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Note

Isotachophoresis and isoelectric focusing of soil humic substances in polyacrylamide gel

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Classically, soil humic substances have been divided into three main fractions: (a) fulvic acid (FA); (b) humic acid (HA); and (c) humin[1]. However, there are no clear differences among these three fractions, as a continuous gradation appears to exist within FA, HA and humin fractions. Further, it has been postulated that no two humic substances are exactly similar[2]. Modern analytical techniques such as chromatography, gel permeation and electrophoresis have been used to study this problem, but only a few sub-fractions have been obtained so far. We report in this paper the separation of FA and HA into several sub-fractions by isotachophoresis and isoelectric focusing, which is helpful in the elucidation of the structure and physical, chemical and biological properties of these soil humic substances.

EXPERIMENTAL

Three milligrams of dry FA or HA, extracted with dilute sodium hydroxide solution from the A_1 horizon of a Brunizem soil[3], were dissolved in 1 ml of 0.1 *M* Tris, and 300 mg of sucrose were added. The FA and HA solutions (about 400 μg per run) were fractionated in polyacrylamide gel tubes using, with minor variations, the following analytical techniques: disc electrophoresis[4]; isotachophoresis[5], with either a mixture of amino acids and organic acids, or Ampholine carrier ampholytes (LKB, Stockholm, Sweden), p*I* range 3–10; and isoelectric focusing[6] with Ampholine carrier ampholytes, p*I* range 3–10. A high degree of reproducibility was obtained in all the replicate runs made at 5 mA per tube.

RESULTS AND DISCUSSION

The results obtained in the fractionation of FA and HA by disc electrophoresis were essentially not different from those previously obtained by other workers[7–9], or recently with sodium dodecyl sulphate[10]. Thus, in our work also, three bands were given by HA and two by FA (Fig. 1). With both FA and HA, the band with the highest mobility was yellow and fluorescent, and apparently it has the lowest molecular weight. We took advantage of the analytically variable parameters that disc electrophoresis exhibits, combining variations in the upper and running gel pH and the pore size of the molecular sieve[11,12]. Even under those conditions, disc electrophoresis was

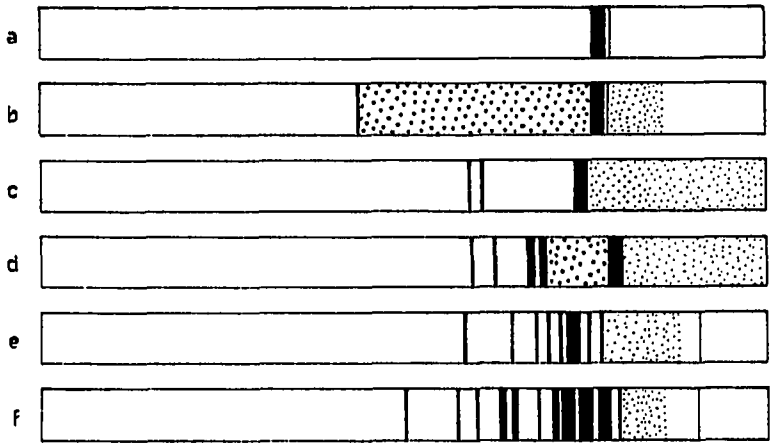

Fig. 1. Diagramatic interpretation of the electrophoretic separation of FA and HA on polyacrylamide gel tubes 12 cm long (the anode front is on the right). Disc electrophoresis on a 7.5% gel tube, pH 8.9: (a) FA; (b) HA. Isotachophoresis with amino acids and organic acids as spacers: (c) FA; (d) HA. Isotachophoresis with Ampholine carrier ampholytes, p*I* range 3–10, on a 3.75% gel column, pH 6.7: (e) FA; (f) HA. The yellow band is the nearest to the anode.

not useful for separating the possible components of the brown bands, probably owing to their similar electrical mobilities. Only with the use of 20% gel was a really appreciable difference observed in the mobilities of the bands.

Isotachophoresis proved to be an excellent method to resolve FA and HA into their individual components. In our preliminary studies we did not use Ampholine carrier ampholytes. Using a mixture of amino acids and organic acids as spacers and a 3.75% polyacrylamide gel column, 12.0 × 0.6 cm, pH 6.7, we separated HA into five bands and FA into three bands (Fig. 1). With the aid of an apparatus similar to that described by Maizel[13] and the mixture of amino acids and organic acids we were able to collect these sub-fractions. With Ampholine carrier ampholytes, the numbers of sub-fractions we obtained were ten for FA and thirteen for HA. In Fig. 1 (e and f) are shown: (a) a yellow region, the nearest to the anode, consisting of a neat yellow band followed by a light yellow zone, both fluorescent, corresponding to the disc electrophoresis yellow band; and (b) a brown region, consisting of several bands, close together owing to their similar electrical mobilities, corresponding to the disc electrophoresis brown bands.

The separation between bands was increased when isotachophoresis was performed with a longer polyacrylamide gel tube (40.0 × 0.6 cm), a high-voltage power supply and a proportionally larger amount of Ampholine. We believe that preparative electrophoresis carried out under those conditions will permit the elution of distinct bands without contamination.

The results with isoelectric focusing carried out in a polyacrylamide gel tube (35.0 × 0.6 cm) were similar to those obtained by isotachophoresis with respect to the number and expected arrangement of the bands along the natural pH gradient (Fig. 2). We believe that the behaviour of FA and HA in this system could be interpreted by assuming that most of the sub-fractions have an ampholytic nature probably due to their amino acid moiety[14,15]. The comparison between the arrangement of the

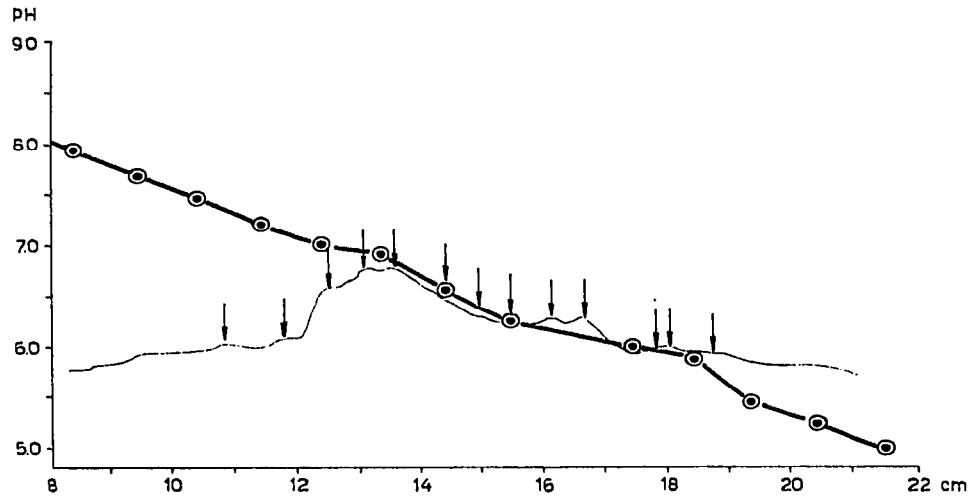

Fig. 2. Isoelectric focusing separation of humic acid in a 35-cm polyacrylamide gel tube with Ampholine carrier ampholytes, p*I* range 3–10. The steadily falling curve shows the natural pH gradient formed in the region of the tube where the bands were disposed. pH values were taken on 1-cm portions of the polyacrylamide gel after 6-h equilibration in 2 ml of distilled water. The thin line is the densitometric trace; arrows show the positions of the bands, and p*I* values can be read from the pH gradient curve.

bands and the stationary pH gradient formed gives a first physico-chemical characterization of the components with regard to their instability at different pH values. In a preliminary test Ampholine of the widest p*I* range was used. As show in Fig. 2, it appeared to be excessive. Recently different range ampholytes could not be used for their high expenses.

The elution of the bands obtained by isotachophoresis, followed by an isotachophoretic separation using Ampholine of various p*I* values under defined operating conditions, and subsequent elution of the sub-fractions obtained, will permit components to be obtained sufficiently pure for their physical, chemical and biological characterization.

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