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# Comparative study for separation of aquatic humic substances by capillary zone electrophoresis using uncoated, polymer coated and gel-filled capillaries

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**Abstract**

Several comparative capillary zone electrophoresis (CZE) experiments were carried out by means of uncoated, polyvinyl alcohol (PVA) and polyacrylamide (PAA) coated silica open tubular capillaries and gel-filled capillaries (linear non-cross-linked polyacrylamide, PAGE, by a pre-coated PAA capillary) using different kinds of background electrolytes (BGEs) and organic modifiers for characterization of aquatic dissolved humic matter (DHM). Organic compounds, such as acetic acid, acetate buffer, methanol, ethylene glycol, acetonitrile, dimethylsulphoxide, 5 M urea and sodium dodecyl sulphate (SDS) were tested as sample modifiers to improve the separative power. The fractionation mode by a PVA coated open tubular capillary using 40 mM phosphate buffer at pH 6.8 and 5 M urea–water as the sample modifier turned out to be fairly practical as well as its PAA homologue. Linear non-cross-linked PAGE with 10% gel concentration and 5 M urea–water as the sample modifier using 40 mM phosphate buffer at pH 6.8 produced the most reliable results as to the adaptation of physical gels, especially if the interactions of humic solutes with the gel matrix are not critical. The addition of SDS in the linear PAGE gel increased the interaction of humic solutes with the gel matrix but also improved the separative power and strengthened the chaotropic effect of the urea modifier.
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*Keywords:* Physical gels; Humic substances; Poly(vinyl alcohol); Polyacrylamides

## 1. Introduction

The capillary zone electrophoresis (CZE) is a relatively new and continually growing analytical technique, which has already proved its usefulness in effective separation of many organic biomacromolecules, such as nucleic acids, peptides, proteins, carbohydrates and lipids [1,2]. Capillary zone electrophoresis of natural organic matter (NOM) is a continuously increasing approach in searching analytical conditions for reliable and straightforward results, as reported in a recent review by Schmitt-Kopplin and Junkers [3]. The capillary electrophoretic behaviours of humic matter (HM, a more general synonym for humic substances) and other NOM have been studied rather frequently using bare silica open tubular capillaries with numerous borate, acetate, phosphate, alanine, etc. (BGEs) at different acidities [4–7].

In bare silica capillaries where the movements of analytes are based on the electro-osmotic flow (EOF), the separation of HM solutes is often more or less poor and typical electropherograms may look like strongly tailing wavy lines containing only little information, e.g. [8]. A difficult problem is also the sorption of HM onto uncoated silica capillaries, and for that reason relatively high sample concentrations or long injection times are usually needed. It is essential to note that concentrating the HM mixture increases the aggregation degree of the possible individual humic solutes. It has been suggested [5] that the few broad humic fractions mostly observed in electropherograms may thus represent different sizes of aggregates or oligomers of dissolved HM solutes. It is also notable that so far the best CZE separation patterns of HM with uncoated silica open tubular capillaries have been obtained with borate buffers, which are known to form borate complexes with 1,2- and 1,3-dihydroxy structures present in the humic substances. Therefore, the separate peaks observed in the electropherograms do not necessarily represent any distinct humic fractions, but may be just

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artifacts caused by interactions of borate ions and HM [9–11].

For improving the resolution in CZE studies of HM, attempts have been made to reduce problems related to aggregation, wall sorption and EOF in bare silica capillaries. Major approaches include the use of several organic dis-aggregating agents to break hydrogen bonds between possible HM aggregates and capillary wall modifications by different permanent or dynamic coatings. Egeberg and Bergli [12] recently used 5 M urea as a BGE additive for obtaining a more characteristic outcome of HM fingerprinting by splitting the broad humic electropherogram into some specific fractions.

The most important peculiar characteristic for different kinds of humic like constituents is the occurrence of acidic functional (mainly carboxylic) groups which render them into polyelectrolytes. The acid constants ($pK_a$) of humic solutes lie approximately between 3 and 10 [13,14]). The $pK_a$ values of different humic solutes arising from carboxylic groups are around 3–7.5, and the remaining range (ca. 8.5–10) is accounted for phenolic OH groups. Because the most acidic carboxylic groups account for about 62% of their total amount, the humic solutes around pH 4 are largely anionic in character. At the acidity around pH 7 practically all acidic carboxylic groups of humic solutes are deprotonated (negatively charged constituents). The presence of negative charge permits, using coated capillaries, to separate humic solutes by electrophoresis in electrical field with inverse polarity $(-) \rightarrow (+)$ contrary to uncoated capillaries $(+) \rightarrow (-)$ in which the EOF is responsible for the movement of solutes. This being the case no other chemical additives are theoretically required and the coating of silica capillaries affords an opportunity to more reasonable and physical electrophoretic separation of humic solutes.

According to Altria [1] the utilization of coated capillaries would be advisable due to two reasons: (1) to reduce the sample-wall interactions and (2) to eliminate the EOF effect. Those very few covalently bound or physically adhered neutral phases, which have been applied in the HM studies for permanent deactivation of the inner capillary wall are mostly polyacrylamide (PAA) and epoxy-coatings [5,15].

The molecular sieving effect, especially the utilization of non-cross-linked polymer solutions, has been introduced into capillaries by means of different kinds of polymers, such as polyvinyl alcohol (PVA), polyethylene glycol (PEG), polyacrylamide (PAA), hydroxyethyl cellulose (HEC) and even cyclodextrin- or oligosaccharide-derivatives [15–18]. The use of these, so-called physical gels (or entangled polymer solutions, adaptation of capillary gel electrophoresis, CGE) has gained increasing attention also in the humus chemistry. This modification, generally applied by uncoated silica capillaries, increases buffer viscosity, mostly generates better separative power through the sieving effect and also serves dynamic deactivation against the inner capillary wall.

Aquatic NOM contains many UV absorbing chromophores which can be applied to their detection and as a result, the absorbance of light by natural waters is a semi-quantitative indicator of the concentration and nature of NOM in the water. Based on the occurrence of $\pi$–$\pi^*$ electron transition for phenolic arenes, benzoic acids, aniline derivatives, polyenes and polycyclic aromatic hydrocarbons with two or more rings, the UV absorbance at 254 nm has been used frequently as a proxy for the concentration and nature of NOM, e.g. [19].

In the present study, the selection of the CZE conditions, including the running buffer, was based on the criterion that the separation must be performed close to the natural conditions, i.e. at a relatively low concentration of DHM and at a rather neutral acidity. Less attention was given to complexing agents, such as borates, or very basic running buffers and bare silica open tubular capillaries to reduce the sample-wall interactions and eliminate the EOF effect. An aquatic humic sample was used as a reference in all experiments, and some additional humic samples were applied for verifying the results. Different kinds of running buffers, including linear non-cross-linked PAGE, were tested by PVA and PAA coated capillaries. Different PAGE concentrations (2.5, 5 and 10%, w/v) were applied for obtaining the effect of the gel concentration on the separative power and on the distribution of the heterogenous humic mixtures. Likewise, the effect of SDS was tested in linear non-cross-linked PAGE. The PVA coated silica open tubular capillaries with phosphate and other running buffers have never been, according to the knowledge of the authors, tested to characterize the natural DHMs.

## 2. Experimental

### 2.1. Background electrolytes

All the BGEs were prepared from analytical (p.a. or HPLC) chemicals and ultra pure water (Elgastat UHQ-PS). Phosphate buffers were prepared from sodium dihydrogen phosphate and disodium hydrogenphosphate, acetate buffers from sodium acetate, borate buffers from boric acid and Tris–buffers from tris(hydroxymethylaminomethane). In the case of Tris–buffers, acetic acid was used for the pH adjustment. The BGE solutions were degassed in an ultrasonic bath and filtered through 0.45 μm PTFE membrane filters (Titan) before the experiments.

### 2.2. Sample preparation

The International Humic Substances Society reference samples of Nordic aquatic fulvic acid (No.FA, code IR105F), applied as the test humic sample, and humic acid (No.HA, code IR105H), used as an additional reference sample, were isolated (1986) by the standard XAD-8 technique from the runoff water of a Norwegian mire. Another additional humic sample SS.DEAE was isolated (1994) by the so-called DEAE-cellulose technique

from the Lake Savojärvi situated in southwestern part of Finland.

The isolation procedures and the chemical characteristics of the freeze-dried No.FA, No.HA and SS.DEAE humic fractions have been reported in detail previously [20,21]. The isolation procedure of a river water humic sample (Pirita humic, the River Pirita of Tallinn, Estonia, used also as an additional reference) has been reported previously [22,23].

The solid No.FA, No.HA and SS.DEAE samples were dissolved in ultra pure water or different modifier solutions in concentration of $150\,mg\,l^{-1}$ (if not otherwise stated), filtered through 0.45 μm filter and degassed in an ultrasonic bath. In the present study, the most frequently applied concentration of $150\,mg\,l^{-1}$ corresponds to the dissolved organic carbon (OC or humic carbon) content for these three samples 75.1, 77.3 and $75.4\,mg\,l^{-1}$, respectively. The liquid Pirita-humic sample (OC content $97.5\,mg\,l^{-1}$) in its original elution water was only filtered (0.45 μm) and degassed before CZE analyses.

The organic chemicals tested as modifiers were acetonitrile, dimethyl sulphoxide, ethylene glycol, methanol and urea.

### 2.3. Instrumentation

CZE experiments were performed on $HP^{3D}CE$ capillary electrophoresis system (Hewlett-Packard) equipped with a diode array detector at 254 nm. Operation of the instrument, data collection and analyses were controlled by ChemStation system software. The polarity was negative to be mostly −10 kV in the experiments of PVA and PAA capillaries. The experiments of bare silica open tubular capillaries were performed using the positive polarity mode (voltages ranged from 15 to 30 kV depending on the buffer composition). Samples were injected hydrodynamically at the temperature of 25 °C using pressure of 5000 Pa (injection time was 10 s, if not otherwise stated).

Relative $UV_{254}$-absorbances $\{(\text{UV abs at 254 nm/OC of the original sample, mg}\,l^{-1}) \times 100\}$ were calculated for the electropherograms irrespective of the fact that the actual OC concentration of the migrated sample is not exactly known but the injection volumes were assumed to be constant. The standardized relative $UV_{254\,nm}$-absorbances make the results more comparable which each other. The common meaning of the $UV_{254\,nm}$-OC ratio in the water chemistry is to indicate the aromaticity of the sample.

### 2.4. Capillaries

The dimensions of the uncoated silica open tubular capillary (Agilent G1600-64411) were: 100 μm internal diameter, 64.5 cm total length and 56 cm effective length. Before sample injections the capillary was daily washed for 15 min with running buffer and conditioned for 2 min with analysis voltage (15–30 kV). Between the injections the capillary was rinsed with 0.1 M NaOH (2 min), with water (2 min) and finally with the running buffer (4 min).

The dimensions of the PVA coated silica open tubular capillary (Agilent G1600-61419) were: 100 μm internal diameter, 64.5 cm total length and 56.0 cm effective length. The daily washing procedure included the rinse with the running buffer (15 min) after which the system was stabilized with the analysis voltage (2 min). Between the different injections the rinse with the running buffer (4 min) was performed. At the end of experiments the capillary was rinsed with the running buffer (5 min), with water (15 min) and finally dried with air flow (10 min). For more thorough cleaning the capillary was rinsed with 10 mM phosphoric acid.

The procedure reported by Hjerten [24] was applied for preparing the covalently bound layer of polyacrylamide (PAA) on the surface of the silica capillary. The silica capillary was first treated with 1 M NaOH for 1 h, then the capillary was rinsed with water, with 0.1 M HCl and with pure water. The capillary was filled with a solution containing 30 μl of 3-(trimethoxysilyl)propyl methacrylate and 1 ml of 60% (v/v) acetone in water. After the reaction time of 1 h the capillary was rinsed with water and filled with degassed solution of 3% acrylamide in water containing $2\,mg\,ml^{-1}$ ammonium persulphate and 1 μl *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED). After polymerization time of 1 h the capillary was rinsed with water and dried overnight with nitrogen gas flow.

Linear non-cross-linked, polyacrylamide gel (PAGE) was prepared as reported by Widhalm et al. [25]. And 1.88 g acrylamide was dissolved in 15 ml of distilled water, 20 mg of TEMED was added and the mixture was degassed for 10 min. After that, 23 mg of ammonium persulphate in 10 ml of water was added into the mixture, and the gel obtained was degassed for 20 min in an ultrasonic bath. No cross-linker was applied. The gel was dissolved in the phosphate buffer (40 mM, pH 6.8) for obtaining running buffers with 2.5, 5 and 10% (w/v) gel concentrations which were applied as different sieving mediums with the PAA pre-coated capillary.

Some experiments were also performed with the PAA coated capillary with the linear non-cross-linked PAGE running buffer containing anionic SDS. Solid SDS was first added in the phosphate buffer (40 mM, pH 6.8) to give a final concentration of 0.5% (w/w), and then the linear PAGE gel was added in this mixture for obtaining an SDS containing running buffer with 10% (w/v) PAGE gel concentration.

## 3. Results

### 3.1. General

It has been stated [26] that phosphate running buffers will cause system peaks (detection at 214 nm) in neutral and weak alkaline solutions of CZE experiments with PAA coated capillaries. Therefore, also blanks were analysed in the present study to check the possibility of the occurrence

of additional peaks originating from the applied phosphate buffer itself. The results confirmed that the phosphate electrolyte produced absolutely no extra peaks by using the PVA and PAA coated and non-coated capillaries.

The spectral properties of organic constituents were also verified at lower wavelengths (210 and 222 nm) which generate somewhat stronger intensities than those at 254 nm being consistent with the statements reported in the literature [27,28]. The presence of a urea modifier in the sample caused extra peaks in electropherograms when the detection was carried out at 210 or 222 nm. However, these extra peaks were not visible at 254 nm. The absorbance intensities were slightly greater (ca. 1.26-fold) at 254 nm than those detected at 280 nm but the resolutions and shapes of the electropherograms were symmetrical. The final data were recorded at 254 nm according to the general practice of the water chemistry. All samples were analysed in triplicate and the relative standard deviations were <5%. The results are demonstrated in Figs. 1–11 according to the applied parameters.

### 3.2. PVA coated silica open tubular capillary

#### 3.2.1. Buffer concentration

Fig. 1 demonstrates the effect of the phosphate buffer concentration (10–50 mM at pH 6.8) on the separation of the No.FA reference dissolved in pure water. The phosphate buffer concentration of 40 mM at pH 6.8 was chosen as the reference BGE which was also applied to the Pirita-humic sample.

#### 3.2.2. Sample concentration

Fig. 2 shows the effect of the sample concentration on the resolution and separative power. As the standard, a DHM concentration of 0.15 mg ml$^{-1}$ was used in further experiments.

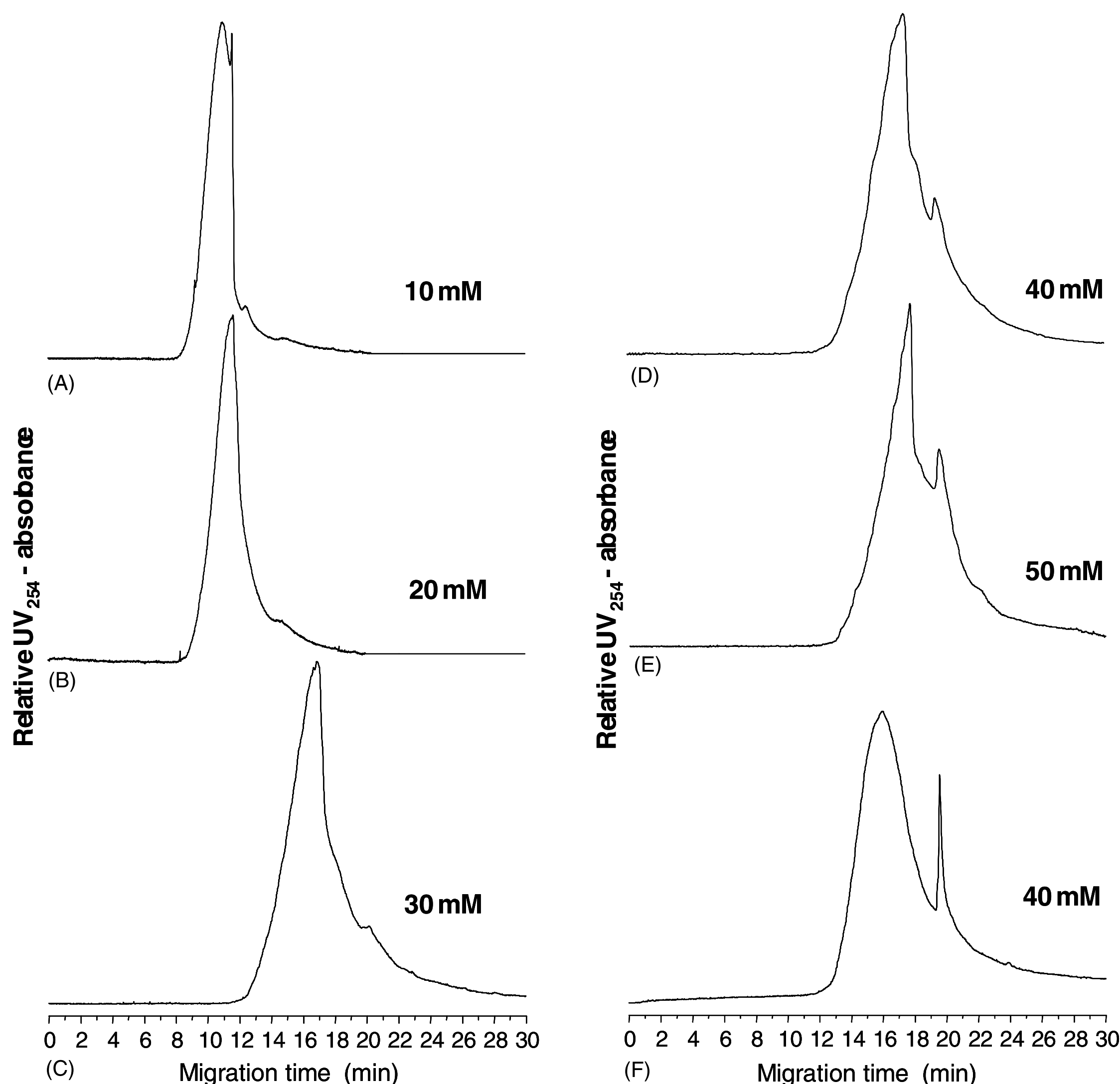


Fig. 1. Effect of phosphate buffer (pH 6.8) concentration on separative power of No.FA (A–E) and Pirita (F) humic samples. Concentration (OC, mg l$^{-1}$) of No.FA humic in pure water 75.1 and Pirita humic in original elution water 97.5. PVA coated open tubular capillary; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–F is 14.5 units.

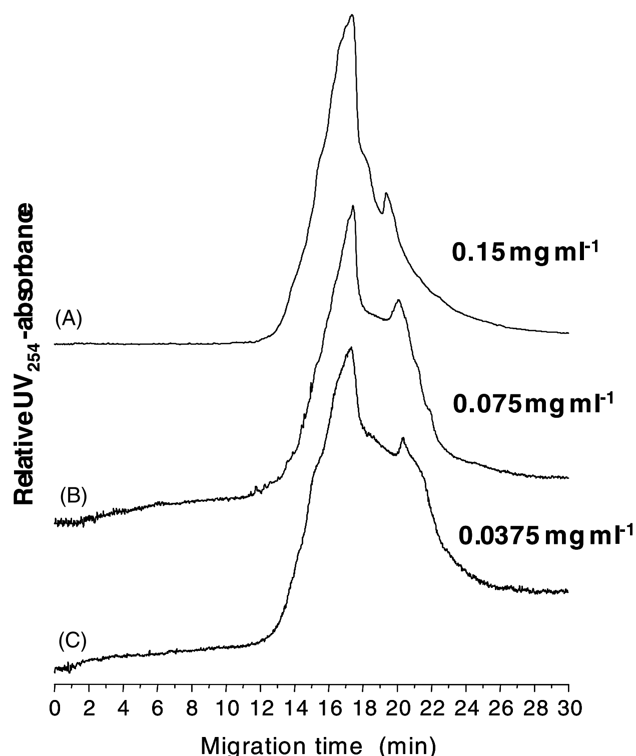


Fig. 2. Effect of sample concentration. No.FA humic dissolved in pure water. 40 mM phosphate buffer at pH 6.8 as BGE. PVA coated open tubular capillary; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–C is 14.4 units.

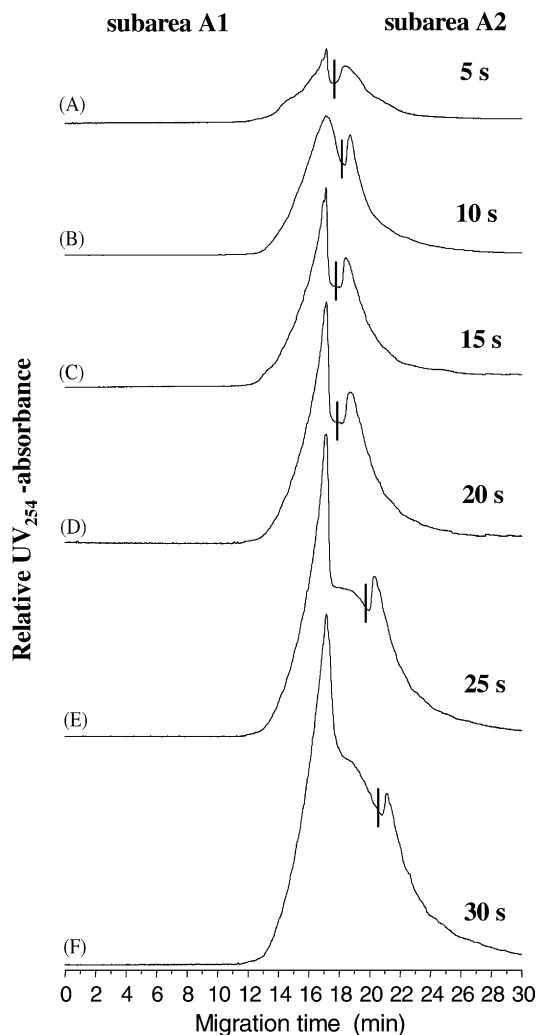


Fig. 3. Effect of injection time. Concentration of No.FA humic in pure water 0.15 mg ml$^{-1}$ (OC, 75.1 mg ml$^{-1}$). 40 mM phosphate buffer at pH 6.8 as BGE. PVA coated open tubular capillary; voltage −10 kV. The maximum absorbance-scale for A–F is 36.7 units.

### 3.2.3. Injection time

Fig. 3 indicates the effect of the hydrodynamic injection time (5–30 s) on the possible overloading of the capillary using 40 mM phosphate buffer at pH 6.8. The injection time of 10 s was routinely used in further experiments.

### 3.2.4. Sample modifiers

Fig. 4 shows the effect of the sample modifier on the separative power. The modifiers of 10 mM acetate buffer at pH 7.0, 0.1 mM acetic acid at pH 4.4, 5 M acetonitrile–water, 5 M methanol-water, ethylene glycol–water (2:8), 5 M DMSO-water and 5 M urea–water were applied for dissolving the No.FA reference sample. The concentration (0.15 mg l$^{-1}$) of No.FA was the same in all solvents and 40 mM phosphate buffer at pH 6.8 was applied as the running BGE.

### 3.2.5. Acidity of BGE

Fig. 5 demonstrates the clear effect of the acidity (40 mM phosphate, pH: 6.2, 6.8, 7.4 and 8.1) on the separative power of the No.FA reference dissolved in 5 M urea–water solvent. As one of the aims of the present study was the separation of DHM in rather neutral acidities, the phosphate BGEs under pH values of 6 were not tested. The pH value of 6.8 was applied in further experiments.

### 3.2.6. Sample age

Fig. 6 visualizes the effect of the aging of the sample dissolved in pure water. Electropherograms were taken using 40 mM phosphate BGE at pH 6.8 just after preparation and on each day up to one week.

### 3.2.7. Urea addition in 40 mM phosphate BGE at pH 6.8

Fig. 7 shows the effect of the urea addition on the separative power of different DHMs. Concentrations of the urea in the BGE were 5, 50 and 500 mM for the No.FA reference dissolved in pure water. Two humic samples (No.HA and SS.DEAE) dissolved in 5 M urea–water were also tested before and after urea addition (50 mM) in the BGE.

### 3.2.8. Additional BGEs

*3.2.8.1. Tris–phosphate BGEs.* Strongly basic Tris–phosphate running buffers (20 and 50 mM, pH 8.5) were ap-

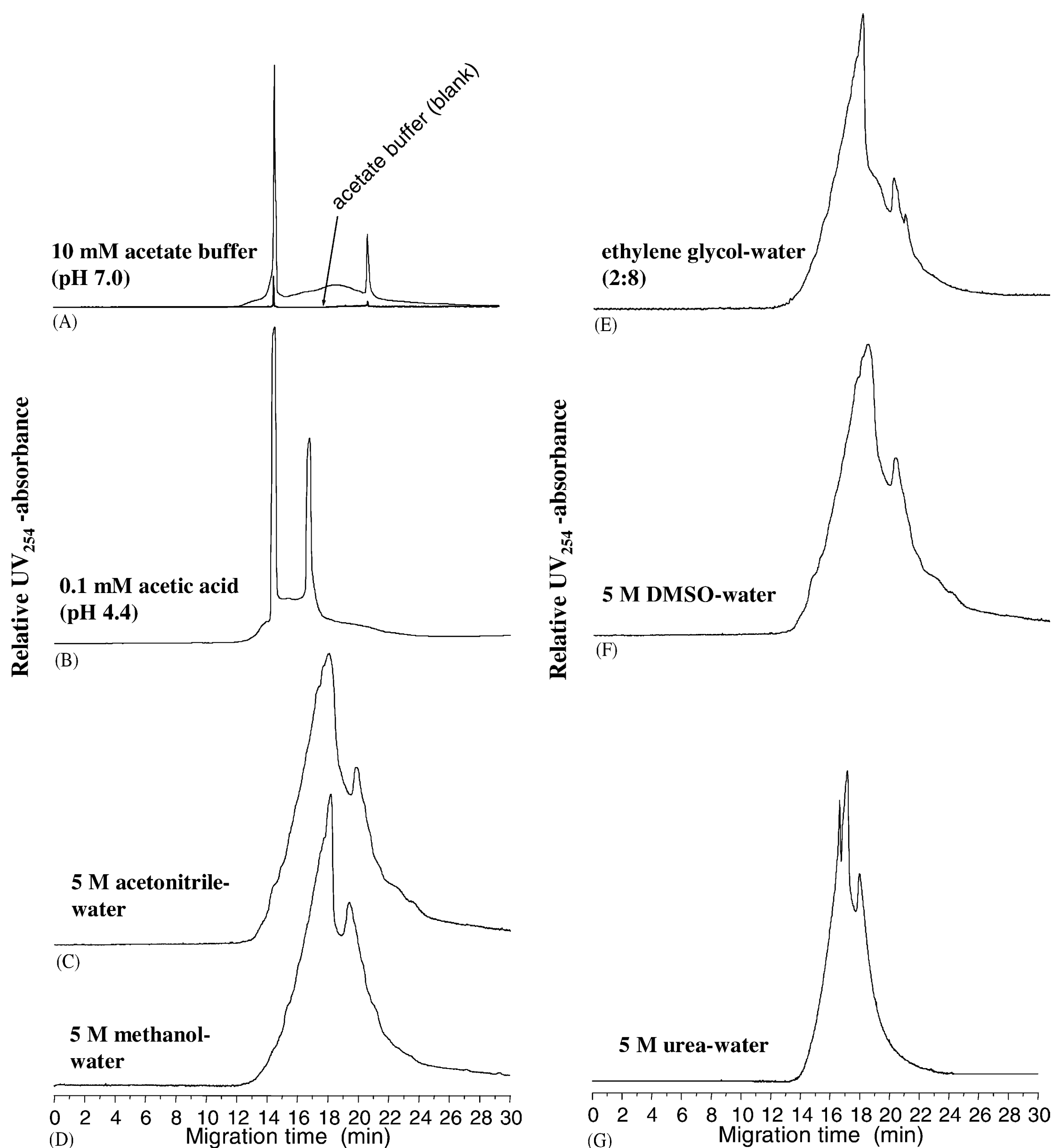


Fig. 4. Effect of different sample modifiers. Concentration of No.FA humic dissolved in sample modifiers 0.15 mg ml$^{-1}$ (OC, 75.1 mg l$^{-1}$). 40 mM phosphate buffer at pH 6.8 as BGE. PVA coated open tubular capillary; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A, B is 43.8 and for C–G 14.5 units.

plied for the separation of the No.FA reference (OC, 75.1 mg l$^{-1}$ in pure water) and the Pirita-humic sample (OC, 97.5 mg l$^{-1}$ in original elution water). Run conditions: PVA coated silica open tubular capillary; injection time 10 s; −10 kV voltage. The obtained electropherograms looked like Gaussian curves with increasing migration times for the peak maxima when the concentration of the BGE was increased to 50 mM (results not shown). Since Tris–phosphate buffers did not improve resolution in comparison to only phosphate containing BGEs, this Tris–BGE was not further optimized and used.

*3.2.8.2. Acetate BGEs.* Three different acetate running buffers were tested for the separation of DHM using the PVA coated silica open tubular capillary. In the case of 10 mM acetate buffer at pH 7.6 the No.FA reference was dissolved (0.15 mg ml$^{-1}$) both in pure water and 5 M urea–water for obtaining a possible action of the urea modifier. Run conditions: injection time 10 s; −10 kV voltage. Both electropherograms resembled Gaussian curve shapes indicating that the sample modification with 5 M urea did not markedly improve resolution. Similar results were obtained when the No.FA reference was dissolved (0.15 mg ml$^{-1}$) in the 10 mM acetate buffer (pH 7.0). Run conditions: injection

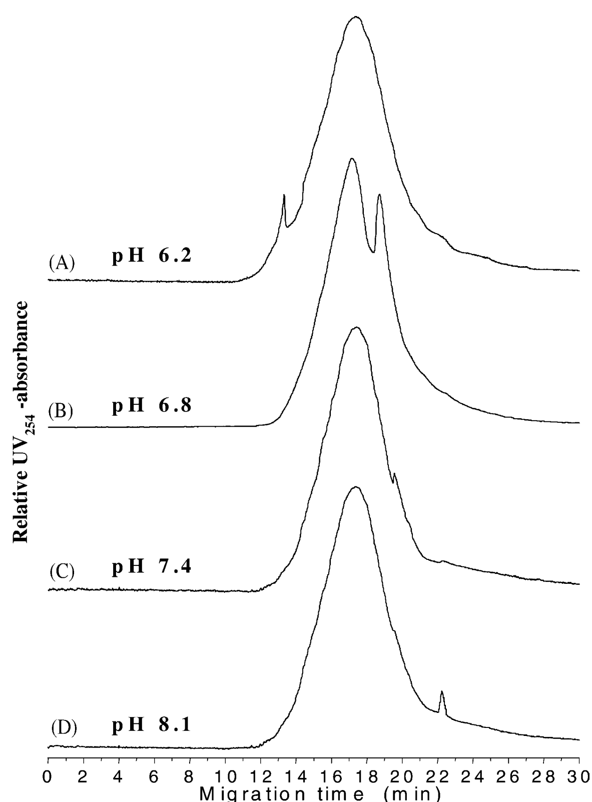


Fig. 5. Effect of acidity of 40 mM phosphate BGE. Concentration of No.FA humic in 5 M urea–water 0.15 mg ml$^{-1}$ (OC, 75.1 mg l$^{-1}$). PVA coated pen tubular capillary; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–D is 14.6 units.

time 10 s; −10 and −30 kV voltages. The increase of the voltage did not change the Gaussian-like shapes of the electropherograms and only a shift in the migration time was observed. The effect of a volatile BGE (5 mM ammonium acetate) on the separative power was also tested with the No.FA reference humic dissolved (0.15 mg ml$^{-1}$) in pure water. Run conditions: injection time 10 s; −10, −20 and −30 kV voltages. Similar results were obtained as in all above acetate containing BGEs, i.e. Gaussian shape electropherograms and no improved resolution with increased voltages.

### 3.3. Uncoated silica open tubular capillary

Fig. 8 demonstrates the electropherograms obtained by means of different adaptations of BGEs and sample solvents. In Fig. 8A the No.FA reference was dissolved (1.0 mg ml$^{-1}$) in pure water and the applied running buffer was 40 mM phosphate at pH 6.8. In Fig. 8C and D the No.FA reference was dissolved (1.0 mg ml$^{-1}$) both in pure water and 5 M urea–water for obtaining a possible action of the urea modifier and the applied BGE was 20 mM phosphate buffer containing 20% ethylene glycol. Fig. 8F demonstrates the effect of the 20 mM phosphate buffer containing 20% ethylene glycol on the separative power when the sample was the

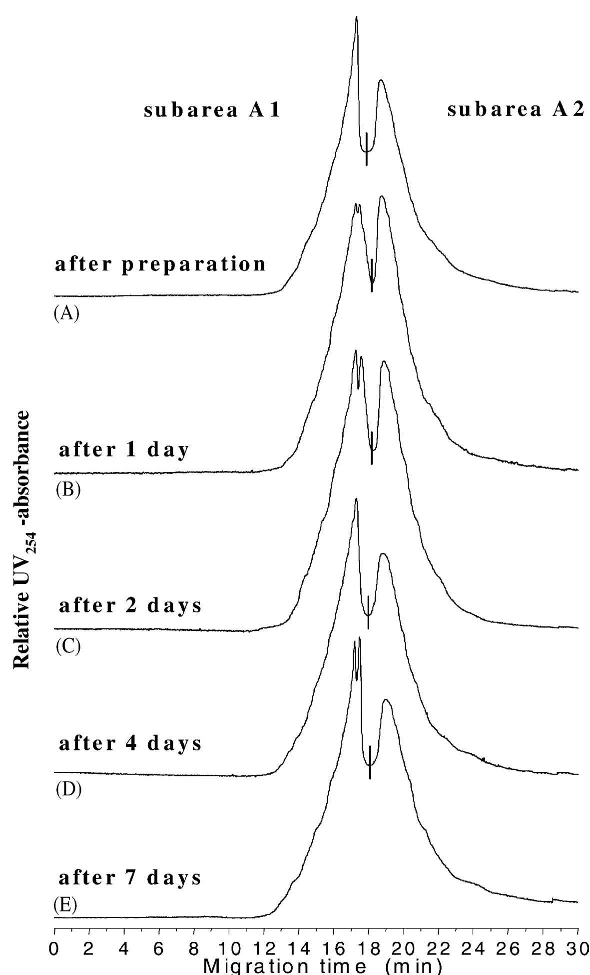


Fig. 6. Effect of sample age. Concentration of No.FA humic in pure water 0.15 mg ml$^{-1}$ (OC, 75.1 mg l$^{-1}$). PVA coated open tubular capillary; 40 mM phosphate BGE at pH 6.8; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–E is 14.2 units.

Pirita humic. Fig. 8B and E show the effect of the strongly basic borate buffer (pH 9) on the separative power when the No.FA reference was dissolved (1.0 mg ml$^{-1}$) both in pure water and in 5 M urea–water, respectively, for obtaining a possible action of the urea modifier.

### 3.4. PAA coated silica open tubular capillary

Fig. 9A–E show the effect of PAA coated silica open tubular capillary on the separative power using 50 mM Tris–phosphate (pH 8.5) and 40 mM phosphate (pH 6.8) BGEs. The No.FA reference was dissolved (with concentrations of 0.15 and 1.0 mg ml$^{-1}$) both in pure water and 5 M urea–water. Pirita humic (OC, 97.5 mg l$^{-1}$) was in its original elution water.

### 3.5. Linear non-cross-linked PAGE

Fig. 10A–F illustrate the changes of the electropherograms as the function of the increasing gel concentration

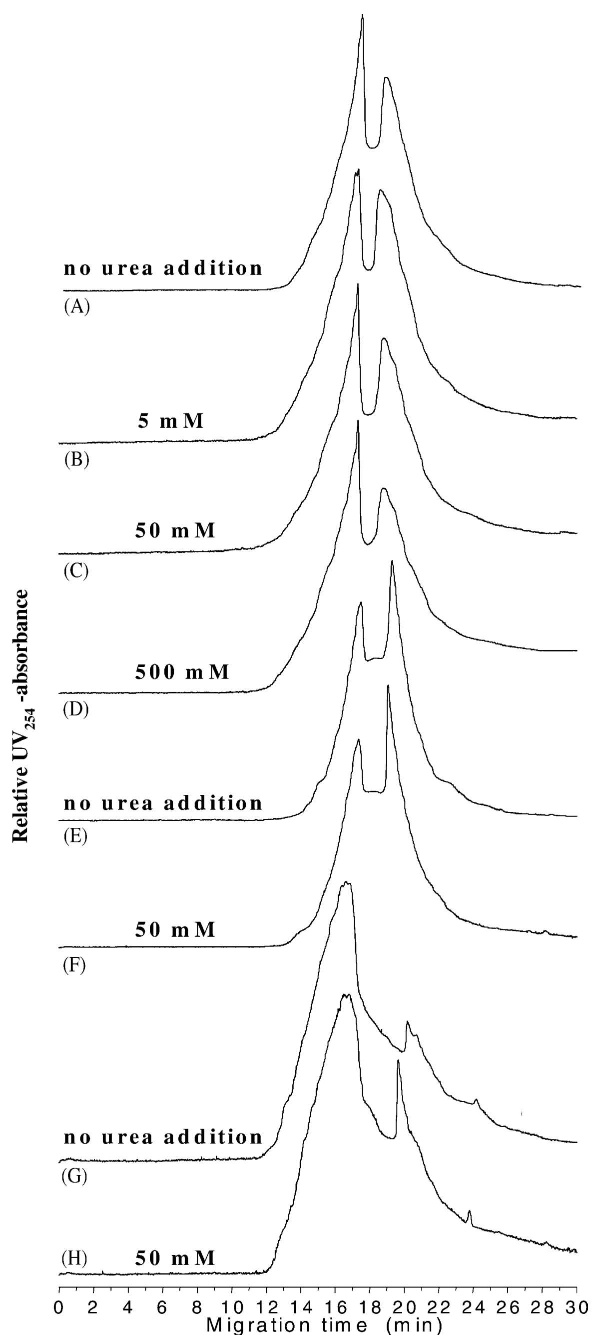


Fig. 7. Effect of urea addition in 40 mM phosphate BGE (pH 6.8). Concentrations of No.FA humic (A–D) in pure water, No.HA (E and F) and SS.DEAE (G and H) humic in 5 M urea–water 0.15 mg ml$^{-1}$. PVA coated open tubular capillary; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–F is 14.2 and for G and H 11.7 units.

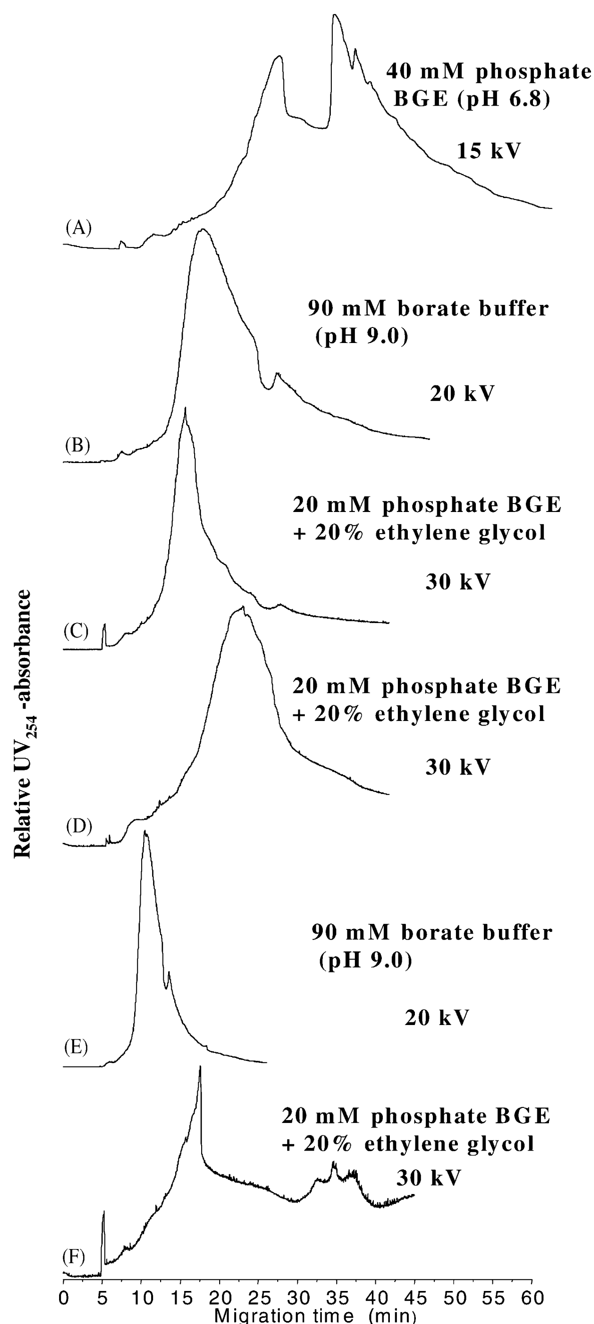


Fig. 8. Effect of different BGEs using uncoated silica open tubular capillary. Concentration (OC, mg l$^{-1}$) of No.FA humic in pure water (A–C) and in 5 M urea–water (D and E) 500.5 and Pirita humic (F) in original elution water 97.5. Injection time 10 s. The maximum absorbance-scale for A–F is 3.5 units.

(2.5, 5 and 10%, w/v) using 40 mM phosphate BGE at pH 6.8. Fig. 10G demonstrates the effect of 50 mM Tris–phosphate BGE at pH 8.5 on the separative power using 10% gel concentration. The No.FA reference was dissolved (1.0 mg ml$^{-1}$) in each individual case in pure water or 5 M urea–water.

### 3.6. SDS-linear PAGE

Fig. 11A and B show the effect of the SDS addition (0.5%, w/w) on the separative power of the No.FA reference (dissolved, 1.0 mg ml$^{-1}$, both in pure water and 5 M urea–water) using 10% (w/v) PAGE gel and 40 mM phosphate BGE at pH 6.8. Fig. 11C visualizes the effect of the SDS addition on the separative power in the case of the Pirita-humic sample.

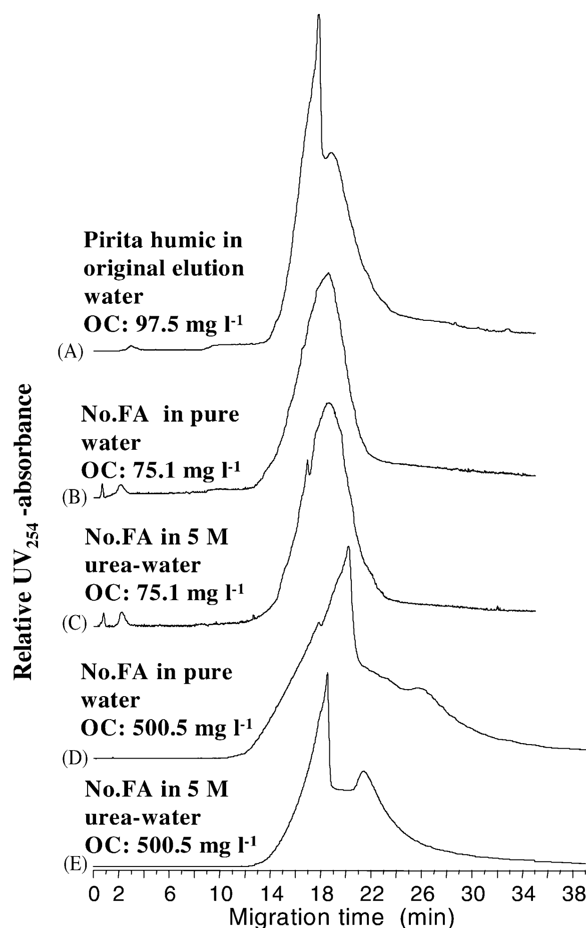


Fig. 9. Effect of different BGEs using PAA coated open tubular capillary. Running buffers 50 mM Tris–phosphate (A–C) at pH 8.5 and 40 mM phosphate (D and E) at pH 6.8. Injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–E is 15.5 units.

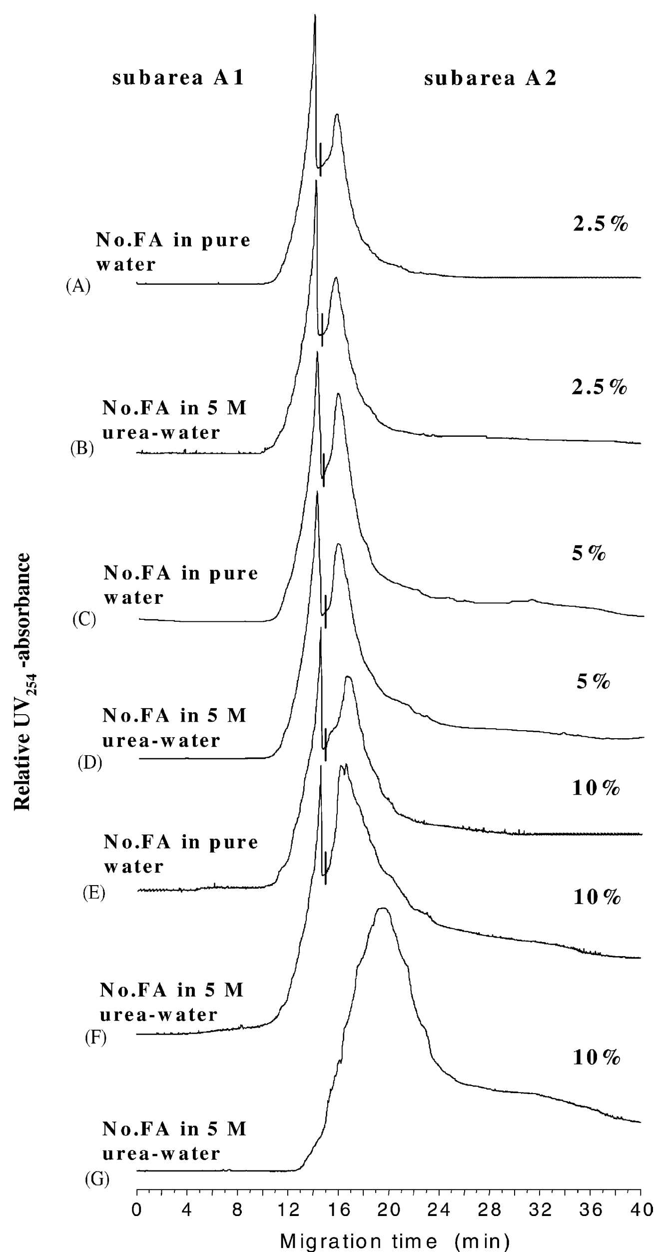


Fig. 10. Effect of gel concentration using linear non-cross-linked PAGE. Concentration of No.FA humic in pure and 5 M urea–water 1.0 mg ml$^{-1}$ (OC, 500.5 mg l$^{-1}$). Running buffer 40 mM phosphate at pH 6.8 (A–F) and 50 mM Tris–phosphate at pH 8.5 (G). PAA pre-coated capillary; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–G is 3.7 units.

## 4. Discussion

### 4.1. General

On CZE experiments analytes migrate with different electrophoretic mobility which can considerably affect the detector response, i.e. analytes with higher velocity stay on the detector cell for a shorter time than those with lower velocity. Likewise, to long response times of the detector can result in broadened and distorted peak shapes. On the other hand, since high acquisition rates and short response times increase the noise, they should be adjusted to the peak widths of the specific analyses. In the present study a relatively high response time of 1.3 s and a peak width of 0.1 min were applied in all experiments for optimizing the noise. The range of the migration times of all analytes in the present study was quite narrow placed within about 15 min. Accordingly, the comparison of the different electropherograms could be considered quite quantitative.

### 4.2. DHM separation with PVA coated silica open tubular capillary

De Nobili et al. [29] have found interaction between the gel-forming PVA polymer and humic solutes using so-called linear polymer-gel method in molecular sieving studies by uncoated silica capillaries (the PVA polymer was suspended (2.5% w/v) in the phosphate background electrolyte). However, in the present study the situation is totally different: the

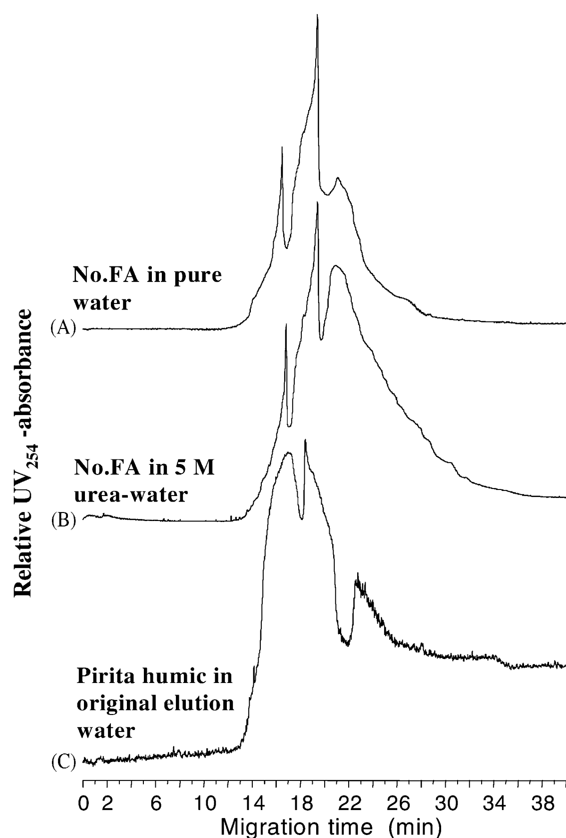


Fig. 11. Effect of SDS in linear non-cross-linked PAGE. Concentration (OC, mg l$^{-1}$) of No.FA humic in pure and 5 M urea–water 500.5 and Pirita humic in original elution water 97.5. Running buffer 40 mM phosphate at pH 6.8 +0.5% SDS. Linear PAGE concentration 10%; PAA pre-coated capillary; injection time 10 s; voltage −10 kV. The maximum absorbance-scale for A–C is 2.0 units.

hydrophilic PVA polymer was permanently fastened inside the silica column walls. Several parallel experiments with the same humic sample (from the initiation with the new column to the end of the whole sample set) speak for the fact that the interactions (sorption–desorption phenomenons) of the PVA coating with humic solutes were practically non-existent (electropherograms of the reference humic sample were continuously identical). Since no tailing was observed in Fig. 1, it is justifiable to postulate that neither ionic nor hydrophobic interactions occur during separation. Electropherograms had quite similar shapes at BGE concentrations of 10–20 mM. The increase of the ionic strength of the phosphate buffer (pH 6.8) resulted in the peak broadening and enhanced resolution. The utilization of an additional humic sample proved that the electropherograms have their characteristic shapes depending of humic solutes in question.

It was possible to separate humic solutes with very low concentrations (up to 0.04 mg ml$^{-1}$) even though the shape of the electropherogram becomes somewhat distorted (Fig. 2). This is an important advantage because so far the separation has been usually performed from very concentrated mixtures (e.g. 5 mg ml$^{-1}$ [12]) that are not normal in natural aquatic systems. Due to the capacity for low concentration ranges, the separation by PVA coated capillaries may find applications in natural organic (and humic) matter separation, characterization and fingerprinting without the concentration procedure that can alter the structural properties of the heterogenous mixtures.

It is generally stated [30] that if the sensitivity is not the limiting factor, the injection times must be so short as possible for avoiding the column overloading when hydrodynamic injection is used (injection times up to 5 s are sufficient). This rule is certainly true, especially whit very high sample concentrations (e.g. 5 mg ml$^{-1}$), a usual case in humic studies. In the present study, however, very low humic concentrations were applied (0.15–1.0 mg ml$^{-1}$) with a PVA coated capillary. For that reason the effect of the injection time from 5 to 30 s was tested using hydrodynamic mode with the pressure of 5000 Pa (Fig. 3). The results indicated that the total area of the electropherograms was a linear function of the injection time (correlation 0.982). This proves that if more humic solutes were injected into the capillary they do not show any interactions with the PVA coated capillary walls. However, the situation is not so optimal if the ratios of two different subareas are compared (Fig. 3: $A1$: from 0 to 18.5 min; $A2$: from 18.5 to 30 min). The $A1/A2$ ratio was relatively constant (1.64) when the injection time was between 10 and 20 s. The subarea $A2$ was strongly enhanced ($A1/A2 = 0.38$) when the injection time was only 5 s. On the contrary, when the injection time was 25 s the subarea $A1$ became enhanced ($A1/A2 = 1.93$), and when the injection time was 30 s it was greatly overemphasised ($A1/A2 = 2.60$). These results clearly indicate that the column overloading became significant when the injection time exceeds 20 s. The injection time of 10 s was considered to be sufficiently valid in the light of the detection sensitivity and the obtained electropherogram.

For generating possibly a better resolution for DHM several organic compounds (acetic acid, acetate buffer, methanol, ethylene glycol, acetonitrile, dimethylsulphoxide and 5 M urea) were tested as sample solvents and modifiers (Fig. 4). Alcohols, such as methanol, ethanol, isopropanol and aprotic solvents, such as acetonitrile and DMSO are useful in assisting the solubility and in modifying the selectivity in CZE [31]. It has been suggested [12] that methanol can disrupt humic aggregates but in the present study the electropherogram obtained by 5 M methanol (Fig. 4D) was almost similar to that where the humic sample was dissolved in pure water. It seems that methanol has minimal effect as a modifier although very slight improvement in resolution was detected (somewhat sharper peaks and less tailing) compared with 5 M acetonitrile (Fig. 4C) and 5 M DMSO (Fig. 4F) as solvents. The electropherogram obtained by ethylene glycol (Fig. 4E) resembled roughly that obtained by methanol. It has been stated [32] that hydrophobic interactions between –$CH_2$–$CH_2$– groups of poly(ethylene glycol) and non-polar groups of organic macromolecules are entropy favourable, and the presence of aromatic structures

is important for these hydrophobic interactions. The formation of humic-ethylene-glycol adduct apparently leads to the broadening of the electropherogram. With the acetate containing modifiers two sharp peaks (Fig. 4A and B) were detected for DHM and the modifier itself caused also weak blank peaks. It has been demonstrated [33,34] that supramolecular structure of humic aggregates may be disrupted in smaller-sized associations by organic acids which is clearly supported by Fig. 4A and B. The best resolution was obtained with 5 M urea modifier (Fig. 4G, three sharp peaks with no tailing), and slightly better resolution was obtained when the DHM samples were allowed first to stabilize for ca 6 h (conformational stability) before the CZE analyses. The above given $A1/A2$ ratio decreased about 37% when the pure water (Fig. 3B; $A1/A2 = 1.64$, split-up at ca 18.5 min) was replaced with the 5 M urea solvent (Fig. 4G; $A1/A2 = 1.04$, split-up at 17.7 min) indicating that the relative content of humic constituents with longer migration times was slightly increased. Likewise, the total migration time slightly decreased and the overall profile of the electropherogram became sharper. The replacing of pure water with 5 M urea–water as the sample solvent seems to work also in the case of humic solutes being in accordance with the utilization of urea as a chaotropic agent in the protein chemistry for disrupting the intermolecular, e.g. peptide–peptide hydrogen-bonds without significant side-reactions even at very high urea concentrations. Besides, it has been demonstrated previously that the disaggregating effect of 7 M urea is not particularly strong for humic solutes [35].

The acidity of the humic solution is the most critical parameter. Fig. 5 verifies the clear effect of acidity on the separative power and shape of the electropherogram. The best resolution was obtained at pH 6.8.

An important question in the DHM characterization is when to analyse the samples, especially those previously freeze-dried and back dissolved. Fig. 6 presents the conformational changes detected as a function of time using a freeze-dried humic sample which was back dissolved into the pure water. The critical examination of Fig. 6 showed that the above-defined $A1/A2$ ratio (split-up at 18.5 min) significantly changed during the age of the sample (giving the values of 1.64, 1.83, 1.88, 1.76 and 1.61 for the experiments A–E in Fig. 6, respectively). The $A1/A2$ ratio became normalized after about 4 to 5 days being at least practically the same as that obtained for a just dissolved sample. In order to achieve reproducible results with DHM samples, it is advisable to measure the samples either just after preparation (only few hours for equilibrium) or after 5 days. This observation for the aging of an aquatic humic sample was comparable with those reported previously by Pacheco et al. [36] and Pokorná et al. [37] for soil and peat humic samples.

The addition of urea to the phosphate buffer (up to 500 mM urea in the buffer) as an extra BGE modifier did not significantly improve the resolution of the DHM samples in the present study when using a PVA coated capillary (Fig. 7). On the other hand, it is reported [12] that the addition of 5 M urea to the phosphate buffer as an extra BGE modifier resulted in longer migration times accompanied by some minor changes in the so-called broad humic peaks when using uncoated silica open tubular capillaries. This dissimilarity verifies the essential effect of the coating for masking the surface charges of free silanol groups resulting in reduced EOF and preventing of wall interactions with the sample, thus giving the actual impact of a modifier exclusively on the sample in question.

### *4.3. DHM separation with uncoated silica open tubular capillary*

The utilization of 40 mM phosphate buffer at pH 6.8 with uncoated silica open tubular capillary for the reference humic sample, dissolved in pure water, resulted in an electrophoretic fraction with rather wide migration time range (up to 50 min) and strong fronting and tailing effect (Fig. 8A). This phenomenon speaks for the interactions with the capillary walls. On the other hand, the resolution was quite good, possibly due to the high ionic strength of the buffer which reduces at some extent ionic interactions. The modification of the same reference humic sample with 5 M urea, however, resulted in total retention of the sample onto the capillary.

Dihydroxy compounds are reported [38] to be more efficient than alcohols in increasing the viscosity within the double layers, thus decreasing the EOF. The addition of ethylene glycol in the phosphate buffer produced from the No.FA reference, dissolved in pure water (Fig. 8C), a rather uncomplicated electropherogram with a relatively short migration time indicating that sorption of humic solutes onto the capillary walls is obviously minimized. This may be caused by interactions of ethylene glycol with charged sites of humic solutes or with capillary walls. The urea modifier increased exceptionally the migration time of the No.FA reference (Fig. 8D) but the shape of the electropherogram was still just uncomplicated. The electropherogarm of the Pirita-humic sample (Fig. 8F) was extremely broad and its disturbed base-line indicates structural changes and strong interactions with capillary walls. The effect of ethylene glycol on the separative power was practically non-existent. Likewise, a phenomenon depending extremely strongly on certain structural peculiarities of humic solutes (Fig. 8F versus Fig. 8C) does not support the utilization of ethylene glycol. Ethylene glycol has been also previously added in the sample solutions in connection with separations of human serum proteins [39] and humic acids [23] in trying to improve the resolution and the reliability. De Nobili et al. [18,29] have also tried to test polyethylene glycols for the same purpose.

Fig. 8B and E demonstrate the effect of the conventional borate buffer (pH 9.0) on the resolution of the No.FA reference dissolved in pure water and 5 M urea, respectively. The resolution was considerably better than that produced by ethylene glycol adaptations. Likewise, the reliability seems

to be better because the effect of the urea modifier was reasonable (decreasing migration time) and the general shape of the electropherogram remained uniform.

### 4.4. DHM separation with PAA coated silica open tubular capillary

The resolution and basic properties of the PAA coated capillary were quite similar to those of its PVA homologue resulting in decreased effective capillary wall charges and increased viscosity at the capillary wall [40]. When the electropherograms obtained by the PAA coated capillary (Fig. 9) were compared with those of PVA (e.g. Fig. 9D versus Fig. 1D and Fig. 9E versus Fig. 4G), it could be concluded that the migration times were about 1.1-times longer. Likewise, the effect of the urea modifier was quite uniform (slightly better resolution and decreased migration time), especially in the case of 40 mM phosphate buffer at pH 6.8. The ability of the PAA coated capillary to retain humic solutes was slightly stronger than that of PVA (i.e. longer migration times and slightly broader and lower shapes of electropherograms) but the total areas of electropherograms remained practically the same (only about 1.04-times greater for the PAA). This means that no organic matter was irreversible retained onto either the PAA or the PVA coating. The $A1/A2$ ratio (0.84, split-up at 19.7 min) obtained with the PAA coated capillary (Fig. 9E) was now about 20% smaller than that (1.04, split-up at 17.7 min) with the PVA coated capillary (Fig. 4G) for the same humic sample dissolved in 5 M urea–water. This phenomenon indicates increased relative content of humic constituents with longer migration times speaking for slightly better separative power of PAA as compared to that of PVA. The effect of 50 mM Tris–phosphate buffer at pH 8.5 (Fig. 9A–C) on the separative power was as negligible as that obtained by the PVA coated capillary (electropherograms not shown).

### 4.5. DHM separation with linear non-cross-linked PAGE

Fig. 10 demonstrates that the application of the molecular sieving effect using the pre-coated PAA capillary filled with non-cross-linked PAGE polymer generates quite well-separated electropherograms from the No.FA-humic reference, especially with the 40 mM phosphate buffer at pH 6.8. The separative power was now significantly better than that obtained by the PVA and PAA coated silica open tubular capillaries for the same reference dissolved in pure water (Figs. 1D and 9D, respectively) or in 5 M urea (Figs. 4G and 9E, respectively) using 40 mM phosphate buffer at pH 6.8. The overall migration time of humic solutes was now shorter than with PVA or PAA coated silica open tubular capillaries.

Table 1 lists the most important findings of Fig. 10 with the average reference values obtained by PVA or PAA coated silica open tubular capillaries. It is clearly seen that in the case of pure water as the sample solvent when using 2.5% gel concentration about 84% of humic solutes retained onto the gel matrix. The increasing gel concentration decreased the sorption of humic solutes onto the gel matrix it being only about 54% at the 10% gel concentration. Using 2.5% gel concentration the proportion of the subarea $A2$ of the electropherogram was about 1.7-times greater than that obtained in the reference experiments. The gel concentration increased linearly ($r = 0.996$) the relative content of the $A2$ subfraction, and its proportion was at the 10% gel concentration about 2.1-times greater than those obtained in the reference experiments.

Table 1 verifies also the effect of the urea modifier (5 M urea–water as sample solvent). The sorption of humic solutes onto the gel matrix was clearly smaller in the experiments with the urea modifier being about 70% for the 2.5% gel concentration. The increasing gel concentration significantly decreased the sorption of humic solutes onto the gel matrix, being only about 14% at the 10% gel concentration. The urea modifier also decreased the proportion of the subarea $A2$ of the electropherogram which was about 1.2-times greater at the 2.5% gel concentration than those obtained in the reference experiments. The gel concentration also increased, in accordance with the results when pure water was the sample solvent, the relative content of the $A2$ subfraction being at the 10% gel concentration about 1.7-times greater than those obtained in the reference experiments.

Fig. 10G proves that the running buffer of 50 mM Tris–phosphate at pH 8.5 is not suitable as the BGE for the

Table 1
Effect of gel concentration on separative power of DHM using linear non-cross-linked PAGE

| Gel% | No.FA humic in pure water | | | | No.FA humic in 5 M urea–water | | | |
|---|---|---|---|---|---|---|---|---|
| | $A$ (total area) | $A2$-% (percent of subarea $A2$) | Sorption-% onto gel matrix | Relative increase of subarea $A2$ | $A$ (total area) | $A2$-% (percent of subarea $A2$) | Sorption-% onto gel matrix | Relative increase of subarea $A2$ |
| 0 | 73.4[a] | 35.9[a] | | | 43.5[b] | 48.0[b] | | |
| 2.5[c] | 11.9 | 61.8 | 83.8 | 1.7 | 13.0 | 57.4 | 70.1 | 1.2 |
| 5[c] | 17.6 | 65.1 | 76.0 | 1.8 | 20.3 | 68.6 | 53.3 | 1.4 |
| 10[c] | 33.4 | 75.4 | 54.5 | 2.1 | 37.4 | 82.8 | 14.0 | 1.7 |

[a] Average reference value obtained from Figs. 1D and 9D (pure water as sample solvent).
[b] Average reference value obtained from Figs. 4G and 9E (5 M urea–water as sample solvent).
[c] Values obtained from Fig. 10.

application of physical gels either. The obtained electropherogram was as uninformative as in the case of the PAA coated open tubular capillary in Fig. 9C. The sorption of humic solutes onto the gel matrix was about 54% as compared to the experiment with the PAA coated silica open tubal capillary in Fig. 9C. The tailing of the electropherogram was also considerably stronger than that obtained by 40 mM phosphate buffer at pH 6.8.

In summary, the sieving effect of the applied PAGE as a physical gel operated moderately well in the present study. The increasing gel concentration improved the sieving effect and the resolution of humic solutes but influenced fairly little on the overall migration time of the electropherogram. The chaotropic effect (breaking up intermolecular hydrogen-bonds of a humic macromolecule) of urea was minor, i.e. the urea modifier did not result in either an aggregating or a completely disaggregating effect of the humic solutes leading toward an extraordinarily larger or smaller molecular size. This result is on line with the previous findings that the major function of the urea modifier is the prevention of sorption of humic solutes onto the gel matrix [35].

In general, the electrophoretic mobility is directly correlated with the charge/mass ratio and, almost without exception, the smallest size organic solutes have lower migration time. This fact is also prevailing in connection with different molecular sieving adaptations of CGE, as proved, e.g. by different DNA and protein separations [41,42]. This phenomenon implies that other factors (e.g. charge/mass ratio) than true sieving based solely on molecular sizes are prevailing in CGE.

The mechanism of the separations obtained for natural humic solutes in CZE using physical gels is still unknown. De Nobili et al. [29] have stated that in the absence of interactions, electropherograms of humic solutes have a bell-shaped featureless appearance, and different molecular size fractions of HMs have nearly the same electrophoretic mobility. This suggests that relatively small differences in charge/mass ratios occur within fairly wide molecular size fractions of HMs. It is well-known that the heterogenous HM consists of components with wide molecular ranges, and these components possess phenolic and carboxylic groups which can act as either hydrogen donors or acceptors thus favouring interactions with hydrophilic polymers.

It has been demonstrated [43] that in PAA coated capillaries the migration times of HM fractions were, in the first place, a function of their molecular sizes both in open tubular and gel-filled (polyethylenglycol or methyl cellulose) capillaries. Dunkel et al. [17] have also reported a similar finding that PAA gel provides a sieving medium for CGE which should cause a separation of humic solutes according to the molecular mass, with the low-molecular-mass fractions first. These outcomes speak for the fact that a certain analogue exists between protein chemistry and natural humic solutes under given CGE conditions, thus permitting a coarse evaluation of molecular mass distribution of HM mixtures.

### 4.6. *Effect of SDS in linear non-cross-linked PAGE*

Fig. 11A and B prove that the addition of SDS improves the separative power compared with the experiments with PVA or PAA coated open tubular capillaries (Figs. 1A–E, 4G and 9D–E) and even with their gel-filled analogues without this micelle forming agent (Fig. 10). However, the critical examination indicates that humic solutes are now in a noticeable degree retained onto the gel matrix. In the case of No.FA dissolved in pure water (Fig. 11A) about 80% of humic solutes were retained onto the gel matrix as compared to the experiments with PVA and PAA coated open tubular capillaries in Figs. 1D and 9D, i.e. about 1.6-times more than its corresponding analogue without SDS in Fig. 10E. The urea modifier decreased somewhat the sorption of humic solutes onto the gel matrix, which was about 67% as compared to the open tubular capillary experiments given in Figs. 4G and 9E but about 4.8-times greater than the interaction obtained for its corresponding analogue without SDS in Fig. 10F. The urea modifier had no effect on the overall migration time of humic solutes in SDS experiments although it was somewhat longer than in analogous experiments without SDS.

The separative power was also better for the Pirita-humic sample (Fig. 11C) as compared to that obtained with the PVA coated open tubular capillary in Fig. 1F but the sorption of humic solutes onto the gel matrix was exceptionally high, about 91%. Furthermore, the tailing of the electropherogram was strong and its base-line was also disturbed.

In summary, the SDS addition in the applied PAGE physical gel improves the sieving effect and the separative power but significantly increases the interaction of humic solutes with the gel matrix. The urea modifier prevented the sorption of humic solutes onto the gel matrix but its protective effect was much weaker than was the case without SDS addition. However, the chaotropic effect of urea was obvious, i.e. the relative proportion of humic solutes with migration times longer than 20 min increased significantly (2.3-fold) in comparison with the water as the solvent.

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