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Electrophoretic aggregation of humic acid

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Abstract

Capillary electrophoresis (CE) has been used to characterize humic acid (HA) aggregation. It was found that when pumping HA solution through the capillary at a constant flow rate with no electric field, the number of spikes could be reduced by filtration of the solution. Applying high voltage (30 kV), the amount of spikes increased again. This is associated with the formation of new aggregates caused by electric field. Aggregation is influenced by the concentration of HA, applied voltage and presence of imidazole in the solution. It is supposed that this phenomenon is characteristic of all solutions that can form colloidal particles: in β-cyclodextrin solution, similar spikes appear in a high-voltage electric field.

Keywords: Aggregation formation; Humic acids

1. Introduction

Humic substances (HSs) are found in soil, lake and sea sediments, peat, natural waters and other natural materials as a product of degradation of organic matter [1–3]. HSs are believed to be complex heterogeneous and polydisperse mixtures of nonstoichiometric composition [4]. Due to the enormous number of different precursor materials and the complexity of the degradation processes, an exact structural formula of HSs cannot be drawn. Humic acid (HA) is defined as a fraction of the HSs that is not soluble in water below pH 2, but becomes soluble at higher pH [5].

HAs behave as supramolecules that are able to polymerize (oligomerize), aggregate, form micelles and might form supramolecular ensembles with other compounds [6,7]. Interpretation of the obtained signals of HA with any analytical approach may appear difficult because of the high polydispersity in the structure of these materials [4,8].

Capillary electrophoresis (CE) is one of the recent methods for the characterization of HA [6,8–11]. In general, on the electropherograms of HA, two (or sometimes multiple) "humps" are observed [4,12,13]. Frequently, on these "humps", multiple randomly scattered sharp peaks ("spikes") appear (see, e.g. electropherograms in papers [6,8,11,13–15]). Usually, in CE, spikes can be associated with air bubbles in the buffer and as artefacts they are of no interest. It has also been suggested that the spikes correspond to the defined compounds present in all HA which are liberated from the supramolecular structure of the HA by the action of different buffer constituents (e.g. boric acid [6,9,10], phosphate [6], cyclodextrins (CD) [13]). Also, it has been proposed that the peaks obtained with boric acid buffer represent individual monomeric fragments of HA [9] or presence of aggregates in the unfiltered buffer solutions [4]. In most cases, the observed spikes are considered artefacts and are usually neglected. So, the nature of spikes in HA electropherograms is never well explained.

Likely, many different mechanisms may result in the appearance of spikes. Recent studies of CE of bacteria associate spikes, on bacterial electropherograms, unanimously with the formation of aggregates in the CE buffer under applied voltage. It was demonstrated by visual observation of the process of clustering of the scattered individual fluorescencing bacteria into a large aggregate using illumination of the whole separation space in capillary by laser [16,17]. However, bacterial aggregates provide good insight to understanding aggregation phenomenon in electric field. First, the appearance of spikes on electropherograms indicates (as one cause) the presence of aggregates. Second, the addition of

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some components (like imidazole) into HA solution changes presumably surface chemistry (like polyethylene oxide in the case of bacterial solutions [16,17]). These studies have demonstrated that CE could be a powerful method to investigate aggregate formation in solutions under an electric field.

CE measurements have confirmed the micellar properties of HSs. Schmitt et al. [18] determined the concentration at which the HSs associates behaved like ionic micelles. Those micellar associates are probably responsible for aggregation phenomenon between the HA molecules.

Lee and Lin [19] have found that the best for separation of metal cations was achieved with UV-absorbed component (imidazole) and complexing agent (different carboxylic acids). We suggested that HSs (in the structure are many carboxylic groups) could also act as complexing agents together with imidazole in separating metal cations. However, the interpretation of the recorded electropherograms failed because of many randomly distributed spikes appeared. Frequency of the appearance of spikes depended on the applied voltage and on the concentration of the HA in the buffer. Also, a clear dependence on the sample preparation conditions and buffer composition was evident. For this reason, the properties of imidazole–HA buffer were investigated. In this study, an attempt was made to investigate the phenomenon of spike formation in a more systematic manner.

2. Experimental

2.1. Equipment and capillary treatment

All CE experiments were performed with an ISCO CV^4 Capillary Electropherograph model 3850, which was cooled with forced air by fan. The bare fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) had a total length of 80 cm (48 cm to the detector). The capillary (75 μm i.d.) was rinsed for 5 min with 0.1 mM NaOH, Milli-Q water (Millipore) and working solution at the beginning of experiments each day and between the runs. Detection wavelength was at 226 nm. Experiments were performed at room temperature.

Measurement procedure consisted of flushing the working solution through the capillary first by applying vacuum (∼0.5 MPa) at the outlet of the capillary. The working solution was pumped through the capillary at a constant rate, using two linear flow rates 0.64 or 3.2 cm s^{-1}, correspondingly. The UV absorbance signal of the solution was simultaneously recorded. After a certain time interval (about 10–11 min), high voltage was switched on and a change in UV signal pattern was recorded. The UV detector signal was digitized by "Mini-16" analogue to digital converter (Keithley, Mertabyte, Taunton, MA, USA) and stored on Pentium PC hard disc for later processing by the procedures written in-house in Matlab (MathWorks, Natick, MA). During all the experiments, the current values were in the range of 3–23 μA.

Since the velocity of the electroosmotic flow (EOF) increases linearly with the applied voltage, the apparent frequency of spikes increases even if the concentration of possible absorbing species remains unchanged when voltage increases. To obtain a quantitative measure of absorbing species concentration as a function of applied voltage and correct results for the EOF influence, the number of spikes, n, the height of which exceeded the preset threshold, was counted after a fixed time interval Δt. The threshold value was three times the baseline noise standard deviation. This number was averaged against the overall measurement time. Then, the aggregates concentration can be estimated as follows:

$$C_{\text{aggr}} = \frac{n}{(F_{\text{EOF}} + F_{\text{pump}})\Delta t} \quad (1)$$

where F_{EOF} and F_{pump} are EOF and pump flow velocities. Buffer bulk velocity under the applied vacuum (without applied voltage), net EOF velocity and F_{total}, total velocity (EOF and bulk) were estimated by using of nitromethane as marker compound. Electrophoretic mobility was calculated according to Schmitt-Kopplin et al. [10]. From the results of EOF flow rate measurements, the calculated EOF mobility was equal to $(1.44 \pm 0.02)\cdot 10^{-3}$ cm^2 V^{-1} s^{-1}.

2.2. Materials

Imidazole (99%, ACS grade) and β-CD (analytical-reagent grade) were purchased from Sigma; NaOH from BDH (UK) and HCl, analytical grade, from Riedel-de Haën (Germany).

The HA was extracted from the sediment of the Baltic Sea (Haapsalu Bay, Estonia) [20]. Natural sediment (water content, 60–70%) was extracted with 0.2 M NaOH (one part of dry mass of sediment to six parts of 0.2 M NaOH) at room temperature for 5 h under nitrogen gas. The suspension was centrifuged at 6000 × g for 30 min. The alkaline extract obtained from the sediment was acidified to pH 2, by addition of 6 M HCl. This solution was allowed to settle about 20 h and then centrifuged at 6000 × g for 15 min, the supernatant discarded. The sediment was repeatedly washed with distilled water and centrifuged, discarding the supernatant until Cl^- free (control with $AgNO_3$). Solid residue was treated with 96% ethanol to remove hymatomelanic acid and centrifuged. This treatment was repeated until the ethanol solution became colourless. Solid residue (gel, contains only HA) was collected and stored at 4 °C. In present study, the non-lyophilised HA was used to avoid changes in their molecular structure. Stock solution of HA was prepared by weighing a given amount of HA gel and diluting it to a fixed volume using Milli-Q water. The value of pH was adjusted with 0.2 M NaOH solution to pH 7. Colloidal stock solution was stored for more than 10 days at 4 °C.

The HA fraction have 33.0% of ash in dry mass and 50.3% C, 7.5% H, 7.2% N, 35.0% O in organic matter [20].

Table 1
Working solutions composition

Solution	HA concentration ($mg\,ml^{-1}$)	Imidazole concentration (mM)	Filtration
A	0.05	0	No
B	0.05	10	No
C	0.05	10	Yes
D	0.05	0	Yes
E	0.2	10	No
F	0.2	10	Yes

2.3. Preparation of working solutions

All working solutions were prepared by diluting the HA stock solution with Milli-Q water and adding imidazole to obtain 10 mM concentration, pH was 8–9. From six prepared working solutions, three were filtered through 0.45 μm biocompatible, sterile filter (Sarstedt, Germany). Solutions composition and filtering conditions are listed in Table 1. Additionally, the working solutions containing imidazole (10 mM) and β-CD (0.2 mM, 15 mM) were prepared.

3. Results and discussion

The typical wavelength for the determination of HSs in CE is 254 nm. However, in many occasions, different other wavelengths are used [8,18,21–23].

We have been traditionally interested in determination of metal cations and metal/HS complexes. For that reason, we used 226 nm in CE experiments. The same wavelength was used in the described experiments to get comparable results. Lower wavelengths (226 nm) may generate somewhat stronger intensities than those at 254 nm [24].

When an unfiltered HA solution A was pumped through the capillary at a constant flow rate without applying voltage, the UV detector signal from some of the working solutions revealed several spikes (Fig. 1). It follows from Fig. 1 that filtration (solutions C, D and F) reduced the number and intensity of the spikes. The dependence of results on HA concentration indicate trivially that the appearance of spikes from unfiltered solutions can be associated with the presence of possible aggregate particles in the HA solution which either absorb or scatter UV light from the detector. Further, basic imidazole possibly increases considerably the solubility of HA in water and reduces aggregate size, which is confirmed by a significant reduction of the amount of spikes on the filtered solution B signal.

Next, when the high voltage +30 kV was applied to the solution A bulk flow in capillary, the frequency of the spikes increased to some extent, starting from the moment when voltage was applied (see Fig. 2, solution A, between 10 and 25 min). However, this increase can be fully associated with the effect of the increased flow velocity of the solution due to the EOF. Indeed, measuring the mean number of spikes over a time span from 0 to 10 min (pump only on, voltage not applied) and from 15 to 25 min (pump and voltage, both applied) with 1 min increment (10 determinations over the interval $\Delta t = 1$ min) results in $n_{pump} = 15.3 \pm 2.2$ and $n_{EOF+pump} = 25.2 \pm 6.8$, where n_{pump} and $n_{EOF+pump}$ are the mean numbers of spikes with voltage off (from 0 to 10 min) and voltage on (from 10 to 25 min), respectively. Flow rates were calculated as:

$$F_{pump} = \tfrac{1}{4} v_{pump} \pi d^2 \quad (2)$$

$$F_{total} = \tfrac{1}{4} (v_{pump} + v_{EOF}) \pi d^2 \quad (3)$$

The flow rates for +30 kV were $F_{pump} = 2.83 \cdot 10^{-7}\ ml\,s^{-1}$ and $F_{total} = 5.03 \cdot 10^{-7}\ ml\,s^{-1}$.

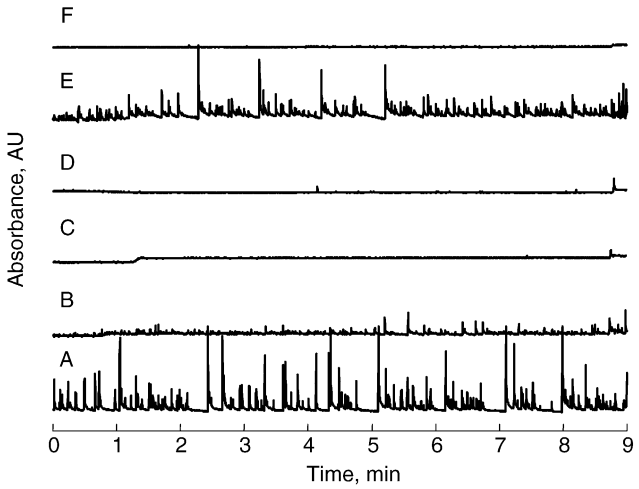

Fig. 1. UV absorbance signals using pump at constant rate (fused-silica capillary, 80 cm × 75 μm, 0.17 ml min^{-1}). No high voltage applied. A, B, C, D, E and F correspond to the different solutions. UV at 226 nm.

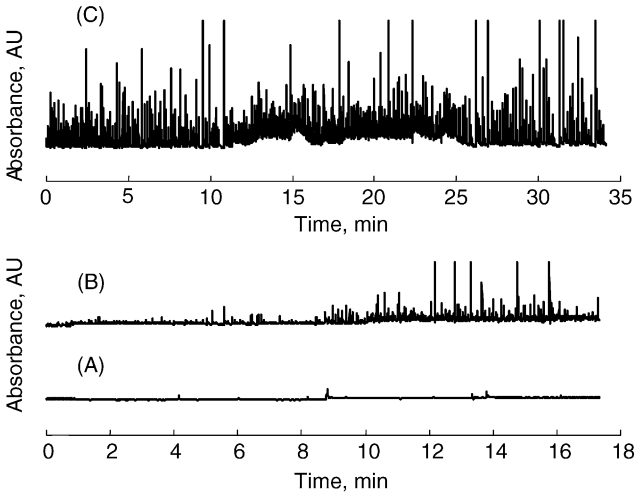

Fig. 2. UV absorbance signals using pump at constant rate (fused-silica capillary, 80 cm × 75 μm, 0.17 ml min^{-1}) without and with applied voltage (30 kV). (A) Solution D, voltage applied at 9 min; (B) solution B, voltage applied at 9 min; (C) solution A, voltage applied at 11 min, switched off at 24 min. UV at 226 nm.

By use of Eq. (1), aggregate concentration is $c_{\text{pump}} = 9.0{\cdot}10^5\,\text{ml}^{-1}$ without applied voltage and $c_{\text{pump+EOF}} = 8.4{\cdot}10^5\,\text{ml}^{-1}$ with applied voltage.

Considering relative standard deviations of flow rate and time measurements negligible compared to the relative standard deviation of a spike number, the standard deviation for aggregate concentration during the flow without applied voltage, s_c^{pump}, estimated by Eq. (1) was:

$$s_c^{\text{pump}} = c_{\text{pump}} \frac{s_n^{\text{pump}}}{n_{\text{pump}}} \tag{4}$$

where s_n^{pump} is a standard deviation of spikes number. The standard deviation from Eq. (4) was $1.3{\cdot}10^5\,\text{ml}^{-1}$.

A similar calculation gives $s_c^{\text{pump+EOF}} = 2.3{\cdot}10^5\,\text{ml}^{-1}$. Calculating the Student's t-value for the difference $c_{\text{pump+EOF}} - c_{\text{pump}}$ results in $t = 0.71$, which is much less than the corresponding critical value, i.e. for two-sided $t_{\text{crit}}\,(18, 0.95) = 2.08$. Thus the difference of 7% between $c_{\text{pump+EOF}}$ and c_{pump} is statistically insignificant. Filtration of the solution A (resulting in solution D) removed almost all spikes in electropherograms (Fig. 2, solution D).

In case of solution B (HA in the presence of imidazole), when applying electric field, a profound effect of aggregation was observed. The increase of the frequency of the spikes (Fig. 2, solution B, beginning from 10 min) is evident and can be associated with an increase in aggregate concentration without any statistical scrutiny. A similar phenomenon was observed for colloidal particles in the electric field where even clear hysteresis cycle was obtained when high voltage at different values was applied [25]. The figures reveal that the aggregation is enhanced by the presence of imidazole. This result may be connected with the formation of imidazolium complexes with HA molecules. Blank imidazole solution (10 mM imidazole) did not exhibit any spikes on electropherogram being just a baseline without any features.

The rest of the HA measurements were performed only with working solutions containing imidazole. The used buffer is similar with those that used Lee and Lin [19] for determination of metal cations. As expected, the frequency of spikes in a more concentrated solution was higher (Fig. 3). A similar result was observed for filtrated solutions as well (Fig. 4), however, spike frequency was lower. It follows again from Figs. 3 and 4 that the appearance of spikes is strongly influenced by the applied voltage. This fact might well explain the nature of spikes encountered in many published electropherograms of HA capillary electrophoresis (see, e.g. [13]). At high HA concentrations (>0.2 mg ml^{-1} used, e.g. in [6]), spikes on "hump" might have appeared because of the aggregation of the sample subjected to high voltage during the CE run. The filtration did not remove particles that might aggregate at high voltage.

Although, the aggregation is influenced by the concentration of HA, the value of applied voltage has a profound effect on aggregate formation, as it was stated already above.

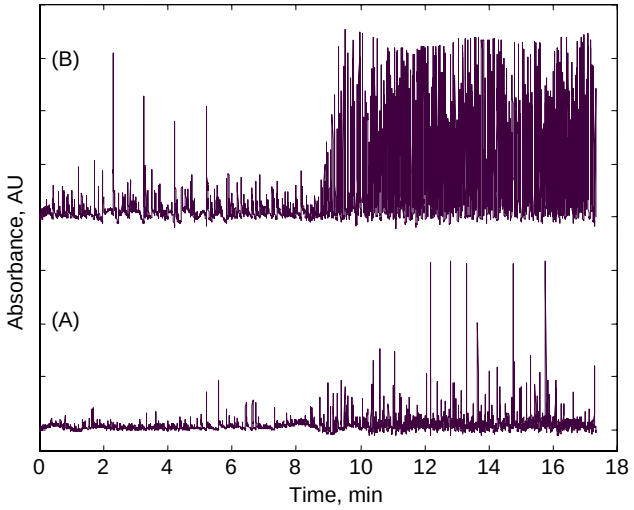

Fig. 3. UV absorbance signals using pump at constant rate with applied voltage 30 kV at 9 min. (A) Solution B and (B) solution E. UV at 226 nm.

By pumping of solution E through the capillary at a constant rate and applying voltages from +5 to +30 kV, the frequency of spikes increased considerably. The obtained result is presented in Fig. 5. From this result, the concentration of aggregates as a function of the applied voltage was calculated by Eq. (1). The results are presented in Fig. 6. It follows from Fig. 6 that an increase in voltage clearly supports aggregation. It is still not clear what type of mechanism is responsible for this behaviour. There may either be a distribution of size to the charge ratio of the aggregates or the applied electric field may change the double layer on the aggregate surface, thus reducing electrical repulsion of aggregates. Also, as speculated by Zheng and Yeung [17], particles may have different shapes and orientation in relation to the electric field in the capillary. All of those facts could result in different mobility of different aggregate par-

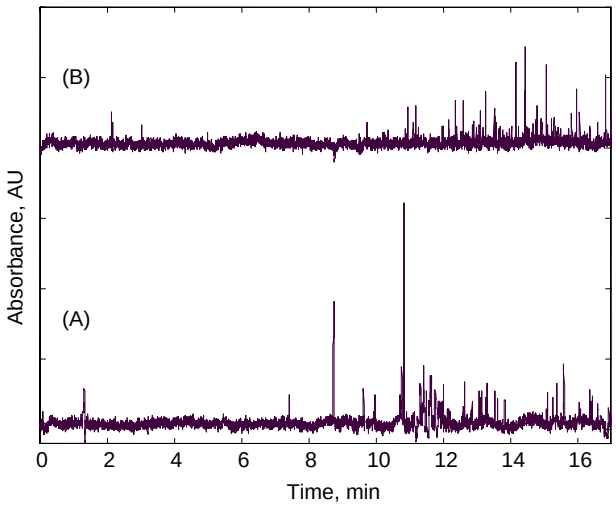

Fig. 4. UV absorbance signals using pump at constant rate with applied voltage 30 kV at 9 min. (A) Solution C and (B) solution F. UV at 226 nm.

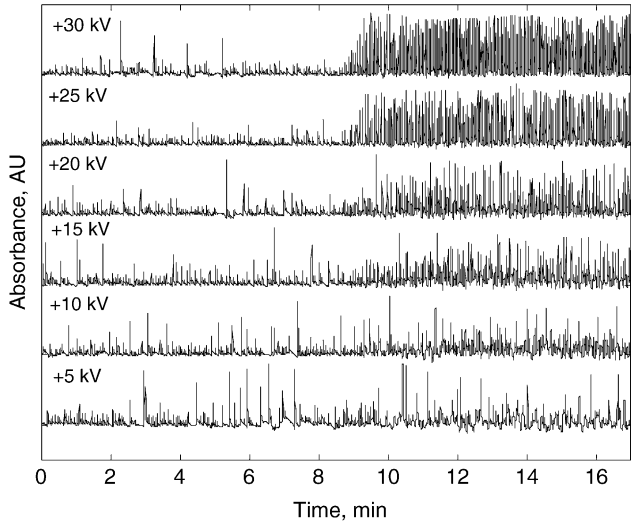

Fig. 5. UV absorbance signals of solution E using pump at constant rate, different voltages applied at 9 min. UV at 226 nm.

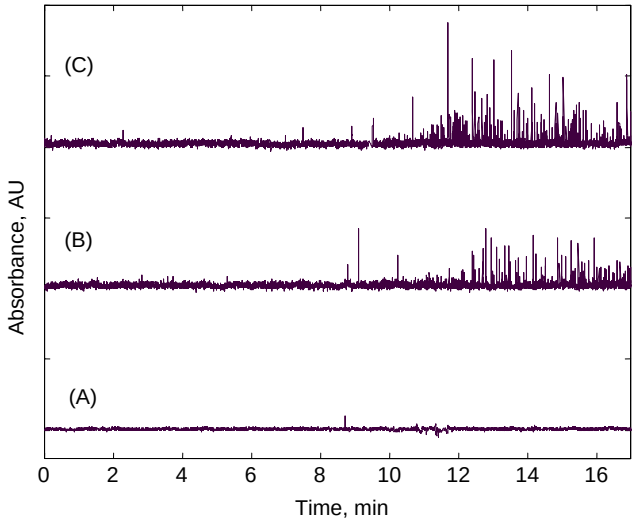

Fig. 7. UV absorbance signals of β-CD solution using: (A) 0.2 mM solution; (B) 15 mM solution and (C) 15 mM solution in 10 mM imidazole, 30 kV applied at 9 min. UV at 226 nm.

ticles and this, in turn, could result in their colliding and clustering to the larger aggregates.

Obtained results are in good accordance with the suggestion made earlier by different authors: the aggregates are formed during a CE run and each aggregate can randomly be retarded in the capillary [26,27].

Our HA solution have colloidal properties at higher pH (pH < 9). At the same conditions, other HS fractions (hymatomelanic and fulvic acid) having no colloidal properties did not give the spikes (electropherograms reveal only baseline, and therefore, are not added to the article).

Pokorna et al. [13] suggested that the spikes correspond to the different compounds present in all HAs that are liberated from the supramolecular structure of the HAs by the action of CDs forming inclusion complexes. However, the aggregation phenomenon is not connected only with HAs. The aggregation of associated organic molecules in the high-voltage electric field was observed recently for colloidal particle [25]. We suggested that the aggregation of colloidal solutions in CE capillary at high-voltage electric field is more general phenomenon and not limited only with the HA solutions in order to prove that we looked for more definite colloidal solutions. Oligosaccharides are typical molecules that form colloidal systems in higher concentration. We were interested in more general characteristics of the phenomenon and if the spike formation is also observed in a solution of regular chemical molecules like CD (i.e. without HA in separation solution). For that purpose, we pumped β-CD solution (0.2 and 15 mM) through the capillary at 30 kV voltage. At lower concentration, no spikes were observed (Fig. 7). However, at higher concentration, like in case of HA, similar spikes appeared when high voltage was applied. Indeed, in the CD solution it is unlikely that imidazole causes the appearance of charged particles (e.g. salt formation).

Thus, these results show that the appearance of spikes is due to the aggregation of different organic molecules, which is not necessarily associated with the properties of HS.

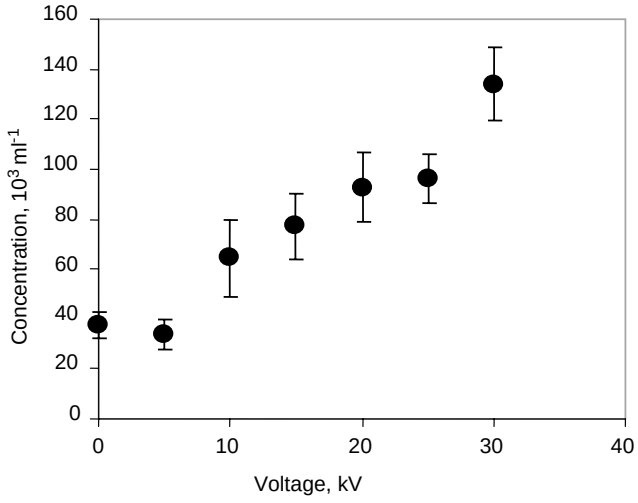

Fig. 6. Dependence of aggregates concentration of applied voltage in HA solution E.

4. Conclusions

The results of this work indicate that formation of spikes in HA colloidal solution electropherograms depends on the applied voltage as well as on the concentration and filtration procedures applied to the HA solutions. The last observations trivially indicate the presence of the particle aggregates in the solution, which either scatter or absorb UV radiation before detector, leading to the appearance of spikes on electropherograms. The electric field strongly promotes the formation of new aggregates. The presence of imidazole in

the buffer solution profoundly supports aggregation. A similar observation was made in a bacterial aggregation when a buffer solution contained polyethylene oxide as an ingredient [16,28]. It is too early to suggest any possible mechanisms of the aggregation. However, the method can be used as a new possibility to characterize the different HS fractions, other non-regular natural substances and colloidal solutions.

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