved (1 mg/ml) in 50 m*M* NaOH and filtered through a Millipore 0.2 μm filter. The quantity of HA material injected in the capillary was identical in all experiments. The electroosmotic flow (EOF) was determined using the mesityl oxide (Fluka) as neutral marker. Typical day-to-day changes in EOF were controlled by washing the capillary with 0.1 *M* NaOH for 5 min between each run. The electropherograms were analysed by BioFocus integration software 6.00 and the effective electrophoretic mobility (EEM) was calculated according to: $EEM = EL \cdot TL/(T_{eof} - T_m) \cdot V$ (where EL=length of the capillary to the detector in cm, V=applied voltage in V, T_{EOF} is the migration time of neutral compounds in s, calculated on migration time of mesityl oxide, T_m=migration time of separate compound in s, TL=total length of capillary in cm). All samples were analysed in triplicate and the relative standard deviations were less than 5%.

3. Results and discussion

3.1. SEC–PAGE

The HAs, originated from Chernozem soil, were fractionated by SEC on Sephadex G-75, using 7 *M* urea as eluent (Fig. 1). All HA materials, applied on the column, were eluted within the total column volume without any adsorption on the Sephadex gel matrix. The analysis of the chromatographic aliquots by PAGE revealed the presence of the main electrophoretic zones A, B and C+D (Fig. 2). Fraction A, corresponding to the excluded peak, formed on the electropherogram start zone that did not move into the gel (10% polyacrylamide gel). Fraction B formed an intensively coloured zone in the middle part of the gel. The fraction C+D formed two main intensively coloured bands with relatively close EM in the

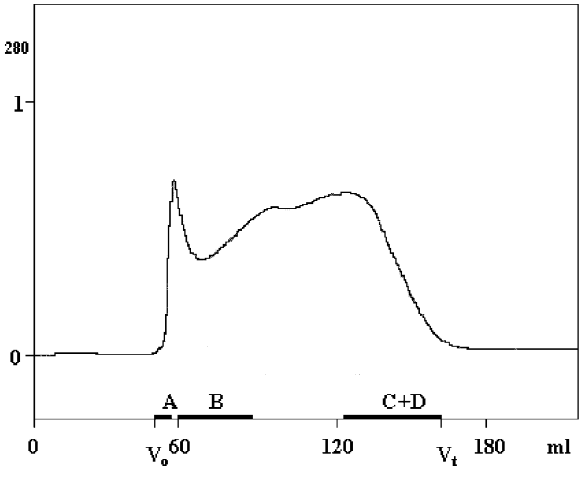

Fig. 1. Size-exclusion chromatography of 5 mg Chernozem HA on Sephadex G-75 column (100×1.5 cm) using 7 *M* urea as eluting system. Black boxes on the *x*-axis shown the combined fractions "A", "B" and "C+D", obtained on the basis of electrophoretic analysis of chromatographic aliquots. *y*-axis is optical density.

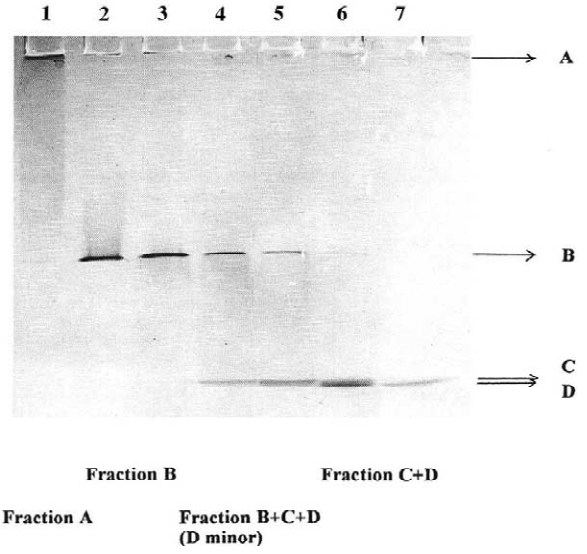

Fig. 2. PAGE analysis of Chernozem HA chromatographic aliquots: lane 1 correspond to elution volume (V_e) 47–55 ml (fraction A), lanes 2 and 3 to V_e 58–70 and 70–80 ml, respectively (fraction B), lanes 4 and 5 to V_e 81–102 and 103–124 ml, respectively (mixture of fractions B and C+D), lanes 6 and 7 to V_e 124–142 and 143–160 ml, respectively (fraction C+D).

bottom of the polyacrylamide gel slab. The chromatographic aliquots, containing mixture of fractions B and C+D were not used in the further investigation. On the basis of fraction elution volumes it may be suggested that molecular size of fraction A>molecular size of fraction B>molecular size of fraction C+D. All the soil HA fractions, obtained in preparative quantities by SEC–PAGE, have been used for further investigations by CZE in the alkaline buffers.

3.2. CZE by alkaline buffers

For CZE studies of Chernozem HA and fractions A, B and C+D, several low alkaline buffers (sodium carbonate, pH 9.0; sodium phosphate, pH 8.5; sodium borate, pH 8.3) were used in the typical experimental conditions (see Experimental section). These buffers were utilised by several authors [11,13,14,16,19] for ''finger-print'' characterisation of HAs and a typical ''humic hump'' in the anionic region of electropherograms with a distribution of around an average electrophoretic mobility (AEM) has been shown [13].

With carbonate and phosphate buffers the whole HA and fractions originated a typical ''humic hump'' with an increasing of average migration time from fraction A to fraction C+D (Fig. 3). The effective electrophoretic mobility (EEM) of the whole HA and fractions obtained with both buffers were similar (Table 1). The humic peak of the whole HA had the AEM between those values of the fractions. This is in agreement with fractions mass distribution (whole Chernozem HA contained about 24% (w/w) content of fraction A, 30% of fraction B and 46% of fraction C+D [21]). The differences in the absorbance intensity of humic peaks corresponding to the fractions A, B and C+D could be explained by the differences in their extinction coefficients, which increased from A to C+D fractions [21]. On the other hand, the more wider peak C+D in comparison with A and B peaks might be explained by assuming that fraction C+D could be more heterogeneous relative to fractions A and B. However, much work need before a firm conclusion can be drawn.

The CZE characterisation of HA and fractions with borate buffer showed a main sharp peak on electropherograms instead of typical ''humic hump'' (Fig. 4). The sharp peaks does not obligatory indicate the distinct molecular size fractions of HAs. This effect is well known and explained due to the interactions between the borate ions and diols and/or carboxylic groups presented in HAs [12–14]. Nevertheless, under these conditions the average migration time of the sharp peaks, which corresponded to fractions A, B and C+D, and their EEMs (in absolute values) also increased with a decrease of fractions molecular sizes (Table 1).

Experiments in carbonate, phosphate and borate buffers shown that independently on buffer conditions migration times and AEM of HA fractions were in good agreement with their molecular size and an increase of AEM during CZE corresponded exactly to the increase of electrophoretic mobility of fractions during PAGE [2,3]. It should be noted that in other experimental studies in sodium phosphate buffer, pH 8.5 [11], Tris–phosphate buffer, pH 8.3 [19] or L-alanine–HCl buffer, pH 3.2 [15], HA fractions obtained by ultrafiltration (UF) and differing in molecular size did not show any essential

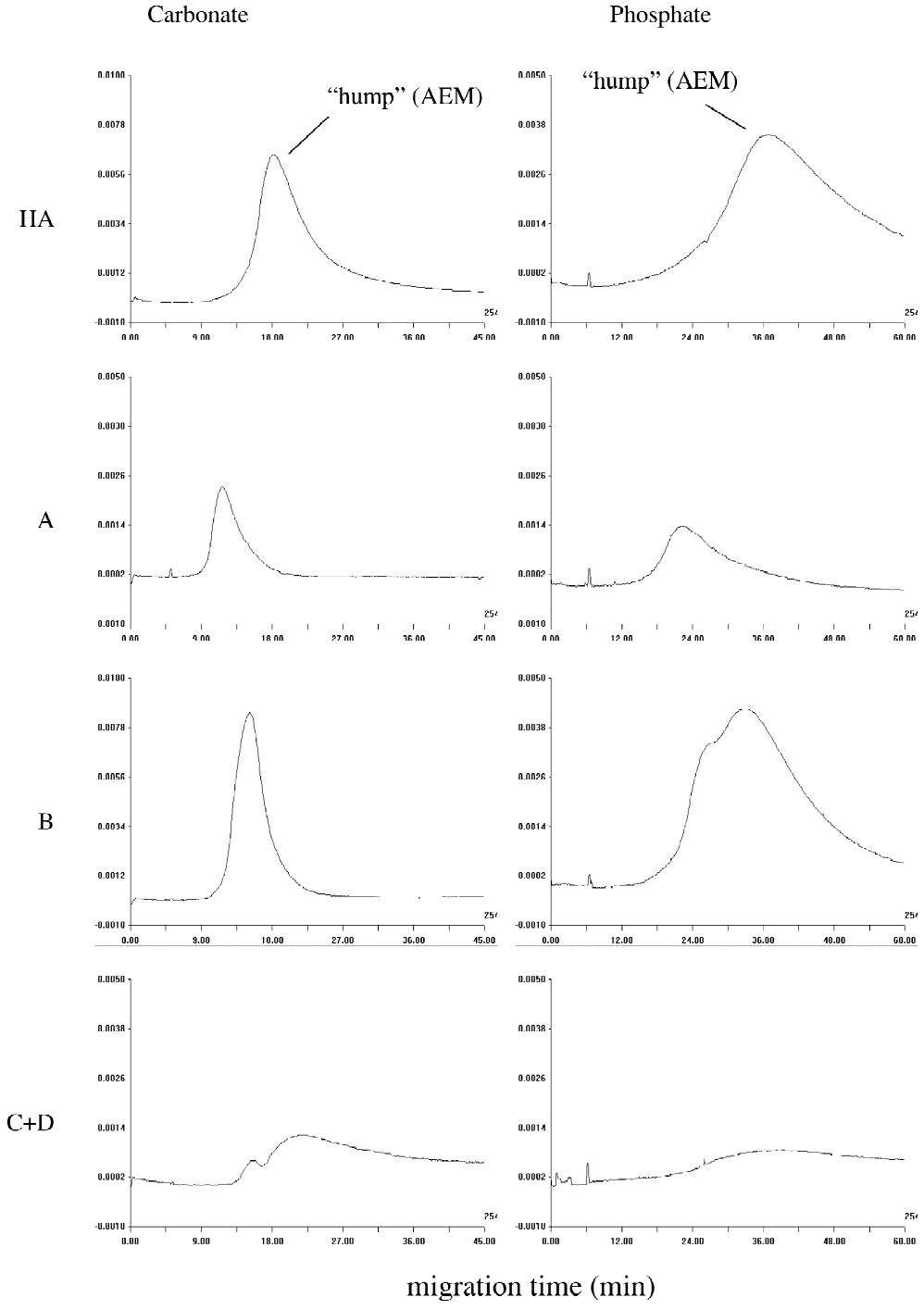

Fig. 3. Electropherogram of original Chernozem HA and fractions A, B and C+D obtained using carbonate (50 m*M*, pH 9.0) and phosphate (50 m*M*, pH 8.5) as the running buffer (capillary 50 cm×50 μm I.D., 15 kV, 20 °C), *y*-axis is absorbance at 254 nm.

Table 1
Effective electrophoretic mobility of separate peaks obtained with different buffers

Buffer	Effective electrophoretic mobility (10^{-4} cm^2 V^{-1} s^{-1})			
	HA original	Fraction A	Fraction B	Fraction C+D
Carbonate 50 mM, pH 9.0				
hump[a] (AEM[b])	−3.36	−2.79	−3.21	−3.66
Phosphate 50 mM, pH 8.5				
hump (AEM)	−3.24	−2.74	−3.06	−3.38
Borate 50 mM, pH 8.3				
hump (AEM)	−3.25	−3.20	−3.30	−3.41
TBE 50 mM+7 M urea+0.1% SDS, pH 8.3				
peak (°)[c]	−1.66	nd[d]	−1.62	−1.53
hump (AEM)	−2.82	−1.30	−2.32	nd[d]
peak (*)[c]	−3.12	−3.13	−3.05	−3.14

[a] See Figs. 3,4.
[b] AEM, average electrophoretic mobility.
[c] See Fig. 4.
[d] nd, not detectable.

changes in migration times during CZE in free solution. Ciavatta et al. [11], using borate buffer, pH 9.0, found different resolution of the peaks depending on their molecular size without any changes in migration times. A good correlation between migration times of UF humic acid fractions and their molecular size was recently reported by De Nobili et al. [19], using CZE as soon as a long-chain hydrophilic polymer polyethyleneglycol at concentration above the entanglement threshold had been added to Tris–phosphate buffer. After this procedure the migration times of the UF fractions increased with a decrease of their molecular size.

3.3. CZE by Tris-borate-EDTA buffer with disaggregating agents

For the enhance the resolution of the whole HA sample the buffer with several disaggregating agents (urea, SDS, EDTA), which prevented different types of inter- and intramolecular interactions, has been used. It should be noted that these reagents were used in our previous PAGE research [2,3].

The CZE electropherograms obtained by using Tris-borate-EDTA (TBE) buffer with urea and SDS are shown in Fig. 4. The whole HA showed two sharp little peaks (°) (migration time about 9 min), a wide hump (migration time from 11 to 20 min), and a big sharp peak (*) (migration time about 21 min) on electropherogram. The fraction A originated the humic hump and sharp peak (*) (migration time was about 8 and 18 min, respectively). The fraction B resolved into the three little sharp peaks (°) and one big sharp peak (*) (migration time was about 9 and 17 min, respectively), and a hump with increasing migration time from 9 to 17 min. The electropherogram of C+D fraction was similar to the whole HA, but the wide hump was less intensive and it is not possible to detect a maximum to calculate the AEM of this fraction. Therefore, if take into account the different contents of fractions in the whole HA and their different optical density, it is possible to assess that the humic hump is mainly constituted by the fractions A and B, while the big sharp peak (*) by C+D fraction. It seems that the TBE buffer with disaggregating agents provided a better characterisation of soil HAs than the alkaline buffers used. On the other hand distinct peaks (°) and (*) does not necessarily indicate a humic fraction with exact molecular size, but it may be artefacts caused by interaction of borate ions with 1.2 and 1.3 diols and/or carboxylic group present in HAs.

Swift et al. [25] divided soil HAs into four subfractions by SEC on Sephadex and found by NMR that with a decrease of subfraction molecular sizes the content of carboxylic groups increased significantly and might be, in our case, to a large extent responsible for the interaction with borate ions. Kopàcek et al. [26] used CZE to investigate soil HA fractions, obtained by gel chromatography on

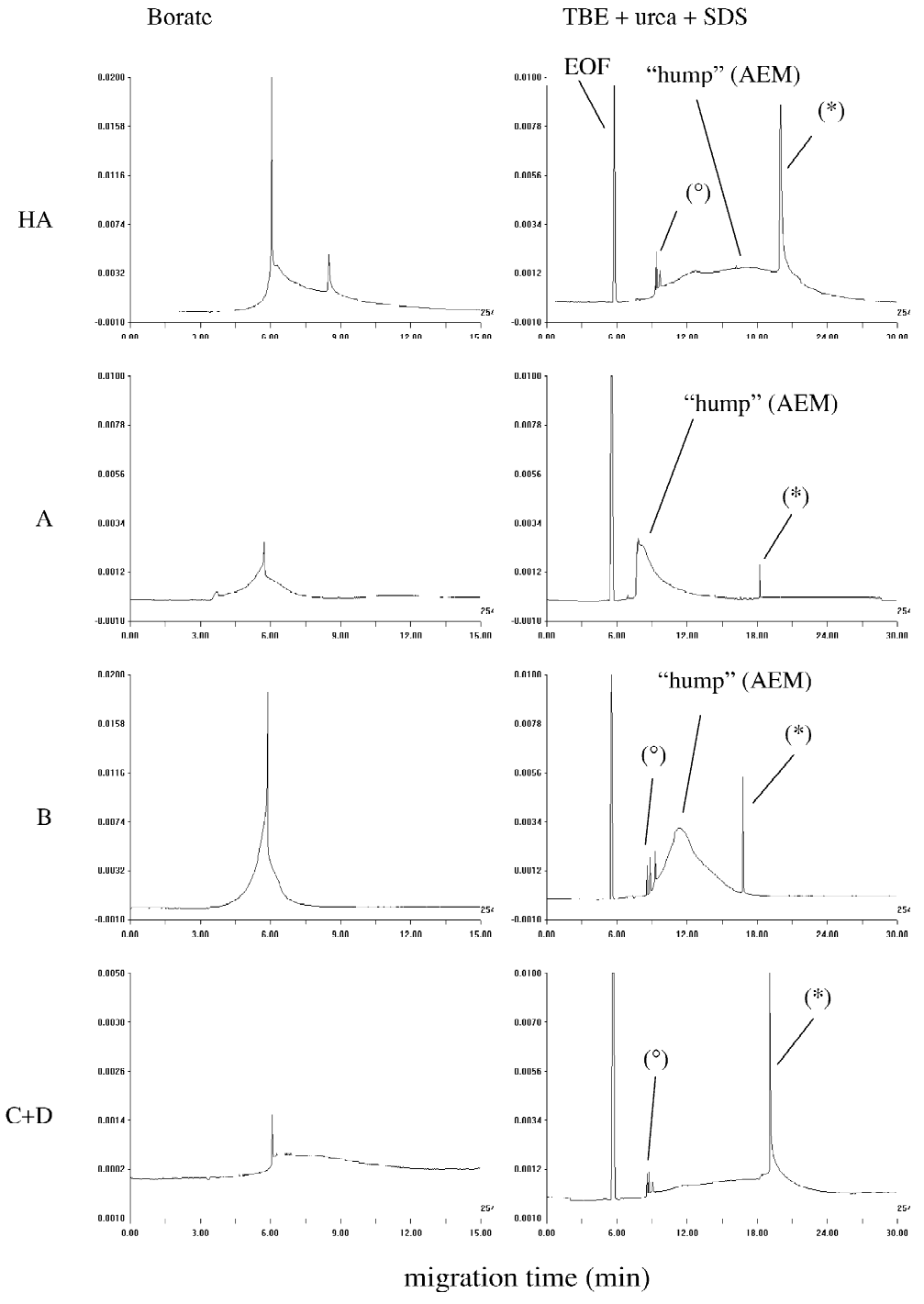

Fig. 4. Electropherogram of original Chernozem HA and fractions A, B and C+D obtained using borate (50 m*M*, pH 8.3) and TBE+7 *M* urea+0.1% SDS (pH 8.3) as the running buffer (capillary 50 cm×50 μm I.D., 15 kV, 20 °C), *y*-axis is absorbance at 254 nm.

Sephadex G-50, and found that HS fractions with higher molecular sizes had lower effective electrophoretic mobilities than the ones with the lower molecular sizes. However, some low molecular size fractions had the lower electrophoretic mobility, that was explained by the content of aromatic structures in low molecular size HAs. In our investigations this contradiction was not observed and the distribution of CZE fractions was in agreement with PAGE and SEC results.

4. Conclusions

The CZE in several low alkaline buffers (sodium carbonate, pH 9.0; sodium phosphate, pH 8.5; sodium borate, pH 8.3; TBE buffer with urea and SDS, pH 8.3) allowed us to characterise the soil HA fractions, obtained by SEC–PAGE, on the basis of their molecular sizes and electrophoretic mobility. However, the better resolution of whole HA has been observed in the TBE buffer with urea and SDS. The distribution of CZE fractions was in agreement with PAGE and SEC results. The CZE method might be successfully used for separating soil HAs in fractions with different molecular sizes.

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