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Capillary electrophoresis in the analysis of humic substances Facts and artifacts

Ph. Schmitt-Kopplin[a,*], A.W. Garrison[b], E.M. Perdue[c], D. Freitag[a], A. Kettrup[a]

[a]*GSF – National Research Center for Environment and Health, Institute for Ecological Chemistry, Ingolstädter Landstrasse 1, D-85758 Neuherberg, Germany*

[b]*National Exposure Research Laboratory, US Environmental Protection Agency, 960 College Station Road, Athens, GA 30605, USA*

[c]*School of Earth and Atmospheric Sciences, Georgia Institute of Technology, Atlanta, GA 30332, USA*

Abstract

Humic substances, extracted as mixtures from soil and surface waters according to their solubility in acids and bases, are relatively high-molecular-mass polyelectrolytes containing aromatic, aliphatic and heterocyclic subunits. The degree of ionization of their phenolic and carboxylic groups is governed by the capillary electrophoresis (CE) buffer pH. In CE, fulvic acids exhibit a consistent and characteristic set of sharp peaks (phenolic acids), extending from a humic ''hump'' whose average electrophoretic mobility (AEM) depends on humic structure and buffer composition; humic acids give only the ''hump''. Special attention must be given to the interpretation of CE electropherograms when fingerprinting humic substances with borate buffers because observed peaks do not necessarily indicate distinct humic fractions, but may be artifacts caused by the interaction of borate ions with 1,2- and 1,3-diols present in the humic mixtures. Depending on the molarity of borate ions in the separation buffer, humic acids exhibit electropherograms with sharp peaks extending from the ''humic hump'' and corresponding to borate complexes. The potential of capillary zone electrophoresis for the comparison of electropherogram patterns is illustrated for the Suwannee River reference humic substances extracted according to the recommendations of the IHSS compared with a fraction of the same source concentrated with the more recent reverse osmosis (RO) technique. The Suwannee River RO fraction behaved like the extracted Suwannee River humic acid fraction under these experimental conditions.

Keywords: Water analysis; Soil; Humic substances; Humic acids; Fulvic acids

1. Introduction

Dissolved humic substances (HSs) are the main constituents of the dissolved organic carbon (DOC) pool in surface, ground and soil pore water and commonly impart a yellowish–brown color to the water system. They are known to be among the most important natural sunlight-absorbing components of soil surfaces and aquatic environments and constitute about half of the organic and nearly all of the colored matter in all of these natural environments. Their structural chemistry, however, is much less known than the chemistry of any biopolymer of animal origin [1]. HSs are known to be high-molecular-mass polyhydroxycarboxylates comprised of polyaromatic and aliphatic subunits. The degree of ionization of these macromolecules is governed by the amount of ionized phenolic and carboxylic groups of the humic core, which is a function of solution pH. Compared to the fulvic acids (FAs – soluble in both alkali and acid solutions), the humic acids (HAs – soluble in

*Corresponding author.

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alkali, insoluble in acid solutions) are of higher molecular mass and lower acidity.

Because the pK_a values of the carboxylic acid functional groups of HSs are in the range of 3.5 to 5 [2], the HS molecules are anionic and migrate in a capillary electrophoresis (CE) system if the separation buffer is above a pH of about 3.5. Regardless of the apparent match between HS analytes and CE technology, only few researchers have tried to apply any techniques of CE to the study of HSs [3–9], coal extracts [10] or lignin related compounds [11,12]. CE techniques for the study of the interaction of HSs with pollutants are only in their infancy [13–17]. In addition, our first work in this area [6] indicated that borate buffers interact with HAs, drastically changing the electropherograms while only slightly altering the pH. We then showed with complemental ^{11}B NMR spectrometry experiments that these changes were caused by the complexation of borate ions with *cis*-diol groups within the HA mixtures [18].

This article is intended to illustrate the present knowledge and limitations of CE in the characterization of HSs and to give an example of the use of CE in the rapid analysis of different humic mixtures by comparison of electropherogram pattern.

2. Experimental

2.1. Instrumentation

Instrumentation consisted of a BioFocus™ 3000 CE system from Bio-Rad Labs. (UV-scanner detection) and a Beckman P/ACE 2100 Series HPCE (UV-filter used at 254 nm for HS analysis) with the Beckman System Gold Chromatography Software.

2.2. Capillary zone electrophoresis (CZE)

Uncoated fused-silica CZE columns [57 cm (50 cm length to the detector)×75 μm I.D.×375 μm O.D.] were obtained from Bio-Rad Labs. and Beckman Instruments. Typical CZE conditions for separation of the various HS fractions were: separation buffer, 50 m*M* acetate (pH 4.95), 40 m*M* borate (pH 9.3) or 50 m*M* carbonate (pH 9.3); temperature, 30°C; voltage, 20 kV; detector wavelength, 254 nm or scanning from 200 nm to 360 nm (2 nm step); hydrodynamic injection, 5 or 10 s. CZE gave good reproducibility of migration times – a standard deviation of 0.06 min (0.89%; $n=5$) with Scheyern HAs. The sample concentration in HSs had no significant influence on the average electrophoretic mobility (AEM). Day-to-day changes in migration times occurring because of relative changes in the electroosmotic flow (EOF) (different capillary surface conditions) could be controlled by washing the capillary with 0.1 *M* NaOH for 2 min between each run.

2.3. Humic substances

Soil humic and fulvic acids were extracted and isolated according to procedures of the International Humic Substances Society (IHSS); for structural information on Scheyern–, Bouzule– and Belle–Fontaine HSs refer to Ref. [15,19,20]. Standard soil and water HSs were obtained from the IHSS, Dr. P.R. Bloom, Department of Soil, Water and Climate, University of Minnesota, St. Paul, MN, USA. The Suwannee River reverse osmosis (RO) fractions were concentrated according to Serkiz and Perdue [21].

For CE analysis, the humic fractions were dissolved in 0.1 *M* sodium hydroxide to a concentration of about 1 mg/ml. The lowest measurable concentration (without stacking conditions) was 50 μg/ml.

3. Results and discussion

Acetate and carbonate have been previously proposed by the authors to be good buffers for fingerprinting HSs [6]. With these buffer systems HSs show a homogeneous ''humic hump'' in the anionic region with a distribution around an AEM. The electrophoretic mobilities μ_{mes} ($cm^2/V\,s$) are calculated taking account of the electrophoretic velocity v_e (cm/s) and the applied electric field strength E (V/cm): $\mu_{mes}=v_e/E=(L_d/t_m)/(V/L_t)$ (with L_d, length of the capillary to the detector in cm; L_t, total length of the capillary in cm; V, the applied voltage; t_m, migration time in min). The effective mobility is calculated relative to the EOF: $\mu_{eff}=\mu_{EOF}-\mu_{mes}$. The AEMs (average μ_{eff}) were always lower with

the HAs than with the FAs, indicating lower charge densities which are determined by the degree of ionization at the separation buffer pH and the average molecular size.

The plot of absorbance versus μ_{eff} (Fig. 1) shows the Gaussian-like distribution of the mobilities around an average value, the AEM; the polydispersity of the humic sample can be evaluated by σ from the electropherograms for each buffer pH. This representation of the primary electrophoretic data in the μ_{eff} domain is a useful visualization of the effective mobility because it takes into account the changes in EOF that can occur from one measurement to the other.

FAs always showed higher polydispersities (wider peaks) than HAs. Several separated sharp peaks, corresponding to lower-molecular-mass compounds (with low charge-to-mass ratios) were found in the FA fractions but never in the HA mixtures. Fig. 2 illustrates the three-dimensional electrophoretic profiles of the Scheyern FA (acid brown soil – cultivated soil) and the Belle–Fontaine FA (rendzina – forest soil) measured with the Biofocus 3000 UV scanner in the wavelength range of 200 nm to 360 nm. The sharp peaks rising out of the humps were identified as syringic (a), vanillic (b) and *p*-hydroxybenzoic (c) acids by spiking the fulvic samples and comparing the UV spectra. These phenolic acids could have been released in solution by partial hydrolysis of the FA core or/and coextracted from the natural soil matrix; they ultimately result from the oxidation of lignic structures (soft or hard wood origins) and are found in different amounts characteristic of the vegetation of the studied soils. Furthermore, Fig. 3 shows the total FAs of the Scheyern soil (upper electropherogram) and a lower-molecular-mass fraction, (non ampholyte-complexing fraction, focused at pH 2.0 during preparative solution isoelectric focusing (IEF) of Scheyern FAs using Servalyte 3–10 ampholytes [22]). This low-molecular-mass fraction, which represents up to 30% of the dissolved organic carbon of the FA mixture could be separated here with CZE into single molecules instead of the humic hump; studies are in progress for the structural identification of these ionised hydroxycarboxylates. The coupling of electrospray

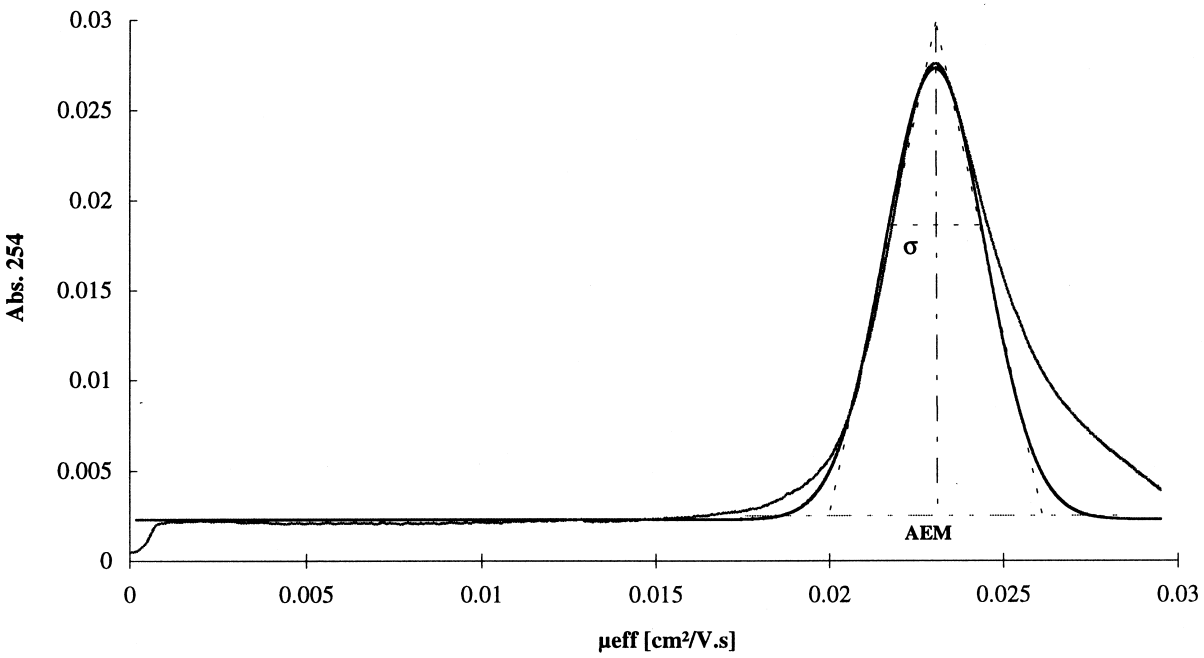

Fig. 1. Plot of the absorbance versus the μ_{eff} of the IHSS reference soil HAs (50 m*M* acetate buffer, pH 5.0, 20 kV, 30°C column 57 cm×75 μm I.D.).

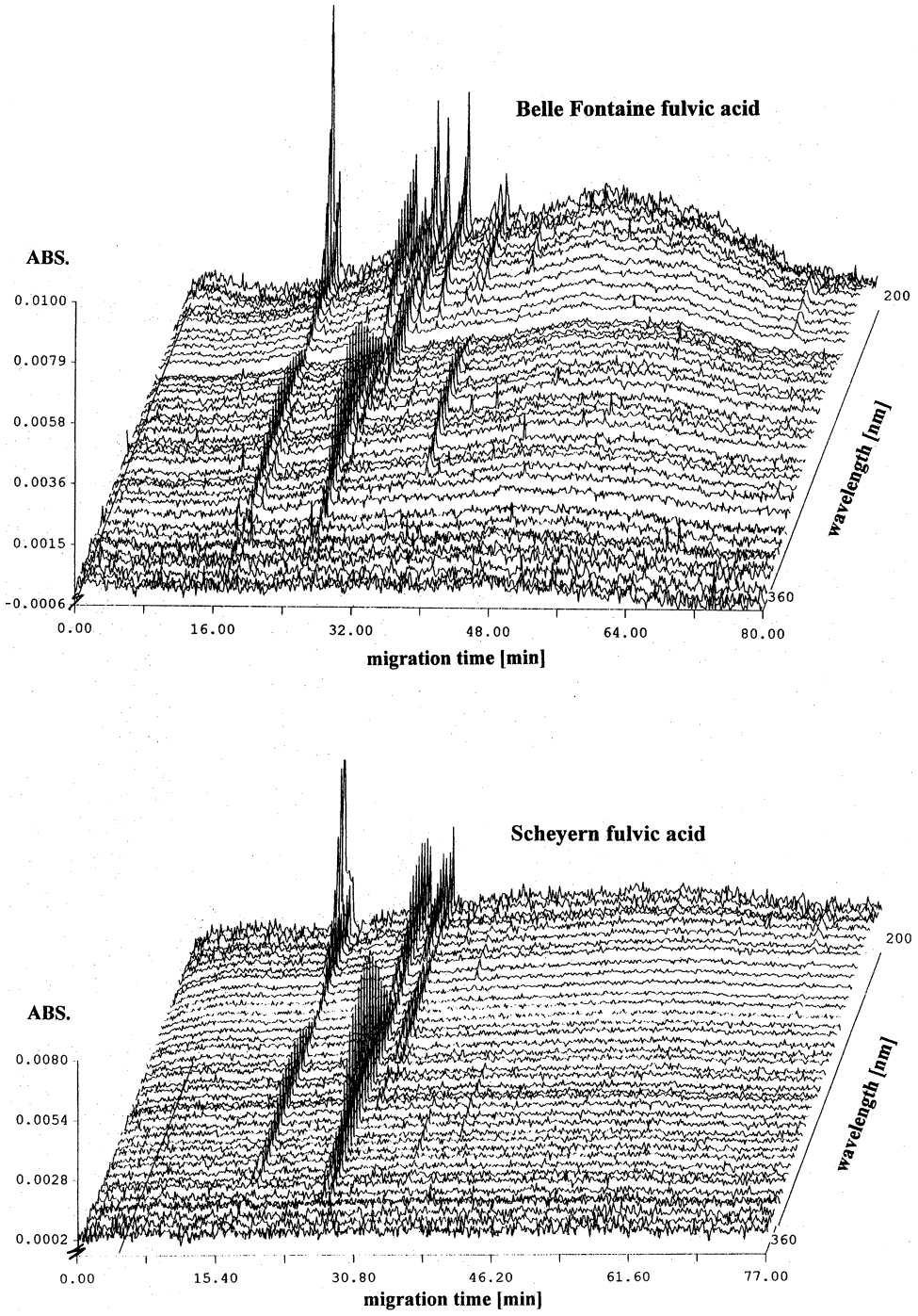

Fig. 2. Three-dimensional electropherogram of the Scheyern and the Belle–Fontaine FAs and the corresponding phenolic acid profiles (50 m*M* acetate buffer, pH 5.0, 25 kV, 30°C, 254 nm, column 100 cm×50 μm I.D.).

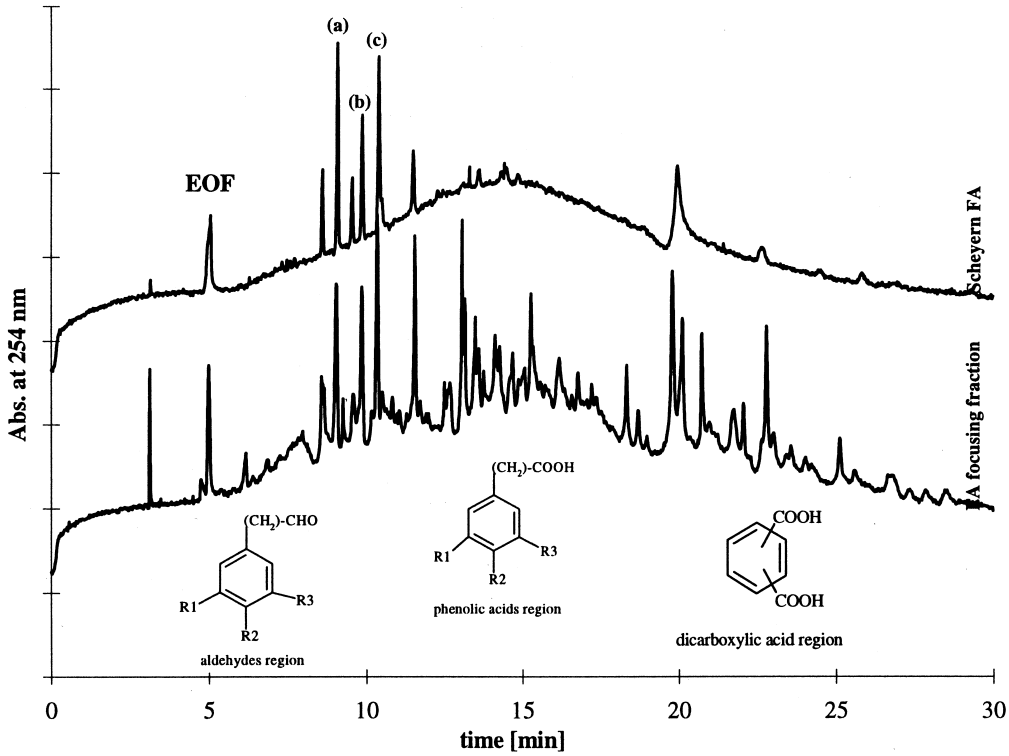

Fig. 3. Scheyern total FAs and a corresponding fraction obtained by isoelectric focusing [22] showing the presence in the fraction of low-molecular-mass single components. The chemical structures indicate time-regions where molecules of this type can migrate (R1, R2, R3=H, OH or OCH_3) (50 m*M* acetate buffer, pH 4.95, 20 kV, 30°C, column 57 cm×75 μm I.D.).

ionization–mass spectrometry (ESI–MS) to the CE system will be very useful in these structural investigations.

3.1. Borate buffer concentration effects

Special attention has to be given to the interpretation of the HS electropherograms, depending on the buffer system used: borate buffer, for example, may form complexes with 1,2- and 1,3-diols of HSs giving additional peaks of HS–borate complexes as a function of the pH and the concentration of tetraborate ions [6]. The influence of the borate concentration in the separation buffer on the electrophoretic patterns of humic mixtures has been studied in detail elsewhere [18]. An example of pattern changes with the Suwannee River reference HAs upon increasing the borate concentration in the buffer is illustrated in Fig. 4. The peak (*) appears with a high absorbance above the humic hump even at low borate molarity (0.5 m*M*). This peak was believed to be caused by formation of bidendate monoesters between borate and the most reactive 1,2-diols as well as by tetradendate diesters formed by intermolecular reaction [8]. Peak (○) beginning at 5 m*M* borate was interpreted to be caused by complexation with the less reactive diols that complex only at higher borate concentration. Studies are in progress on the structural identification of these complexes using semipreparative CZE fractionation followed by NMR and MS. This borate complexation property was used previously in a comparison of FA samples, where it was shown in an IEF and capillary IEF study of humic substances [22] that

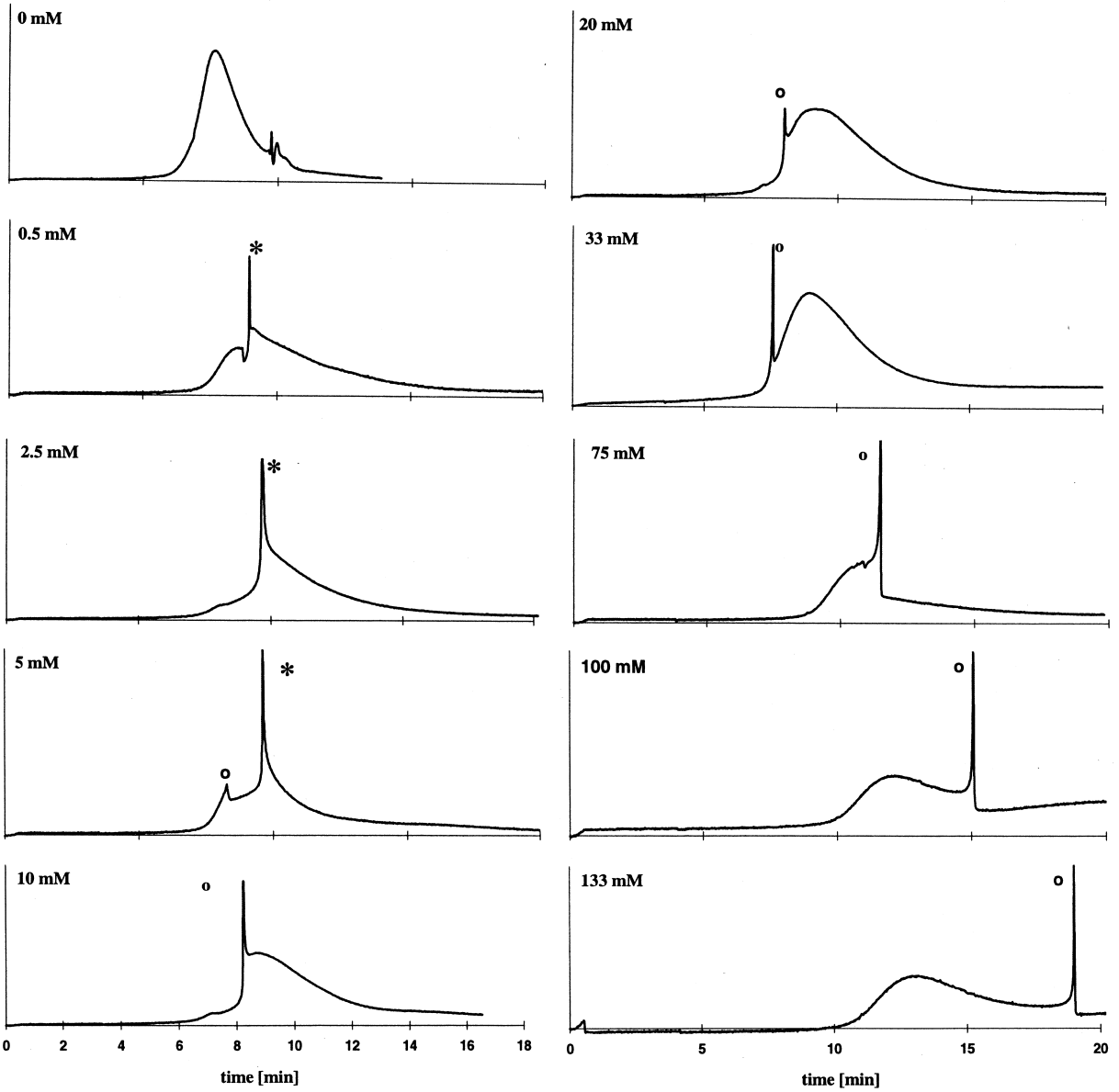

Fig. 4. Electropherograms of Suwannee River reference HAs as a function of borate molarity in the separation buffer (10 m*M* carbonate buffer, pH 9.3, 20 kV, column 57 cm×75 μm I.D.).

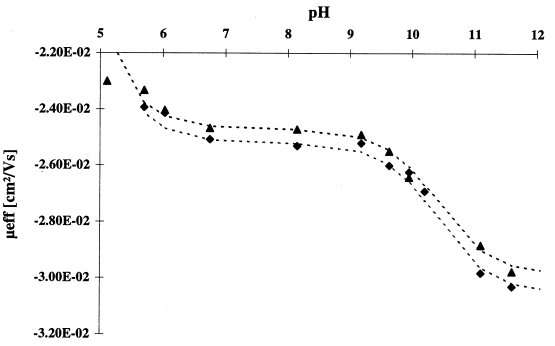

Fig. 5. Changes of AEM of the Suwannee River reference FAs and HAs with increasing pH of the separation buffer (20 kV, column 57 cm×75 μm I.D.).

complexation increased with increasing acidity of the FAs.

3.2. Buffer pH effects

The changes of the AEM of Suwannee River reference HSs are illustrated in Fig. 5 when varying the pH of the separation buffer from pH 4.7 to pH 12.5. The fitted curves were calculated assuming the humic substances behave as ''diprotic acids'' with an average carboxylic acidity (pK_{a1} 4.2 and 4.4 for HAs and FAs, respectively) and an average phenolic acidity (pK_{a2} 10.35 and 10.5 for HAs and FAs, respectively). This is a very crude approximation because the AEM is a function of both acidity (ionization degree at a given pH) and molecular size characteristics of the humic mixtures (controlling the charge densities) at the different pH.

Even though this behavior of the humics is still under investigation, three buffer systems can be used to describe rapidly the electrophoretic behavior of HSs that have to be compared: an acetate buffer pH 5.0 and two carbonate buffers pH 9.0 and 11.4. The electropherograms which were obtained with these buffers are illustrated in Fig. 6. Additionally a borate buffer can be used at pH 9.0 to compare the presence in the humic mixture of borate complexing sites. A graphical representation is given in Fig. 7 by expressing the AEM of the four humic fractions at the three buffer pH. One notices the lower mobility of the soil HAs as compared to the Suwannee River HAs at alkaline pH. This can be interpreted in terms of higher molecular size of the soil HAs and/or lower phenolic acidity. The RO fraction and the Suwannee HAs have similar electrophoretic patterns for these three pH values and their AEMs are not significantly different. The same conclusion can be found when comparing the four borate buffer fingerprints: FAs have the highest borate complexing capacity, followed by the Suwannee HAs, which is very similar to the pattern of Suwannee RO. The soil HAs are only slightly affected by the borate ions, showing the lowest amount of borate complexing substances in the sample. The exact nature of these complexing molecules is still under investigation.

The RO sample is considered to be representative of unfractionated dissolved organic matter in a water sample [21]; however, humic and fulvic acids are only operationally defined components which are separated from one another during the concentration of HSs from waters. The RO sample is electrophoretically more related to the HAs than to the FAs from the same water. This result is surprising, in that the RO sample more closely resembles the FA sample with respect to elemental composition and ^{13}C NMR spectra [23]. The current results indicate that the strong acids and bases which are used in the classical extraction procedures may alter the molecular structure (FA/HA ratio) of the dissolved humic substances present in the natural water (hydrolysis and oxidation), leading to an overestimation of the lower-molecular-size fulvic component.

4. Conclusions

These results show clearly the potential of CE for the rapid screening and characterization of different dissolved HS fractions. By using appropriate separation buffers, the electrophoretic patterns can be compared and interpreted in terms of changes in structural characteristics of the humic mixtures. CE is particularly useful when comparing series of humic fractions involved in processes such as degradation or pollutants interactions. The choice of the separation buffer plays a key role in interpretation of the electrophoretic patterns and in the use of this method for the study of interactions of humics with pollutants. Significant advantages of these CE techniques include the capabilities to work with very small amounts of sample and to attain rapid sepa-

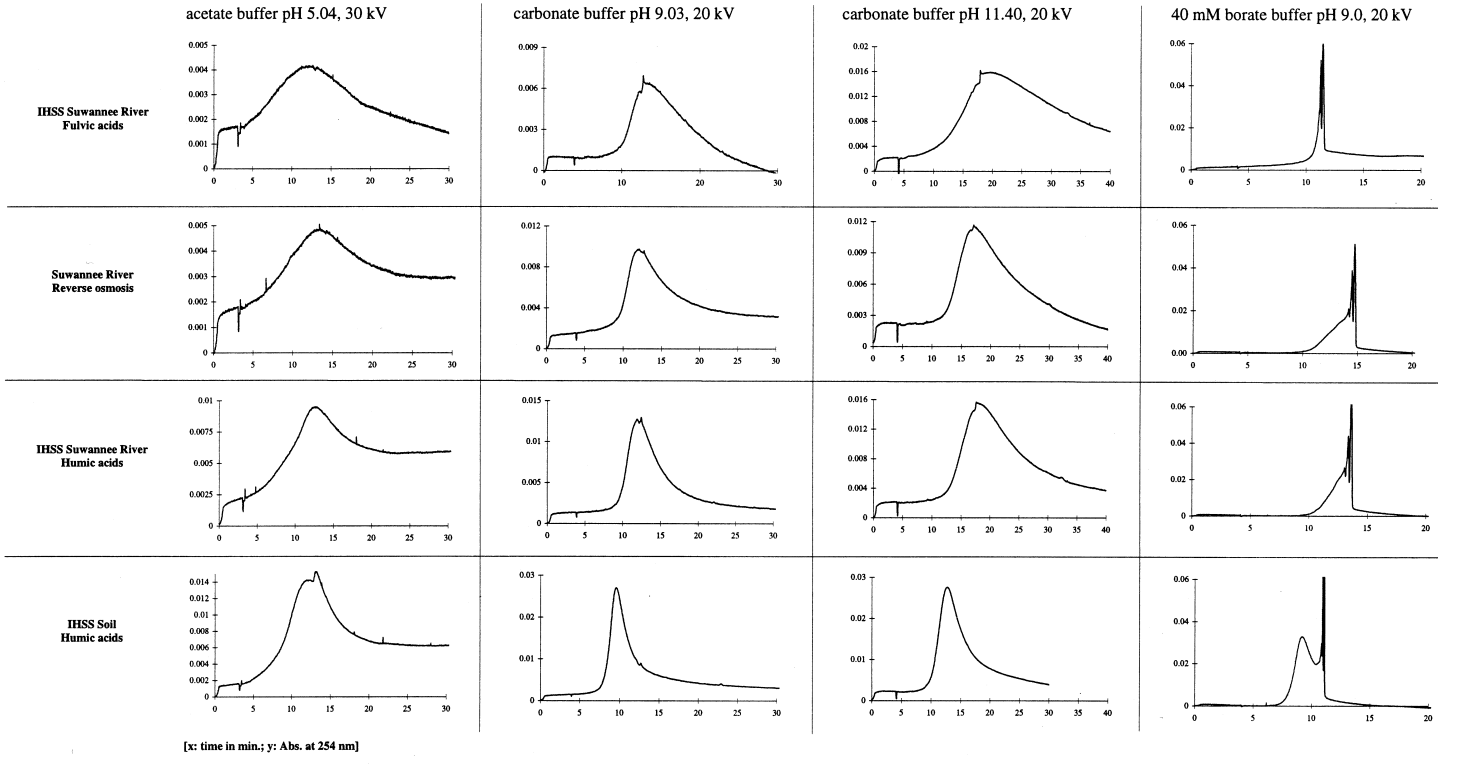

Fig. 6. Electrophoretic patterns of Suwannee River reference FAs and HAs, a Suwannee River (RO) fraction obtained by the reverse osmosis procedure and an IHSS soil reference HAs compared using four different separation buffer systems.

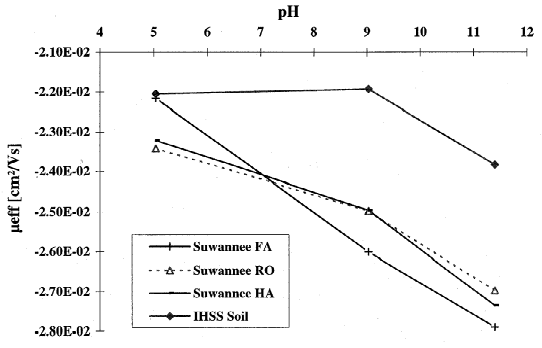

Fig. 7. Expression of the AEM of Suwannee River reference FAs and HAs, Suwannee River RO fraction, and the IHSS soil reference HAs at varying pH and IHSS soil reference HAs (data from Fig. 6).

rations in aqueous media closely resembling natural systems.

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