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# Polyacrylamide gel electrophoresis of soil humic acid fractionated by size-exclusion chromatography and ultrafiltration

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**Abstract**

A humic acid (HA) from chernozem soil has been fractionated either by size-exclusion chromatography (SEC) on Sephadex G-75 using water, Tris–HCl or 7 *M* urea as eluents, or by ultrafiltration (UF) in the presence of 7 *M* urea or water. UF-fractionated HA was classified as 100K (nominal retention size >100 000); 30K (100 000–30 000); 10K (30 000–10 000); 5K (10 000–5000). Several pools from each chromatography and all ultrafiltration retentates were assayed by polyacrylamide gel electrophoresis (PAGE). The results indicate that SEC fractionation gave a better separation of HA on fractions differing in electrophoretic mobility and molecular size (MS), and that apparently SEC is a more feasible technique than UF for soil HA fractionation; nevertheless the UF in 7 *M* urea solution might be useful for MS evaluation of HA fractions obtained by association SEC–PAGE. PAGE in the presence of denaturing agents can be successfully used for checking the purity of HA fractions obtained by both SEC and UF.

*Keywords:* Soil; Humic acids

## 1. Introduction

Soil humic acids (HAs) are dark-colored, partly aromatic, chemically complex, polyelectrolyte-like materials with molecular sizes (MSs) ranging from several hundred to several hundred thousands [1]. Since HAs exhibit MS heterogeneity, many attempts have been made to fractionate them on the basis of MS. Thus, size-exclusion chromatography (SEC) and ultrafiltration (UF) have been extensively employed as rapid and versatile techniques enabling preparative quantities of samples to be obtained. However, much of the experimental data published on SEC and UF of HAs are contradictory and cannot be systematized, and it is not clear which method has more advantages for soil HA fractionation [2–8].

Up to now, optimal conditions have not been obtained for SEC of HAs, especially with regard to the elution system. Water and various elution systems containing NaCl, sodium-acetate, NaOH, pyrophosphate, etc., were used without success due to adsorption of HA. The use of buffer containing a large organic cation such as Tris or urea, which prevents

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adsorption of HA on Sephadex, is recommended [2,9–13].

The use of UF as a technique for estimating MS data and obtaining different MS fractions of HAs can result in erroneous data [14]. Broad, nominal MS cutoffs and solute interactions with membrane surfaces make the analysis of ultrafiltration data for these solutes very difficult [7,8,14].

Sometimes, fractionation of HAs has been achieved by using polyacrylamide gel electrophoresis (PAGE) [15–17], where electrophoretic mobility is related to both size and charge of HA fractions. Association of PAGE with SEC or UF seems to be useful for obtaining fractions with identical MS and electrophoretic mobilities (EM). However, attempts to discover the relationship between PAGE zones and chromatographic peaks or ultrafiltration retentates have previously been unsuccessful [13,17–19]. Recently some correlations between UF soil humic matter retentates and their EMs have been observed but not exactly defined, electrophoretic zones have been investigated [20], yet the question still remains as to what method (SEC or UF) is more preferable for soil HA fractionation.

Trubetskoj et al. [21] have developed HA fractionation by means of PAGE in the presence of denaturing agents, which allowed separation of soil HAs into four distinct naturally colored bands with different EM. The aim of this paper is to investigate the relationship between PAGE and SEC or UF, and the feasibility of these techniques for fractionation of soil HAs.

## 2. Experimental

### 2.1. Extraction and purification

The sample used in this study was taken from the A horizon (10–20 cm) of a typical chernozem soil (Kursk region, Russia). The soil characteristics are reported elsewhere [21]. The sample was air-dried and powdered to pass through a 2 mm sieve. Before extraction, plant debris was removed by flotation. 100 g batches of the sieved dry sample were extracted with a 1:10 cold solution of 0.1 *M* sodium pyrophosphate and 0.1 *M* NaOH under nitrogen gas. The extraction was repeated with fresh extractant until no appreciable amounts of HA could be removed (about 6 treatments). The extracts were centrifuged at 45 000 *g* to remove mineral residues, acidified to pH 2 with HCl, and the precipitated HA removed by centrifugation at 10 000 *g*. The extract was dissolved again in 0.1 *M* NaOH, centrifuged at 10 000 *g* for 30 min, then acidified and washed with 0.1 *M* HCl until colorless, dialyzed against distilled water and then lyophilized. Analytical characteristics of chernozem HA obtained were as follows: C 62.6%; N 3.2%; H 2.8%; ash 2.2%.

### 2.2. Size-exclusion chromatography

The HA was dissolved by adding the appropriate eluent (water, 0.1 *M* Tris–HCl, pH 9.0 or 7 *M* urea), made up to 1 ml and loaded onto a Sephadex G-75 (Pharmacia, Sweden) column (45×1.5 cm in the case of analytical and 100×1.5 cm in that of preparative SEC) with the same solution as eluent. Water HA samples were prepared by adding sufficient 0.1 *M* NaOH to give a solution of pH 7 and made up to 1 ml with distilled water. In the case of urea, HA samples were prepared by adding sufficient 0.1 *M* NaOH to give a solution of pH 7, made up to 1 ml with 7 *M* urea, and then dialysed against 7 *M* urea for 48 h. The samples applied to the column usually contained 5 mg HA in the case of analytical or 10 mg in that of preparative chromatography, and varied between 1–10 mg, when concentration effects were being investigated. Dark brown material adsorbed on Sephadex gel was eluted by 7 *M* urea. The flow-rate was 20 ml/h. The void column volume ($V_0$=20 ml for the analytical column and $V_0$=47 ml for the preparative column) was determined using Dextran Blue 2000. The total volume ($V_t$) was 65 ml for the analytical and 160 ml for the preparative column. For comparison of the fractionation results obtained on the analytical and preparative columns using 7 *M* urea the fractions were generally characterized by their $K_{av}=(V_e-V_0)/(V_e-V_t)$ ($V_e$=an elution volume corresponding to maximum absorbance of the peak). Column effluent was collected as 2 ml aliquots. The elution curves were determined by measurement of optical density at 280 nm with an UA-5 detector (ISCO, USA) and recorded with an automatic recorder. The fractions were collected into several pools, each of which was dialyzed against

distilled water, lyophilized and assayed by PAGE according to Trubetskoj et al. [21].

### 2.3. Ultrafiltration

Ultrafiltration of 10 mg HA dissolved in 100 ml 7 *M* urea or water was carried out using a series of Amicon Diaflo Ultrafilters of various MS retentions and an Amicon filtration device. The ultrafilters used and their MS retentions were as follows: YM100 (nominal retention size >100 000); YM30 (>30 000); YM10 (>10 000); YM5 (>5000). The membranes used were 62 mm in diameter. HAs and their fractions were passed through each filter starting with the highest MS retention (YM100) and then through progressively smaller MS retainers in a nitrogen gas atmosphere at a pressure of 10 p.s.i. (1 p.s.i.=6894.76 Pa). The resulting HA fractions were classified as HA (100K) MS>100 000; HA (30K) 30 000–100 000; HA (10K) 10 000–30 000; HA (5K) 5000–10 000. All ultrafiltration retentates were collected, dialyzed, lyophilized and assayed by PAGE according to Trubetskoj et al. [21]. Molecular size distributions of chromatographic HA fractions between UF membranes were determined by comparing of optical densities at 465 nm of retentates to those of initial amounts, using a Beckman DU-8 spectrophotometer equipped with an automatic recorder.

### 2.4. Electrophoresis

The method used for fractionation of HA has been reported previously [21]. 9.7% acrylamide and 0.3% bisacrylamide were dissolved in 89 m*M* Tris–borate, pH 8.3, with 1 m*M* EDTA and 7 *M* urea. The apparatus was a vertical electrophoresis device (LKB 2001 Vertical Electrophoresis, Sweden) with gel slab (20×20 cm). Electrophoresis was carried out for 1 h at a current intensity of 25 mA. For analytical electrophoresis we have applied 0.05–0.25 mg of each sample completely dissolved in 50 μl of sample buffer containing 89 m*M* Tris–borate, pH 8.3, 7 *M* urea, 1% SDS and 1 m*M* EDTA on the gel. Preparative electrophoretic fractionation of HA, isolation and purification of HA fractions from the polyacrylamide gel were carried out using a previously published method [22].

## 3. Results and discussion

Chernozem HA was fractionated by SEC on Sephadex G-75 with water, 0.1 *M* Tris–HCl, pH 9.0, or 7 *M* urea as eluents to discover which eluting system is preferable for HA fractionation.

Several absorbance peaks with shoulders between them were resolved in the elution profiles of HA samples in all cases (Fig. 1a–c). Chromatographic pools from each experiment were collected and assayed by 10% PAGE in the presence of denaturing agents (Fig. 2a–c). The unfractionated HA, used as a reference, was divided into four discrete fractions: (A) a start zone that did not move into the gel; (B), (C) and (D) three narrow intensely colored zones. Zone B differed greatly from zones C and D in electrophoretic mobility, zones C and D were combined in fraction C+D due to relatively close electrophoretic behavior (Fig. 2d).

In the case of SEC separation with water or Tris–HCl buffer as eluents (Fig. 1a,b) essential interaction between Sephadex-gel and solute still takes place, a great deal of HA material was eluted after the total column volume. The electropherograms show that the excluded peaks (pools C1) formed fraction A and B; pools C2 produced fraction B with some admixture of fractions A and C+D; pools C3–C8 (water eluting system) and C3–C7 (Tris–HCl eluting system) formed fractions B and C+D. Most of the brown colored material, adsorbed on the top of the column, was washed off with 7 *M* urea eluent and combined in pools C9 and C10 (water eluting system) or pool C8 (Tris–HCl eluting system), which formed on electropherogram fractions A and B with some admixture of fraction C+D or fractions B and C+D with some admixture of fraction A, respectively (Fig. 2a,b).

In the case of urea SEC separation all humic matter, loaded on the column, was eluted from the gel within the total column volume and there was no adsorption of HA on the gel matrix (Fig. 1c). Moreover, pools C1 to C7 obtained better separation of HA on electrophoretic zones: pool C1 formed fraction A; C2 and C3, fraction B; C4 and C5 contained mixture of previous fraction B and the following fraction C+D; C6 and C7, fraction C+D (Fig. 2c). It should be noted that when HA was fractionated on the column equilibrated with the

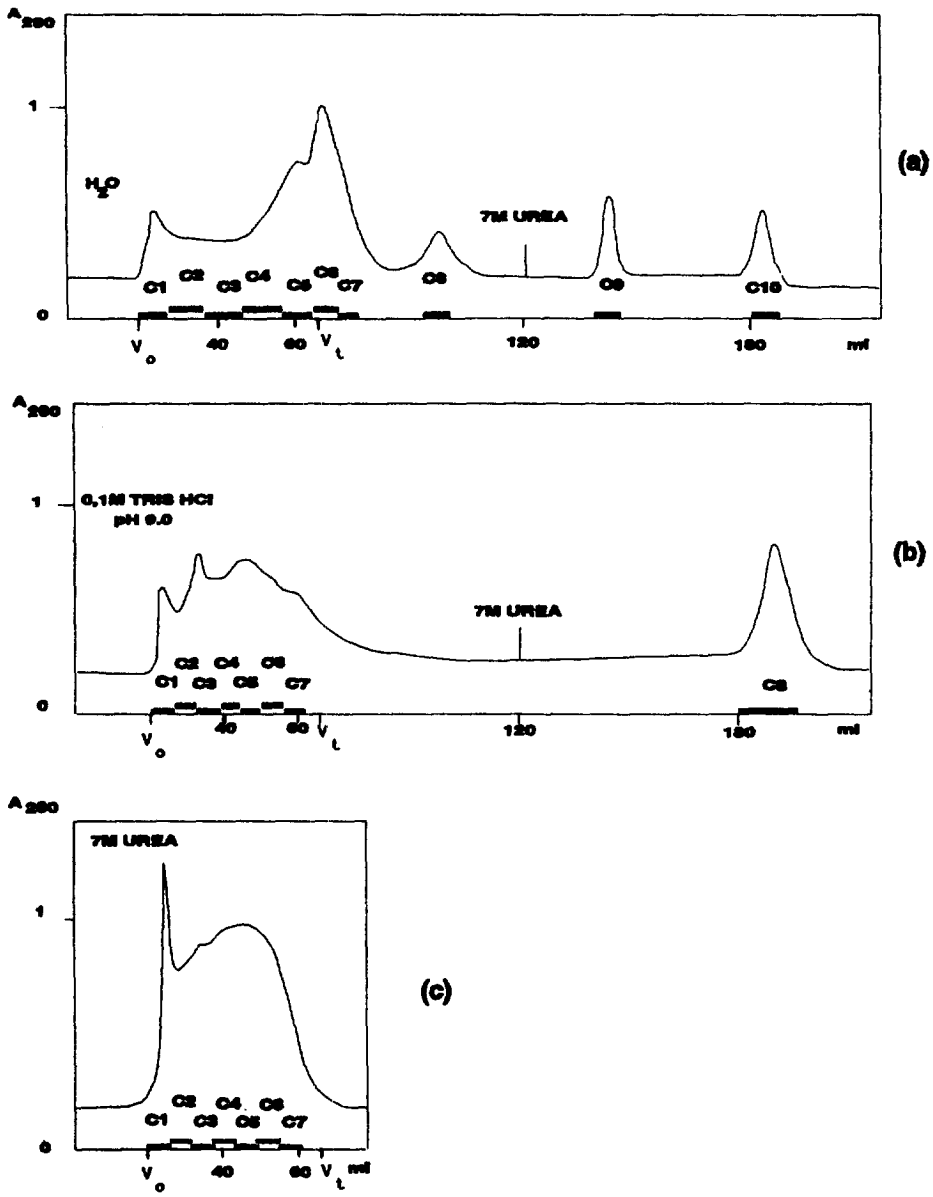


Fig. 1. Fractionation of 5 mg chernozem HA on Sephadex G-75 column (45×1.5 cm) and chromatographic pools collected from size-exclusion chromatography using (a) water (C1–C10), (b) Tris–HCl (C1–C8), (c) 7 *M* urea (C1–C7) as eluting systems.

buffer containing 0.1 *M* Tris–HCl, pH 9.0, plus 7 *M* urea, the elution profile and electrophoretic analysis of the pools C1–C7 gave identical results with those obtained by using 7 *M* urea. These results suggested that Tris–HCl buffer does not improve the conditions for fractionation of HA by SEC on Sephadex and the column equilibrated with 7 *M* urea is preferable, therefore we used 7 *M* urea as eluent for concentration and flow-rate effect investigations.

When 1 mg, 5 mg or 10 mg HA was applied on the same column, all loaded humic matter was eluted from the gel within the total column volume in-

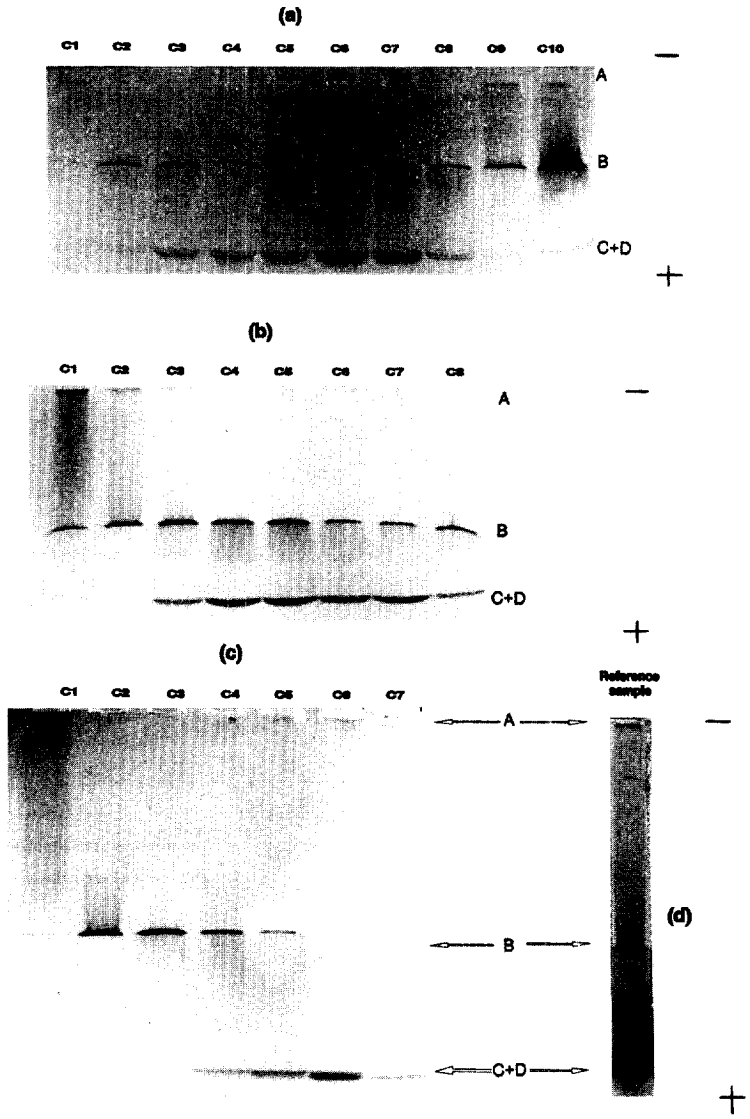


Fig. 2. Electropherograms of chromatographic pools collected from size-exclusion chromatography of chernozem HA using (a) water (C1–C10), (b) Tris–HCl (C1–C8), (c) 7 *M* urea (C1–C7) and (d) unfractionated chernozem HA as a reference. The A, B, C+D are discrete colored zones which have been obtained by PAGE in the presence of denaturing agents.

dependently of sample concentration. The C1–C7 pools from each chromatography, which were checked by electrophoresis, showed identical results, but the intensity of the electrophoretic bands increased with the increasing of quantity of HA loaded on the column.

A 5 mg and 10 mg HA sample was loaded on the column in order to determine the influence of flow-rate on SEC fractionation. The elution profile and electrophoretic analysis did not change, even when three different flow-rates (10 ml/h, 20 ml/h and 30 ml/h) were used.

According to these results it could be concluded that HA fractionation in 7 *M* urea is based soley on MS differences, because: (a) the whole of applied sample is eluted within the total column volume and

there is no interaction between gel matrix and the macromolecules fractionated; (b) fractionation is largely independent of sample concentration and flow-rate. On the other hand, the electrophoretic analysis showed that 7 *M* urea is preferable for obtaining chromatographic fractions, which formed only one electrophoretic zone in the PAGE matrix.

After preparative HA fractionation (Fig. 3a) the chromatographic elution profile was assayed by PAGE and aliquots, which formed only one electro-

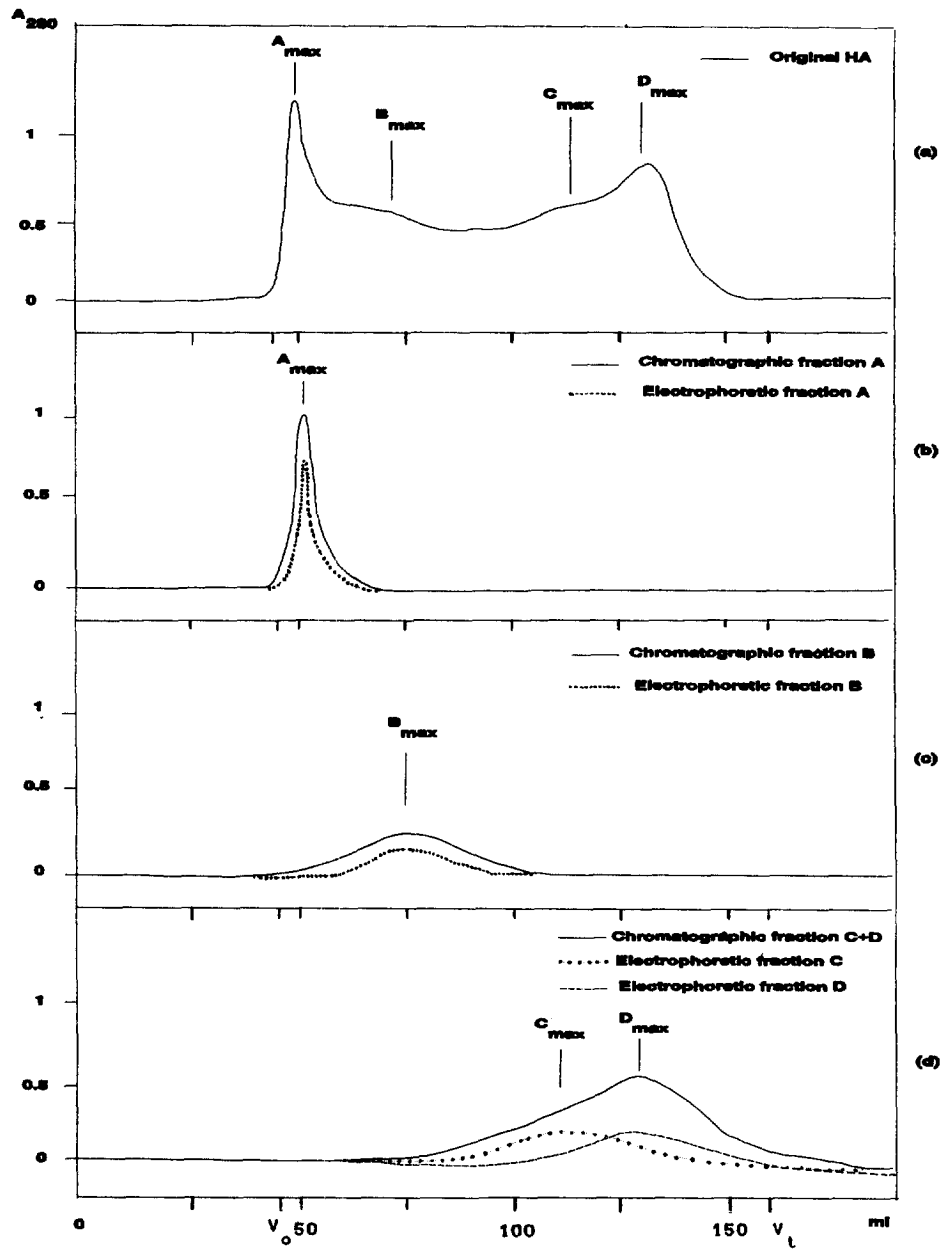


Fig. 3. Fractionation of 10 mg chernozem HA on Sephadex G-75 column (100×1.5 cm) using 7 *M* urea eluting system (a); rechromatography of chromatographic and electrophoretic fractions on the same column (b, c, d).

phoretic zone, were combined into pools; A (excluded peak, elution volume 47–55 ml, $K_{av}$=0.03), B (elution volume 58–80 ml, $K_{av}$=0.25), C+D (elution volume 110–150 ml, $K_{av}$=0.73), concentrated to 1 ml on YM5 ultrafiltration membrane and applied on the same column. It should be noted that $K_{av}$ for A, B and C+D fractions were similar as for preparative and analytical columns. Rechromatography of fractions A, B and C+D yielded the same elution patterns as the original chromatogram (Fig. 3).

After preparative electrophoresis according to Trubetskoj and Trubetskaya [22] electrophoretic zones A, B, C and D were isolated by PAGE and applied on the chromatographic column for comparison with the corresponding SEC fractions. Electrophoretic fractions gave identical elution patterns, which corresponded to the same positions as the SEC fractions (Fig. 3b–d). Hence, the electrophoretic and corresponding chromatographic fractions had identical MS values [23]. This data confirms the assumption [20,22] that distribution of HA in the PAGE matrix is mainly by MS.

Another method for MS determination is ultrafiltration. In theory, UF is a simple process. In practice, however, a number of problems concerning to broad, nominal MS cutoffs and solute interactions with membrane surfaces have been noted [14]. In this work we used our method of PAGE for analysis of HA fractions obtained by UF.

Chernozem HA dissolved in 7 *M* urea or distilled water was fractionated using a series of Amicon Diaflo Ultrafilters of different MS retentions. The resulting HA retentates were classified as HA 100K (nominal retention size >100 000); HA 30K (100 000–30 000); HA 10K (30 000–10 000); HA 5K (10 000–5000). All retentates were collected, dialyzed, lyophilized and assayed by PAGE (Fig. 4). When 7 *M* urea was used, retentate 100K originated on electropherogram fractions A and B with some admixture of fraction C+D; retentate 30K yielded fractions B and C+D; retentate 10K yielded fraction C+D with admixture of B, and retentate 5K gave only fraction C+D (Fig. 4). When water was used, retentates 100K and 30K produced fractions A and B with some admixture of fraction C+D; retentate 10K yielded fractions B and C+D and retentate 5K gave traces of fractions B and C+D (Fig. 4). It seems that

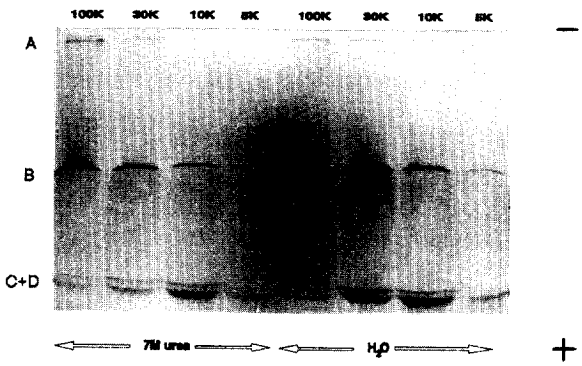


Fig. 4. Electropherograms of retentates obtained by ultrafiltration of chernozem HA dissolved in 7 *M* urea or water. The resulting HA retentates are HA 100K (nominal retention size >100 000); HA 30K (30 000–100 000); HA 10K (10 000–30 000); HA 5K (5000–10 000).

fractionation in urea originated better separation of HA into fractions according to MS because of 7 *M* urea destroys hydrogen bonds and prevents the formation of humic aggregates. However, neither urea nor water provided obtaining electrophoretic pure fractions (with the exception of 5K retentates dissolved in urea). All UF retentates contained mixture of different MS fractions.

Based on the electrophoretic analysis of SEC pools and UF retentates, the SEC in 7 *M* urea appears to allow us to obtain a preparative quantity, comparatively homogeneous with respect to electrophoretic mobilities, of soil HA fractions with different MS, and for this purpose SEC is a more effective method than UF.

Nevertheless, we have used the UF method for approximate evaluation of MS of HA fractions obtained by tandem SEC–PAGE. Fractions A, B and C+D dissolved in 7 *M* urea were passed through a series of ultrafiltration membranes starting with the highest MS retention (YM100) and then through progressively smaller MS retainers. 83% of fraction A was retained by the 100K membrane and 17% by 30K; 22% of fraction B was retained by 100K, 59% by 30K and 19% by 10K membranes; 7% of fraction C+D was retained by the 30K membrane, 60% by 10K, 30% by 5K and only 3% passed through the 5K membrane. Based on these results it can be suggested that the MS of fraction A is high, likely >100 000 (nominal retention size), fraction B mainly

ranges between 100 000 and 30 000 and fraction C+D ranges from 30 000 to 5000.

The MS values obtained by UF are comparable to those obtained by size-exclusion chromatography on Sephadex G-75, because fraction A was eluted in the void column volume and the fractionation for Sephadex G-75 ranges from 80 000 to 3000 for proteins and from 50 000 to 1000 for polysaccharides.

Summarizing the results of this study it can be stated that:

1. Urea (7 *M*) is preferable to water or Tris–HCl elution systems for fractionation of soil HA on Sephadex G-75.
2. The combination SEC–PAGE indicated a good correspondence between both fractionation systems; the increase of elution volume closely corresponds to an increase of electrophoretic mobility of soil HA.
3. Electrophoretic and corresponding chromatographic fractions yield the same elution pattern as the original chromatograms of HA and therefore PAGE fractionation of soil HA is based mainly on MS differences.
4. PAGE in the presence of denaturing agents can be successfully used for checking of the purity of HA fractions obtained by SEC or UF.
5. SEC in 7 *M*. urea allowed us to obtain a preparative quantity, comparatively homogeneous with respect to electrophoretic mobilities, of soil HA fractions with different MS, and for this purpose SEC is a more feasible method than UF. Nevertheless, the UF method might be useful for MS evaluation of HA fractions obtained by SEC–PAGE.

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