

## Mobility Distribution of Synthetic and Natural Polyelectrolytes with Capillary Zone Electrophoresis

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Parameters are proposed to describe the distribution of the effective electrophoretic mobilities of various anionic polyelectrolytes such as poly(styrene-4-sulfonates) and humic substances of different origins analyzed by capillary zone electrophoresis. The first step of the data treatment is a baseline correction and conversion of the electrophoretic raw time data to effective electrophoretic mobilities ( $\mu$ -scale), taking into account the electroosmotic flow. With this new  $\mu$ -scaling, the electropherograms are more representative of velocity-based separation phenomena than those using migration times, and a direct comparison of electropherograms is possible. Four different average electrophoretic mobilities were defined: the number-average effective mobility ( $\mu_n$ ), the weight-average effective mobility ( $\mu_w$ ), the z-average effective mobility ( $\mu_z$ ), and the peak-average electrophoretic mobility ( $\mu_p$ ). The polydispersity of the mobility of mixtures was described by the  $\mu_w/\mu_n$ ,  $\mu_z/\mu_w$ , and  $\mu_p/\mu_w$  ratios. These parameters were used to describe the electrophoretic mobility distributions of different fractions of restricted molecular size: those obtained by ultrafiltration from a soil humic acid at neutral pH and reference humic substances of the International Humic Substances Society at different values of pH and ionic strength. The data clearly show the influence of both molecular size and charge distribution of the analyzed mixtures on the mobility distributions.

Polyelectrolytes of high molecular weight are the subject of much interest in modern chemistry and biochemistry. Not only are functionalized colloids small entities presenting a huge exchange surface, but they also have pH-dependent aggregation potentials, creating "compartments" with their own specific architectures and functions. The best examples are macromolecular systems like collagen, hyaluronic acids, or lignin in living systems (1). Synthetic poly(carboxylates) are an example of polyelectrolytes that are used on a technical scale to remove heavy metals from contaminated waters or to avoid calcareous deposits in water-based coolers (2). In the environment, poly(hydroxycarboxylates) like dissolved humic substances (HS) are the main constituents of the dissolved natural organic carbon pool in surface, ground, and soil pore water. They represent about 25% of the total organic carbon on earth and 50% of the organic carbon in oceans and fresh waters (3), but less is known about their structural chemistry than about the chemistry of any biopolymer of living origin (4). The degree of ionization of these macromolecules is governed by the ionization of the phenolic and carboxylic groups of the humic core, which is a function of solution pH. Compared with the operationally defined fulvic acids (FA), which are soluble in both alkali and acid solutions, the humic acids (HA), which are soluble in alkali and insoluble in acid solutions, are of higher molecular size and lower acidity. Capillary electrophoresis (CE) methods were shown to be ideal investigative techniques for charged macromolecules; choosing the appropriate buffer and CE methodology allows fingerprinting of these complex mixtures (5, 6) and description of their structural properties (7, 8) and of the oligomeric distributions (9, 10).

Traditionally, CE electropherograms are represented as a plot of a measured property (e.g., absorbance) versus migration time. However, the use of a time-scaled  $x$ -axis has some serious disadvantages. First, the fundamental characteristic of the electrophoretic behavior of a species is its migration velocity rather than its migration time. Because the relationship between migration time and velocity is nonlinear, the use of a time-scaled  $x$ -axis results in some "deformation" of the electropherogram. Second, migration times are strongly dependent on electroosmotic flow (EOF), the velocity of the

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buffer in the capillary, which reflects the surface condition of the inner walls of the capillary and often varies from one measurement to another.

To remedy these problems some researchers proposed representing electropherograms as a function of the quantity of the charge (11) or in the 1/time domain (12). In our opinion, the best remedy is to use the effective electrophoretic mobility scale (13). Effective electrophoretic mobility is the most fundamental integral property that combines information about the molecular size and net charge of a component under given separation conditions (buffer pH, ionic strength). Under conditions of counterosmotic flow, its value is obtained by subtracting the EOF mobility from the measured mobility calculated from the migration time.

Complex mixtures possessing wide dispersity in molecular size and in net charge can be characterized by the description of the signal distribution, i.e., the effective mobility distribution. Parameters were defined to describe such polydispersity by analogy with parameters from polymer chemistry. This paper is intended to illustrate the use of polydispersity descriptors to characterize polyelectrolytes such as well-defined poly(styrene-4-sulfonates) and more heterogeneous natural HS.

## Experimental

### Instrumentation

A Beckman P/ACE 2050 Series CE with UV filter at 254 nm was used for HS analysis, with Beckman System Gold Chromatography Software.

### Capillary Zone Electrophoresis (CZE)

Uncoated fused-silica CZE columns (75  $\mu\text{m}$  id, 375  $\mu\text{m}$  od, 50 cm length to detector, total length 57 cm) were obtained from Laser 2000 (Weßling, Germany). Typical CZE conditions for separation of the various HS fractions were: separation buffer, 50 mM acetate (pH 5.05) and 50 mM carbonate (pH 9.03, and adjusted to pH 11.4 with NaOH); temperature, 30°C; voltage, 20 kV; detection wavelength, 254 nm; hydrodynamic injection, 5 or 10 s. Analyte concentration in HS had no significant influence on the peak-average electrophoretic mobility (AEM,  $\mu_p$ ). Day-to-day changes in migration times occurring because of relative changes in the EOF (different capillary surface conditions) were limited by washing the capillary with 0.1M NaOH for 2 min between each run.

### Data processing

The raw electrophoretic data were treated with program "GelTreat" developed by A. Kudryavtsev (Lomonosov Moscow State University, Department of Chemistry, Russia). The program is described by Kudryavtsev et al. (14) and was initially designed for the treatment of size-exclusion chromatograms of HS. To make the program applicable to the treatment of electropherograms using a mobility scale, a modification was added to block the linear transformation of the x-axis of the initial electropherogram, which allowed its conversion from time-scale to  $\mu$ -scale (13). The input parameters

**Table 1. Weight-average ( $M_w$ ) and peak-average ( $M_p$ ) molecular weights of the poly(styrene-4-sulfonates) standards**

Standard	Manufacturer's guaranteed dispersity <sup>a</sup>	Molecular weight, kD		
		$M_p$	$M_w$	$M_w/M_p$
1	<1.2	1.12	1.1	1.018
2	<1.2	6.71	6.78	0.990
3	<1.2	13.4	13.2	1.015
4	<1.2	46.4	48.6	0.955
5	<1.1	82.8	77.4	1.070
6	<1.2	150	149	1.007
7	<1.1	356	350	1.017

<sup>a</sup> Phenomenex (Hörsbach, Germany).

<sup>b</sup> MWD = dispersity in molecular weight as guaranteed by the manufacturer and measured with size-exclusion chromatography.

needed for the transformation were total column length ( $L_t$ ), column length to the detector ( $L_d$ ), separation voltage ( $V$ ), and time of the EOF peak ( $t_{\text{eof}}$ ). Data transformation was done for each data point ( $t_m$ ) of the electropherogram according to Equation 1:

$$\mu_{\text{eff}} = \frac{L_d \times L_t}{t_m \times V} - \frac{L_d \times L_t}{t_{\text{eof}} \times V} \quad (1)$$

### Humic Substances

Scheyern soil HA were extracted and isolated according to procedures of the International Humic Substances Society (IHSS); for structural information on the Scheyern HS, refer to refs. 15 and 16. The standard soil, water, peat, and Leonardite HS were obtained from the IHSS (P.R. Bloom, University of Minnesota, Department of Soil, Water and Climate, St. Paul, MN). The Suwannee River natural organic matter (NOM) fractions were concentrated with reverse osmosis (RO) according to the procedure reported by Serkiz and Perdue (17) and were kindly provided by E.M. Perdue (Georgia Institute of Technology, School of Earth and Atmospheric Sciences, Atlanta, GA).

Ultrafiltration of Scheyern HA was performed in phosphate buffer, pH 7.1, with 4 Amikon DIALFO membranes, XM300, YM100, XM50, and YM10, at cutoff limits of 300, 100, 50, and 10 kD, respectively (pore sizes of 200, 50, 15, and 12 Å, respectively) at a pressure of  $\leq 10$  psi. The membranes were soaked several times in a beaker of deionized water before use; rinsed membranes were stored in water-ethanol (90 + 10) and refrigerated. The fractions obtained were designated <10 kD, 10–50 kD, 50–100 kD, and >300 kD. Fraction 100–300 kD was not available in sufficient amounts to be analyzed in this study. Filtration was stopped when the solution coming through the membrane was colorless. The solutions were then dialyzed (cutoff 1000 D), protonated by cation exchange on Dowex 50WX8, and finally freeze-dried. After the

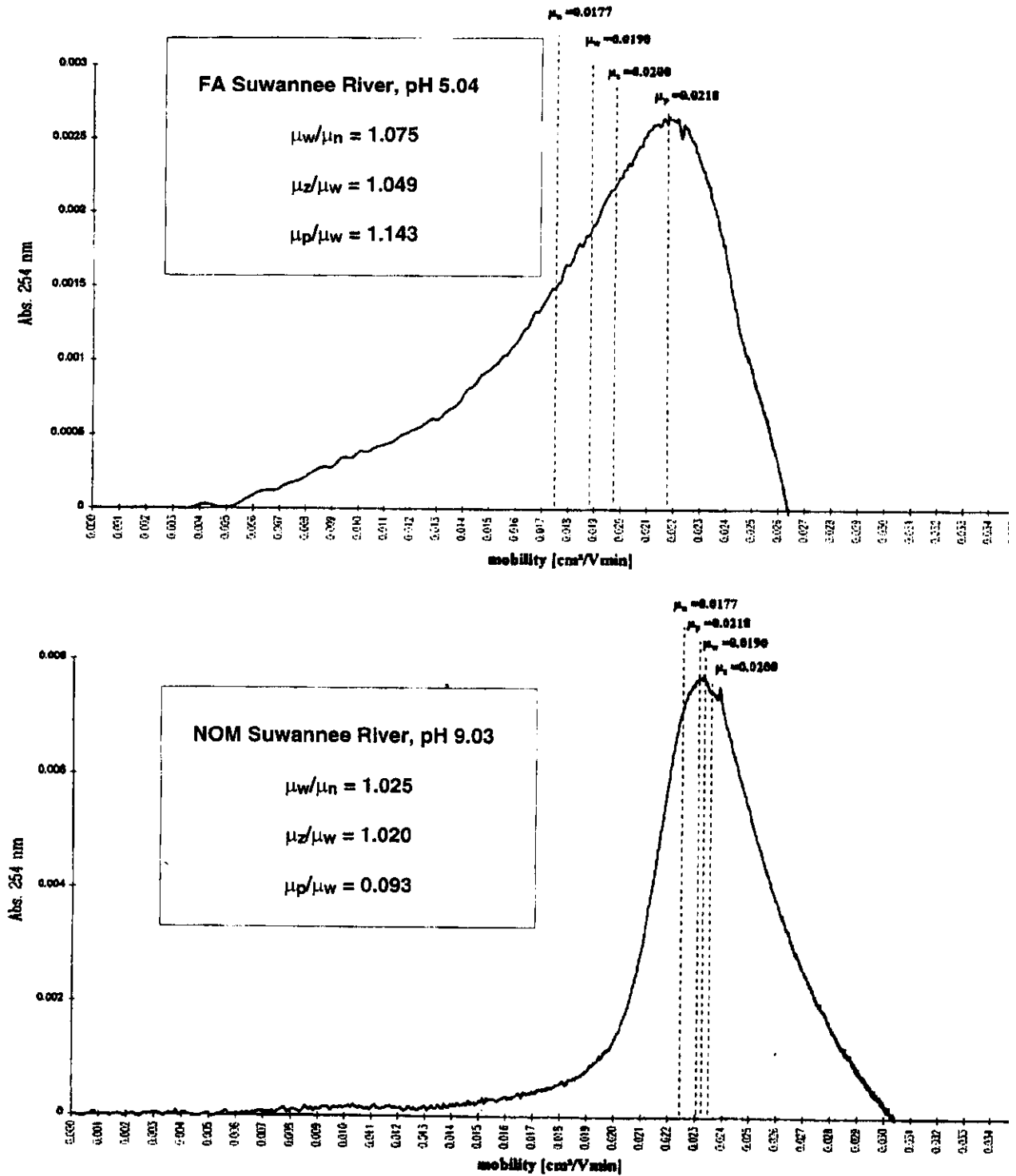
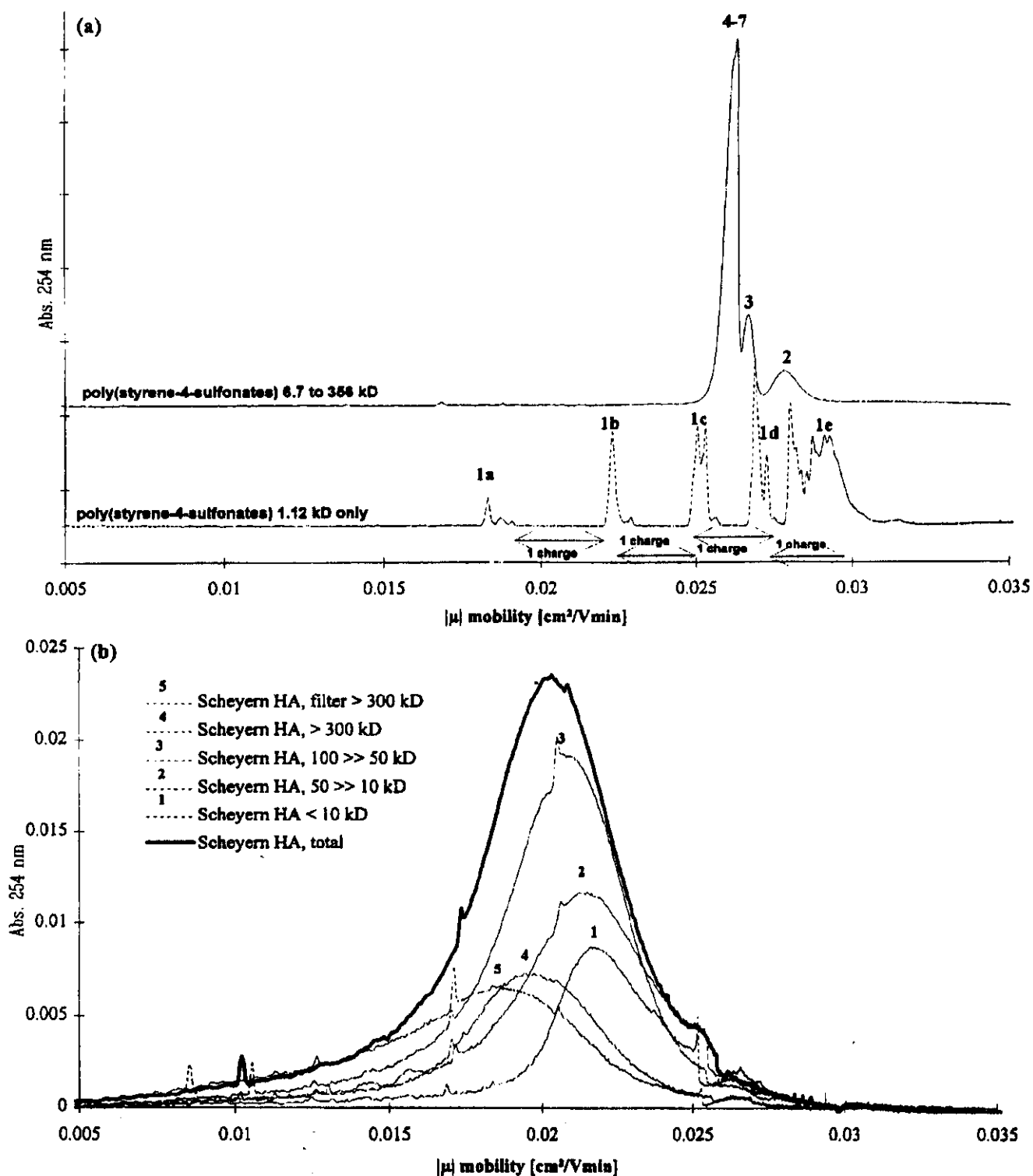


Figure 1. Illustration of the significance of the defined number-average effective mobility ( $\mu_n$ ), the weight-average effective mobility ( $\mu_w$ ), the z-average effective mobility ( $\mu_z$ ), the peak-average effective mobility ( $\mu_p$ ), and the corresponding ratios  $\mu_w/\mu_n$ ,  $\mu_z/\mu_w$ , and  $\mu_p/\mu_w$  ( $\mu_p/\mu_w > 1$  means left-sided asymmetry, and  $\mu_p/\mu_w < 1$  means right-sided asymmetry).



**Figure 2.** (a) Poly(styrene-4-sulfonates) standards measured in 25 mM carbonate buffer, pH 9.03, after  $\mu$ -scale correction (1: 1.12 kD, 2: 6.71 kD, 3: 13.4 kD, 4: 46.4 kD, 5: 82.8 kD, 6: 150 kD, 7: 356 kD Mp); peaks 1a–e correspond to the fractions of close size (1.12 kD) and different charges (see text). (b) Total Scheyern HA and molecular size fractions obtained by ultrafiltration under the same CE separation conditions as in (a).

first filtration (300 kD filter), some highly colloidal material stacked on the membrane; this fraction was collected separately and called "filter >300 kD."

The fractions were dissolved in 0.1M NaOH and diluted to a concentration of ca 1 mg/mL for CE analysis. The lowest measurable concentration (without stacking conditions) was 50  $\mu\text{g/mL}$  at 254 nm.

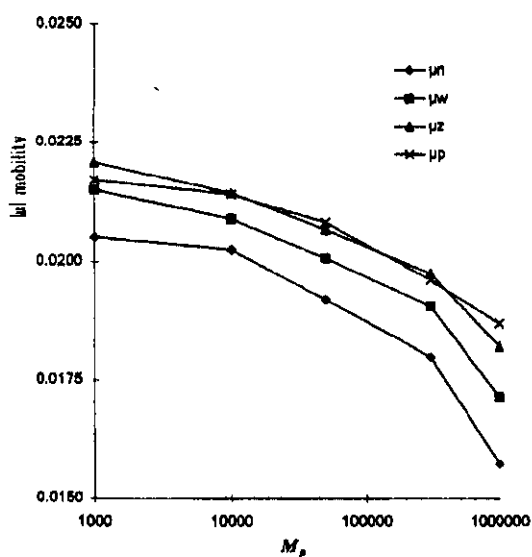
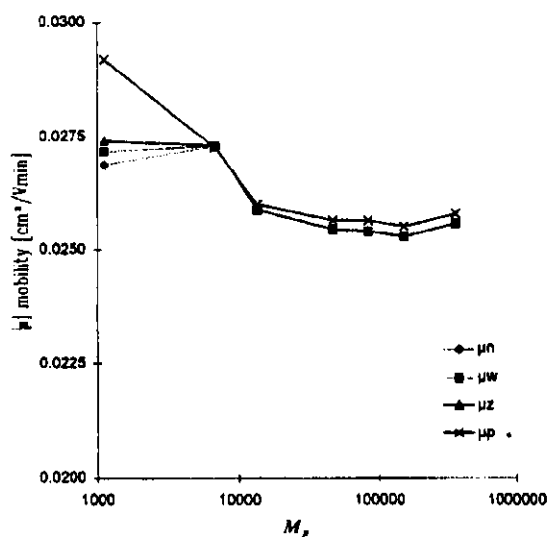
### Chemicals

The poly(styrene-4-sulfonates) are size-exclusion chromatography standards of restricted molecular weight ranges and

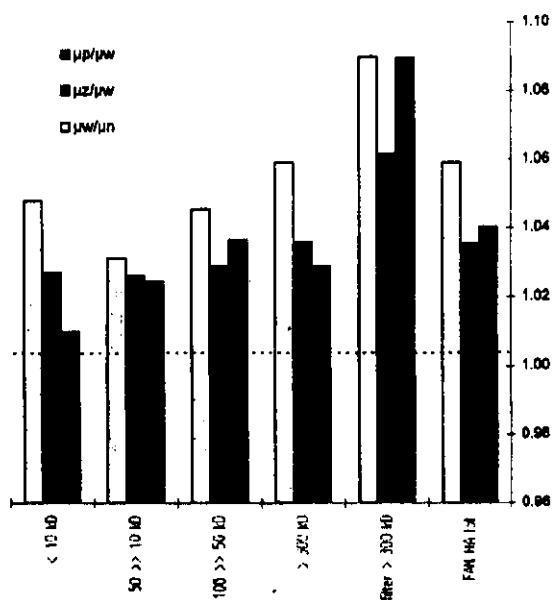
were purchased from Phenomenex (Hörsbach, Germany). The molecular weight distribution characteristics [weight-average ( $M_w$ ) and peak-average ( $M_p$ ) molecular weights] are given in Table 1. MWD ( $M_w/M_n$ ) is the dispersity in molecular weight as guaranteed by the manufacturer and measured with size-exclusion chromatography (Phenomenex).

### Theoretical Background

In polymer chemistry a number of average statistical parameters are used to describe the molecular mass distribution of a sample (18). In general, they are calculated as



(a)



(b)

Figure 3. Variation of the average mobilities and defined mobility ratios as a function of the molecular size of the samples. (a) Poly(styrene-4-sulfonates). (b) Scheyern HA and ultrafiltration fractions (the cutoff of the ultrafiltration membrane was taken as  $M_p$ ).

$$M_i = \frac{\int_0^{\infty} M^i p(M) dM}{\int_0^{\infty} M^{i-1} p(M) dM} \quad (2)$$

where  $p(M)$  is a signal (physical property) proportional to the weight fraction of a polymer with molecular mass  $M$ . Most commonly used are  $M_0$ ,  $M_1$ , and  $M_2$ , which are called number-average, weight-average, and z-average molecular mass, respectively.

By analogy, we propose average parameters of effective electrophoretic mobility defined as follows:

(a) Number average effective mobility:

$$M_0 = \mu_n = \frac{\int_0^{\infty} A(\mu) \times d\mu}{\int_0^{\infty} A(\mu) \times \mu^{-1} \times d\mu} \quad (3)$$

(b) Weight-average effective mobility:

$$M_1 = \mu_w = \frac{\int_0^{\infty} A(\mu) \times \mu \times d\mu}{\int_0^{\infty} A(\mu) \times d\mu} \quad (4)$$

(c) z-average effective mobility:

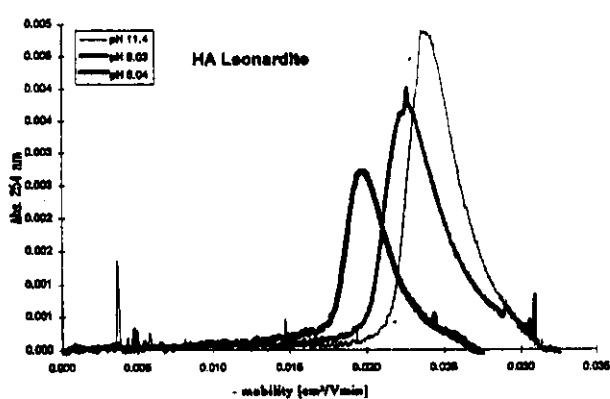
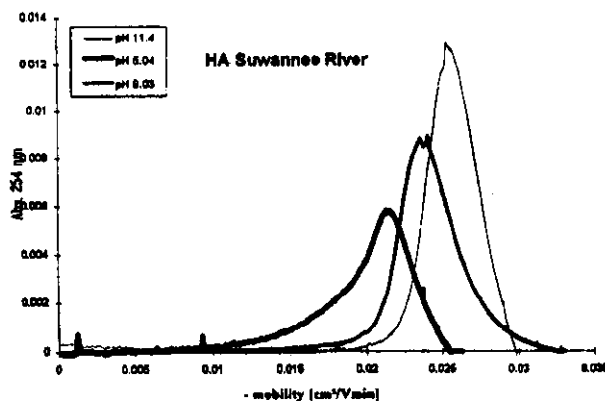
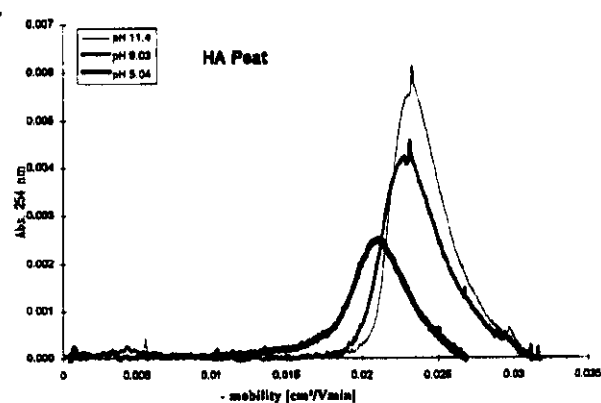
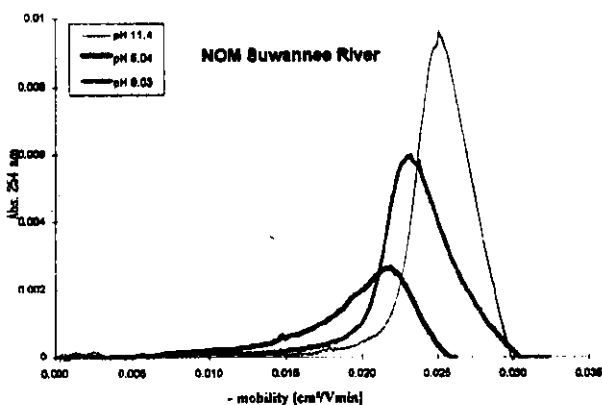
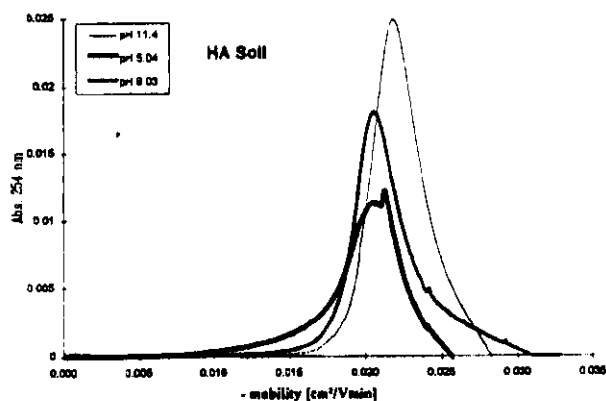
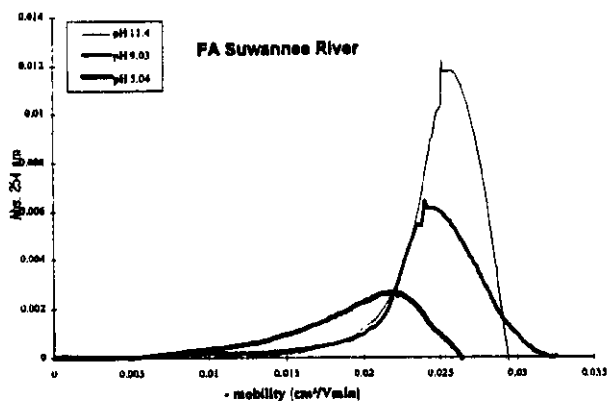


Figure 4. Electropherograms in  $\mu$  scale of 6 IHSS standard HS at 3 different pH values.

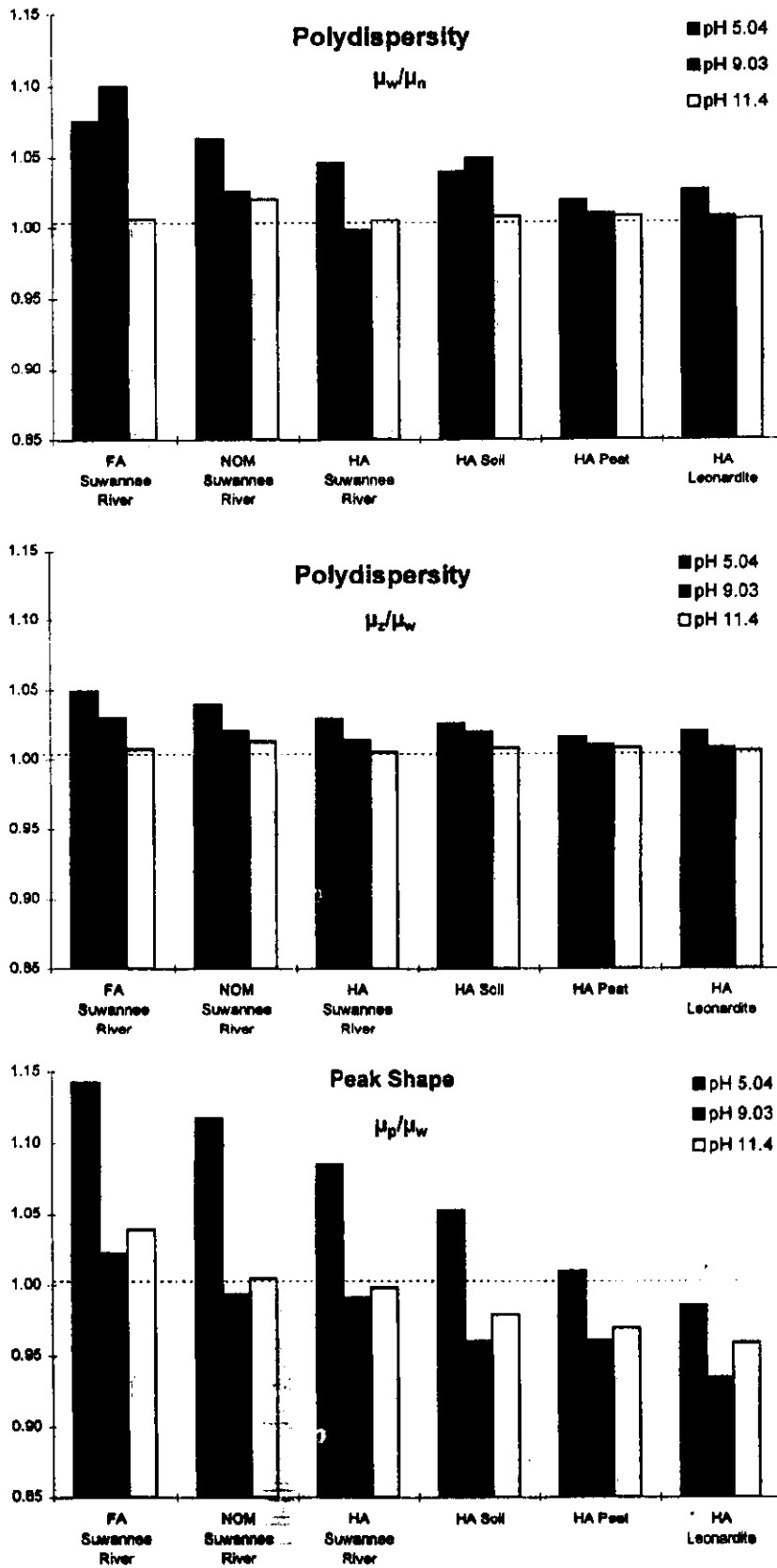


Figure 5. Variation of mobility ratios with pH of the 6 IHSS standard HS.

$$M_2 = \mu_z = \frac{\int_0^\infty A(\mu) \times \mu^2 \times d\mu}{\int_0^\infty A(\mu) \times \mu \times d\mu} \quad (5)$$

where  $A(\mu)$  corresponds to the UV-absorbance signal at 254 nm at a given effective mobility,  $\mu$ . In addition, peak-average effective mobility,  $\mu_p$ , may be defined by analogy with peak-average molecular weight,  $M_p$ , as the mobility calculated from the migration time of the peak maximum.

For any distribution  $|\mu_n| \leq |\mu_w| \leq |\mu_z|$  (the mobility of anionic compounds is negative in sign); more generally,  $M_i \leq M_{i+1}$ . Strict equality takes place for infinitely narrow distribution (monodisperse system) only. Thus, the ratios  $\mu_w/\mu_n$  and  $\mu_z/\mu_w$  ( $\geq 1$ ) can be used to describe the degree of polydispersity in mobility. The ratio  $\mu_p/\mu_w$  can serve as an indicator of the shape of the distribution. For symmetrical (e.g., Gaussian) distributions  $\mu_p = \mu_w$  and  $\mu_p/\mu_w = 1$ . For nonsymmetrical distributions this ratio differs from 1, being  $>1$  for left-sided asymmetry and  $<1$  for right-sided asymmetry. Therefore, the value of  $\mu_p/\mu_w$  reflects the relative contributions of different fractions to the entire electrophoretic peak:  $\mu_p/\mu_w > 1$  means a greater contribution of less mobile fractions and  $\mu_p/\mu_w < 1$ , a greater contribution of more mobile fractions. Figure 1 illustrates usage of the defined parameters for description of mobility distributions. In the case of a wide distribution of mobilities (higher polydispersity in mobility), e.g., with the Suwannee River FA at pH 5.04, the calculated average mobilities are noticeably different from each other. When the humic hump presents a more homogeneous distribution, e.g., with the Suwannee River NOM at pH 9.03, the calculated average mobilities are closer in value.

## Results and Discussion

### Poly(Styrene-4-Sulfonates) Standards

Seven poly(styrene-4-sulfonates) standards (Table 1) of restricted molecular weights (from 1.12 to 356 kD) with a certified degree of sulfonation  $> 90\%$ , were first investigated with CZE at pH 9.03. Figure 2a shows electropherograms of the individual standard 1.12 kD and of the mixture of 6.71–356 kD standards. The lower molecular weight standard ( $M_p = 1.12$  kD) showed not only 1 peak in CZE but a set of peaks (Figure 2a, peaks 1a–e) corresponding to individual fractions of close molecular weights (guaranteed MWD  $< 1.2$ ) but different mobilities. With the effective mobility scale the differences in mobility of the individual peaks of poly(styrene-4-sulfonates) 1.12 kD are linear functions of the net charge. The variation in charge could correspond to different degrees of sulfonation and the splitting of each peak into different isomers as illustrated in Figure 2a. The equal distances between these peak groups correspond to a difference of 1 charge unit only, i.e., they are not due to fractions of different molecular weights. Figure 2a also shows that in the mixture of standards those of higher molecular weight, standards 4–7, coelute in 1 peak, indicating that each standard has the same or a very similar charge-to-size ratio. This can be explained as a coiling of the macromolecules caused by the pres-

ence of the nonsulfonated, more hydrophobic monomers ( $< 10\%$  in number), which shield some of the charged monomers and limit the number of exposed charge sites, decreasing the net charge of the molecule. Additional experiments with individual standards were performed. The variation in the calculated mobilities versus molecular weight ( $M_p$ ) is shown in Figure 3a. The polydispersity in mobility of these compounds as measured by the  $\mu_w/\mu_n$  and  $\mu_z/\mu_w$  ratios, shown in the right panel of Figure 3a, is very close to 1; only the  $\mu_p/\mu_w$  ratio increases with increasing molecular weight, showing slight changes in peak shape. Only for the lower molecular weight standard ( $M_p = 1.12$  kD) is the  $\mu_p/\mu_w$  ratio much higher, because  $\mu_w$  is calculated over the entire mobility range of the different peaks of this fraction, and  $\mu_p$  corresponds to the peak mobility of the fully sulfonated polymer (peak 1 in Figure 2).

### Size-Fractionated Humic Acids

When HS are analyzed with CZE in noncomplexing buffers, the electropherograms show a wide distribution of the signals around a peak-average effective mobility ( $\mu_p$ ; 8). The relationship of this measured average mobility to structural characteristics of the humic mixture was investigated in a previous study (7), which gave information on average net charges and average size values, but not on the distribution of those parameters. Ongoing studies are trying to correlate the charge and size distribution information as measured by potentiometric titration or size-exclusion chromatography with the mobility distribution of such polyelectrolyte systems.

The electropherograms of 6 molecular weight-restricted humic fractions obtained by ultrafiltration, as well as that of the original nonfractionated HA, are superimposed and shown in Figure 2b. Compared with the poly(styrene-4-sulfonates) that are in the same range of molecular size, the peaks are very wide. The electropherograms correspond to a distribution of the mobilities of thousands of different components and not to peak broadening due to diffusion phenomena (6). The lower the molecular weight of the fraction, the higher in value is the average mobility; the variation of the calculated mobilities with molecular weight (lower cutoff limit) is shown in Figure 3b. Great care has to be taken in the interpretation of molecular weight data obtained by ultrafiltration because of broad nominal cutoffs and possible membrane/solute interactions, which are a function of the structural characteristics (hydrophobe/hydrophile) of the humic materials analyzed. The micropores of the membranes are not uniform in size (the cutoff represents the particle size that will be 90% retained), and ultrafiltration processes are dependent on pressure, ionic strength, and concentration gradient (19, 20). The curves in Figure 3b are thus specific for the HA (Scheyern) in our study, and the analysis of HS from other origins would not give the same relation between the mobilities and the  $M_p$  (each molecular weight-restricted fraction has its own charge distribution). However, the trend in increasing molecular sizes in successive fractions is significant, and these data clearly show higher polydispersity of fractions of higher molecular weight (overall higher calculated mobility ratios in Figure 3b). This can be explained either by the higher polydispersity in net



charges and/or sizes of these fractions of higher molecular weight, or by an instability of the "aggregate" distribution obtained with ultrafiltration that can lead to "contamination" with fractions of lower molecular weight. The dynamic equilibria between humic molecules of different sizes, structures, and charges are known as "aging" processes; this leads to the formation of more or less stable aggregation systems different from one natural environment to another (type of soils). During ultrafiltration separation, these dynamic equilibria are broken and the separation in very narrow size classes, as would happen with proteins or other biomolecules, cannot be achieved with HS.

### Mobility Description of the IHSS Humic Standard Materials

Until now the use of CZE in the characterization of HS has been restricted to observation of changes in the "CZE-fingerprint." A numerical approach to describe changes in electropherograms of humic mixtures that may be attributed to abiotic (16) or biological processes (21) has not yet been achieved. The conversion of the time-scaled  $x$ -axes into the effective mobility scale and the calculation of the different mobilities and polydispersity descriptors are useful tools for comparing the mobility distributions of these complex mixtures. Six standard HS are available from the IHSS, including surface water, soil, leonardite, and peat HS. In a previous study we showed the influence of borate buffers on electropherogram shapes and the similarity of the electrophoretic behavior of the Suwannee River NOM extracted by reverse osmosis (RO; 16) to that of the Suwannee River HS (22). The electropherograms in  $\mu$ -scale of these 6 standard materials are shown in Figure 4 at 3 different separation pH values. The pH of the separation buffer directly influences the net charge of the molecules and their mobilities; at pH 5.04 most of the carboxylic groups are ionized, but the phenolic groups are not, and the carboxylic acidity contributes mostly to the net charge of the molecules. At pH 11.4, most of the carboxyl and phenolic groups are expected to be ionized, and the higher mobility reflects the total charge increase of the molecules in the mixture.

The average mobility ratios  $\mu_z/\mu_w$  and  $\mu_w/\mu_n$  indicate sample polydispersity in terms of the charge-to-mass ratio. These ratios are noticeably pH-dependent (Figure 5). At pH 11.4, where most of the acidic groups are ionized, those values are very close to 1, showing quite homogeneous charge distribution. Therefore, the average number of acidic groups per mass unit is approximately the same for all fractions constituting each humic sample. At lower pH when the groups are only partially ionized, the  $\mu_z/\mu_w$  and  $\mu_w/\mu_n$  ratios are essentially  $>1$ , indicating nonhomogeneous distribution of the functional groups of different acidity within each sample. It should be noted that for each given sample the ratio  $\mu_w/\mu_n$  is in general  $> \mu_z/\mu_w$ ; therefore,  $\mu_w/\mu_n$  is more sensitive to sample polydispersity. Meanwhile, the general tendency of polydispersity to decrease with an increase in pH is expressed more distinctly when the ratio  $\mu_z/\mu_w$  is used—maybe because the error for  $\mu_n$  is essentially larger than that for  $\mu_w$  and  $\mu_z$ . The

variation of  $\mu_z/\mu_w$  with pH ( $\Delta(\mu_z/\mu_w)/\Delta\text{pH}$ ) decreases in the order FA Suwannee River  $>$  NOM Suwannee River  $>$  HA Suwannee River  $>$  HA soil  $>$  HA peat  $>$  HA leonardite. The surface water FA and the NOM have the highest polydispersity ( $\mu_z/\mu_w$  ratio) when compared with the more homogeneous peat and leonardite HA. This behavior is thus dependent on the source of the HS (water, soil, peat, leonardite).

The peak shape as characterized by the  $\mu_p/\mu_w$  ratio also changes with pH, and this ratio is the lowest at pH 9.03. These changes with pH can be explained by (1) the presence at low pH of some lower-charged molecules with mobility lower than the average mobility of the hump ( $\mu_p/\mu_w > 1$  means left-sided asymmetry), and (2) the presence at high pH of highly charged molecules with significant higher mobility values than the average mobility of the hump ( $\mu_p/\mu_w < 1$  means right-sided asymmetry).

### Conclusions

Until now, CZE could be used only as a fingerprinting technique to visually compare the changes in electropherograms of complex HS mixtures obtained from different sources or during degradation processes (abiotic or biotic). The conversion of the time scale to an effective mobility scale ( $\mu$ -scale) is the first step toward a better comprehension of the electropherograms of polyelectrolyte solutions. This conversion allows the calculation of statistically defined "descriptors" like number-, peak-, weight-, and z-average mobilities that can be directly compared, as well as mobility ratio characteristics of the dispersity, and shapes of the mobility distributions. Such descriptors are essential in attempts to analyze processes involving HS that affect the distribution of their net charges or sizes (metal interactions, sieving in physical gels).

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