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Journal of Chromatography A, 807 (1998) 275–284

JOURNAL OF CHROMATOGRAPHY A

Ultrafiltration–capillary zone electrophoresis for the determination of humic acid fractions

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Received 28 July 1997; received in revised form 21 January 1998; accepted 28 January 1998

Abstract

Ultrafiltration and capillary zone electrophoresis were combined to obtain the molecular-mass distribution of one commercial and four natural humic acids. The molecular-mass fractions derived from the cut-offs used in the ultrafiltration process were 10 000, 30 000, 50 000, 100 000 and 300 000. The same electrophoretic behavior was observed in all the fractions, which enabled us to quantify the humic acid content in each case. In obtaining reliable values of humic acid distribution, the influence of gas pressure, time-dependent concentration of the solute in the ultrafiltrate and solute concentration in the feed solution were evaluated.

Keywords: Ultrafiltration; Soil; Humic acids

1. Introduction

Humic acids (HAs) are organic macromolecules exhibiting a wide variety of molecular-mass (M_r) distributions, substructures and functional groups. These compounds are of most interest in environmental systems since they have considerable influence on the bioavailability of toxic elements because of their high complexation capability. Fractionation and characterization of HAs have been studied in recent years using chromatographic [1], spectroscopic [2] and electrophoretic techniques such as isoelectric focusing in polyacrylamide gel [3] and capillary isotachophoresis [4]. However, few methods have been developed for the determination of these compounds, so that the quantification of the dissolved organic carbon (DOC) after the isolation of the HAs from the original sample is the most widely applied method for this purpose. To date, only two new methods have been developed for the determination of HAs directly following the extraction process. The first of these is based on high-performance liquid chromatography (HPLC) with fluorimetric detection [1], while the second uses capillary zone electrophoresis (CZE) with UV detection for the determination of the HA content in soil samples, after a single extraction–precipitation–solubilization process and without requiring further purification steps [5]. However, none of these methods has been applied to the quantification of the HA fractions associated with a given M_r range in soil samples.

Ultrafiltration (UF) in a stirred cell is increasingly being used as a separation technique and its applications in environmental studies include, among others, the determination of formation constants of metal ion–HA complexes [6,7], and the isolation and fractionation of HAs and fulvic acids (FAs) from

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0021-9673/98/$19.00
PII: S0021-9673(98)00088-0

natural waters [8,9]. Therefore, the combination of UF–CZE may be considered as a method for the determination of HA distribution, according to the M_r of HAs. To date, studies carried out on the UF technique applied to HAs have highlighted the influence of certain parameters such as pH, ionic strength and solute concentration on the fractionation pattern of humic substances [6,7,10].

However, in all the studies cited, focused mainly on aquatic systems, the HA content was finally quantified through the DOC [6,9]. In a recent study, the CZE technique was used following UF to characterize the different M_r fractions of a HA derived from a peat soil, though here no attempts were made at quantification [11].

In this paper we present the combined use of UF–CZE for the determination of the HA fractions in soils. First, the dependence of the shape of the electropherogram on the M_r was tested in commercial and natural HAs, since this fact was not clarified in previous studies. Once it was demonstrated that the electropherogram obtained did not depend on the M_r of the HAs, under given experimental conditions developed in a previous work [5], the determination of the HA content could be carried out for all the fractions obtained by UF. The second objective of this study was to obtain the M_r distribution of HAs derived from commercial and natural samples. In order to obtain reliable values, the influence of various experimental parameters on the percentage of the ultrafiltered HA fraction, including gas pressure, time-dependent concentration of the solute in the ultrafiltrate during the separation process and solute concentration in the feed solution, were considered.

2. Experimental

2.1. Humic acid samples

2.1.1. Commercial humic acid

Commercial HA from Fluka (Ref. 53680) was used as a reference for quantification as in previous studies [12,13]. The elemental composition of this HA was 45.9% C, 3.7% H and 0.6% N presenting an absorptivity of 0.0229 expressed as absorbance at 400 nm per mg l^{-1} of C per cm of cell. A 100 mg l^{-1} stock solution was prepared by dissolving a given mass of HA in 0.1 mol l^{-1} NaOH (Merck, analytical-reagent grade) and diluting it to 10^{-3} mol l^{-1} NaOH. This stock solution was stored at 4°C and further solutions, which were later injected into the CZE system for calibration purposes, were obtained by dilution in 10^{-3} mol l^{-1} NaOH.

2.1.2. Natural humic acids

Four HA samples were obtained following extraction from the $A_{h\ (0-10\ cm)}$ horizon of a forest soil (Montseny), the humus horizon of another forest soil (Prades), an arable soil (Bragin) and a meadow (Hatton), using a simplified extraction procedure detailed below. The soil classification and certain characteristics of the original samples such as pH, total organic carbon (% C org.), cation-exchange capacity (CEC) and percentage of HAs are shown in Table 1.

2.2. Extraction of natural humic acids

HAs were extracted by applying a simplified

Table 1
Some characteristics of soil samples

Label	Soil classification	pH^{a}_{KCl}	% C org.	Cation-exchange capacity ($cmol_c\ kg^{-1}$)	Humic acids[b] (%)
Montseny	$A_{h\ (0-10\ cm)}$ horizon of a Chromic Luvisol forest soil, Montseny, Spain	4.2	4.2	9.0	2.6
Prades	Humus horizon of a Eutric Cambisol forest soil, Prades, Spain	5.9	18.1	62.3	3.5
Bragin	Terric Histosol peat soil derived from schists, Bragin region of Gomel, Belarus	5.1	29.0	103	19.6
Hatton	Peat of the Hatton Association developed over Middle Old Red Sandstone, Moss of Fishrie, Scotland, National Grid Reference NJ 823592	2.4	44.8	237	6.0

[a] Obtained in 0.1 mol l^{-1} KCl with a ratio of 2.5 ml of solution per gram of soil.
[b] Determined following a method previously described [5] and expressed as grams of HA per gram of soil (for further details see Section 2.4).

method adapted from the literature [14]. The humus and soil samples, ranging from 1 to 4 g depending on the HA content, were shaken with 0.1 mol l^{-1} NaOH (Merck, analytical-reagent grade) for 16 h with a ratio of 40 ml of solution per gram of soil. The dark colored supernatant was then separated from the residual soil by centrifugation at 13 000 *g* for 20 min.

To isolate HAs, the alkaline extract was first filtered through a 0.45-μm cellulose acetate filter, then acidified to pH 1 with 6 mol l^{-1} HCl (Merck, analytical-reagent grade) and allowed to stand at room temperature for 16 h. The soluble material (attributable to the FA fraction) was separated from the coagulated material (attributable to the HA fraction) by centrifugation at 13 000 *g*. The HA fraction was then washed with 1 ml of double-deionized water and centrifuged again. Finally, it was dissolved in 10^{-3} mol l^{-1} NaOH without further purification or drying.

A further sample of HAs extracted from the Montseny soil by the method proposed by the International Humic Substances Society (IHSS) was also studied, in order to compare the two extraction procedures.

2.3. Ultrafiltration

UF was performed under nitrogen pressure, in an Amicon 8050 stirred cell with 43 mm I.D. and 50 ml capacity. Filtron Omega membranes (polyethersulfone) with different M_r cut-off levels (10 000, 30 000, 50 000, 100 000 and 300 000) were used in the UF process. Different aliquots of the same feed solution, which was defined as the HA sample in 10^{-3} mol l^{-1} NaOH, were ultrafiltered through the different cut-offs. The concentration of HAs in the ultrafiltered volume and in the feed solution was determined by CZE (see Section 2.4), the ratio between these two concentrations allowing the definition of the percentage of HAs with a M_r lower than the cut-off. Then, five fractions were obtained in each case with M_r <10 000, <30 000, <50 000, <100 000 and <300 000 and the M_r distribution for the HA sample was obtained by difference between each pair of consecutive fractions. UF was carried out by placing the feed solution directly in the stirred cell after rinsing the membrane with distilled water. The first 2 ml of ultrafiltrate were discarded in each case to avoid the dilution effect because of the water remaining in certain parts of the cell after rinsing. The pressure of UF and the volume ultrafiltered were adjusted to obtain an optimal, constant concentration of HAs.

2.4. Capillary zone electrophoresis

A CZE system from Applied Biosystems Model 270A was used with a 72 cm×50 μm fused-silica capillary filled with the buffer. This instrument is equipped with a UV detector with a deuterium lamp operating between 190 and 700 nm. The electropherograms were recorded using a Hitachi Model D-2500 integrator.

The method used in the separation process was developed in a previous study [5]. The capillary was first washed with a 0.1 mol l^{-1} HCl solution (Merck, analytical-reagent grade) for 2 min. A buffer solution, 59.9 mmol l^{-1} in L-alanine (Merck, analytical-reagent grade) and 8 mmol l^{-1} in HCl at pH 3.2, was used for 5 min to condition the capillary. Some tests were carried out to discard precipitation of HAs into the capillary. The low concentration of HAs into the capillary prevented them from coagulation. The capillary was flushed using a built-in vacuum system at $6.8 \cdot 10^4$ Pa. The sample was injected for 12 s by the vacuum technique resulting in a volume of 48.6 nl, and separation was achieved using a voltage of 15 kV with anodic injection and cathodic detection. Each sample was injected in triplicate and detection was carried out at 215 nm by means of an optical window for UV located 50 cm from the injection site. The column temperature was set at 40°C.

As shown in the previous study [5], four peaks were obtained for the HAs in these conditions. The small peaks around 10 and 12 min were considered artifacts since their area did not show any relationship with the concentration of HAs and they sometimes appeared when blank was injected. On the contrary, peak at 9 min and band at 16 min were attributable to HAs because of the correlation between the area of these peaks and the HA concentration, with the following equations: peak $\text{area}_{9\ \text{min}} = 0.0194[\text{HA}] - 0.0081$ ($r > 0.998$, $\text{R.S.D.}_{\text{slope}} = 7\%$) and band $\text{area}_{16\ \text{min}} = 0.0795[\text{HA}] +$

0.1358 (r>0.998, R.S.D.$_{slope}$=8%). Among these two peaks, peak at 16 min was also discarded for quantification purposes, since it underwent some changes in shape when working with natural HAs, which could suggest some undesirable compounds being present in this peak for these samples. Therefore, the peak at 9 min was finally used in estimating the HA content, since it was identical and very reproducible for both commercial and natural HAs. The R.S.D. of the migration time for this peak was lower than 3% and peak area measurements were chosen since they were more precise than peak height measurements with a R.S.D. lower than 5%. A calibration plot method with a concentration range from 5 to 100 mg l^{-1} was used for quantification [5].

3. Results and discussion

3.1. Influence of molecular mass range on the electropherogram

Several parameters might influence the electropherogram pattern of HAs in CZE. For example, the influence of the buffer solution and its pH have already been established. Using the experimental conditions described in the Section 2.4, the electropherogram obtained with HCl–L-alanine at pH 3.2 differs from those obtained with other buffer solutions shown in the literature, such as phosphate at pH 6.3 [15] or 9.0 [11], borate at pH 8.5 [11], dihydrogenphosphate–tetraborate at pH 8.9 [16] and acetate at pH 4.9 [15]. For the same buffer solution significant changes were also observed with little variation of pH [15]. With respect to the origin of the HAs, some authors state that it might also influence the electropherogram [15]. On the contrary, similarities in the electropherogram shape and mobilities were observed for commercial and natural HAs under our experimental conditions [12,13], which agreed with other findings from the literature [16]. However, less is known about the influence of the M_r of HAs on the electropherogram obtained, especially if what is sought is the relationship between the peaks obtained and the M_r range. This aspect has to be studied in considerable depth in order to ensure quality in the quantification process. To establish the influence of M_r on the electropherogram, a feed solution of the commercial HA was ultrafiltered through the various cut-off membranes, and the resulting fractions were injected to the CZE system.

Fig. 1a shows the electropherograms of the M_r fractions obtained from a feed solution of 75 mg l^{-1} of the commercial HA. As can be seen, there were no qualitative changes in the electropherograms of the ultrafiltrates obtained from the various M_r cut-off membranes, indeed peaks and migration times were similar in all the electropherograms. This fact is especially relevant for the first peak, which is used for quantification purposes. In that case, changes in migration times for different M_r samples were of the same order that for replicates of the same sample (R.S.D.<3%). The constancy in the shape of the electropherograms indicated that charge density had a negligible effect on the mobilities of the HAs under the experimental conditions used, as obtained by other authors under different experimental conditions [17]. For the M_r <10 000 fraction, a considerable widening in the wide band was observed. However, this can be attributed to the low concentration of HAs in this fraction, since this widening also occurred in highly diluted solutions (under 20 mg l^{-1}) without UF (electropherograms not shown here), but it was not an impediment for quantification.

The influence of M_r on the electropherogram was also tested for the HA from the Montseny soil using a feed solution of 120 mg l^{-1}, in order to check if similar conclusions could be drawn for natural HAs. Fig. 1b shows the electropherograms obtained for a number of the M_r fractions studied (<100 000 and <50 000). First, it can be observed that similar electropherograms were obtained for the two commercial and natural HAs. Besides, the electropherograms did not depend on the M_r range for natural HAs, although a slight distortion of the peak used for quantification was observed when using the M_r 50 000 membrane cut-off. This distortion seemed to be related to the nature of the HAs and not to the separation technique, since it did not appear with the commercial HA. Moreover, this distortion lost significance when working with more diluted feed solutions (data not included). At this stage there is no apparent explanation for this behavior. Therefore, for natural HAs, working with feed solutions with lower concentrations than when using commercial HAs may be recommended, although the concentration

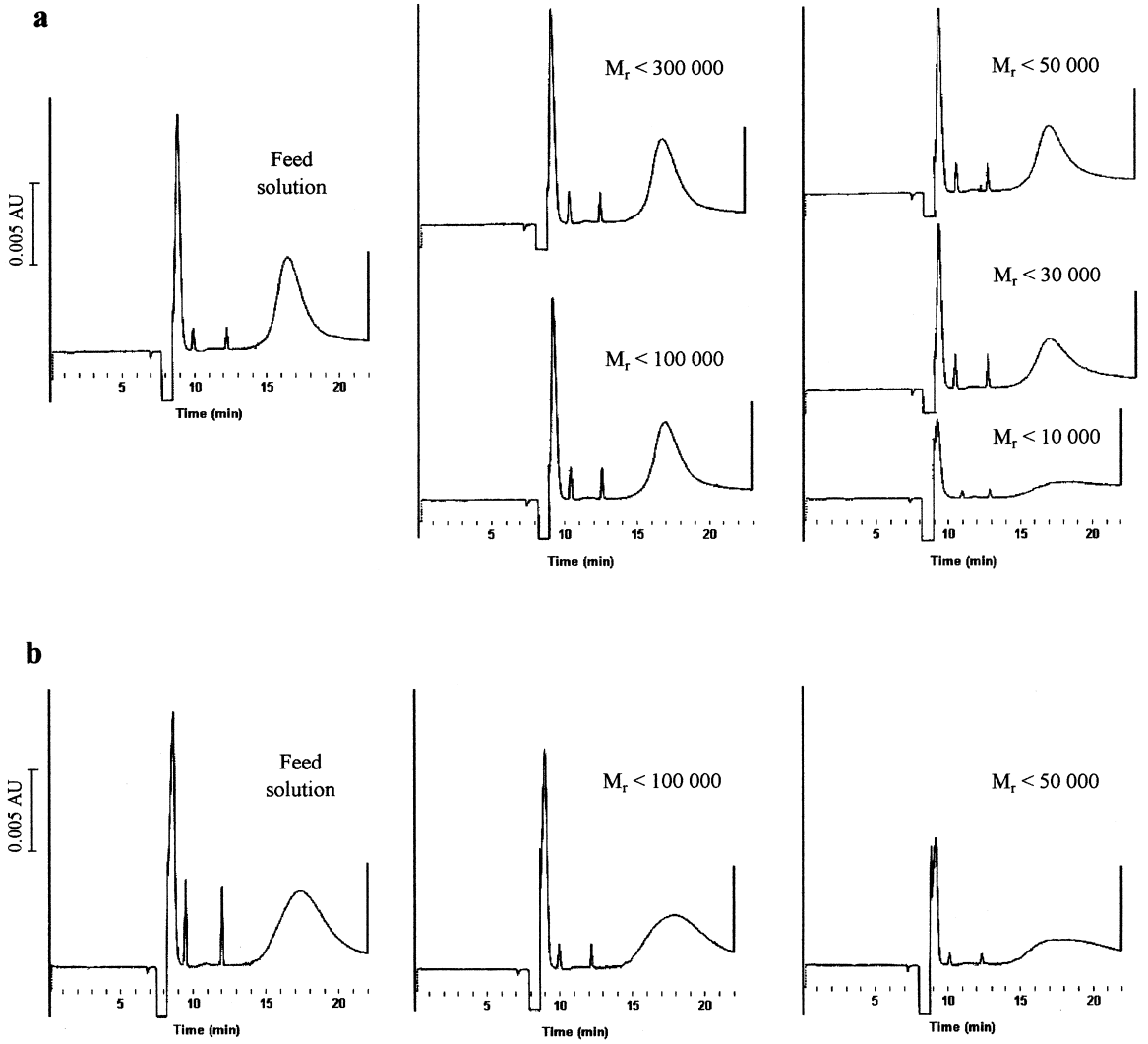

Fig. 1. Electropherograms of the M_r fractions obtained from the UF of (a) a feed solution of 75 mg l^{-1} of the commercial HA through M_r 300 000 (75 mg l^{-1}), 100 000 (65 mg l^{-1}), 50 000 (60 mg l^{-1}), 30 000 (42 mg l^{-1}) and 10 000 (15 mg l^{-1}) and (b) a feed solution of 120 mg l^{-1} of the Montseny HA through M_r 100 000 (80 mg l^{-1}) and 50 000 (45 mg l^{-1}).

cannot be drastically lowered to ensure quantification for cut-offs of M_r lower than 50 000.

The lack of dependence of the electropherogram on the M_r range observed in this study is in agreement with other studies that show that similar mobilities could be expected for HAs of different M_r when working in free solution and that to enhance separation according to M_r the use of polymer matrices such as polyacrylamide gel could be proposed to act as a molecular sieve [17]. From another study, a similar conclusion was derived since a single broad band with similar migration times for HA fractions of different M_r was also obtained using a phosphate buffer at pH 9 [11]. However, we should emphasize that this lack of dependence cannot be extrapolated to other experimental conditions. Thus, the later authors, using borate buffer at pH 8.5, have found changes in the electropherograms in accord-

ance with the M_r range considered and with better resolution of the peaks, but also with similar migration times [11].

Since non-ultrafiltered solutions were decided to be used for calibration, the potential influence of the different M_r ranges of the HAs in the relationship between concentration and absorbance was also tested, to discard changes in sensitivity that may lead to errors in the quantification step. In short, several feed solutions of different concentrations were ultrafiltered through a given M_r cut-off and the ratios between the peak area of the ultrafiltrate and the peak area of the feed solution were calculated. These ratios were quite constant (R.S.D.$<$5%) regardless of the concentration of the feed solution, thus indicating a similar sensitivity of the non-ultrafiltered and the ultrafiltered samples.

Therefore, it can be concluded that in using electrophoretic separation under the experimental conditions of this paper, both commercial and natural HAs showed the same pattern and the same response independently of their M_r, and that the peaks in the electropherogram were not related to a given M_r fraction. Then, the experimental conditions proposed in this paper could be considered unsuitable for characterization purposes, but allow a proper quantification of the HA content in all cases using the commercial HA as a reference.

3.2. Experimental parameters influencing ultrafiltration efficiency

After demonstrating that the CZE–UV quantification step is not influenced by the previous UF separation, the optimization of the UF step is illustrated in this section. The fractions derived after an UF separation are usually related to M_r ranges because of the characteristic nominal M_r cut-off levels of the membranes, which are operationally defined as the mass of a model solute whose retention is 90% on this membrane. However, even though the membranes are classified by the manufacturers according to their M_r, it must be emphasized that solute molecules are separated according to molecular size in UF. Converting molecular size to M_r is by no means straightforward, particularly in the case of macromolecules where, given their flexibility, molecular shape is a very important factor. For HAs, various parameters, mainly pH and ionic strength may lead to changes in molecular size for similar M_r, thus exhibiting very different behavior. In general, high pH values are recommended for decreasing adsorption on the membrane surface [6,10], although some authors state that ionic strength and pH do not have a significant effect on the UF of HAs when extreme conditions were not used [8,9]. In the present study, the influence of pH and ionic strength was not considered, since all the feed solutions used in UF had a constant pH and ionic strength due to the dilution of the sample in 10^{-3} mol l^{-1} NaOH. Furthermore, all the comparisons were made on the basis of the M_r operationally defined by the cut-offs of the membranes. Several interfacial problems might be present during UF, such as interactions macromolecule–membrane and self-coagulation of colloids at the membrane surface [18]. These two effects are difficult to remove but they can be minimized by using a continuous stirring of the solution during the UF process and, as shown in the following sections, obtaining a small volume of ultrafiltrate.

In order to obtain reliable values of the concentration of HAs according to their M_r, three parameters were considered as being of great significance in the present study. These were gas pressure, time-dependent concentration of the solute in the ultrafiltrate during the separation process and concentration of the feed solution. Their influence on the percentage of the HAs associated with each fraction is shown in the following sections.

3.2.1. Influence of gas pressure on the humic acid content

The percentage of ultrafiltered HAs, calculated from the ratio of the HA concentration in the ultrafiltrate with respect to the HA concentration in the feed solution, was determined for the commercial HA at increasing gas pressures and for a set of membrane cut-offs. Fig. 2 shows the changes in the percentage of HAs associated with a given M_r range, depending on the gas pressure applied (solid lines for a 300 mg l^{-1} feed solution). As can be seen, there was a significant influence of gas pressure on the fractionation. The percentage of HAs related to a M_r fraction increased when increasing the gas pressure, until a plateau was achieved. The optimal gas

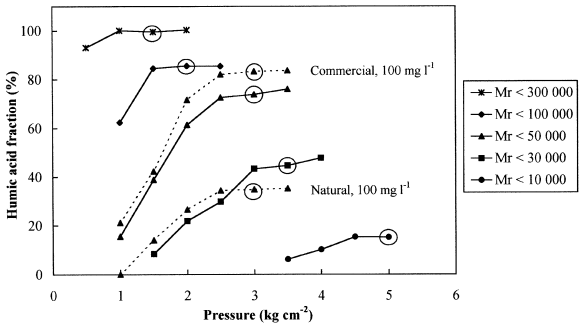

Fig. 2. Influence of gas pressure on the percentage of HAs obtained by UF of a 300 mg l^{-1} feed solution of the commercial HA through the different membrane cut-offs (solid lines) and by UF of a 100 mg l^{-1} feed solution of the commercial and Montseny HAs through an intermediate cut-off of M_r 50 000 (dotted lines).

pressure for each cut-off was deduced by considering the second pressure value of the plateau to ensure the maximum percentage for the corresponding HA fraction. Pressures below the optimal would lead to the establishment of unreal M_r distributions of the HAs or even to an inability to quantify some of the fractions. An optimal and different gas pressure should be used therefore for every membrane – the gas pressure increasing as the M_r cut-off of the membrane decreased. The pressures established for subsequent analysis were 1.5, 2.0, 3.0, 3.5 and 5.0 kg cm^{-2} for M_r 300 000, 100 000, 50 000, 30 000 and 10 000, respectively.

Considering the potential dependence of optimal pressure on the concentration of the feed solution or even on the nature of the sample, a lower concentration of the feed solution for the commercial HA (100 mg l^{-1}) and also a natural HA taken from the Montseny sample were tested using an intermediate cut-off of M_r 50 000 (dotted lines). The results from these experiments are also shown in Fig. 2. As can be seen, the same optimal pressure was obtained regardless of the concentration of the feed solution of the commercial HA, although small differences in the quantification of the HA fraction were observed, a finding that is explained bellow. On the other hand, the optimal pressure was also the same for both the commercial and the natural HAs. Therefore, it can be stated that, for our samples and with constant pH and ionic strength, it is the cut-off that rules the optimal pressure, and there is no influence of the feed solution concentration and nature of the sample.

3.2.2. Influence of gas pressure on the ultrafiltrate flow

Two interfacial effects may appear during the UF process: concentration polarization, which is the formation of a gel layer due to the accumulation of rejected solute, and fouling, which is the deposition and accumulation of submicron particles and solute on the membrane surface. These two processes have a great influence on the flow obtained in the UF and consequently, they may interfere with the efficiency of the separation. When the process is membrane controlled (i.e., when the resistance of the gel layer is much smaller than that of the membrane), the flow-pressure relationship is lineal. When the process is controlled by polarization, the flow reaches a plateau and might actually decrease as the pressure is increased [19].

Fig. 3 shows the variation in the UF flow observed for a 300 mg l^{-1} feed solution of the commercial HA when increasing the gas pressure for the different membrane cut-offs. It can be seen that for the membrane with the highest M_r cut-off (300 000), a constant increase in flow was experienced with increasing gas pressure with a steep slope for the correlation, which suggests that the process is controlled by membrane porous size. However, a drastic decrease in the slope was observed for lower M_r cut-offs, being almost flat for the M_r 10 000 membrane. In this case, an additional contribution of

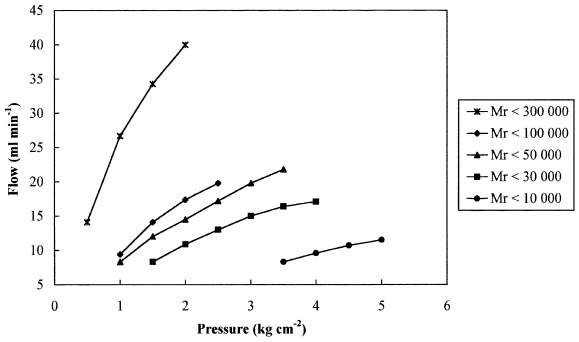

Fig. 3. Influence of gas pressure on the UF flow for a 300 mg l^{-1} feed solution of the commercial HA in the various M_r ranges.

concentration polarization should also be considered. From these results, it is clear that the interfacial effects may be significant for medium-low cut-offs when working at optimal gas pressure, thus influencing the HA concentration in the ultrafiltrate and, consequently, the percentage of HAs lower than the given cut-off. The optimal experimental conditions in order to minimize these effects are analyzed in the following section.

3.2.3. Time-dependence of the humic acid concentration in the ultrafiltrate

To minimize the influence of the interfacial effects on the UF process, the time-dependence of the concentration of HAs in the ultrafiltrate was studied at different concentrations of the feed solution and different gas pressures in order to establish the optimal ultrafiltrate volume for quantification purposes. For this experiment and after rejecting the first 2 ml, five volumes of 5 ml each (aliquots 1, 2, 3, 4 and 5) were taken successively during the UF process through a membrane with a cut-off of M_r 50 000, and subsequently quantified.

Fig. 4 represents the changes in the percentage of HAs in the ultrafiltrate, determined as indicated above, with the aliquot considered for quantification, working with various concentrations of the feed solution of the commercial HA (60, 100, 200 and 300 mg l^{-1}) at the optimum gas pressure for this cut-off (3.0 kg cm^{-2}) and with a feed solution of 300 mg l^{-1} at a lower pressure (1.0 kg cm^{-2}). As can be seen, the concentration of the feed solution

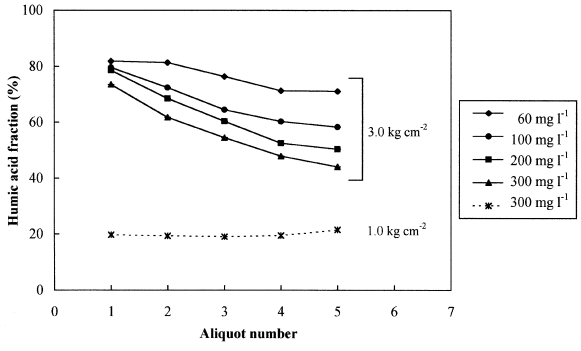

Fig. 4. Influence of the concentration of the feed solution and gas pressure on the percentage of HAs in the aliquot of ultrafiltrate used for quantification, for the commercial HA and a M_r 50 000 cut-off.

plays a certain role in the eventual percentage of HAs obtained in the ultrafiltrate, and starting from the second aliquot, at the optimal pressure, there was a clear decrease in the HA percentage obtained in the ultrafiltrate during separation, though this was less drastic in decreasing the concentration of the feed solution from 300 to 60 mg l^{-1}. These results can be explained by the fact that as the concentration of retained solutes increases during UF, the resistance of the gel layer becomes significantly greater than that of the membrane, while flow becomes independent of membrane permeability, and actually decreases during separation (data not included in the figure).

In Fig. 4 it can also be seen that different behavior was observed at a lower pressure than that of the optimum, with the HA concentration in the ultrafiltrate remaining constant during separation. However, the percentage of HAs associated with this fraction was very low, thus indicating that although it seems that interfacial effects might be controlled by operating at lower pressures, the percentage of HAs obtained in the ultrafiltrate would not be correct in these conditions. In contrast, at optimum pressures the percentage was correct, but highly dependent on the volume of ultrafiltrate. Therefore, the volume of ultrafiltrate considered for quantification is critical when working at high, optimum pressures. After rejecting the first 2 ml, the following 5 ml may be proposed since no quantitative differences were observed at different concentrations of the feed solution considering the standard deviations of the measurements (<5%), interfacial effects being then negligible for such low volumes, excepting the 300 mg l^{-1} feed solution. Therefore, a feed solution concentration below 200 mg l^{-1} is also recommended.

3.3. Reproducibility

The precision of the determination of HA content in each fraction obtained in the combination UF–CZE was calculated at optimal conditions for the commercial HA sample, thus avoiding the uncertainty associated with the heterogeneity of the natural HA sample.

The R.S.D.s of the HA determination in the M_r fractions studied were determined in five replicates

using a feed solution of 100 mg l^{-1}. The values were <2% for the M_r <300 000 and <100 000 fractions; <5% for the M_r <50 000 and <30 000 fractions; and <10% for M_r <10 000 fraction. If we consider that the R.S.D.s obtained for CZE were <2% for short-term measurements and <5% for long-term measurements, it can be concluded that there was only a significant contribution of the UF process in the reproducibility of the results at the M_r 10 000 cut-off, since lower cut-off membranes are more closely influenced by the boundary-layer and interfacial effects, and they also lead to lower concentrations of HAs.

3.4. Molecular mass distributions of humic acid samples

Several restrictive properties of HAs such as dissociation of functional groups, limited solubility, heterogeneous composition and lack of standard materials hinder the precise determination of their M_r. Besides, and it can be deduced from the literature, the M_r distribution of humic substances is very dependent on the method used for the separation [7,20,21]. Concretely for UF, a factor that is very important is the ionic strength of the solution, and thus extremely different M_r distributions may be obtained by ultrafiltration at different values of ionic strength [22]. Therefore, the M_r distribution should be operationally defined as function of the method and the experimental conditions used.

In the present study, after the UF–CZE method was optimized, it was applied for the determination of the M_r distribution of different HA samples with two main objectives: first, the comparison between the extraction method proposed by the IHSS and the simplified method applied in this work and second, the comparison of the M_r distribution of HA samples with different origin.

Table 2 shows the M_r distributions obtained for commercial and natural HAs. The percentage of each M_r fraction was calculated from the difference between the percentage obtained for a given membrane cut-off and that of the immediately lower cut-off. As can be seen, the M_r distribution of HAs seems to be dependent on the type of sample, although the most remarkable differences were between commercial and natural HAs. For the commercial HA a significant percentage of molecules in the M_r <10 000 fraction was noticed, whereas higher M_r fractions were observed for natural samples, which were even found to contain molecules with M_r >300 000. It has to be considered that these results may not be extrapolable to other experimental conditions and methods. However and although the comparison of results obtained with different methods is difficult, the M_r distribution found for the natural soils agree with the one obtained for Ceccanti et al. [22] in a water solution of a soil HA (no presence of molecules with M_r <10 000 and 40% with M_r >100 000), although an increase in the HA fractions with lower M_r was observed by these authors in increasing ionic strength (10, 50 and 100 mmol l^{-1} NaCl).

The data included in Table 2 also allow comparison between the IHSS and the simplified methods for the extraction of HAs, since the M_r distribution for the HAs of the Montseny sample, extracted applying both methods, are compared. It can be seen that the distributions obtained are quite

Table 2
M_r distributions obtained for commercial (R.S.D.<5% for M_r>10 000 and R.S.D.<10% for M_r<10 000) and natural HAs (R.S.D.<10% for M_r>30 000 and R.S.D.<20% for M_r<30 000)

	M_r					
Humic Acid	>300 000	300 000–100 000	100 000–50 000	50 000–30 000	30 000–10 000	<10 000
Commercial	<L.Q.[a]	11	8	25	33	23
Montseny (IHSS)	21	18	27	20	14	<L.Q.
Montseny (simplified)	14	22	32	17	15	<L.Q.
Prades (simplified)	12	20	39	22	7	<L.Q.
Bragin (simplified)	8	26	34	19	13	<L.Q.
Hatton (simplified)	10	38	30	13	9	<L.Q.

[a] L.Q.: Limit of quantification.

similar and thus not dependent on the extraction procedure applied to isolate the HAs.

4. Conclusions

Commercial and natural HAs, the latter having been obtained by extraction from soil samples, may be separated into a set of M_r fractions by UF, which associates each M_r range with a given membrane cut-off. All the fractions have the same electropherogram pattern when applying a CZE system. The lack of dependence of the electropherogram on the M_r of the HAs and the equal electrophoretic response for all the M_r fractions allow each of them to be quantified by UF–CZE with the non-ultrafiltered HA from Fluka as reference.

The experimental conditions in the UF fractionation are of considerable importance in obtaining reliable concentration values of HAs according to their M_r ranges. It is necessary to work at an optimal pressure for each membrane, with feed solutions of moderate concentrations and to quantify a controlled, optimized UF volume.

The operationally-defined M_r distributions of HAs were dependent on the origin of the sample, the commercial sample containing a higher proportion of molecules of low M_r, and the natural HAs having a fraction with $M_r > 300\,000$. The similarity in the distributions obtained with HAs extracted following the IHSS and the simplified methods suggests that the simplified method is a viable alternative for the extraction of HAs.

Acknowledgements

This work was part funded by the CICYT (AMB96-0950). The authors thank C.A. Shand and M. Cheshire from the MLURI (Aberdeen, UK) for their help with the ultrafiltration technique and for supplying the Hatton sample and J.M. Alcañiz for supplying the Montseny soil and the humic acid extracted form it by the IHSS scheme. A.R. also thanks MEC for the grant received.

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