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Capillary electrophoretic behaviour of humic substances in physical gels

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Abstract

We investigated the principles of the capillary electrophoretic behaviour of humic substances (HSs) in physical gels. Long chain (M_r 4000, 6000 and 20 000) polyethylene glycols (PEGs) at concentrations above their entanglement threshold caused the separation of HS fractions according to molecular size differences. Close linear relationships between effective mobilities and mean apparent molecular masses were observed at PEG concentrations between 2.5 and 15%. The efficiency of the separation does not increase in gels of increasing polymer concentrations. The possibility of interactions between HSs and gel-forming polymers was also investigated. Short chain (M_r 400) PEGs, added to the buffer at concentrations from 2.5 to 12.5%, increased the migration times of all HS fractions, but no separation was obtained even at large polymer concentrations, showing that gel formation was essential for the separation. In 2.5% polyvinyl alcohol (PVA) 49 000 all fractions show two unresolved, but well defined peaks. This separation is probably artefactual and depends on the relative concentration of HSs and PVA, as the relative abundance of the peaks changes with the sample concentration.

Keywords: Buffer composition; Entangled polymers; Humic substances; Poly(ethylene glycol); Poly(vinyl alcohol)

1. Introduction

Humic substances (HSs) are the ultimate residual products of the decomposition of plant and animal remains in soils, sediments and natural waters. They are often found also in geological deposits such as carbonaceous shales, lignites and brown coals. HSs are not only quantitatively important, being among the main pools in the carbon cycle ($60 \cdot 10^{13}$ kg organic C as compared to $7 \cdot 10^{13}$ kg in living organisms), but have many different important roles in the geochemistry of many elements and in the fate of both organic and inorganic pollutants in the environment [1]. HSs are in fact actively involved in the transportation of trace metals, and can either reduce or enhance the toxicity and bioavailability of toxic organic compounds and heavy metals. They also influence the sorption capacity of soil to act as a sink for S and N oxides in the atmosphere [2]. In spite of their well-known environmental importance, HSs are still ill defined [2,3] and their structures, which include a large number and variety of functional groups (mostly COOH and phenolic OH), are largely unknown. Reasons for this are the huge molecular mass polydispersion of HSs and the wide

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range of molecular properties values that change only slightly from one molecular species to another. All this has to be kept in mind when attempting fractionation of HSs: not only is the isolation of pure, identifiable compounds practically impossible, but even the isolation of well defined fractions must be considered with caution [3,4].

In comparison with other techniques, conventional electrophoresis itself has been sparingly applied to HSs and in most cases with the only aim of achieving separation into distinct fractions [5]. Polyacrylamide gel electrophoresis (PAGE) of HSs in a single buffer system does not achieve fractionation into defined bands, nevertheless a meaningful separation is obtained and HSs taken from the front of the migrating band display significant different structural characteristics as shown by IR and pyrolysis–gas chromatography–mass spectrometry (GC–MS) [4]. The electrophoretic mobility of HS fractions of reduced molecular mass polydispersity extracted from the same soil is in the first place a function of the apparent molecular size as calculated from the 95% cut-off limits of the ultrafiltration membranes from which the fractions were prepared [6]. Up to now, relatively few studies have dealt with capillary zone electrophoresis (CZE) behaviour of HSs: mostly with the aim of obtaining a fingerprint characterisation of HSs or the fractionation and quantitation of humic acids [7,8]. A more systematic approach to the study of factors affecting the CZE mobilities of HSs has been carried out by Schmitt-Kopplin et al. [9]. It showed that the heterogeneity displayed by HS preparations in borate buffers was caused by interactions of HSs with borate. In the absence of interactions, HS electropherograms have a bell-shaped featureless appearance, and different molecular size fractions of HSs have almost the same electrophoretic mobility [10,11]. As for other macromolecules, such as nucleic acids and sodium dodecyl sulphate (SDS)–protein complexes, which do not exhibit significant mobility differences in free zone electrophoresis, HSs are conveniently separated in the presence of a sieving matrix. Gel-filled capillaries, however, have several serious disadvantages, such as shrinkage, degradation by hydrolysis, bubble formation, etc., that lead to inconveniently short lifetimes of the sieving matrices and allow a limited number of injections. For these reasons the use of the so-called physical gels or entangled polymer solutions has gained increasing attention. Because of the wide molecular range exhibited by HSs and the fact that these molecules possess phenolic and carboxylic groups, which can act as either hydrogen donors and acceptors, favouring interactions with hydrophilic polymers [12–14], the mechanism of the separations obtained for these natural organic compounds in CZE in the presence of physical gels is still unknown.

Hydrophilic polymer solutions show two distinct regimes: a diluted regime in which polymer chains are isolated from each other and a regime, above a specific concentration called entanglement point or entanglement threshold (c^*), in which polymer chains become to overlap [15,16]. Above this threshold, polymer chains form a dynamic porous matrix with sieving properties. The entanglement point c^* is geometrically defined by the molecular mass M_r and the radius of gyration R_g of the polymer chain and is a function of M_r^{-1}. However, above this point, as long as the polymer is entangled, the pore size of the matrix decreases with increasing polymer concentration and do not depends on M_r. Experimentally c^* can be obtained for a given polymer by determining the point of departure from linearity of the plot of viscosity vs. concentration.

Barron et al. [17] demonstrated that hydroxymethylcellulose (HEC) shows a sieving power also below its entanglement threshold. Similar findings were obtained in the separation of SDS–protein complexes by polyvinyl alcohol (PVA) [18]. The behaviour of HEC solutions was explained by their capability to form a transient entangled network by coupling polymer chains with large sample molecules, whereas the hydrogen bonding effect was proposed for PVA solutions.

In the presence of a true sieving effect, when the pore size of the matrix is comparable to the hydrodynamic radius of the sample molecules, size-based separations are generally explained according to the Ogston model [19,20]. The reptation model and the reptation with stretching model [17] are, on the contrary, used to describe the migration of long polyionic molecules or micelles that have molecular radii larger than the size of the matrix pores and can eventually stretch under the effect of a high electric field.

Interactions of analytes with gel-forming polymers, leading to separations due to molecular size differences, have also been demonstrated for phenol carboxylic acids [13,14]. The aim of this work was to investigate the principles governing the capillary electrophoretic behaviour of HSs with respect to the possibility of interactions between HSs and gel forming polymers. The effects of the kind and molecular size of the polymer used and of its concentration in the electrophoretic buffer were considered.

2. Experimental

2.1. Apparatus

CZE was performed with an ABI 270A-HT unit (Applied Biosystems, Foster City, CA, USA) equipped with a UV–Vis detector and a software system for data acquisition on a personal computer. Two sets of fused-silica capillaries (375 μm O.D.) were used: (i) uncoated capillaries of 55 cm (length to the detector 30 cm)×75 μm I.D. (Composite Metal Services, Hallow, UK); and (ii) μSIL DB-WAX polyether-coated capillaries (J&W Scientific, Folsom, CA, USA) of 100 μm I.D with a length of either 55 cm (30 cm to detector) or 100 cm (75 cm to detector).

2.2. Chemicals and solutions

Sodium hydroxide, sodium dihydrogenphosphate, sodium pyrophosphate and phosphoric acid were obtained from Carlo Erba (Milan, Italy). Tris-hydroxymethylaminomethane (Tris) and polyethylene glycol (PEG) with mean molecular masses of 400, 4000, 6000 and 20 000 were purchased from BDH (Poole, UK). PVA of M_r 49 000 and mesityl oxide (MSO) were obtained from Fluka (Buchs, Switzerland). All reagents used were of analytical grade.

The electrophoretic buffer was a solution of 50 m*M* Tris and 50 m*M* sodium dihydrogenphosphate adjusted to pH 8.3 and filtered through a Whatman 0.2-μm filter. PEG was added to the buffer at concentrations ranging from 1 to 20% (w/v), whereas PVA 49 000 was added at a concentration of 2.5% (w/v). The MSO was dissolved in the electrophoretic buffer to a concentration of 1% (v/v) and it was used as a neutral marker for electroosmotic flow (EOF) determination.

2.3. Procedure

CZE was performed at a constant temperature of 30°C. Before each analysis, the capillary was rinsed with 0.1 *M* NaOH for 5 min (uncoated capillaries only) and with the buffer, eventually containing PEG or PVA, for 5 min. Humic molecules are negatively charged at the pH of the experiment and in coated capillaries they move to the anode. When CZE was carried out in coated capillaries, samples where introduced at the cathodic end of the capillary by hydrodynamic injection for 3 s, run under a potential difference of −14 kV and detected at 360 nm.

In uncoated capillaries humic molecules are driven to the cathode by the EOF. Samples were then introduced at the anodic end of the capillary for 3 s and electrophoretic separations were performed from the anode to the cathode at a voltage of +14 kV. The EOF was determined using MSO – detected at 210 nm – at the end of each series of measures. Carrying out CZE in uncoated capillaries, the electropherograms show a baseline disturbance in correspondence with the peak of MSO [21]. Such disturbance was routinely used to measure the EOF in each run. All measurements were made in triplicate.

The apparent electrophoretic mobility, μ_{app}, was calculated with the equation:

$$\mu_{app} = (L_d/t_m)/(L_t/V)$$

where L_t is the total length of the capillary in cm; L_d the length of the capillary to the detector in cm; V the applied voltage; and t_m the migration time of peak maximum [20,23]. The effective mobility, μ_{eff}, was determined taking into account the mobility of EOF (μ_{EOF}), according to the equation:

$$\mu_{eff} = |\mu_{app} - \mu_{EOF}|.$$

2.4. Extraction and fractionation of humic substances

The HSs were extracted from a commercial sample of *Sphagnum* peat (Novobalt, Lithuania), dried and milled to pass a 0.5-mm sieve, and from the A_1

horizon of a Spodosol, sieved at 2.0 mm after air drying. Extraction was done for 1 h at room temperature with 0.5 *M* NaOH under nitrogen flux. Extracts were filtered through a 0.2-μm cellulose nitrate filter (Whatman, Maidstone, UK) and treated with Amberlite IR 120 H^+ (Carlo Erba) cation-exchange resin in order to lower the pH to 7 and to remove excess sodium.

The extracts were ultrafiltered on Diaflo YM and XM membranes (Amicon, Beverly, MA, USA). Fractionation was carried out with 0.1 *M* sodium pyrophosphate, adjusted to pH 7.1 with concentrated H_3PO_4. Five fractions of the following M_r ranges, deduced from the 95% cut-off limits of the membranes, were obtained: 1000–5000, 5000–10 000, 10 000–30 000, 50 000–100 000, as membranes are calibrated by means of globular proteins, these values are to be considered only indicative. In the present work the mean molecular mass of HS fractions has therefore been indicated as an apparent molecular mass. The ultrafiltration of each fraction ended when the effluent coming out of the cell looked completely colourless. All fractions were diafiltered on the same membrane with distilled water until the effluent had a conductability of about 30 μS cm^{-1} to eliminate pyrophosphate, concentrated to a final volume of about 10 ml and a final concentration of about 1 mg organic C per ml, and stored at −18°C.

3. Results and discussion

When the negative charge due to the ionisation of silanol groups on the inner capillary surface is not shielded or suppressed by the use of appropriate EOF modifiers or by chemical treatment, HSs are driven to the cathode in spite of their negative charge. In free solution, when there are no interactions with the buffer, these macromolecules migrate as a single symmetric broad band (Fig. 1) and the different fractions obtained by multistage ultrafiltration display very close effective mobilities [7,10]. The same behaviour is obtained in polyether-coated capillaries, where the EOF is suppressed (data not shown). The fact that all fractions display practically the same effective electrophoretic mobilities in free solution suggests that relatively small differences in charge/mass ratios occur within the fairly wide molecular range examined. The relatively small differences in charge might also be masked by enhanced hydration of molecules with higher charge densities, leading to a more or less constant charge-to-mass ratio. The electrophoretic velocity is in fact inversely propor-

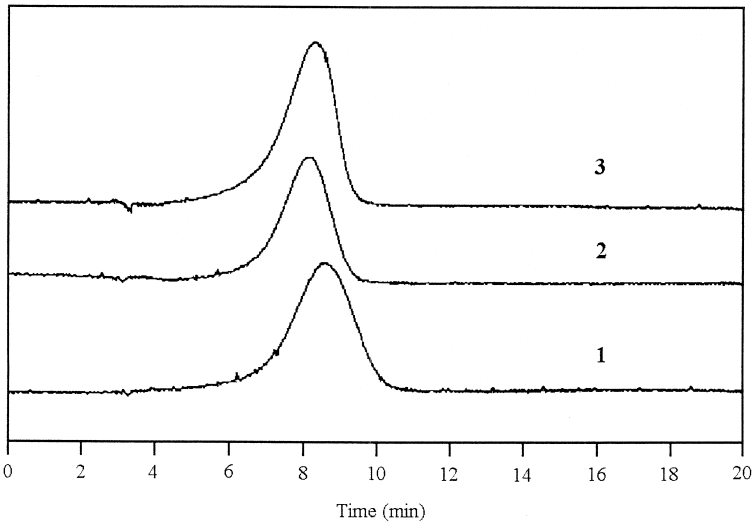

Fig. 1. Electropherograms of HS fractions of reduced molecular mass polydispersity extracted from peat in an uncoated capillary (total length 55 cm, length to the detector 30 cm) filled with 50 m*M* Tris–phosphate buffer, pH 8.3. Mean apparent molecular size of HS fractions: 1=7500; 2=20 000; 3=75 000. Electrophoretic conditions: voltage=+14 kV, current=16 μA. Hydrodynamic injection at the anode (3 s).

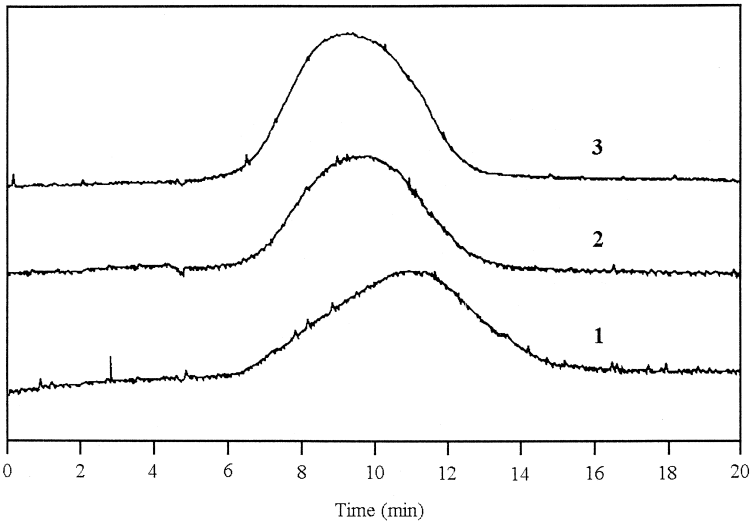

Fig. 2. Electropherograms of HS fractions of reduced molecular mass polydispersity extracted from peat in an uncoated capillary (total length 55 cm, length to the detector 30 cm) filled with 50 m*M* Tris–phosphate buffer, pH 8.3, containing 5% PEG 4000. Mean apparent molecular size of HS fractions: 1=7500; 2=20 000; 3=75 000. Electrophoretic conditions: voltage=+14 kV, current=16 μA. Hydrodynamic injection at the anode (3 s).

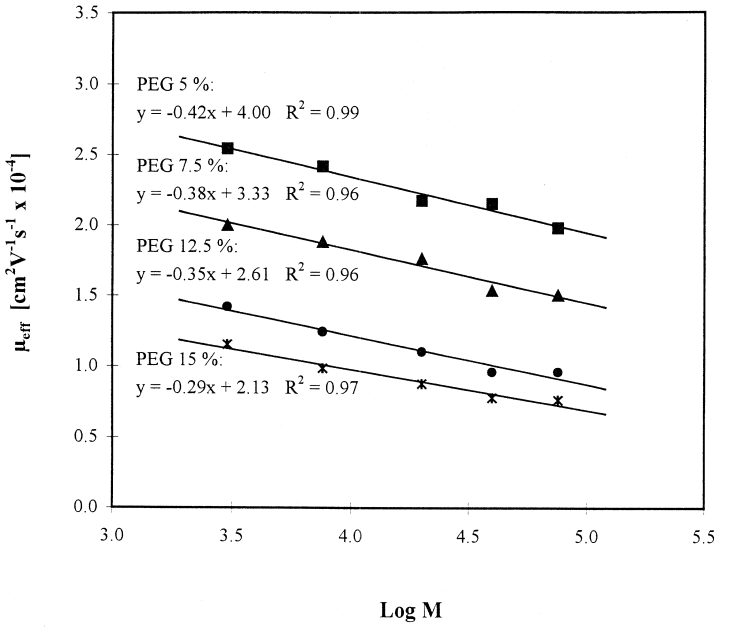

Fig. 3. Relationship between the effective mobilities (μ_{eff}) of HS fractions extracted from a spodosol and the logarithm of the mean apparent molecular sizes (*M*) in an uncoated capillary (total length 55 cm, length to the detector 30 cm) filled with 50 m*M* Tris–phosphate buffer, pH 8.3, containing increasing concentrations of PEG 4000. Electrophoretic conditions: voltage=+14 kV, current=16 μA. Hydrodynamic injection at the anode (3 s).

tional to the effective hydrodynamic size, which includes solvation shells. By increasing the separation efficiency by using a longer capillary (length to the detector 75 cm, instead of 30 cm), it is possible to observe that a relatively slight but regular variation of charge-to-mass ratio actually occurs with molecular size [23].

When the capillaries are filled with a solution containing long-chain PEGs at a concentration above its entanglement threshold, which is around 2.5% for PEG 4000 [13], the mobilities of HS fractions decrease (Fig. 2). In the presence of a physical gel, a strong, inverse linear relationship exists between the effective mobilities and the apparent mean molecular mass of fractions deduced from the cut off limits of the ultrafiltration membranes used for their preparation. This might be explained by the fact that, in the presence of PEG, the translational frictional coefficient could amplify the effect of the differences in molecular size on hydrodynamic mobility. Increasing the polymer concentration (Fig. 3) causes a decrease in the effective mobilities of the different fractions in uncoated capillaries. The efficiency of the separations is, however, rather low and does not improve with the increase of PEG concentration. Nevertheless a close linear relationship between the CZE mobilities and the mean apparent molecular mass of the fractions still exists at all PEG concentrations ex-

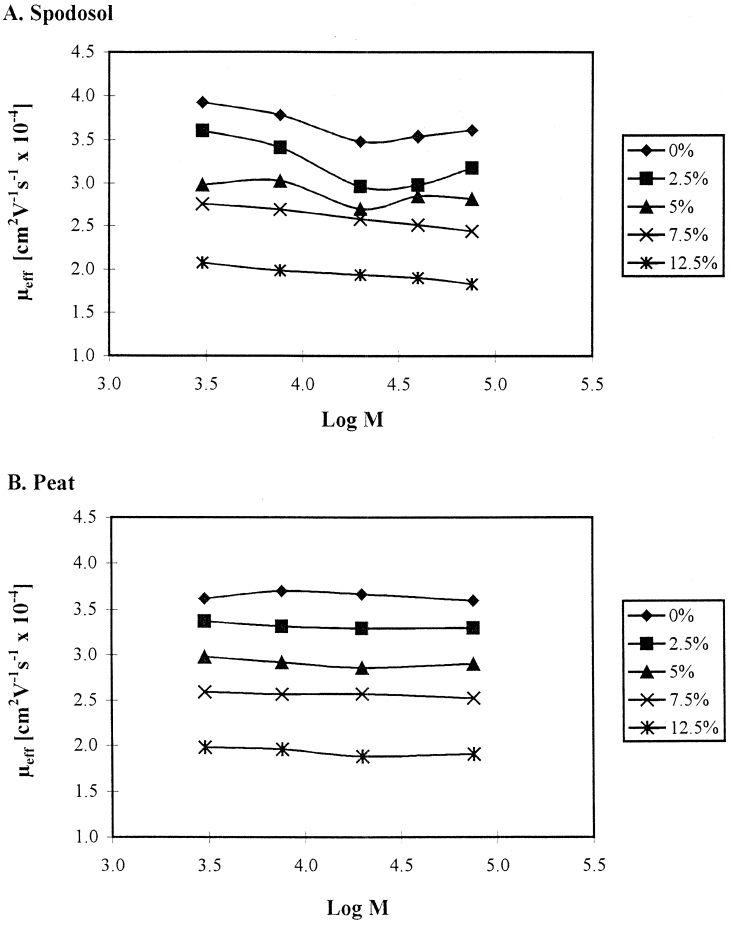

Fig. 4. Effective mobilities (μ_{eff}) of HS fractions extracted from the spodosol (A) and from peat (B) in an uncoated capillary (total length 55 cm, length to the detector 30 cm) filled with 50 m*M* Tris–phosphate buffer, pH 8.3, containing increasing concentrations of PEG 400. Electrophoretic conditions: voltage = +14 kV, current = 16 μA. Hydrodynamic injection at the anode (3 s).

amined. The existence of such relationships in the presence of PEG 4000 and of PEGs of longer chain lengths would suggest that a purely sieving mechanism is involved [24]. However the selectivity coefficient decreases linearly ($r^2 = 0.96$) from 0.45 in a 2.5% gel to 0.29 in a 15% gel for the spodosol fractions and from about 0.20 to 0.11 for the peat fractions. Because the pore size of the matrix deceases with increasing polymer concentrations, this fact suggests that even at 2.5% PEG concentration, HS molecules are excluded from the gel pores and reptate in the space between the entangled polymer chains [15,20].

Another possibility that must be considered is that the separation could be the result of H bonding interactions between HS molecules and PEG. Interactions of other H donor molecules, such as phenol carboxylic acids, with PEG, leading to separations apparently based on the molecular size, have actually been observed in CZE [13,14]. Polyethers, acting as electrostatic electron donors, in fact diminish the effective mobility of negatively charged interacting molecules migrating contrary to the direction of the EOF, whereas they increase migration times for interacting molecules migrating under the only effect of the electric field. This is indeed the behaviour observed, respectively, in uncoated and coated capillaries for HSs, which possess both phenolic and carboxylic groups. To check the mechanism of separation, we studied the behaviour of HSs in the presence of varying concentrations of PEG 400, that, owing to its limited chain length does not cause the formation of physical gels in the concentration range examined. The addition of PEG 400 at concentrations from 2.5% to 12.5% decreases the effective mobilities of all fractions proportionally to the polymer concentration, but at a given PEG 400 concentration all fractions migrate at about the same velocity (Fig. 4). In the concentration range up to 15% the relatively short polymeric chains of PEG 400 do not become entangled as shown by the regular decrease of volumetric flow-rate of the EOF (Fig. 5). On the contrary, PEG 4000 at only 2.5% causes an abrupt change of the EOF flow-rate; after the occurrence of entanglement the decreasing flow-rate becomes again linear with the polymer con-

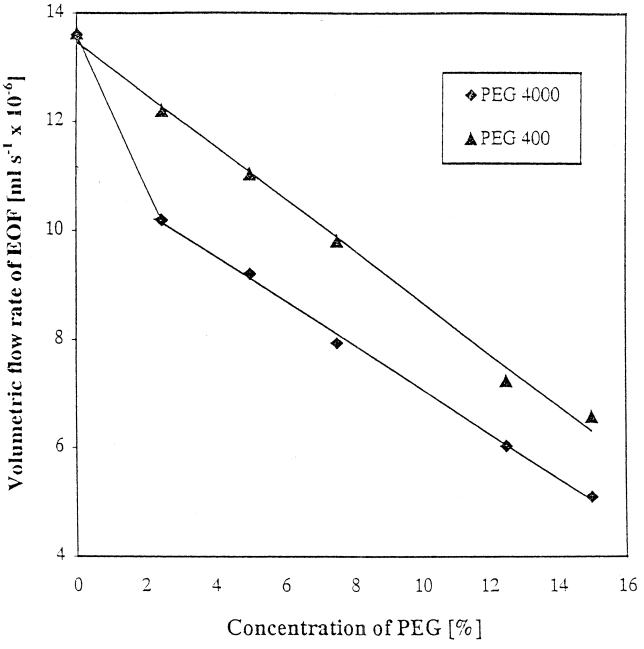

Fig. 5. Volumetric flow-rate of EOF in an uncoated capillary (total length 55 cm) filled with 50 m*M* Tris–phosphate buffer, pH 8.3, and with the same buffer containing increasing concentrations of either PEG 400 and PEG 4000. Electrophoretic conditions: voltage = +14 kV, current = 16 μA.

centration. This seems to exclude the possibility of describing the CZE behaviour of HSs in a PEG matrix in terms of hydrogen bonding interactions. The influence of long chain PEGs appears to be the result of two factors: a decrease in the EOF caused by the corresponding increase in viscosity upon addition of PEG and a sieving effect caused by the formation of a physical gel. In matrices containing PEG 400, where there is no formation of a physical gel [22], the only effective factor appears to be the non specific action of increased buffer viscosity on the EOF. A linear relationship exists in fact between the apparent mobility of HSs and the EOF for both high- and low-M_r fractions in solutions containing different concentrations of PEG 400 whereas the apparent mobilities of the same fractions deviate from linearity in solutions containing PEG 4000 above its entanglement threshold (Fig. 6). The deviation is larger, particularly at low gel concentrations (2.5–5%), for molecules of larger apparent molecular size, which are evidently more strongly retarded in their migration to the anode having a larger probability of contact with the gel network. Similar results were obtained with PEG 6000 and PEG 20 000 (data not reported).

Another UV-transparent and low-viscosity polymer that has proved to produce an efficient sieving of SDS–protein complexes is PVA [18]. Contrary to PEG matrices, where HSs migrate as single peaks, in 2.5% PVA 49 000 the electropherograms of all fractions show two unresolved, but well defined peaks (Fig. 7). A linear inverse relationship (Fig. 8) still exists between the logarithm of the apparent mean molecular mass and the migration time of the second peak, whereas the migration time of the first peak (from 3.6 to 3.9 min) is practically the same for all fractions. The relative abundance of the two peaks changes with the apparent molecular size of the fraction and with sample concentration. The first peak in fact is proportionally larger for fractions of larger molecular size and increases by increasing the sample concentration. It is therefore evident that PVA interacts with HS, possibly via an H-donor mechanism. The separation observed is therefore

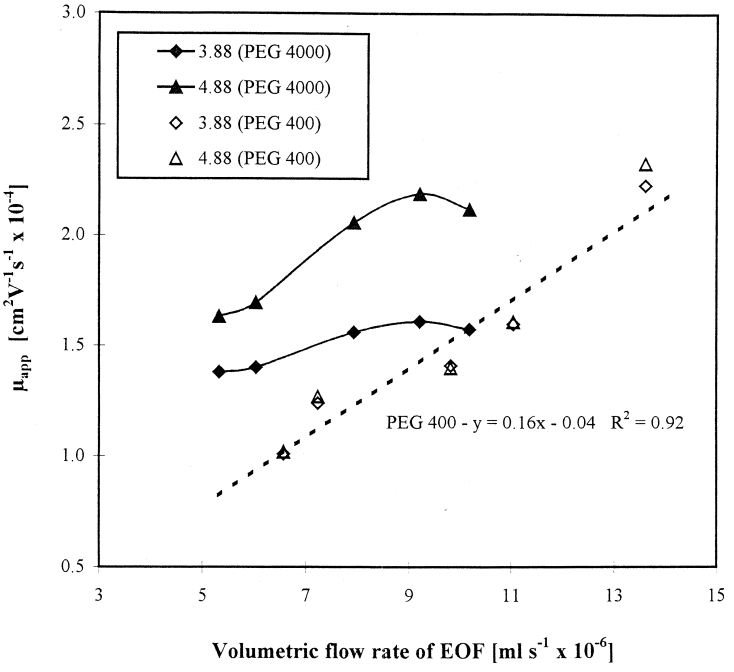

Fig. 6. Relationship between the apparent mobilities (μ_{app}) of spodosol HS fractions of mean apparent molecular sizes 7500 (log M=3.88) and 75 000 (log M=4.88) and the EOF in 50 mM Tris–phosphate buffer solutions, pH 8.3, containing different concentrations of either PEG 400 and PEG 4000. Electrophoretic conditions: voltage=+14 kV, current=16 μA. Hydrodynamic injection at the anode (3 s).

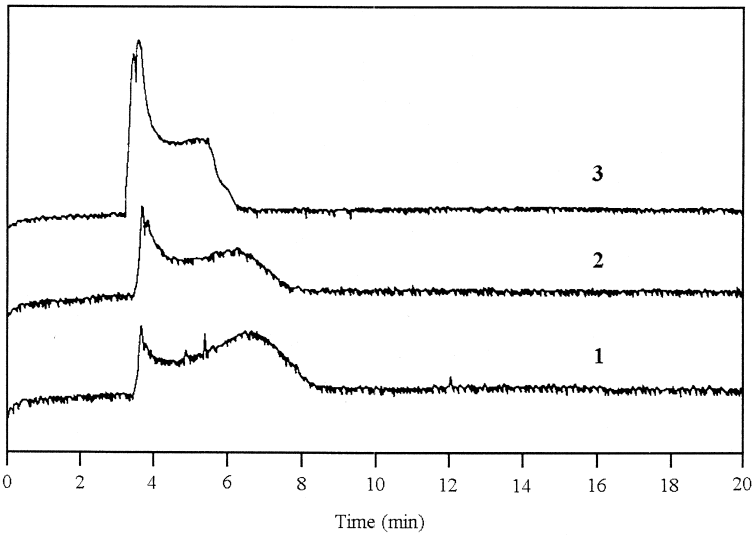

Fig. 7. Electropherograms of HS fractions of reduced molecular mass polydispersity extracted from peat in an uncoated capillary (total length 55 cm, length to the detector 30 cm) filled with 50 m*M* Tris–phosphate buffer, pH 8.3, containing 2.5% PVA 49 000. Mean apparent molecular size of HS fractions: 1=7500; 2=20 000; 3=75 000. Electrophoretic conditions: voltage=+14 kV, current=16 μA. Hydrodynamic injection at the anode (3 s).

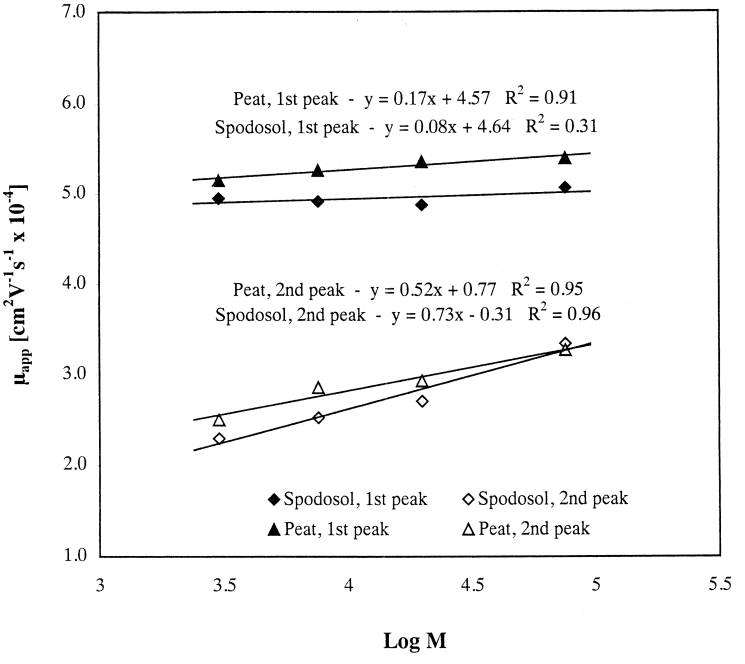

Fig. 8. Apparent mobilities (μ_{app}) of HS fractions extracted from the spodosol and from peat in an uncoated capillary (total length 55 cm, length to the detector 30 cm) filled with 50 m*M* Tris–phosphate buffer, pH 8.3, containing 2.5% PVA 49 000. Electrophoretic conditions: voltage=+14 kV, current=16 μA. Hydrodynamic injection at the anode (3 s).

artefactual and depends only on the relative concentration of HS and PVA. The large number of hydroxide groups on the PVA chain as compared to PEG can be a possible cause for the interaction.

4. Conclusions

The separation of HSs analysed in capillaries filled with physical gels of PEG appears to be size based without contribution of chemical interactions between HSs and PEG. Further work is necessary to ascertain whether the mechanism of separation implies true sieving or reptation. Although the selectivity appears to be rather low, the determination of the molecular size distribution of HSs can be performed, in principle, in either coated or uncoated capillaries filled with entangled PEG solutions. The use of longer capillaries that normally improve the efficiency of the separation is not possible, in this case, as the effect of charge differences would be no longer negligible and the separation would not depend solely on molecular size.

To obtain real molecular mass distributions for HSs a calibration with appropriate M_r standards is, however, necessary. At present such standards are not available for HS, but whenever this problem is solved CE in physical gels can became a convenient method for routine determination of the M_r distribution of HSs.

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