

Note

Fractionation of humic substances by hydrophobic interaction chromatography

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Humic substances comprise mixtures of polyelectrolytes covering a wide range of molecular weights, and constitute the major organic constituents of soil; they also occur in aqueous environments. These complex and biologically resistant compounds are formed from different organic precursors of essentially plant or microbial origin (lignin, carbohydrates, polypeptides, polyphenols) as a result of enzymatic or autoxidative reactions occurring during the humification processes.

Possible structures for these humic compounds have been proposed: Haworth's aromatic core¹ to which are attached chemically or physically polysaccharides, proteins, simple phenols, metals; the molecular sieve type of Schnitzer and Khan² to which phenolic and benzene carboxylic acids are joined by hydrogen bonding, with voids able to trap low-molecular-weight organic as well as inorganic compounds; or the hierarchical structure of Wershaw *et al.*³ with structural elements comprising simple phenolic, quinoid and benzene carboxylic acid groups linked together by covalent bonds into particles which are in turn linked together by weak covalent and non-covalent bonds into aggregates.

The model proposed by Schnitzer and Khan seems more adaptable to the fulvic acid (FA), acid-soluble fraction of extractable humic substances, whereas the two other models reflect the humic acid (HA), acid-insoluble fraction. The acidic character of these compounds is mainly due to carboxyl and phenolic groups.

Taking into account their complexity, attempts to fractionate humic substances into subfractions of less complex mixtures have been performed. Fractionation based solely on molecular size considerations is generally unsatisfactory on account of the large polydispersity of the molecules, but polyacrylamide gel has been employed in the characterization of HA by electrophoresis⁴ or electrofocusing^{5,6}. Fractionation on hydrophobic resins has also been achieved with XAD-2 resin, using a series of eluents having progressively greater pH values⁷ or with XAD-8 resin eluted with a continuously changing pH gradient⁸ or a nearly linear pH gradient⁹.

In this paper, we present a new fractionation procedure involving hydrophobic interaction chromatography on octyl-Sepharose CL-4B.

EXPERIMENTAL

Sample preparation

Two natural samples (terrestrial and sedimentary) of HA and FA, one commercial sample of HA and a synthetic sample of polymaleic acid (PMA) were used for the fractionation assay.

Terrestrial humic substances were extracted from the upper horizon (0–5 cm) of a forest soil using 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ solution. Non-humified organic matter was previously removed by the densimetric method²³. After centrifugation, HA and FA were separated by acidification of the supernatant solution to pH 1.0. The precipitated HA was recovered, redissolved in sodium hydroxide solution and purified by ultracentrifugation (100 000 g for 60 min) followed by dialysis in tubes with a cut-off threshold for 1000 daltons. After dialysis, HA was neutralized with sodium hydroxide and freeze-dried. The FA was purified on Amberlite XAD-8 resin according to Aiken *et al.*¹⁰, dialyzed and freeze-dried in the same way as for HA.

Sedimentary humic substances from the superficial sediments collected in an estuary were extracted with 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$ + 0.1 M sodium hydroxide solution and pretreated with 0.1 M hydrochloric acid. The separation of HA and FA and purification procedures were identical to those for the soil samples.

Commercial HA (Aldrich), whose terrestrial nature was used for comparison purposes, was purified as described by Narkis and Rebhun¹¹, treated on Amberlite IR-120 (H^+), neutralized and freeze-dried.

PMA, an aliphatic polymer highly substituted with carboxylic acid groups, proposed as a model for FA by Anderson and Russell¹² and possessing similar properties to freshwater humus compounds¹³, was obtained according to the method of Braun and Pomakis¹⁴.

Fractionation method

The fractionation was carried out using Octyl-Sepharose CL-4B (Pharmacia). The humic compound (10 mg organic carbon) was initially solubilized in 0.01 M Tris-HCl buffer pH 7.0 and sodium chloride was added to a concentration of 3 M in a total volume of 5 ml. This sample was applied on Octyl-Sepharose (30 × 2.6 cm) and eluted with 3 M sodium chloride in Tris-HCl buffer pH 7.0. The adsorbed compounds were fractionated, first with a decreasing concentration of sodium chloride from 3 M to 0, followed by a continuous flow of pure distilled water, then by raising the pH with an increasing concentration of sodium hydroxide in the eluent up to 0.2 M and finally with 0.2 M sodium hydroxide.

The flow-rate was 40 ml/h, and an UV detector operated at 254 nm was employed. The efficiency of elution was determined by quantifying the amount of organic carbon dissolved.

RESULTS AND DISCUSSION

Sodium chloride was selected to promote the hydrophobic interactions with octyl-Sepharose because its presence in solutions of sodium humates at relatively high ionic strength does not cause their precipitation. Fig. 1 shows, *e.g.*, for a soil sample of HA, that the retention capacity of octyl-Sepharose was satisfactory at 3

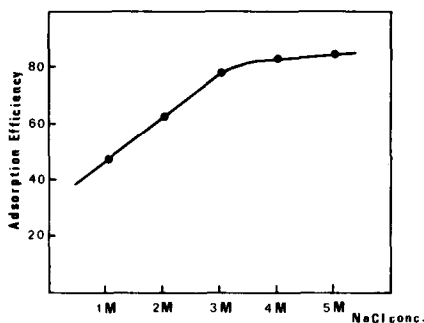


Fig. 1. Adsorption efficiency of soil sample of HA (10 mg organic carbon in 5 ml of 0.01 *M* Tris-HCl buffer pH 7.0) on octyl-Sepharose CL-4B as a function of sodium chloride concentration.

M sodium chloride. In view of the salting-out precipitation in some sample cases, *e.g.*, sedimentary HA, induced by higher concentrations of sodium chloride the above concentration was used for all the fractionation experiments.

After sample application, attempts to elute humic compounds with a linear gradient of decreasing sodium chloride concentration or with a simultaneous gradient of decreasing sodium chloride concentration and of increasing ethylene glycol concentration (final concentrations 0 and 50% respectively) were unsuccessful. Elution was effectively obtained by pure distilled water. A decreasing sodium chloride concentration gradient was used solely to avoid artifact formation through the contact between the salt and pure distilled water.

The remaining tightly bound organic molecules were eluted in part by raising the pH of eluent with 0.2 *M* sodium hydroxyde. Desorption was more readily achieved with higher concentrations of alkali, but these strongly basic eluents were avoided in order to minimize oxidation.

By using this fractionation technique, the recovery of humic substances ranged between 85 and 96% according to the nature of the samples: the recovery was higher for soil samples than for sedimentary samples.

Representative chromatograms of HA and FA (+PMA) fractions are shown in Figs. 2 and 3. The FA sample exhibited weaker hydrophobic interactions than did the HA sample: the amount of non-adsorbed compounds was 87 and 64% for the terrestrial and sedimentary FA respectively, whereas it was 23 and 8% for the corresponding HA. Furthermore, sedimentary humic substances (HA + FA) are more hydrophobic than the corresponding terrestrial compounds. These observations are in accordance with the investigations on surface-active properties of sedimentary humic substances^{15,16}.

The result of the fractionation of the commercial HA indicate its strong similarity with the terrestrial HA sample. The PMA sample resembles more that of FA than HA, but its fractionation is different in that its constituents are eluted wholly by the sodium chloride solution, but with a time lag for some of them. The structural discrimination between PMA and FA emphasized by Spiteller and Schnitzer¹⁷ and Martin *et al.*¹⁸ can explain this result.

As regards the humic compounds retained in the presence of sodium chloride but eluted with distilled water, most are desorbed as soon as the salt is removed, and

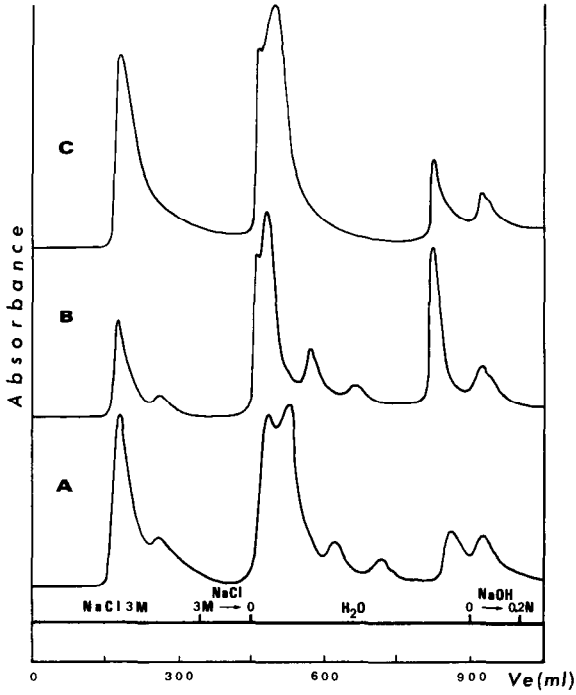


Fig. 2. Fractionation of HA samples on Octyl-Sepharose CL-4B: A, terrestrial HA; B, sedimentary HA; C, Aldrich HA.

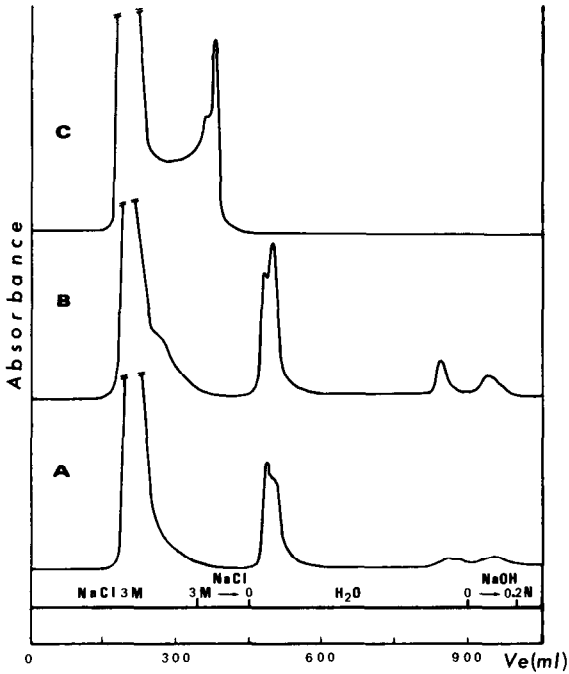


Fig. 3. Fractionation of FA and PMA samples on Octyl-Sepharose CL-4B. A, terrestrial FA; B, sedimentary FA; C, PMA.

are usually fractionated into two distinct peaks, e.g., the soil sample of HA. Another fraction is desorbed later, just before the molecules eluted by sodium hydroxide solution. This fraction, significant for sedimentary HA, may be in part an artifact. Its rechromatography on the same octyl-Sepharose column does not result in a totally reproducible result, one part being eluted as soon as the ionic strength of the eluent is lowered to zero. Experiments involving various samples concentrations or pH values (5.5 instead of 7.0) showed that the amount of this fraction is controlled by these two factors. It is possible that the macromolecular structure of these compounds is strongly influenced by these factors, the importance of which was pointed out by Ghosh and Schnitzer¹⁹.

The properties of humic substances which allow their fractionation on octyl-Sepharose can in part be related to the CH, CH₂ and CH₃ groups suspected to be the active sites for hydrophobic adsorption at the HA surface^{20,21}. Experiments with another cross-linked agarose containing hydrophobic phenyl groups, phenyl-Sepharose CL-4B, did not improve the fractionation of these humic samples. The hydrophobic character of molecular weight-fractionated FA has been studied by reversed-phase liquid chromatography by Hayase and Tsubota²².

This non-degrading method of separation opens up new possibilities for more detailed studies on humic substances, e.g., their characterization and their evolution during humification.

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