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Potential of capillary zone electrophoresis for estimation of humate acid-base properties

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Abstract

Capillary zone electrophoresis (CZE) has been applied for fractionation and characterization of soil-derived humic acids (HAs). Humic acids from soddy-podzolic (HA_s) and chernozem (HA_{ch}) soils were studied as well as hydrophobic high-molecular-weight (HMW) and hydrophilic low-molecular-weight (LMW) HA_s fractions obtained by salting-out with ammonium sulfate at a saturation of 0–40% and >70%, respectively. The possibility of CZE partial fractionation of HAs has been demonstrated. The shape of "humic hump" was shown to depend on the pH of running electrolyte. Almost the whole peak overlapping occurred if alkaline solutions were used for fractionation, but the peak resolution was improved at pH 5–7. Under appropriate fractionation conditions (pH 7), at least three humic acid subfractions with different electrophoretic mobilities were distinguished in the electropherograms of initial HA and HA_s fractions. Such a high peak resolution has never been achieved for humic acids before. The presence of three subfractions in the HA is in agreement with gel-filtration analysis and was confirmed by comparison of the electrophoretic behavior of HA_s with those of its HMW (hydrophobic) and the LMW (hydrophilic) fractions. The potentiometric titration of HA and its fractions was performed and the pK_a of the functional groups were calculated. An attempt was made for the first time to relate the variation of electrophoretic mobility values with acid-base properties of humic acids. It was shown that changes in the humate charge resulting from the variation of the ionization degree of its functional groups as a function of pH can be estimated on the basis of electrophoretic mobility values. Potential of CZE in estimation of HA isoelectric point was demonstrated. The pH value corresponding to the lowest absolute electrophoretic mobility value of about 20×10^{-5} cm^2 V^{-1} s^{-1} can be used for approximate estimation of HA isoelectric point. The data were discussed and agreement with the random coil structural model has been shown.
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Keywords: Capillary electrophoresis; Humic acids; Isoelectric point; Acid-base properties

1. Introduction

Humic substances (HSs) are ubiquitous natural compounds comprising the major part of stable organic matter in the diversity of environments (soils, sediments and waters). They are a product of the chemical and biological transformation (humification) of plant residues or microbiological synthesis [1] and play an important role in soil formation processes and binding of environmental pollutants. Commonly HSs are subdivided according to their solubility in acidic medium (pH 1–2) into humic acids (HAs) and fulvic acids (FAs). HAs comprise high-molecular-weight (HMW) organic substances that are soluble in alkaline media and insoluble in acidic ones.

Despite a number of publications devoted to HS investigation, their structure and reactivity are not well understood yet. Different structural concepts for HSs can be found in many reports ([1,2] and references cited therein). There is an uncertainty as to whether HSs are macromolecules or are an association of relatively small and heterogeneous molecules self-assembled in supramolecular structures stabilized only by weak forces, such as dispersive interactions and hydrogen bonding [3]. Generally HSs are considered as complex heterogeneous and polydisperse mixtures or associations of relatively high-molecular-weight polyelectrolyte-like molecules containing aromatic rings and aliphatic chains with O-, N- and S-containing functional groups

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[4] such as carboxylic, phenolic, hydroxyl and carbonyl ones. The flexible, expanding, random coil model was proposed for HA structure in solution [1]. It considers the humate molecules as long strands that are able to coil randomly. Molecules may be tightly or loosely coiled depending on the conditions (molecule charge density, pH, ionic strength, types of counter ions and solvent) and form structures of roughly spherical shape with Gaussian distribution of molecular weight around the centre.

To provide more complete information about HS and their fractions a combination of various techniques based on different properties of HAs should be used for their fractionation and characterization such as gel permeation chromatography (GPC), hydrophobic interaction chromatography, membrane micro- and ultrafiltration, electrophoresis, isotachophoresis, isoelectric focusing, solvent extraction and others [2]. Advantages of capillary electrophoresis technique include the capabilities to work with very small amounts of sample, to separate natural organic matter in aqueous solutions within the pH range, close to environmental conditions, and to obtain information on their charge based on the electrophoretic mobility data ([2,5] and references cited therein, [6]). To date capillary electrophoresis [6–13] and capillary isoelectric focusing [14,15] were used mainly for HA fingerprint characterization. Some studies have been devoted to quantitation, assessment of changes in electropherogram pattern depending on the molecular weight of HSs fractionated by multistage ultrafiltration [13,16,17] or GPC [18,19].

The main properties that affect HS reactivity in environmental compartments are their size and charge distribution, which in turn govern their hydrophilic/hydrophobic balance [20]. These data are important for understanding the mechanisms of HS interactions with mineral and organic compounds and predicting HS behavior in natural processes. De Nobili et al. [16] showed that the effective electrophoretic mobility is inversely proportional to the molecule size of HS fractioned by ultrafiltration when capillary zone electrophoresis (CZE) performed in background electrolyte containing long-chain polyethylene glycols at concentrations above their entanglement threshold. The agreement between the effective electrophoretic mobility and molecular size of soil humic acids was found [18] on the basis of CZE investigation of humic fractions isolated by combining polyacrylamide gel electrophoresis (PAGE) with size-exclusion chromatography (SEC). Significant differences in structural characteristics of HS fractions were shown [21] when combinations of PAGE with infrared spectroscopy and GC–MS studies were used. It was shown that plots of UV-absorbance vs. electrophoretic mobility look like Gaussian distributions around an average electrophoretic mobility which in turn depends on humic structure and experimental conditions [5]. No attempts were made to relate the variation of electroporetic mobility values with HA acid-base properties. These properties are determined by the pK_a values of functional groups in HA composition. Differences in HA charge due to variation of ionization degree of functional groups with pH should result in differences in electrophoretic mobility values.

The aim of the present work was to study the applicability of CZE for fractionation and characterization of acid-base properties of humates extracted from soddy-podzolic (HA_s) and chernozem (HA_{ch}) soils, to estimate the influence of pH on the HA charge, and to try to make a some step towards the elucidation of the humate structure. Two HA mentioned above were characterized earlier by different methods [22–24]. The elemental composition, the spectroscopic characteristics, the functional group concentration, dissociation constants and molecular-weight distribution were determined. We imply that the comparison of the data resulting from different techniques allows obtaining the more reliable estimation of CZE potential in humate investigation.

2. Experimental

2.1. Materials and chemicals

HA from soddy-podzolic (HA_s) and chernozem (HA_{ch}) soils were used [22–24]. Prior to experiments HA_s were transferred to a water-soluble NH_4^+ form by dissolution in 10% NH_4OH followed by their evaporation on a water bath at 40 °C. High-molecular-weight/hydrophobic and low-molecular-weight/hydrophilic fractions of HA_s were isolated by salting-out with $(NH_4)_2SO_4$ at pH 7.0 using 0–40% and >70% saturation respectively as described in Ref. [24]. Molecular-weight distributions of initial HA_s and its fractions were obtained by GPC as described in Ref. [23].

NaOH, $(NH_4)_2SO_4$, NH_4OH, HCl, Na_2HPO_4 and NaH_2PO_4 utilized for preparing the solutions were of analytical reagent grade (Merck, Darmstadt, Germany). Deionised water (Milli-Q; Millipore, Bedford, MA, USA) was used throughout all the experiments.

2.2. Instrumentation and procedures

Capel-105 capillary electrophoresis system (Lumex, St. Petersburg, Russia) equipped with an on-column UV detector with deuterium lamp-P 701 and UV monochromator operating in a wavelength range of 185–400 nm was used. CHROM&SPEC software with an MS Windows 95/98/NT interface was used. Uncoated fused-silica capillaries of 75 μm i.d. and 360 μm o.d. with a total length of 60 cm and an effective length of 50 cm were used. All separations were performed with the power supply set for positive polarity at applied voltage of 20 kV. The temperature was maintained at 25 ± 0.1 °C during all runs. Hydrodynamic injections of samples by a pressure of 20 mbar were performed at the anode end of the capillary. Triple injection was performed by successive injections of 4% acetone as an electro-osmotic flow marker (5 s), a running electrolyte (5 s) and a humate sample dissolved in a running electrolyte (5 s). Two wavelengths of 220 and 254 nm were selected for peak detection. The highest absorbance value was reached at 220 nm, however, a noticeable noise was shown to appear in this case. The capillary was rinsed with 0.1 M NaOH (5 min) and then with water (5 min) before the first daily experiment and after the last one; it was conditioned by rinsing with the running electrolyte (5 min) before runs. Vials were refilled with a fresh portion of the electrolyte after each run.

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3. Results and discussion

3.1. Electrophoretic separation of initial HA

The effect of pH on the electrophoretic behavior of HA_s and HA_{ch} was studied. Phosphate buffer with a concentration of 5.0 mM and pH ranging between 3 and 11 was used as a running electrolyte. Such buffer concentration is optimal and is a compromise between two opposite effects. On one hand, the lowest buffer concentration (ionic strength) should be preferred to provide the highest charge density under the given conditions and to prevent the humate association. On the other hand, our preliminary experiments showed that at a buffer concentration less than 5 mM the migration time reproducibility became worse for lack of a buffer capacity. The choice of the separation buffer type is of importance for interpretation of the electrokinetic behavior of the humates studied. Buffer ions should not interact with sample constituents. With acetate, phosphate and carbonate buffer systems, HA was shown to give similar electropherograms with "humic hump" [12]. With a borate buffer system, additional peaks can appear in electropherograms due to complexation of borate with 1,2- and 1,3-diol groups in HA depending on the pH and concentration of tetraborate ions [10,12,25,26]. Phosphate buffer system was preferred for investigation of humate electrophoretic behavior because it enables the separation over a wide pH range to be performed. As an example Fig. 1 shows electropherograms of the initial HA_s. The HA_s "humic hump" shape was found to change with pH. When alkaline or acidic buffers are used as a running electrolyte, HA_s gives typical electropherograms with one broad and smooth peak, whereas the tops of two or three peaks appear on a "humic hump" in the pH range between 5 and 7. Similar influence of pH on the "humic hump" shape was discovered when the initial HA_{ch} was tested, although the peak resolution was worse in this case.

The peaks in electropherograms were suggested to arise due to three HA subfractions (A, B and C) differing in their size, hydrophobicity and structure. Each subfraction can contain several compounds with close electrokinetic behavior which cannot be separated by CZE. The average absolute electrophoretic mobilities (μ_{ep}) corresponding with the peak tops in HA electropherograms were calculated taking into account the electro-osmotic mobility (μ_{EOF}) changes as follows:

$$|\mu_{ep}| = \mu_{EOF} - \mu_{app}, \quad \mu_{app} = \frac{V_{app}}{E} = \frac{L_d L_t}{U t_m}$$

with μ_{app} is the apparent average electrophoretic mobility, V_{app} the apparent migration rate, E the electric field strength, L_d the length of the capillary to the detector, L_t the total length of the capillary, U the applied voltage and t_m is the migration time.

The respective curves are presented in Fig. 2. Change of 5.0 M phosphate buffer (pH 7.0) for 5.0 mM acetate buffer (pH 7.1) did not influenced noticeably on the migration behavior of humate subfractions. For example, the average absolute electrophoretic mobilities of A, B and C HA_s subfractions were equal to 28×10^{-5}, 34×10^{-5} and 38×10^{-5} $cm^2\,V^{-1}\,s^{-1}$, respectively. The general regularity is observed: decreasing the absolute electrophoretic mobility of HA with decreasing pH. It is in accordance with decreasing the concentration of humate anions in the same pH range. According to the random coil model [1] we suggested also that an additional contribution to the electrophoretic mobility value may be due to conformation

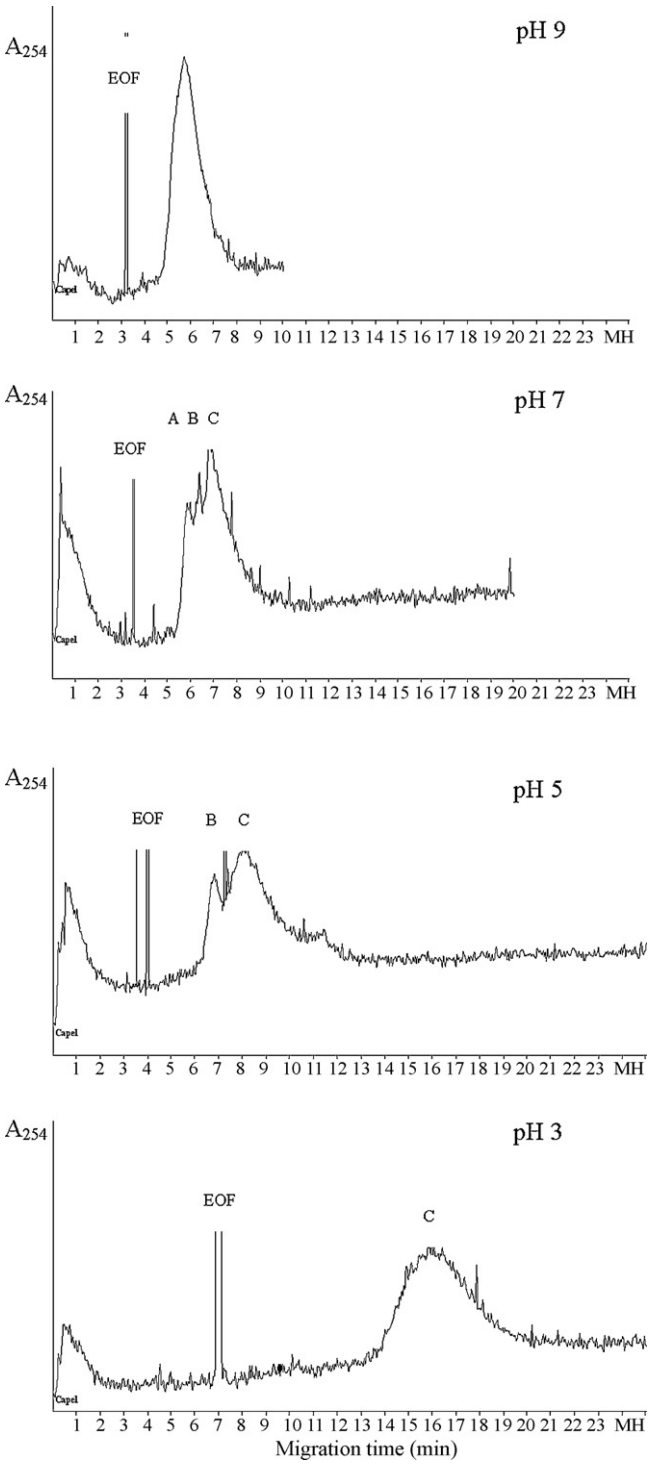

Fig. 1. Effect of pH on the electrophoretic behavior of initial HA_s. Running electrolyte: 5.0 mM phosphate buffer; electric field strength: 330 V/cm; injection pressure: 20 mbar; injection time: 5 s; wavelength: 254 nm; EOF: electro-osmotic flow.

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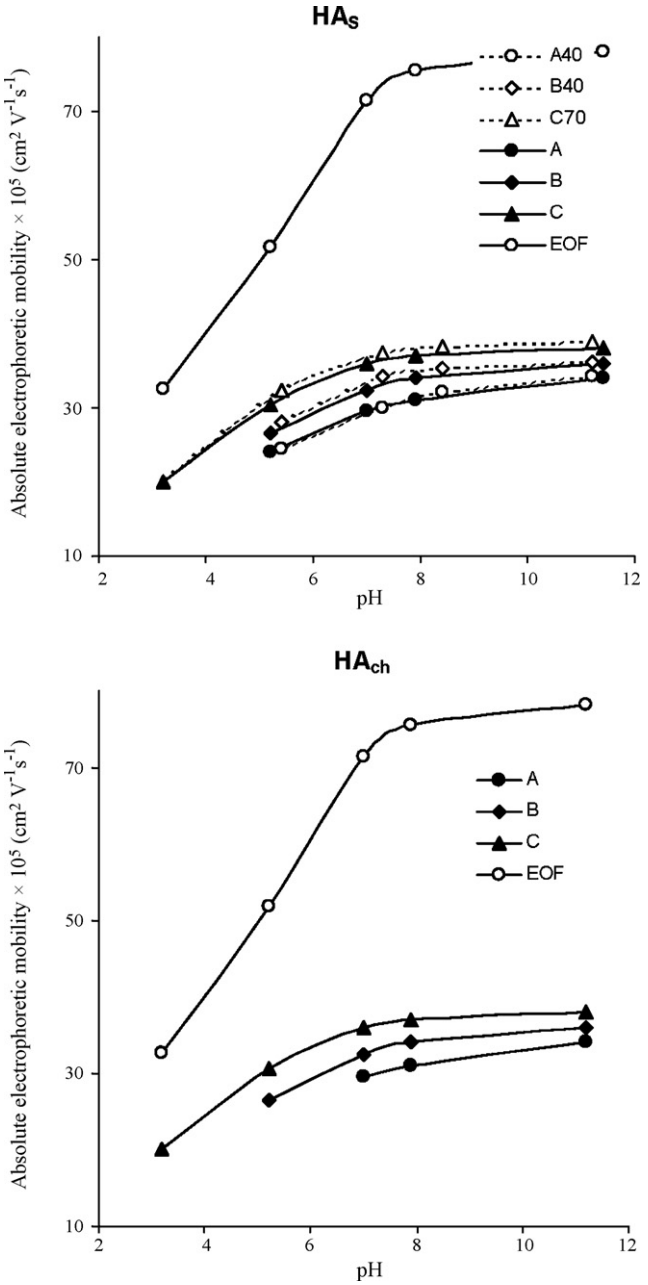

Fig. 2. Effect of pH on the humate absolute electrophoretic mobility. A, B and C: subfractions of initial HA; A40, B40: HMW (hydrophobic) HA_s fraction isolated at 0–40% of $(NH_4)_2SO_4$ saturation; C70: LMW (hydrophilic) HA_s fraction isolated at >70% of $(NH_4)_2SO_4$ saturation; EOF: electro-osmotic flow. All conditions as in Fig. 1.

changes of humate molecules depending on electrolyte pH. With a pH decrease humate coil becomes tighter, its size reduces. It is known that a charge decrease usually results in a decrease of electrophoretic mobility whereas the tendency of electrophoretic mobility alteration with size is uncertain. Therefore, we cannot say anything about the sign of this contribution. Taking into account that the tendency of electrophoretic mobility alteration is in accordance with that of dissociation degree of humate functional groups we think that it is not noticeable in the pH range studied.

3.2. Electrophoretic separation of HA_s fractions salted out with $(NH_4)_2SO_4$

To confirm our suggestion about the occurrence of three subfractions in the HA composition, the electrokinetic behavior of HMW (hydrophobic) and LMW (hydrophilic) HA_s fractions isolated at 0–40% and >70% of $(NH_4)_2SO_4$ saturations respectively was studied. Only a single peak appeared in the electropherograms of the LMW fraction over the whole pH range studied whereas two peaks appeared in those of the HMW fraction in the pH range between 5 and 7. The electrophoretic mobilities corresponding with the each peak in electropherograms of HA_s fractions were calculated. The curves $|\mu_{ep}| = f(\text{pH})$ of HMW subfractions were shown to almost coincide with ones of subfractions A and B of initial HA_s, whereas the curve of LMW subfraction coincides with one of subfraction C of initial HA_s (Fig. 2). The comparison of electropherograms of initial HA_s with those of its fractions allowed us to conclude that the peaks A, B and C arise from different humate subfractions. Variation of the number of peaks in the electropherograms results from resolution changes caused by the influence of the pH value on the relative peak position but it is not an artifact. Thus, our suggestion about the presence of three subfractions in the HA_s composition has been confirmed. Besides, the similarity of electrophoretic behavior of the HA_s subfractions (A, B and C) before and after HA fractionation by salting-out with $(NH_4)_2SO_4$ allows to conclude that this procedure has not noticeably affected the humate nature.

3.3. Comparison of CZE data with fractionation of HA_s by GPC

The conclusion about the occurrence of three subfractions in the HA_s revealed by CZE is in accordance with the results of HA_s fractionation by GPC. Gel-chromatographic profile of initial HA_s on Sephadex G-100 gel consisted of three fractions: a high-molecular-weight fraction (MW > 150 kDa) eluted with a void volume, a middle-molecular-weight fraction (MMW, MW 72 kDa) and a low-molecular-weight fraction (MW 8 kDa). Gel-chromatographic profile of HA_s fraction isolated at 0–40% of $(NH_4)_2SO_4$ saturation consisted of two peaks: HMW with MW of >150 kDa and MMW with MW of 72 kDa. Gel-chromatographic profile of HA_s fraction isolated at >70% of $(NH_4)_2SO_4$ saturation consisted of only one LMW peak with molecular weight of 15 kDa (Fig. 3). It should be noted that although we have followed strictly the salting-out procedure described in [24] the results of GPC revealed that the apparent molecular weight of HA_s fraction isolated at >70% of $(NH_4)_2SO_4$ saturation was lower than reported previously [24] and it was free of HMW impurities.

The GPC data together with CZE results allow us to consider the humates as a mixture of high-molecular-weight electrolyte molecules. Such mixture can be separated into subfractions with similar properties. This conclusion is in accordance with the random coil model [1]. It should be noted that the hydrophilic LMW fraction containing mainly subfraction C possesses a

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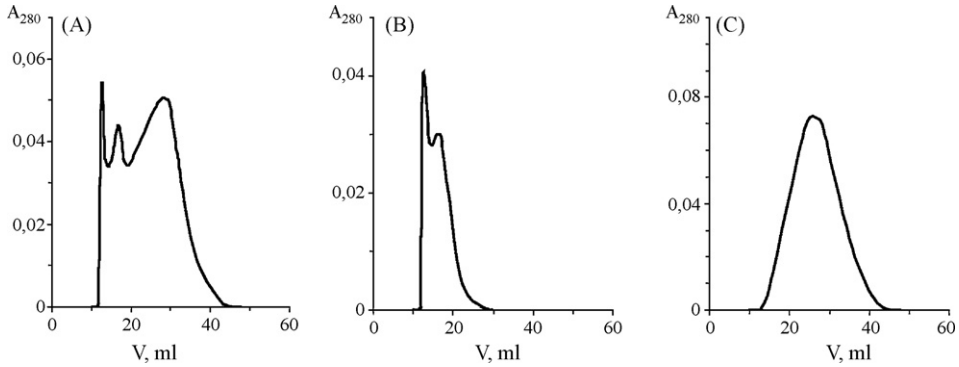

Fig. 3. Molecular-weight distribution of initial HA_s (A) and its fractions isolated at 0–40% (B) and >70% (C) of $(NH_4)_2SO_4$ saturation. Fractionation by GPC; column: 60 cm × 0.9 cm, Sephadex G-100 gel; eluent: 0.025 M Tris–HCl (pH 8.2) with 0.05 M NaCl and 0.1% SDS; elution rate: 3 ml h^{-1}.

higher average electrophoretic mobility in comparison with the hydrophobic HMW fraction containing mainly less mobile subfractions A and B. Thus, under the same conditions the components of hydrophilic LMW fraction bear a higher negative charge.

3.4. Estimation of isoelectric point

It was found that during the pH decreasing step precipitation of HA fractions occurred, as soon as their average absolute electrophoretic mobility achieved a value of less then 20×10^{-5} cm^2 V^{-1} s^{-1}. It means that there is a proper pH value for each HA subfraction corresponding with the electrophoretic mobility value indicated above. We tried to understand whether this characteristic pH value could be used for estimation of humate isoelectric point. Earlier [22] the potentiometric titration of initial HAs was performed and pK_a values of the humate functional groups were calculated. They are 4.6, 7.7, 9.8 and 4.7, 8.1, 9.9 for HA_s and HA_{ch}, respectively. We determined the pK_a of functional groups of HMW and LMW fractions as well. The differences of pK_a corresponding to the same type of functional groups ranged from 0.2 to 0.6 for two fractions. This fact lends further support to De Nobili et al. [21] conclusion that there are differences in structural characteristics of humate fractions. Based on these pK_a values the pH ranges were determined within which HA are present as anions. This pH range was shown to coincide with one where decreasing HA absolute electrophoretic mobility was observed. The anion concentration corresponding to the least pH value equal to 3 at which electrophoretic mobility of the subfraction C can be determined yet has not exceeded 4%. The value of pH 3 seems to be close to a pH value at which the fastest subfraction C losses the charge. This value can serve as approximate estimate of the isoelectric point of C.

3.5. Estimation of functional group content

It was shown earlier that HA_{ch} (in comparison with HA_s) contains a larger number of carboxylic and other functional groups and lower number of aliphatic carbon chains bearing $\equiv CH$, $=CH_2$, $-CH_3$ groups (assessed by the H/C ratio). HA_{ch} is the more aromatic one characterized by the higher absorbtivity values at 465 and 650 nm. Average molecular weight and relative hydrophobicity (estimated by means of hydrophobic interaction chromatography and extraction in aqueous biphasic systems) of HA increase in the order $HA_{ch} < HA_s$. We compared the absolute electrophoretic mobilities of A, B and C subfractions of two HA studied at the pH values equal to their pK_a values. Under these conditions of about 50% HA are dissociated. As shown in Fig. 4 the HA_{ch} possess the higher absolute electrophoretic mobility within the whole pK_a range. This regularity is in accordance with the data pointed above. Thus, the electrophoretic mobility values obtained at pH = pK_a allow to compare the functional group contents in humates and their fractions. The higher the electrophoretic mobility the higher is the content of negatively charged functional groups in a sample. The

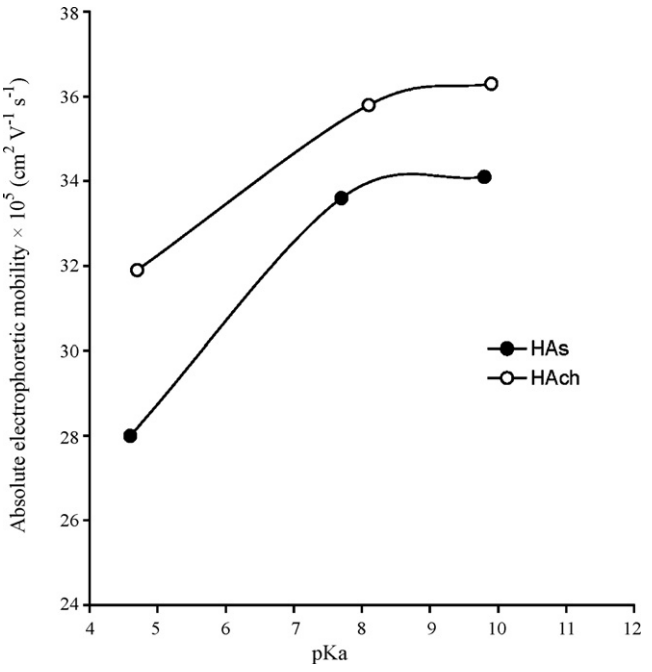

Fig. 4. Effect of HA type on absolute electrophoretic mobility: pH = pK_a. All conditions as in Fig. 1.

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course of curves μ_{ep} vs. pH showed that the molecule charge reduces with pH decreasing that may be due to the dissociation of functional groups. The highest electrophoretic mobility value of C subfraction at the lowest pH confirms the highest hydrophilicity of LMW fraction. This fact is in agreement with the conclusion about significant increase of carboxylic group content in humic acid molecule with decreasing its molecular weight [1,27]. It is important to notice that the inflexions are observed on these curves near pH 5 and 8. Accordingly to humate pK_a [6] determined on the basis of potentiometric titration data, they locate near the pH values at which the number of kinds of dissociated functional group changes that results in a alteration of the migration behavior. In the pH interval mentioned simultaneous dissociation of all kinds of functional groups occurs that provides the favorable conditions for optimal resolution of subfractions.

4. Conclusions

The results presented show the potential of CE for the fractionation and the characterization of acid-base properties of HA. The shape of "humic hump" was shown to depend on the pH of running electrolyte. Under appropriate separation conditions three peaks due to at least three humic subfractions possessing different average electrophoretic mobilities were distinguished in the electropherograms of initial HAs and HA_s fractions. These mobility differences can be due to variation of acid-base properties and contents of functional groups belonging to different HA subfractions. The average absolute electrophoretic mobility of each subfraction was shown to decrease with the decreasing pH. The pH value corresponding to the lowest absolute electrophoretic mobility value of about $20 \times 10^{-5}\,cm^2\,V^{-1}\,s^{-1}$ and the precipitation start can be used for approximate estimation of HA isoelectric point. The electrophoretic behavior of humic acids and their subfractions was compared and interpreted in terms of variations in their acid-base properties and contents of functional groups. Changes in the humate charge resulting from the variation of the ionization degree of its functional groups as a function of pH can be estimated as well on the basis of electrophoretic mobility values. Comparison of CZE and GPC data allowed to conclude in support to the random coil structural model.

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