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Comparative study for the separation of aquatic humic substances by electrophoresis[1]

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Abstract

Various humic and fulvic acids, isolated from natural waters, were investigated by the following electrophoretic techniques: free solution capillary electrophoresis (FSCE), capillary gel electrophoresis (CGE), isoelectric focusing (IEF) in ultra-thin layers, micellar electrokinetic chromatography (MEKC) and SDS gradient-gel electrophoresis. A broad band is dominant in FSCE. The addition of 5 *M* urea to the running buffer facilitates the separation of the complex humic molecular aggregates, resulting in an increase in the number of peaks in the electropherogram. Under comparable denaturing conditions, and with a gel optimised for sieving single stranded deoxyoligonucleotides, CGE gives a separation with lower resolution than for DNA. Best separation is achieved by IEF in ultra-thin layers of polyacrylamide, giving about 20 sharp bands. SDS gradient-gel electrophoresis gave three similar bands for different fulvic acid samples, corresponding to molecular masses of standards of 1000, 6000 and 8000 g/mol. © 1997 Elsevier Science B.V.

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1. Introduction

Humic substances (HS) are widespread in the biosphere. They make up the major part of organic matter. In surface waters approximately half of the total amount of organic substances and nearly all organic substances with chromophores belong to the humic substances. These compounds with unsettled structure strongly influence the chemical–physical properties of soils, such as crumb structure and sorption capacity. Furthermore, humic substances are able to bind nutrients for plants and also remobilize sedimented heavy metals. In spite of its importance for ecology, agriculture and raw water processing and a huge research interest by scientists, the composition and structure of its components is not clear. This is caused by the fact that humic substances were formed by association of high-molecular-mass substances from microbiological, vegetative and animal origin. Molecular masses have been found between some hundreds and thousands of mass units [1]. For its genesis both biotic and abiotic processes play a significant role, and precursor species are mainly polysaccharides, lignins, peptides and lipids. The

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composition is often very heterogeneous and depends on geographical, climatic, physical and biological circumstances, respectively.

Due to their high complexation capability, HS can strongly influence the transport, deposition and bioavailability of trace metals and organic xenobiotica in aquatic systems [2]. The complexation behaviour towards dissolved metal ions, e.g. their complexing capacity, the resulting equilibria or the formation and dissociation reactions of HS–metal species can be characterised by a variety of methods [3]. A number of analytical techniques have been applied for structural research of humic substances: gel chromatography [4], pyrolysis gas chromatography–mass spectroscopy [5], ultrafiltration [6], UV-, IR-, Raman- and NMR spectroscopy [2], but none of this techniques has led to ultimate structure elucidation.

As the humic substances contain polyelectrolytes, electrophoretic techniques should be applicable for their separation. Electrophoresis, especially PAGE, provides a separation power up to several millions theoretical plates [7]. The aim of this paper is to compare the various modes of both slab-gel and capillary electrophoresis (CE) for the separation and characterisation of aquatic humic substances.

2. Experimental

2.1. Instrumentation

All CE data were collected with a Dionex capillary electrophoretic system I equipped with UV- and fluorescence detection. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50–100 μm I.D. and 375 μm O.D. were used for CE analysis. A l.c. polyacrylamide gel-filled capillary MicroSolv (Scientific Resources, Eatontown, NJ, USA) 75 μm I.D. and 375 μm O.D. was used for CGE. IEF-gels (Serva, Heidelberg, Germany) and SDS-gels (Pharmacia Biotech, Freiburg, Germany) were used on a horizontal slab-gel unit FBE-3000 and FBE Imuno (Pharmacia, Uppsala, Sweden). The unit was cooled by a thermostat (Haake, Karlsruhe, Germany) and powered by a voltage supply Statron 4205 (Dresden, Germany).

2.2. Materials

Double distilled water was used in the preparation of CE buffers. Acrylamide, N,N′-methylene-bis-acrylamide, TEMED and ammonium persulfate were obtained from Sigma (St. Louis, MO, USA). SDS, TRIS, CHES[2], MES, CAPS were purchased from Merck (Darmstadt, Germany), sodium tetraborate, L-alanine, sodium acetate and boric acid from Aldrich (Milwaukee, WI, USA). Prestained high-molecular-mass standards (M_r 4000–250 000) for SDS gel electrophoresis were obtained from Serva. Ampholytes ranges pH 2–4 and 3–9 and p*I* standards were also obtained from Serva. All chemicals were of analytical-reagent grade or electrophoresis grade and used without further purification.

2.3. Isolation of humic substances from waters

The humic substances were isolated using XAD-7 (Amberlite) sorption [8]. Samples were membrane filtered (cellulose acetate, 0.45 μm), acidified to pH 2 and enriched on a XAD-column (styrene–divinylbenzene copolymer). The humic substances were eluted with 0.2 *M* sodium hydroxide, acidified and split into humic and fulvic acid fractions; the fulvic acid part is washed on a cation-exchange resin (Lewatit S 1080) to minimise salt content.

Four samples[3] of different origin (Hohloh Lake HO10 FA and HO10 HA, Schwelvollert Lake SV1 FA, Karlsruhe municipal waste water ABV2 FA, and soil seeping water BS1 FA) were investigated.

2.4. Capillary electrophoresis

The fused-silica capillaries used had a total length of 55 cm and an effective length of 50 cm. Bare fused-silica capillaries were initially rinsed with 1 *M* NaOH and water and conditioned between runs with 0.1 *M* NaOH and water. Using different buffers (see Table 1), CE data were collected at 220 nm, +20 kV,

[2]Abbreviations, see Table 1.

[3]Provided by Engler-Bunte-Institute Karlsruhe, Germany.

Table 1
BGE systems and pH values used

Background electrolyte	Concentration (m*M*)	pH
L-Alanine	8	3.17
Acetate	5	4.75
Borate	10	9.24
MES (Morpholinoethanesulfonic acid)	20	6.15
TRIS (Tris-(hydroxymethyl)-aminomethane)	20	8.30
CAPS (3-(cyclohexylamino)-1-propanesulfonic acid)	20	10.40
CHES (2-(N-cyclohexylamino)-ethanesulfonic acid)	20	9.50

room temperature and gravity injection 30 mm for 10 s.

2.5. Capillary gel electrophoresis

The commercial polyacrylamide gel-filled capillary (5% T, 5% C) had a total length of 50 cm (45 cm effective length). It was used with the buffer (100 m*M* Tris–100 m*M* boric acid, 7 *M* urea, 2 m*M* EDTA, pH 8.3) supplied by the company. The capillary was pre-treated with this buffer and an increased voltage of 5–15 kV applied, until the detector signal was stable. CE data were collected at 220 nm, −15 kV, room temperature and electromigration injection (6000 V for 10 s). A home-made polyacrylamide filled capillary was prepared as described elsewhere [9].

2.6. Isoelectric focusing

IEF on ultra-thin (300 μm) polyacrylamide layers was performed on a horizontal slab-gel unit. After prefocusing (1 h at 200 V) 10 μl of the samples were loaded 1 cm above the cathode. The gel was focused for 2–3 h (50–130 V/cm) at 10°C. The gel was fixed with 5% glutardialdehyde–water and stained either with silver [10] or Stains-all [11].

2.7. SDS-gradient-gel-electrophoresis

Ready-to-use gels with a gradient of 8–18% l.c. polyacrylamide were used. Samples and standards (10 μl) were loaded 1 cm above the cathode. Voltage was applied for 1 h at 50 V/cm followed by 1 h at 100 V/cm. No colouring step was used after the electrophoresis. The gel was washed with 5% glutardialdehyde and dried.

3. Results and discussion

3.1. Free solution capillary electrophoresis in different buffer systems

By varying the pH of the buffers, information about the acid dissociation constants of the different fractions should be available. As is known from the literature, variation of this parameter is not only connected to changes of polarity but also causes structural changes of molecule clusters. We investigated isolated HS in certain buffers as listed in Table 1.

A typical electropherogram in a borate buffer is shown in Fig. 1. Narrow peaks are seen for only a small number of components. Most of the material migrates as a broad, unresolved band. Analogous broad bands are seen in reversed-phase chromatography [5]. Changing the buffer type (ionic to zwitterionic) and pH did not provide any improvement in the separations.

The reason for the broad band is, in our opinion, that these substances cluster into aggregates, and because of the polydisperse nature of the aggregates the electrophoretic mobility in free solution is not a sufficient separation criterion. Three possibilities were investigated to improve the separation: (i) the interaction with micelles, (ii) application of a high concentration of urea to destroy aggregation, (iii) the use of sieving media like polyacrylamide.

The addition of 5 *M* urea to borate and Tris buffers effected a slight improvement in the sepa-

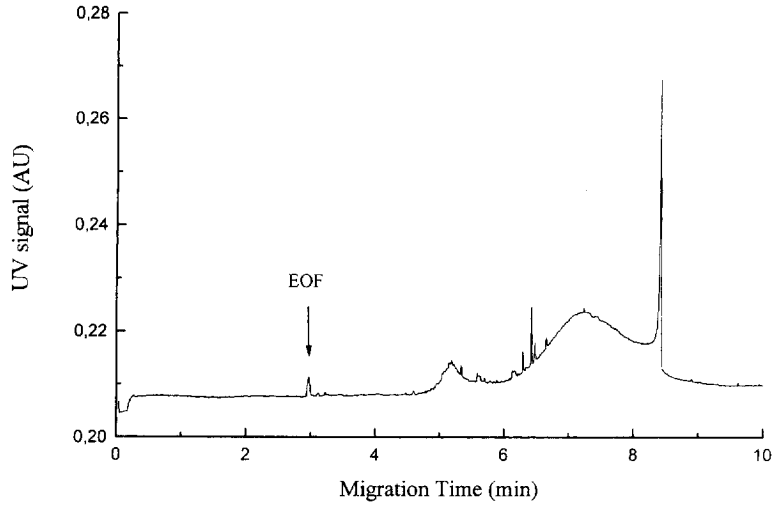

Fig. 1. Typical electropherogram of humic acid HO10 HA in borate buffer (pH 9.3).

ration (see Fig. 2). More small and sharp peaks are visible above the broad band, but the broad band was still not resolved.

3.2. Capillary gel electrophoresis

Polyacrylamide-gel electrophoresis allows excellent separations of oligodeoxynucleotides, with efficiencies of 10^7 plates/m reported [7]. For that reason we applied the PAGE technique for the separation of humic substances, using gel and denaturing buffer conditions appropriate for separation single-stranded DNA oligomers of degree of polymerisation in the range ~10–200. Polyacrylamide gels have major advantages over agarose gels: they can generally accommodate much larger quantities of substance than agarose gels without significant loss of resolution. Polyacrylamide gel provides a sieving medium which should cause a separation of HS according to the molecular mass, with the low-molecular-mass fraction detected first. The separa-

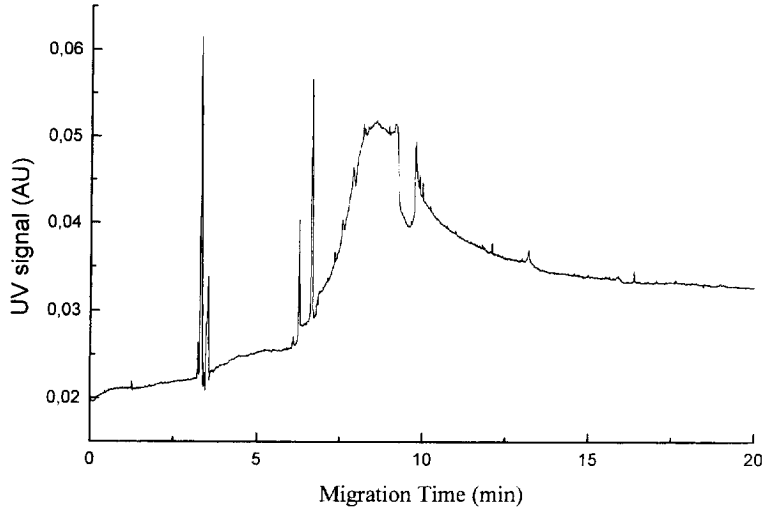

Fig. 2. Electropherogram of fulvic acid HO10 FA in 10 m*M* borate buffer and 5 *M* urea (pH 7.4).

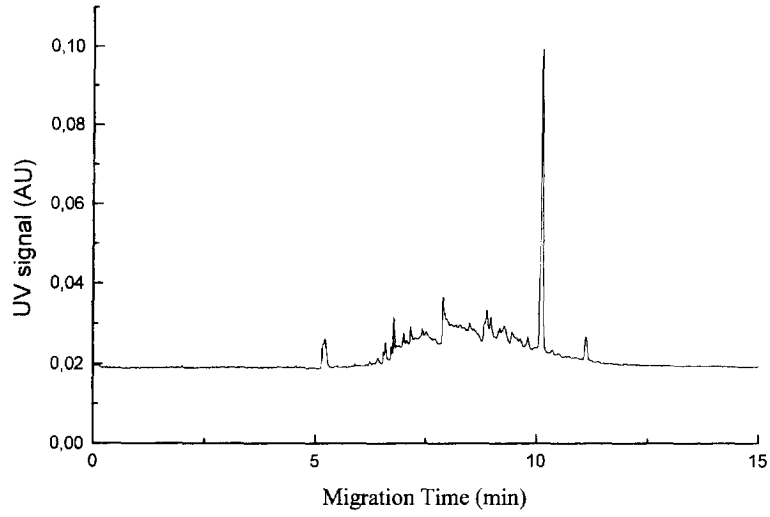

Fig. 3. CGE of the fulvic acid SV1 FA.

tion achieved is shown in Fig. 3. The broad band is much better resolved than by use of FSCE, but the quality of the separation is well below what is achievable with DNA oligomers. We conclude, that the produced polymer network used (5% T, 5% C), with pore size of approximately 20 nm, is not the optimum retardation medium for the polydisperse HS.

3.3. Isoelectric focusing

Focusing is a highly sensitive method which also has a very high resolving power, being able to separate proteins with closely spaced isoelectric points. We applied this technique to the separation of our aquatic humic substances. Following suggestions of Kutsch et al. [12] we varied the experimental conditions involving the prefocusing, focusing and washing steps. For some humic substances, the brown colour of the bands they give is sufficient and no colouring procedure is necessary (see Fig. 4), but we also applied the silver and Stains-all colouring step which is more sensitive. The results are essentially the same, no further bands appeared.

After the focusing procedure we put the gels on a scanner and made a digital copy of the image. From the image we made a densitometric plot. There are many bands on it, most of them corresponding to migration towards the anode and apparent p*I* values of between 3 and 6 (Fig. 5).

Surprisingly, by use of the same gels and ampholytes the same separation pattern was obtained

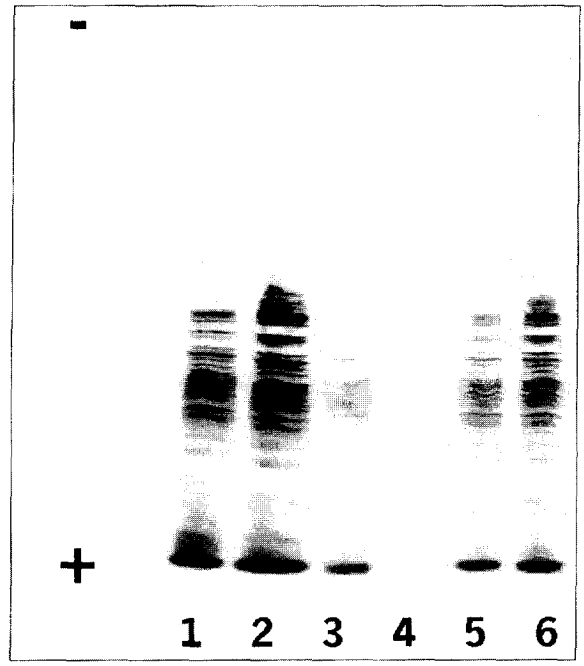

Fig. 4. IEF of humic substances: Lane 1, HO10 FA; Lane 2, SV1 FA; Lane 3, ABV2 FA; Lane 4, blank; Lane 5, BS1 FA; Lane 6, HO10 HA.

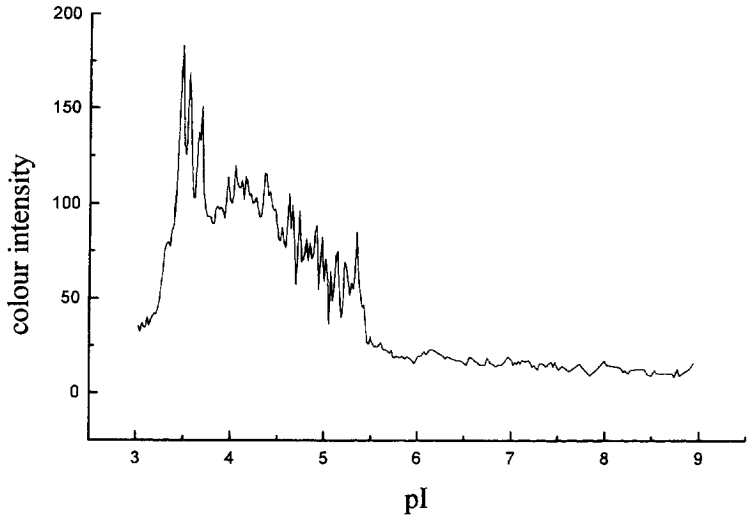

Fig. 5. Densitometric plot of IEF of fulvic acid SV1 FA.

from different samples. Up to now we have no explanation for this, but we believe, that there could be an interaction between ampholytes and humic substances which would give an apparent rather than a true focusing. To check this we used a gel by another manufacturer, but the same result was observed. So the possibility that different aquatic humic substances have similar isoelectric points has to be favoured.

3.4. Micellar electrokinetic chromatography

Micellar electrokinetic chromatography is a powerful technique for the analysis of hydrophobic species, which allows neutral molecules to be separated via their differential distribution between an aqueous mobile phase and a pseudostationary micellar phase. MEKC involves the addition of a surfactant (e.g. SDS) to the aqueous phase at concentrations above its critical micelle concentration. Retention is generally based on hydrophobicity. Fig. 6 demonstrates the influence of variation of concentrations of SDS on the separation of HS. With increasing SDS concentration, the broad peak shows some improvement in resolution, but significant fronting or tailing limits the utility of MEKC as a separation method.

3.5. SDS gradient-gel electrophoresis

SDS gradient-gel electrophoresis is widely used for determining the molecular mass of unknown macromolecular substances. All fulvic acids from different origins show the same three bands at positions of molecular mass standards of approximately 8000, 6000 and 1000 g/mol (Fig. 7)

4. Conclusions

Using different electrophoretic techniques for the separation and characterisation of humic acids from waters we obtained the following results:

- free solution electrophoresis is not suitable for the separation of humic substances
- addition of urea provides a slight improvement in the separation
- micellar electrokinetic chromatography is of limited applicability
- polyacrylamide capillary gel electrophoresis using conditions appropriate for single-stranded DNA oligomers brings about a limited separation
- isoelectric focusing is the only electrophoretic technique which performs a separation of humic acids

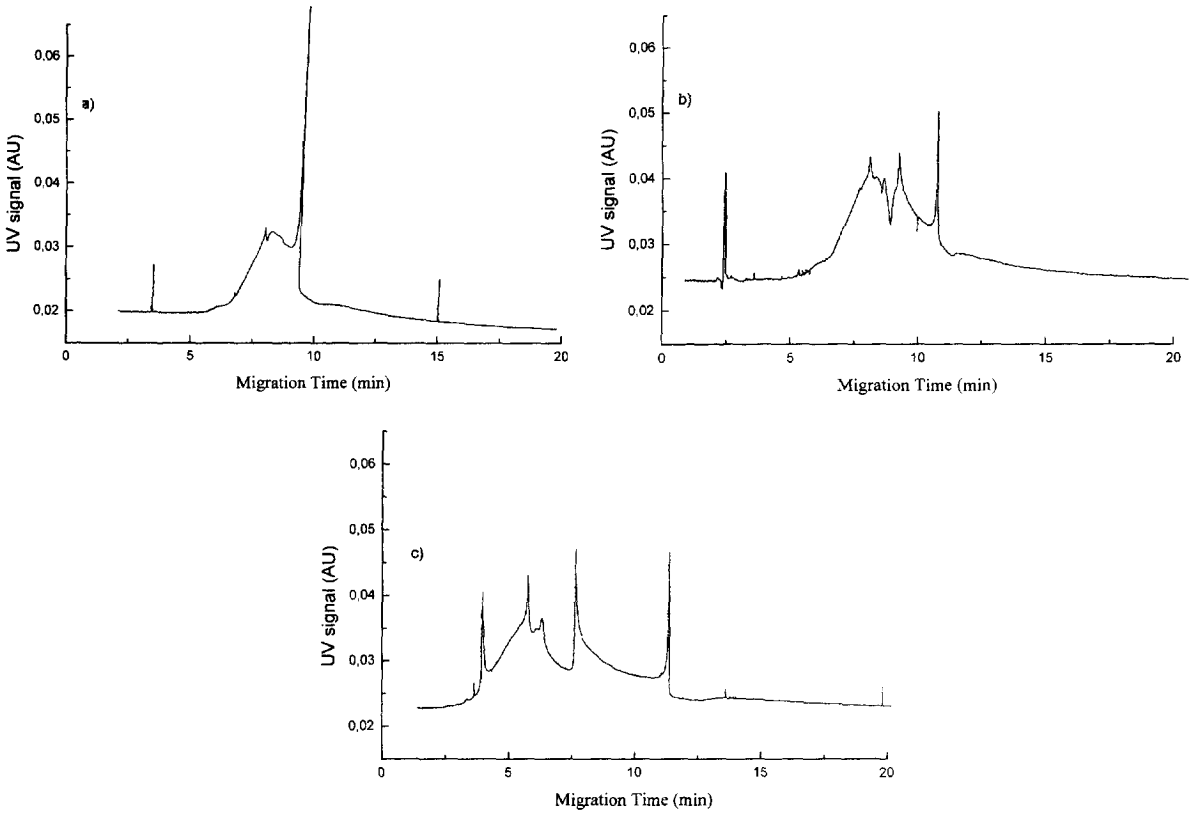

Fig. 6. MEKC of humic acid HO10 HA, buffer 10 m*M* borate (pH 9.2), at different concentration of SDS: (a) 5 m*M*, (b) 10 m*M*, (c) 25 m*M*.

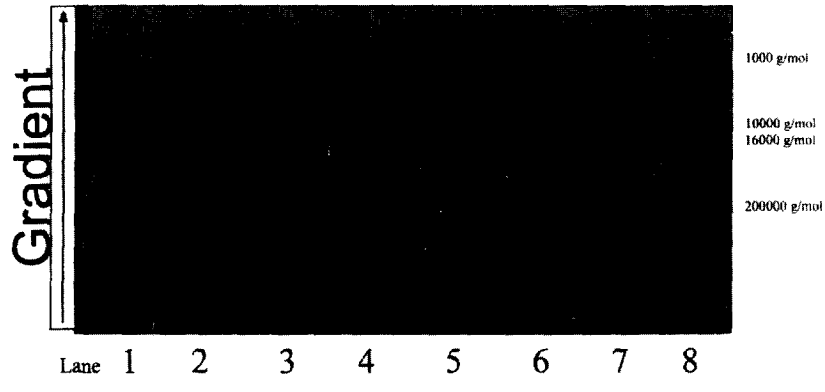

Fig. 7. SDS gradient gel electrophoresis; starting at the left lane: Standard 1, Standard 1, Standard 2, Sample 1, Sample 2, Standard 1, Standard 2, Sample 3; Samples 1–3 were different fulvic acids: HO10 FA, SV1 FA, BS1 FA.

- SDS-PAGE of different samples shows that they have similar molecular mass fractions

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