

Article

Macromolecular Structure of a Commercial Humic Acid Sample

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Abstract: The molecular structure of a commercial sample of humic acids (HA) was investigated by membrane dialysis experiments (MD) and low-pressure size-exclusion chromatography (LP-SEC). MD showed that HA molecules were retained by dialysis membrane with a cut-off of 6–8 kDa, independently from HA concentration (15 or 150 mg L⁻¹), NaHCO₃ concentration (0.005–2.0 mol L⁻¹), and from propan 2-ol (0–5 v/v %). SEC experiments at low pressure gave chromatograms with a broad peak, with an elution volume between those of the globular proteins bovine serum albumin (molecular weight = 66.5 kDa) and lysozyme from egg (molecular weight = 14.4 kDa). The pattern of the chromatogram did not vary with HA concentration, and second-run chromatograms of single eluted fractions showed relatively sharp peaks. From these data, we reveal that the commercial HA sample analysed has a macromolecular structure rather than being a supramolecular aggregate of relatively small molecules, as recently proposed for some samples of HA obtained from different sources.

Keywords: humic substances; molecular structure; supramolecular aggregate; exclusion chromatography

1. Introduction

Humic substances are natural organic substances spontaneously arising from the biological decomposition of organic matter. Humic substances are the main fraction of organic matter in soil and surface water; they profoundly affect the structure and physico-chemical properties of soil and are involved in most soil surface phenomena [1]. Based on differential solubility in water, humic substances are distinguished into humin, humic acids (HA), and fulvic acids. HA are the fraction soluble at neutral and alkaline pH and are the main carriers affecting the mobility of pollutants in the environment [2]; because of this, their chemical structure and adsorbing properties have been investigated extensively. HA consist of aromatic rings and alkyl chains bearing a diversity of functional groups, notably carboxyl, phenol, hydroxyl, and quinone groups [3]. As reported in the literature [3–5] HA can bind cations by electrostatic interactions, as well as non-ionic organic compounds by means of hydrogen bonds, dipole–dipole interactions, and hydrophobic interactions. Because of these properties, HA have been proposed as an adsorbent in wastewater purification plants [5–7]. On the other hand, HA cannot be removed from water by using conventional oxidation processes; however, several approaches are effective for treating recalcitrant compounds, such as adsorption [8,9] and advanced oxidation processes [10,11].

In water, HA molecules tend to aggregate giving colloids [12]. Self-assembly increases with the concentration and in the presence of metal ions, and decreases with increasing pH; the rate is relatively

fast and is consistent with the diffusion-limited colloid aggregation model [13]. Moreover, it has been shown that, because of their amphiphilic character, HA molecules form micelles in basic aqueous solutions, although at relatively high concentrations [14,15]. Because of this behaviour and of the wide molecular mass distribution of HA, an accurate determination of the molar mass of these compounds is no simple task. Indeed, despite the remarkable amount of work devoted to the topic, the molecular mass of HA remains controversial. In contrast with the traditional notion of a macromolecular structure [12], it has been suggested that HA consist of a supramolecular assembly of small units held together by weak intermolecular forces [16]. However, other researchers [17] have observed that this view appears to run counter to certain experimental observations. Baigorri et al. [18] have reported the simultaneous presence of macromolecules and supramolecular assemblies in solution. A central point of the current debate about HA structure is the mass of discrete units forming the aggregates, which ranges from about 600 g mol^{-1} to tens of thousands [19]. In a recent study by molecular dynamics simulations, the authors used condensed-phase models of humic substances obtained by the supramolecular assembly of small molecules [20]. On the other hand, the formation of humic acids with high molecular weight during the composting biodegradation process has been shown [21].

The knowledge of molecular properties of HA is essential for understanding their behaviour in natural processes and depuration plants [22]. Here we report a study on the molecular structure of a HA sample from a commercial supplier. In particular, the aim of the work was to investigate the structure of the commercial humic acid sample in order to understand if it is a macromolecular one or a supramolecular aggregate of relatively small molecules, excluding external aggregation phenomena, which may alternate the structure of the humic acid molecules.

2. Materials and Methods

2.1. Humic Acids (HA) Sample

A sample of HA, produced by the decomposition of plant matter, was purchased from Alfa Aesar (Germany). The elementary composition and the content of carboxylic and phenolic groups are reported in [23]. The sample was purified by acid/base treatment before use. A quantity of 1 g of HA was dissolved in 500 mL of distilled water, and the pH brought to 12.0 with 1.0 mol L^{-1} NaOH. The mixture was stirred for one day, and the pH was periodically checked and readjusted to 12.0 with few drops of 0.1 mol L^{-1} NaOH if necessary. The solution was centrifuged at 10,000 rpm for 20 min, the supernatant was collected, and the pH brought to 1.5 with concentrated HCl. After one night in the fridge at $4 \text{ }^{\circ}\text{C}$, the precipitate was centrifuged at 10,000 rpm for 30 min, washed with a small amount of 0.001 mol L^{-1} HCl and oven-dried at $45 \text{ }^{\circ}\text{C}$. The purification procedure was performed twice, obtaining a final product with an ash content of 1.2% after heating at $550 \text{ }^{\circ}\text{C}$ for 5 h.

The proteins bovine serum albumin and lysozyme from eggs, and all the other reagents employed, were purchased from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Dialysis Experiments

Quantities of 30 mL of 150 or 15 mg L^{-1} HA in 2, 0.2, or $0.005 \text{ mol dm}^{-3}$ NaHCO_3 solution were introduced in a dialysis tube with a cut-off of 6–8 kDa (Spectra/Por, Rancho Dominguez, CA, USA). Propan 2-ol was added in some experiments at 2.5% or 5% (v/v). The bag was put in contact with 30 mL of a solution with the same composition except lacking HA and kept under periodical, gentle shaking. The outside HA concentration was monitored by measuring the absorbance at 450 and 280 nm through a spectrophotometer (Perkin Elmer, Lambda 40, Waltham, MA, USA). The peak centred on 280 nm is the result of absorbance by the aromatic ring portion of HA structure, while HAs have a specific absorption band near 450 nm as widely reported in the literature [3,5,24]. The pH of the tested suspension was 8.6.

It is worth highlighting that the concentrations of 150 or 15 mg L^{-1} were selected because they are fully different values but, at the same time, below the aggregation limit [16]. Moreover, the use of

NaHCO₃ allows preventing the formation of aggregate, which depends on the ionic strength, amongst other factors, avoiding the alteration of the structure of the humic acid molecule [16,25,26].

2.3. Low-Pressure Size-Exclusion Chromatography (LP-SEC)

LP-SEC was performed using a Bio-Gel P30 Gel medium with 2.5–40 kDa fractional range (Bio-Rad Laboratories, Hercules, CA, USA), in a column 30 cm in length and 2.5 cm in diameter, eluted with 0.20 mol L⁻¹ NaHCO₃ (pH 8.6), flow rate 2.0 mL h⁻¹. The HA concentration in the eluate was evaluated from the absorbance at 450 nm and 280 nm through a spectrophotometer (Perkin Elmer, Lambda 40, Waltham, MA, USA).

3. Results and Discussion

All the experiments were carried out at HA concentrations lower than the Critical Micelle concentrations (CMCs) reported for HA of various origin [14,15]. CMC is the concentration above which the solute starts to form micelles.

3.1. Membrane Dialysis Experiments (MD)

The CMC values depend on temperature, the ionic strength of the solution, and on the presence of organic molecules such as alcohols [25,26], hence some MD experiments were carried out in NaHCO₃ solution containing propan-2-ol; moreover, Piccolo [16] suggests that the presence of alcohols disadvantages the formation of monomers. The experimental conditions are reported in Table 1.

Table 1. Equilibrium conditions of membrane dialysis experiments.

HA Concentration Inside the Bag (mg L ⁻¹)	NaHCO ₃ Concentration (mol L ⁻¹)	C ₃ H ₇ OH Concentration (% v/v)	HA Concentration Outside the Bag at the Equilibrium
150	2	0	n.d. ¹
150	0.2	0	n.d. ¹
150	0.02	0	n.d. ¹
15	0.02	0	n.d. ¹
150	0.005	2.5	n.d. ¹
15	0.005	2.5	n.d. ¹
150	0.005	5	n.d. ¹
15	0.005	5	n.d. ¹

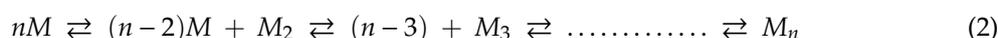
¹ n.d. = not detected.

In all the experiments, the HA level in the outside solution remained undetectable after two weeks, independently of HA, NaHCO₃, and alcohol concentrations. This behaviour is a clear indication that the commercial HA sample analysed consists of molecules with a mass above the 6–8 kDa membrane cut-off, not of micelles or aggregates of relatively small molecules held together by weak intermolecular forces. Indeed, if the latter were true, an equilibrium would be established in solution between different forms. If HA formed micelles (an unlikely hypothesis considering the low concentrations adopted), one would expect the establishment of the following dynamic equilibrium:



where *M* is a monomeric unit and *M_n* a micelle formed by *n* monomeric units.

Alternatively, in the presence of a molecular aggregation involving different grades of association, the following equilibria should be established:



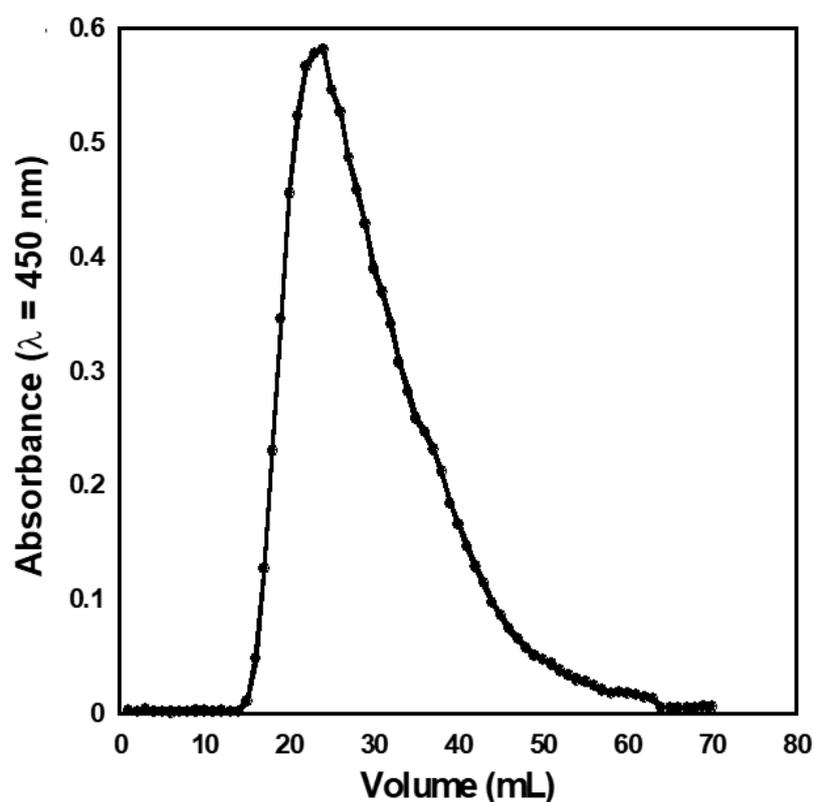
In either case, if the monomeric units could diffuse through the membrane pores, the same HA concentration should have established on the two sides of the membrane after a long dialysis time. In contrast, HA concentration in the outside solution remained below the detectable level (0.1 mg L^{-1}), thereby proving that the monomeric units were larger than the 6–8 kDa membrane cut-off.

3.2. Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography (SEC) is one of the techniques commonly used to separate macromolecules from small molecules, and to characterise them in relation to molar mass; SEC has been employed in the study of HA properties [27–29], including the preparation of humic acid fractions with a definite range of particle sizes [30]. This chromatographic technique separates the analytes on the basis of their hydrodynamic volume [31,32], and therefore it permits an estimation of their molar mass. To verify if the humic acids are bound/complexed to the SE column, we carried out experiments with vanillic acid. Vanillic acid has a structure like that of the monomeric unit of the polymer humic acids. The elution volume was expected for small molecules, indicating no interaction.

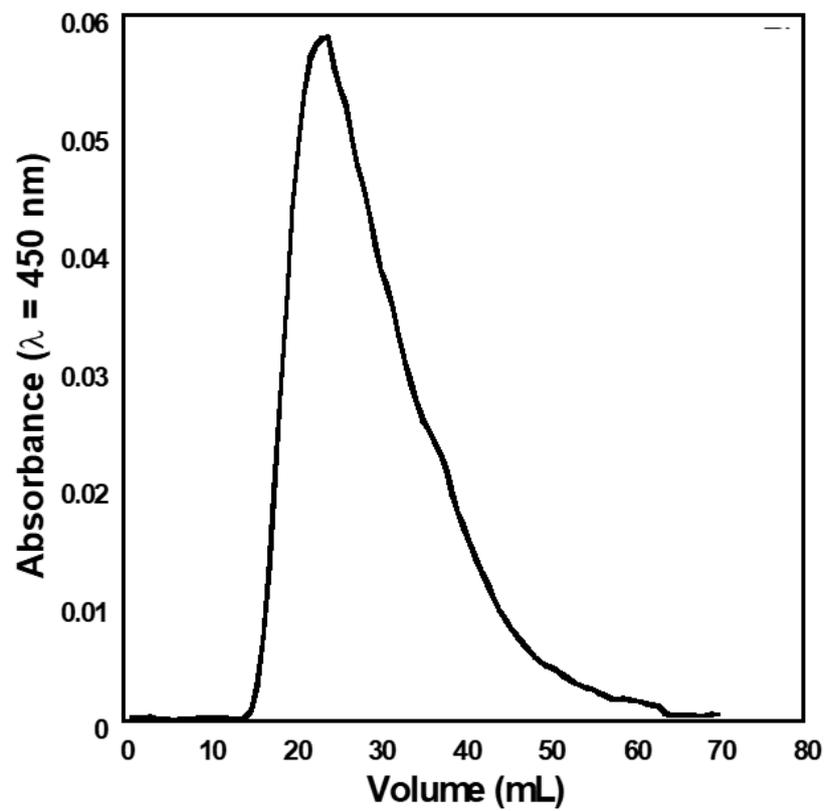
Figure 1 reports the low-pressure size exclusion chromatograms of the commercial HA sample analysed, obtained from HA solutions at three different concentrations.

As can be seen, the chromatograms are very similar, except for the different absorbances. Moreover, when the eluate fraction between the 28th and 30th mL was run again on the same column, the chromatogram obtained had a much sharper peak centred in correspondence to the eluate fraction selected (Figure 2). Analogous results were obtained with other fractions.

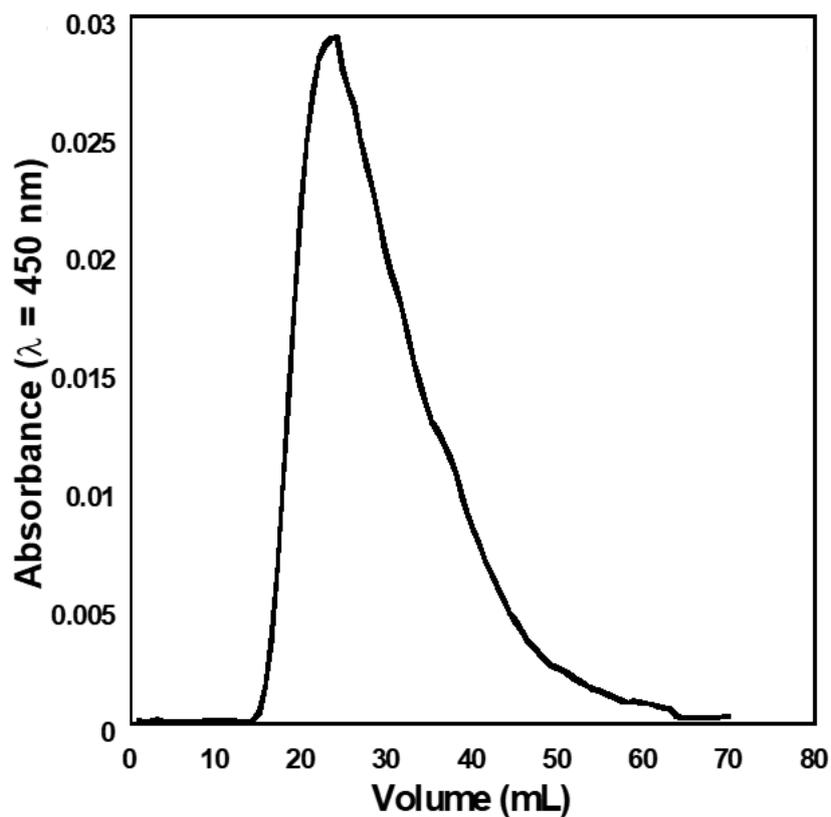


(a)

Figure 1. *Cont.*



(b)



(c)

Figure 1. Low-pressure size exclusion chromatograms of humic acids (HA). Initial HA concentration (2.0 mL): (a) 400 mg L^{-1} ; (b) 40 mg L^{-1} ; (c) 20 mg L^{-1} .

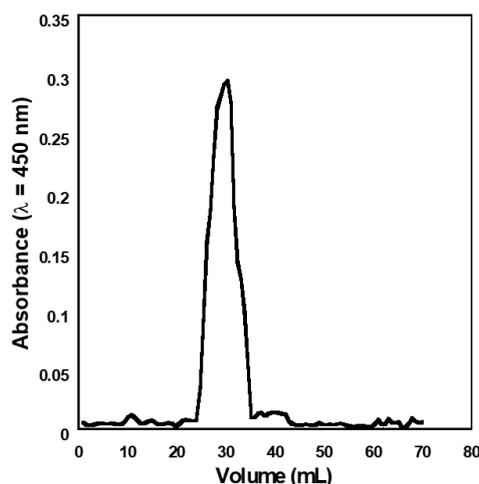


Figure 2. Low-pressure size exclusion chromatograms of the eluate fraction from the 30th to the 31st mL of the chromatogram reported in Figure 1a.

By comparison with the behaviour of bovine albumin serum (molecular mass 66.5 kDa) and lysozyme from egg (molecular mass = 14.4 kDa), we infer that the HA solutions tested contain a population of molecules with hydrodynamic volumes ranging between these two extremes (Figure 3). It is worth noting that the humic acid sample analysed in this study, on the basis of recent studies of Li and coworkers [33], can easily be removed from aqueous solutions by adsorption on nanoscale zero-valent iron and magnetite.

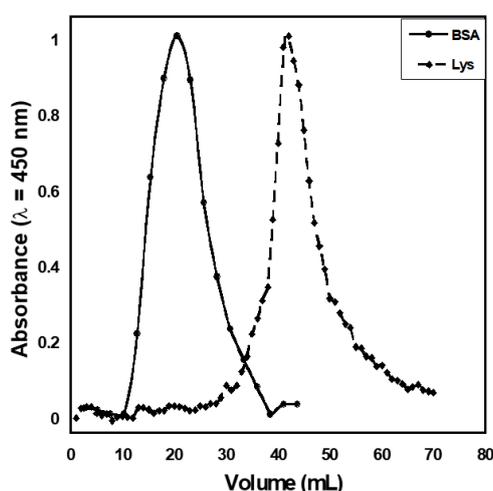


Figure 3. Low-pressure size-exclusion chromatograms of the globular proteins bovine serum albumin (BSA, molecular mass 66.5 kDa) and lysozyme from egg (Lys, molecular mass = 14.4 kDa). The absorbances of each chromatogram have been divided by the value of the peak recorded at the maximum absorptivity in the UV-Vis region.

It is pertinent to note, however, that size-exclusion chromatography does not permit an accurate determination of the molar mass of polyelectrolyte molecules lacking a stable three-dimensional structure, as is probably the case for HA because the hydrodynamic volume of these compounds varies with pH and ionic strength.

4. Conclusions

Although the knowledge of the molecular structure of HA is of great importance in the theoretical and experimental studies in the interaction between HA and environmental pollutants, its definition is

still a controversial topic due to the complexity of humic acid molecules. The aim of this work was to investigate the structure of a commercial humic acid, to understand if it is a macromolecular one or a supramolecular aggregate of relatively small molecules. To exclude external aggregation phenomena, which may alternate the structure of the humic acid molecule, investigations were carried out by varying HA concentration and by adding NaHCO_3 . Our results show unambiguously that monomeric units with a molecular mass of some tens of kDa are the prevalent chemical species present in a water solution of the commercial HA considered, at least at neutral or basic pH and at concentrations $\leq 150 \text{ mg L}^{-1}$. Moreover, the present study points out that size-exclusion chromatography and membrane dialysis are, at the same time, very powerful, cheap, and fast techniques for studying equilibria involving macromolecules or molecular aggregates in solution.

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Conflicts of Interest: The authors declare no conflict of interest.

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