

MOLECULAR ARRANGEMENT AND SECONDARY STRUCTURE OF HUMIC SUBSTANCES

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Abstract

Humic acids were fractionated using universal buffer solutions in the range of pH values 2-12. The methods of dynamic light scattering and gel-permeation chromatography were used to investigate the molecular organization of humic acids in eluates. The obtained results were supplemented by ultraviolet/visible spectrometry, measurement of the zeta potential, conductivity and pH values. Fractionated humic acids had the supramolecular character and their molecular organization was strongly affected by pH value of surrounding medium. Observed changes in the secondary structure of humic acids were connected with the changes in colloidal stability, particle size distribution, and polydispersity.

Keywords: Humic substances, molecular arrangement, conformation, particle size, zeta potential

1. INTRODUCTION

Humic substances have a colloidal character, whose size and negative charge are strictly dependent on surface functional groups [1], [2]. They exhibit different structures at different conditions and participate in both nutrient and contaminant transport, and they support the soil structure and its pH [1]-[4]. They behave as rigid spherocolloids or compacted networks at high humic concentrations, low pH values, or high neutral electrolyte concentrations, but as flexible linear colloids at low humic concentrations and high pH values [5]-[8]. The physicochemical properties of humic substances, such as their biopolymer chain structure and colloidal character, are closely related to the solution chemistry, e.g. the electrolyte and pH. They are able to rearrange and restructure themselves in response to environmental changes such as changes in pH, ionic strength, and moisture. The significance of the secondary structure of dissolved humic substances in their interactions in nature has been attributed to entrapment in structural voids, adsorption on surfaces, and partitioning into the hydrophobic interiors of micelle-like structures and humic aggregates [9]-[11]. Many authors (e.g. [6], [12] and [13]) confirmed that humic molecules in solution are organized in supramolecular associations of relatively small molecules loosely bound together by dispersive interactions and hydrogen bonds. Other results are indicative of the presence of both macromolecules and supramolecules in humic substances in solution [14], [15].

This work is focused on the humic acids, as the part of humic substances corresponding with the division of humic substances into humins, humic and fulvic acids according the solubility [16]. Since the humic substance is not a single well-defined molecule but a mixture of various heterogeneous particles or aggregates containing a variety of functional groups resulting in polyelectrolyte and polyfunctional properties, it cannot be treated as an ordinary complex-forming ligand in the interaction with ions. This complex nature of humic substances makes it difficult to obtain precise information on its chemical structure and properties. One of the ways of overcoming these difficulties is to separate the humic acids into several fractions which reduces their heterogeneity. In this work, the method of extraction procedure of gradient pH values was used to fractionate the humic acids [17], [18].

2. MATERIALS AND METHODS

Humic acids were purchased from the International Humic Substances Society (Leonardite Standard HA 1S104H). They were fractionated by dissolving in the universal buffer solution NaOH-H₃PO₄-CH₃COOH-H₃BO₃ (the pH region 4-12). The finely ground humic sample was mixed with the buffer solution at the lowest pH value (pH = 2) in the ratio 2 g / 100 cm³ and stirred for 24 h. The insoluble residue was (partially) dissolved in the buffer solution with the higher pH value and the whole procedure was repeated [15], [16]. Obtained leachates were analyzed by the UV/VIS spectrometry (Hitachi U-3300), potentiometry (Mettler Toledo Five Easy Plus) dynamic light scattering (Zetasizer Nano ZS) and size exclusion chromatography (SEC) coupled with multiangle static light scattering, differential refractive index and UV/VIS detection (SEC chromatographic system from Agilent Technologies, detectors from Wyatt Technology).

3. RESULTS AND DISCUSSION

On the basis of the previous experiences [15], [16], we prepared several humic fractions and investigated their properties with respect to their molecular arrangement, stability and secondary structure. As estimated, the partial dissolving of humic acids in the buffers caused only negligible changes in their pH values (see **Figure 1**). The solubility of some humic fractions in buffers with acidic pH values was enabled by the presence of strong acidic functional groups. Although typical average values of pK_a of humic acids are between 3 and 5, some fractions can have the pK_a values very low, which enable them to dissociate in relatively acidic solutions, which support the solubility of humic acids at low pH values [15]-[18].

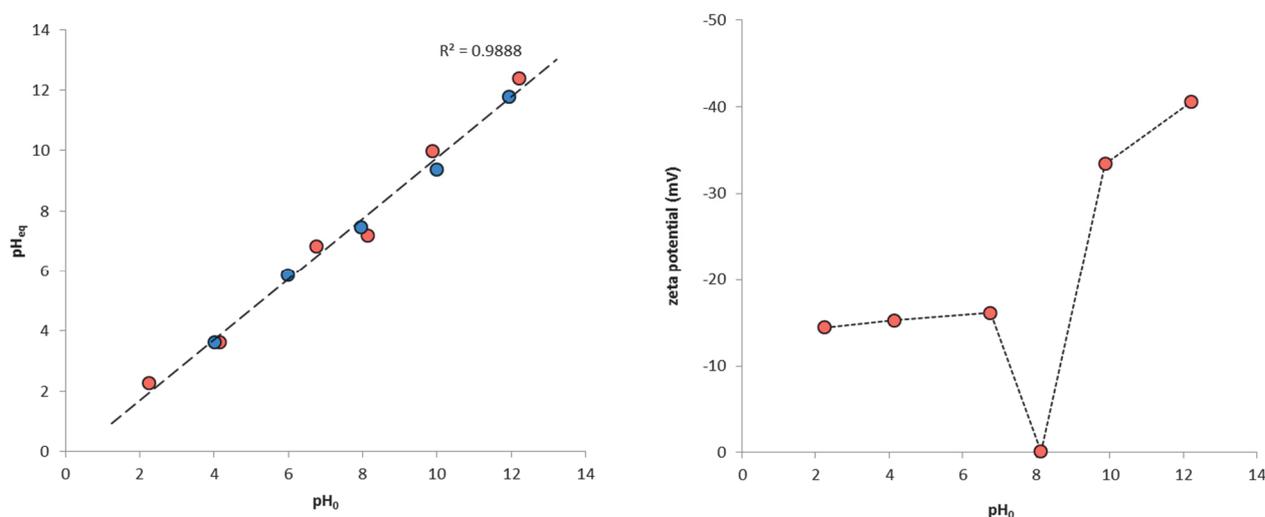


Figure 1 Equilibrium pH values and zeta potential of humic fractions dissolved in buffer solutions (data obtained in ref. [16] are shown for comparison - blue circles)

The colloidal stability of obtained humic fractions was characterized on the basis of their zeta potential. Their values were affected by polyelectrolyte character of humic acids, negative charge and character of surface functional groups. As we can see (**Figure 1**), the increasing pH values of buffer solutions were connected with the decrease of zeta potential resulting in the higher stability. The one fraction, which did not corresponds with this trend is the fraction dissolved at pH 8. Its particles appeared as neutral without a charge. This observation is surprising and its explanation is not easy. In general, two simultaneous processes can influence resulting value of zeta potential and colloidal stability of humic substances. They are the dissociation of acidic functional groups and de-aggregation (breaking of humic aggregates into smaller molecular associations and finally into humic molecules) caused by H-bonds breaking, having opposite effects on the value of zeta potential [18]-[20]. Some authors, e.g. [1], [22] and [23], described gradual decreasing of zeta potential with the increasing

pH value of buffer solution and a plateau in region around pH 5-6. On the other hand, the minimums between pH 5 and 8 (depending on concentration) were detected in [21]. Authors assumed, that the dissociation of acidic groups in the acidic region can be considered as the prevailing process leading to a negative charge increase, more negative values of zeta potential, and finally to its minimum. At higher pH values (from the minimum upward), de-aggregation process likely predominates over dissociation, resulting in decrease of particle sizes. Our results are different. They correspond partially with [1], [20] and [21], but the increase of the colloidal stability in the acidic region is very small. The values of zeta potential are relatively high and obtained systems are not stable (similarly as in ref. [1]). The maximum of zeta potential was detected for pH 8 and the strong decrease of its values was observed in the alkaline region. Comparing with above mentioned studies [1], [21]-[23], we measured leachates of humic acids in buffer solutions. Therefore only a part of humic acids is dissolved and our bulk sample was fractionated into several different parts. The solubility of humic acids and the stability of dissolved particles in acidic mediums is not high [1], [16]-[18]. The drop of colloidal stability around pH 8 is probably connected with the sequential extraction of humic fractions and the fact that the majority of humic fractions soluble in acidic and neutral mediums were exhausted by dissolving in buffer solutions with lower pH values. The increase of colloidal stability in alkaline region corresponds with the increase of humic solubility.

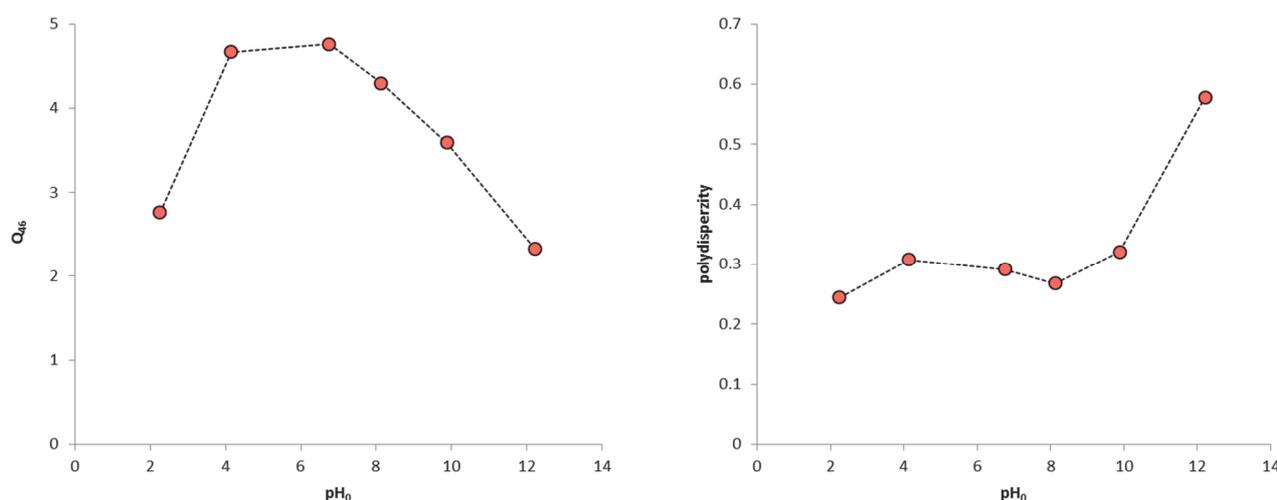


Figure 2 Humification index Q_{46} and polydispersity of humic fractions dissolved in buffer solutions

As mentioned, changes in molecular organization with pH are connected also with changes in size of dissolved particles. The traditional approach to characterization of molecular weight and particle size of humic substances is the use of so called humification index Q_{46} presented in **Figure 2**. It is the ratio of absorbance at 465 nm to at 665 nm and its value is usually for humic acids <5 [18], [23]-[25]. The low value of humification index for strong acidic (pH 2) and strong alkaline (pH 12) solutions showed on the presence of humic fractions with higher molecular weight and humification degree. The higher values may be indicative of the presence of smaller humic particles and oxygen containing functional groups. More details about particle size distribution were obtained using DLS and SEC (**Figure 3**). The humic fraction containing strong acidic groups and humic particles with relatively narrow distribution was dissolved in the buffer solution with pH 2. Results of DLS indicated the presence humic aggregates whose size decreased with the increasing pH value. In middle pH region, dissolved humic fraction had wider particle size distribution, which resulted also in slightly higher polydispersity (**Figure 2**). The strong increase of polydispersity is related to bimodal character of humic fractions extracted at pH 12.

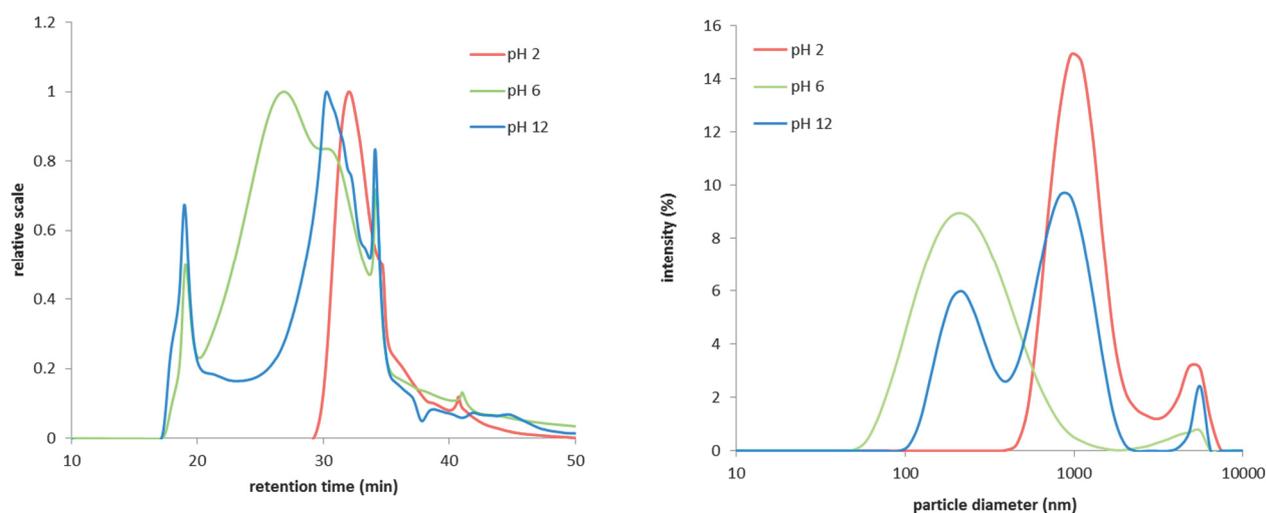


Figure 3 Particle size distribution of humic fractions at pH 2, 6 and 12 determined by SEC (left) and DLS (right)

Although, the average particle size obtained using DLS is highest for the humic fractions obtained at pH 2 and 12 (around 1000 and 550 nm, respectively), their molecular arrangements are very different. In the case of strong acidic conditions (and low concentrations caused by low solubility), the aggregation of humic acids and the formation of large particles were detected. At low pH, protonated functional groups partake in hydrogen-bonding that crosslinking humic chains both inter- and intra-molecularly [1], [14], [22], [26]. The increase of pH promotes the humic solubility, dissociation of their acidic functional groups and expansion of coils as a consequence of high affinity of humic acids to surrounding alkaline solution. As we can see in **Figure 3**, more fractions were dissolved at pH 12. In intermediate region (pH 4-10), the average size increased slightly from 220 to 320 nm. According to results presented in ref. [22], the decrease in the size of humic colloids was affected more by the ionization of phenolic acidic groups, than by that of carboxylic ones, which was probably because, in the case of ionized carboxylic groups, humic colloids were still capable of generating H-bonds. Our results showed that that humic substances appeared to be composed of two main fractions: one which exhibited clear macromolecular behavior in solution, with macromolecules and/or very stable molecular aggregates present; and another fraction that was principally formed by molecular aggregates (supramolecular associations), which also included molecules of low molecular weight and an unclear macromolecular nature. Macromolecules, small molecules, and supramolecular associations all seem to coexist in humic systems which correspond with conclusions stated in [1], [14] and [21].

4. CONCLUSION

In this work, the molecular arrangement of fractionated humic acids was studied. The methods of UV/VIS spectrometry, dynamic light scattering, zeta potential measurement, and size exclusion chromatography were used in order to monitor changes in secondary structures in dependence on pH of buffer solution used for their extraction from the bulk sample. It was found that the conformational arrangement changed with the pH and concentration which was given by different solubility at different pH values. In general, the organization of humic systems can be divided into several parts, but the main change in the secondary structure of humic acids was observed at around pH 8. Very low stability and absence of negative charge was observed for this humic fraction. Humic fractions obtained at acidic conditions showed much lower colloidal stability in comparison with alkaline solutions. The leachates of humic acids in alkaline buffer solutions exhibited different behavior. The reason is the alkaline pH value, which caused the formation of an open unfolded structure available for polar interactions, which was partially restricted with increasing concentration.

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