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On-line fractionation and characterization of aquatic humic substances by means of sequential-stage ultrafiltration

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Abstract. A five-stage tangential-flow ultrafiltration (UF) device equipped with advanced membrane filters (molecular weight cut-off: 1, 5, 10, 50 and 100 kDalton) of the polyethersulfone type is described and applied for the analytical on-line fractionation of a series of aquatic humic substances (HS) originating from surface or groundwaters. Fractionation patterns of HS (6 fractions each) evaluated by this UF device exhibit their particular dependence on the HS concentration, the pH-value and the salt content of the sample (10 ml) to be analyzed. Fundamental parameters (e.g., washing volume) governing the molecular-size fractionation of HS by means of multistage UF are discussed, too. The fractionation of an aquatic reference HS (BOC 3/9.5) by means of the above UF procedure reveals considerable differences preferably characterized by the UV-VIS absorption ratio E_{350}/E_{450} and metal complexing capacity (Cu(II)) of the produced fractions. Moreover, molecular spectroscopy investigations (FTIR, ^{1}H-NMR) of the fraction series of this HS indicate that carbohydrate substructures (preferably found in fractions >50 kDalton) and aromatic ones (preferably in fractions <5 kDalton) are unevenly distributed.

Introduction

Humic substances (HS) are main components of the organic carbon mass in soils and of the DOC (dissolved organic carbon) in natural aquatic environments, particularly in ground and surface waters [1]. In general, HS comprise a complex class of related organic macromolecules exhibiting largely varying molecular-weight distributions, (sub)structures and functionalities which give rise to their manifold interactions in the biosphere [2, 3]. Due to their high complexation capability HS can strongly influence the transport, deposition and bioavailability of trace metals and organic xenobiotica (e.g., pesticides, PAH) [4] in aquatic systems. For the detailed elucidation of such HS interactions subtle analytical information on the distribution of reactive (sub)structures and functional groups among natural HS mixtures is needed which may be obtained by a variety of powerful spectroscopic and chemical methods [2]. An important prerequisite for such investigations of HS is the development of suitable procedures for their effective fractionation and separation.

Owing to the extreme complexity of HS mixtures and the small number of identical molecules theoretically contained in each of an immense multitude of highly resolved HS fractions [5], however, so far no "high-performance (HP)" separation procedures exist for HS molecules (high-resolution mass spectrometry for small ones excepted). In practice, instead of "HP" separation a more or less rough fractionation of dissolved HS can be achieved based on differentiable properties of the contained molecules, for instance their varying hydrophobic/hydrophilic interaction with RP sorbents in HPLC systems [6], affinity to metalcontaining sorbents [7], behaviour in electric fields (e.g., electrophoresis [8]), size-exclusion of non-fitting molecules in special gel phases containing an uniform pore system [9] and molecular sizes split up by adequate ultrafilters [9, 10]. Particularly, fractionation of HS by ultrafiltration (UF) on suitable membrane filters is in principle a simple way to characterize such natural substance mixtures as a function of their molecular-weight distributions. Moreover, relevant physical and chemical properties of dissolved HS (e.g., solubility, adsorption behaviour, capacity for hydrogen and metal ions, distribution of functional groups and reactive substructures) can be supposed to be considerably varying as a function of the molecular size [11].

Nowadays advanced ultramembrane filters consisting of relatively inert materials (e.g., polyethersulfone (Filtron Nova)) and offering a large assortment of relatively precise molecular cut-offs (e.g., 1, 3, 5, 8, 10, 30, 50 and 100 kDalton) enable to split mixtures of water-solu-

Dedicated to Professor Dr. Dieter Klockow on the occasion of his 60th birthday

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ble macromolecules (e.g., proteins) into a corresponding series of fractions [12]. Stepwise UF of macromolecules, for instance aquatic HS, through such membrane filters using a series of conventional stirred cells [13, 14], however, is a time-consuming large-scale procedure. In the present work, a new on-line UF device consisting of up to five different ultramembrane filter units (1, 5, 10, 50, 100 kDalton) which are directly coupled together and run by merely one peristaltic pump is proposed for simple one-step fractionation of aquatic HS. One objective of our investigations on HS fractionation by this UF device was its technical optimization; other ones were to characterize the influence of relevant water parameters (like HS concentration, pH-value and salt concentration) on the HS fractionation pattern. Moreover, the complexing capacity for heavy metals (e.g., Cu(II)), the distribution of metals and the UV-VIS absorption ration $E_{350\,nm}/E_{450\,nm}$ (E_3/E_4) as a function of the molecular HS size were considered as important dependences. An additional feature was the molecular-spectroscopic characterization of low- and high-molecular weight fractions in a well-known aquatic reference HS (BOC 3 from the DFG (Deutsche Forschungsgemeinschaft) multi-level well "Bocholt 3") [15] by means of FTIR and ^{1}H NMR.

2 Experimental

Chemicals and reagents

All chemicals used were of high-purity grade unless otherwise stated. Diluted acids and bases necessary for HS isolation were prepared by diluting 30% hydrochloric acid (suprapur, Merck AG) and sodium hydroxide-monohydrate (suprapur, Merck AG), resp., with high-purity water (Milli-Q system, Millipore). The sorbent XAD 8 p.a. (Serva Feinbiochemica) necessary for isolation of aquatic HS was prepurified by successive soaking with 0.5 mol/l HCl, 0.5 mol/l NaOH and methanol p.a. (24 h, each).

HS isolation by XAD 8

Some HS samples were isolated from aquatic sources according to the XAD 8 procedure, as proposed in [16]. After filtration (0.45 µm membrane filters) HS samples (20 l) to be processed were acidified (pH 2.0) by 10 mol/l HCl (suprapur, Merck) and run over prepurified XAD 8 columns. Subsequently, the HS were eluted from this collector by 0.2 mol/l NaOH (suprapur, Merck) and converted on a strong cation exchanger (Dowex 50 WX 8, 200–400 mesh) into their H^+ form. Finally, the obtained HS solutions were freeze-dried and stored in a refrigerator at +4 °C.

HS enrichment by UF

Parallel to the XAD 8 procedure aquatic HS (30 l sample, 5–50 mg/l DOC, prefiltered through 0.45 µm filters) were concentrated at their natural pH-value by preparative UF using a Millipore Pellicon cassette system in tangential flow mode. The molecular weight cut-off was ≧300 Dalton, the filter area 0.46 m^2 and the flow rate of the penetrate 15–20 ml/min.

Fractionation of HS by multistage UF

Aquatic HS were fractionated on-line by means of a special multistage UF unit constructed at the Vernadsky Institute, Moscow. Scheme and flow chart of this device are illustrated in Fig. 1. Accordingly, it consists of a series of up to 5 UF stages made of high-purity acrylic polymer and attached together by two bolts and nuts. The UF stages are equipped with convenient UF membranes M_1 to M_5 (Filtron NOVA (polyethersulfone), 25 mm, 100 (M_1) to 1 (M_5) kDalton) and simultaneously processed by means of a five-channel peristaltic pump (F_1 to F_5) in tangential-flow mode. Before a fractionation run, 10 ml high-purity water (Millipore Q) were pumped through the UF unit filling the fraction reservoirs R_1 to R_4 (1 ml, each) and cleaning the whole system. Then, the HS sample (10 ml) to be fractionated was pressed by pump (initial pressure: 2.5 bar) through the series of membranes M_1 to M_5 exhibiting a penetrate rate of 1.5 to 2 ml/h (tangential flow of F_2 to F_5: 2 ml/min (each)). Subsequently, 10 ml high-purity water (Millipore Q) was in the same way pumped through the UF unit. Finally, the fractions F_2 to F_5 were eluted from the reservoirs R_n and F_1 (as retentate) from the membrane M_1, respectively, by means of 10 ml high-purity water (each). F_6 was collected as total penetrate of membrane M_5.

After five-stage UF as described above the HS fractions F_1 to F_6 were characterized by means of the following spectroscopic and chemical procedures.

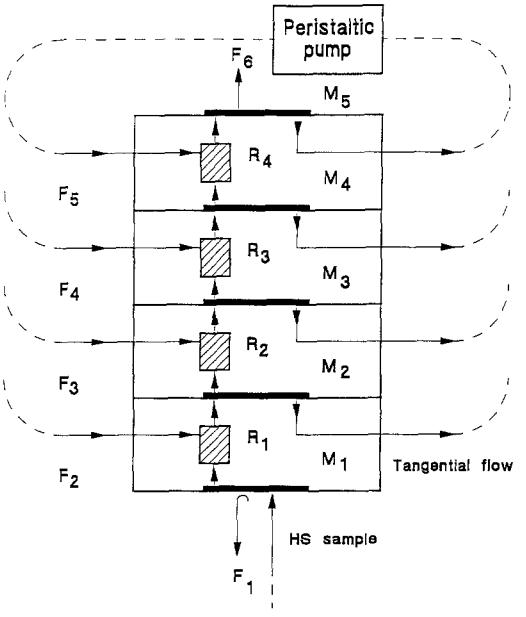

Fig. 1. Flow chart of multistage ultrafiltration (nominal molecular-weight cut-offs of membranes: $M_1 = 1$, $M_2 = 5$, $M_3 = 10$, $M_4 = 50$ and $M_5 = 100$ kDalton; fractions: F_1 to F_6; tangential flow: 2 ml/min, penetrate flow: 2 ml/h. R, reservoir

UV/VIS spectrometry. UV/VIS spectra of aquatic HS and their UF fractions were registered in the range of 225 – 600 nm using a scanning two-beam spectrometer (Varian Cary 1/3).

FTIR spectrometry. FTIR spectra of isolated and dried HS fractions (1 mg each, after drying over di-P_2O_5, 25 °C, 30 mbar, 48 h) were recorded from KBr pellets applying an FTIR spectrometer (Perkin-Elmer 2000) according to routine conditions recommended by the manufacturer.

^{1}H-NMR spectrometry. ^{1}H-NMR spectra of HS fractions (2 to 20 mg samples) dissolved in D_2O were registered by means of a high-field (400 MHz) NMR spectrometer (JEOL GX 400). The following experimental conditions were chosen: pulse-acquisition spectrum (45°), acquisition time: 1.36 s, number of scans: 70 – 4000 (depending on the HS concentration).

Metal determinations. Metal determinations in HS solutions were simultaneously performed by means of energy-dispersive total reflection X-ray fluorescence (TXRF) (Spectrometer: Seifert EXTRA II, 100 ng/ml Ga as internal standard, excitation by a Mo X-ray tube, 500 s measurement time) according to [17]. Flame AAS under recommended conditions served for the determination of Al (Spectrometer: Pye-Unicam SP 9, flame: C_2H_2/N_2O).

Complexing capacity (Cu(II)) of HS. The complexing capacity of HS samples (for Cu(II)) was evaluated by means of a Cu(II)-sensitive electrode (WTW 500) according to the following conditions: 0.3 – 1.0 mg DOC, 100 ml sample, 0.1 mol/l $NaNO_3$, pH 5.5.

DOC. Catalytic combustion of the DOC (dissolved organic carbon) in an oxygen stream and subsequent IR detection served for its determination (Analyzer: Shimadzu TOC 2000).

3 Fundamentals

In general, the performance of UF separations of organic macromolecules is in the first place dependent on the quality of the membrane filters used. For that purpose, advanced membrane filters exhibiting merely small interactions with the molecules to be separated are needed. Membrane filters on the basis of polyethersulfone (e.g., Filtron NOVA and OMEGA) [18] can widely fulfil this requirement, as their application to the fractionation of hydrophilic biomolecules (e.g., proteins) has proved. In the case of dissolved HS, however, the sorption behaviour of these macromolecules on membrane surfaces consisting of polyethersulfone is still to be characterized, in particular as a function of the pH value. Moreover, for efficient fractionation of HS molecules as a function of their molecular weight and size, respectively, membrane filters offering a series of small-size and relatively sharp molecular weight cut-offs in the range of 1 to 100 kDalton are required. According to technical notes of the manufacturer, polyethersulfone-type filters (e.g., Filtron NOVA) can fairly fulfil such prerequisites as Fig. 2 (from [18]) reveals for the fractionation of a variety of dissolved macromolecular dyes.

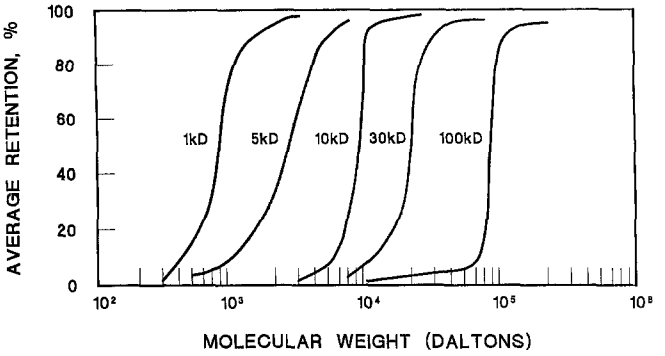

Fig. 2. Retention of macromolecules on FILTRON NOVA polyethersulfone membranes as a function of their molecular weight (according to [18])

The permeation of macromolecules m through a membrane filter by means of UF can be characterized by their permeation coefficient P_m according to equation (1)

$$P_m = C_R/C_F \quad (1)$$

C_R: concentration of m in the retentate, C_F: concentration of m in the filtrate.

In the ideal case of quantitative penetration of m through the membrane, P_m is to be described with the value 1, in the case of quantitative retention with the value 0 as defined in equation (2):

$$0 \leq P_m \leq 1 \quad (2)$$

In reality, however, conventional membranes do not exhibit very sharp molecular weight cut-offs, but more or less broad transition ranges (as shown in Fig. 2 for a series of filters) are observed, characterized by P_m values between 0.05 and 0.95. A similar fractionation behaviour of HS molecules on UF membranes has been proved by [13, 14, 19].

Another point to be discussed in the case of HS fractionation by means of one-stage UF cells is the volume of washing solution necessary for the removal of penetrating molecules m or ions from the cell volume V_R. The concentration decrease of m in such a constant cell volume V_R by means of a washing solution (volume: V_F) can be described according to [14, 19, 20] by the exponential function (3)

$$C/C_0 = e^{-P_m \cdot V_F/V_R} \quad (3)$$

C_0: initial concentration of m in the cell; C: concentration of m in the cell after washing with the volume V_F.

According to equation (3), for example, ratios of $V_F/V_R \geqq 3$ are needed to lower the concentration C_0 of m in the cell volume to a twentieth ($P_m = 1$). In the case of low penetration coefficients P_m (e.g., 0.1 to 0.5), however, the volume of the washing solution (V_F) is to be increased correspondingly to obtain the same separation efficiency for m.

Compared with one-stage UF, the mathematical characterization of analogous separations in a multistage UF system as described in [14] by means of the equation (4) is more complicated:

$$C/C_0\,(n) = (P_m\,(n)^n/(n-1)!) \cdot (V_F/V_R)^{n-1} \cdot e^{-P_m \cdot V_F/V_R} \quad (4)$$

$C/C_0\,(n)$ = relative concentration of m in stage n,
n = number of stage under consideration,
$P_m\,(n)$ = penetration coefficient of m in stage n

In the simple case of a component m contained in stage 1 and penetrating all other membranes of the UF system ($P_m = 1$ on all stages) its transport through the stages 1 to n can be described as a function of the relative washing volume V_F/V_R according to Fig. 3:

At the beginning of washing m through the UF system its relative concentration C/C_0 in stage 1 is 1.0. For the decrease of m down to <0.02 in that stage about 4 volumes of V_R are needed. Moreover, after 1, 2 and 3 volumes of V_R, relative maxima of C/C_0 in the subsequent stages 2, 3 and 4, are obtained. A concentration decrease of m in these stages down to $C/C_0 < 0.02$ can be performed by about 6, 8 and 10 volumes of V_R, respectively.

Accordingly, in the case of the studied UF device consisting of 4 stages with $V_R = 1$ ml (each) about 10 ml washing solution are required for far-reaching removal of small molecules or ions ($\ll 1000$ Dalton) from the stages 1 to 4, presupposed that no sorption effects occur. However, in the case of macromolecular components which are penetrating one of the sequential stages F_2 to F_5 with relatively small permeation coefficients P_m (e.g., $0.1 \leqq P_m \leqq 0.8$) their (nearly) complete permeation through the concerning stage requires correspondingly increased volumes of washing solution. Thus, for the fractionation of complex mixtures like HS containing considerable amounts in the discussed P_m range on every stage by means of multistage UF the conclusions can be drawn, that high V_F/V_R ratios of $\gg 10$ are required for their efficient separation. On the other hand, washing volumes of 50 or more ml (at flow rates of 1 to 2 ml/h) would lengthen the working time of an UF run to an unacceptable period of time. Therefore, in the present study the processed (total) volume of HS samples to be fractionated in the described UF device and the subsequent washing volume (deionized water) was limited to 10 ml each.

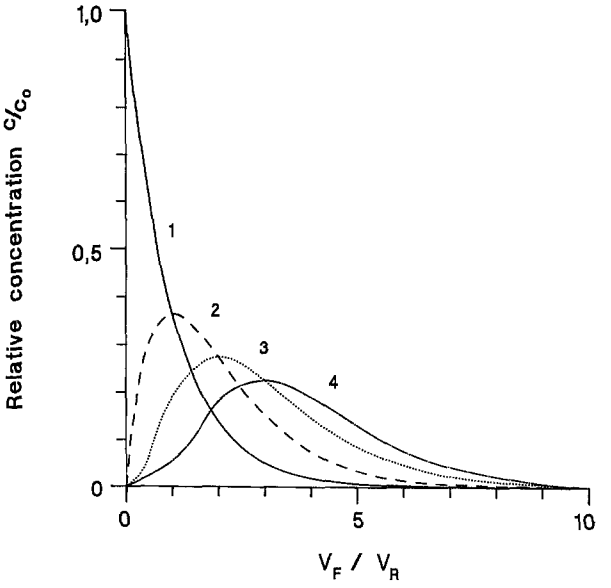

Fig. 3. Concentration decrease c/c_0 of substance m (permeation coefficient $P_m = 1$ for all membranes; c_0: initial concentration of m in R_1 at $V_F = 0$ ml) in the on-line ultrafiltration reservoirs R_1 to R_4 as a function of the washing volume V_F/V_R

4 Results and discussion

The series of aquatic HS fractionated by on-line UF (4 stages, 6 fractions) in this study is described in Table 1. It comprises HS from a typical bog water (Venner Moor) as original sample (after 0.45 µm filtration) and preconcentrated by the XAD 8 procedure, resp., those from river water (Ruhr) preconcentrated by preparative UF (Millipore Pellicon System, molecular weight cut-off: 300 Dalton) and those isolated (XAD 2, anion exchanger) from groundwaters (Bocholt, Fuhrberg/Hannover). In particular, the reference HS BOC3/9.5 isolated from a well-characterized groundwater area has been subject matter of a series of recent investigations [21].

Prior to multistage UF of aquatic HS as described above, the influence of presumably relevant solution pa-

Table 1. Characterization of the aquatic HS under study. The studied HS originate from different aquatic systems (ground, river and bog water)

Label	Origin	Isolation	DOC (mg/l)
BOC 3/9.5 – XAD 2	DFG multilevel well 3, Bocholt	XAD 2 procedure performed by [15]	5.0
VM 4 – original	Venner Moor, Münsterland (NRW)	Original sample (0.45 µm) filtration	48.0 (pH 3.7)
VM 4 – XAD 8	Venner Moor, Münsterland (NRW)	XAD 8 procedure applied to VM 4 – original	48.0
FU 2 – AnEx[a]	Wasserwerke Fuhrberg/Hannover	Technical anion exchanger Lewatit MP 500	6 – 10[b]
R2 – UF	Ruhr/Schwerte	Ultrafiltration (Millipore-Pellicon, cut-off $M \geq 1000$ D)	5.5 (pH 6.8)

[a] From Dr. W. Kölle, Stadtwerke Hannover
[b] According to [4]

rameters, in particular the HS concentration, pH value and salt content, on the distribution and on the recovery of HS molecules in the different stages (F 1 – F 6) are to be clarified. For instance, high HS concentration and low pH-values might promote unfavourable aggregation or sorption effects within the UF cells. Elevated salt concentrations are well-known to change the tertiary and quaternary structure of dissolved HS molecules [11].

The influence of the concentration of HS on their separation pattern and recovery is characterized in Fig. 4 in the case of the reference material BOC 3/9.5 at pH 6.0. At low HS concentrations (0.2 to 1.0 mg/ml BOC 3/9.5) merely small fractions (5 – 15% of the total HS) are adsorbed within the UF device and lost in the recovery balance. Higher HS concentrations (>2 mg/ml), however, cause an increasing loss, preferably in the high-molecular weight fractions F 1 to F 3 which might be attributed to increased sorption capability of these HS molecules caused by aggregation. In the case of the low-molecular weight fractions F 5 and F 6, however, the reverse effect can be observed.

The molecular-weight distribution of HS molecules (1 mg/ml BOC 3/9.5) in the UF stages (F 1 to F 6) as a function of the pH value is shown in Fig. 5. At elevated pH-values merely 2 (pH 8.0) to 14% (pH 6.0) of the fractionated HS are adsorbed on the inner surface of the UF stages. On the contrary, decreased pH-values of <4 cause considerably reduced recovery rates of <60%, preferably in the fractions of higher molecular weight (>5 kD). This effect is to be ascribed to the increasing sorption of HS molecules on hydrophobic surfaces like polyethersulfone (membranes) and polyethylene (tubes) as a function of the pH-value. Accordingly, acidic working pH-values of <4 are to be avoided for HS in the case of the used UF equipment.

Very dramatic is the influence of increasing salt concentrations (e.g., NaCl) on the HS distribution (1 mg/ml BOC 3/9.5, pH 6.0) in different stages (F 1 to F 6) after UF as shown in Fig. 6. Already some g/l NaCl increase the low-molecular weight fractions F 5 and F 6 from about 30 to more than 70%. Moreover, high salt contents of >15 g/l NaCl lead to 90% separation of HS in these

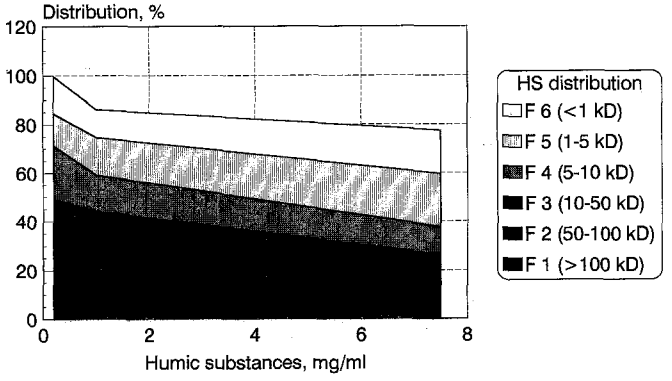

Fig. 4. Molecular distribution pattern (*F 1*: >100 kD, *F 2*: 50 – 100 kD, *F 3*: 10 – 50 kD, *F 4*: 5 – 10 kD, *F 5*: 1 – 5 kD, *F 6*: <1 kD) of an aquatic HS (BOC 3/9.5) attained by five-stage ultrafiltration as a function of its concentration (pH 6.0)

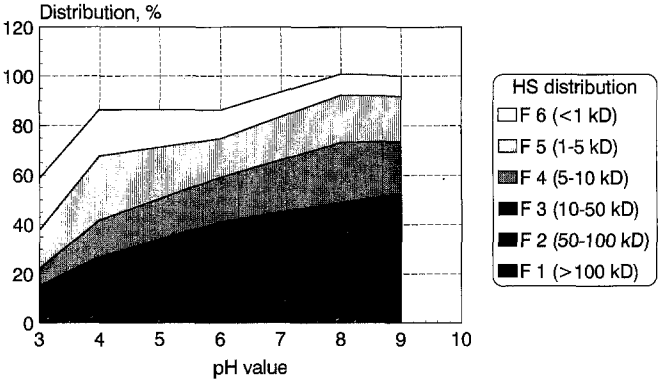

Fig. 5. Influence of the pH-value on the molecular distribution of HS (HS: 1.0 mg/ml BOC 3/9.5; sample volume: 10 ml, washing volume: 10 ml, HS fractions: *F 1* to *F 6*)

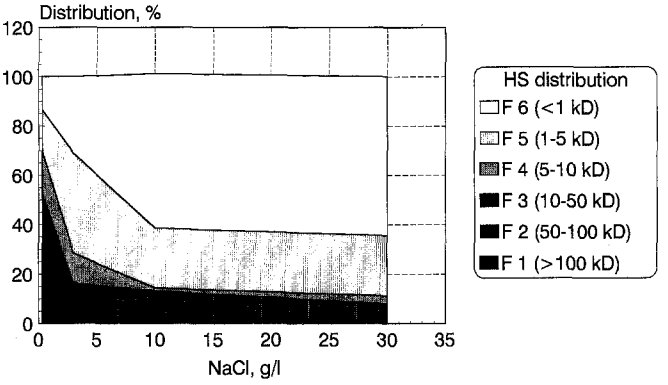

Fig. 6. Salt influence (e.g., NaCl) on the molecular distribution of HS (HS: 1.0 mg/ml BOC 3/9.5, pH 6.0, other conditions as in Fig. 5)

fractions (F 1: 65%). It is obvious that in this case not the molecular weight of the fractionated HS molecules penetrating the pores of the membranes preferably as function of their size and shape, is changed, but their tertiary structure. According to [11] it can be suggested that increasing electrolyte concentrations cause changes of the size of HS macromolecules as the intramolecular charge repulsion power decreases. Thus, HS molecules expanded in solution-state start to eject the held water molecules and to collapse partially lowering their molecular size. Aquatic HS molecules reduced in this manner can penetrate even small-size ultrafiltration membranes (cut-off: 1 kD) as shown above.

The findings according to Figs. 4 to 6 reveal that the sequential-stage UF of aquatic HS results in their effective fractionation, which however is considerably governed by operational chemical and physical parameters like pH-value, salt concentration and sorption effects. Fractionation patterns of HS obtained by this methods are merely comparable under fairly similar separation conditions.

Fractionation patterns of a series of aquatic HS (from Table 1) obtained by on-line UF in five stages (F 1: >100, F 2: 50 – 100, F 3: 10 – 50, F 4: 5 – 10, F 5: 1 – 5, F 6: <1 kDalton) under comparable separation conditions are summarized in Fig. 7. Accordingly, the investigated

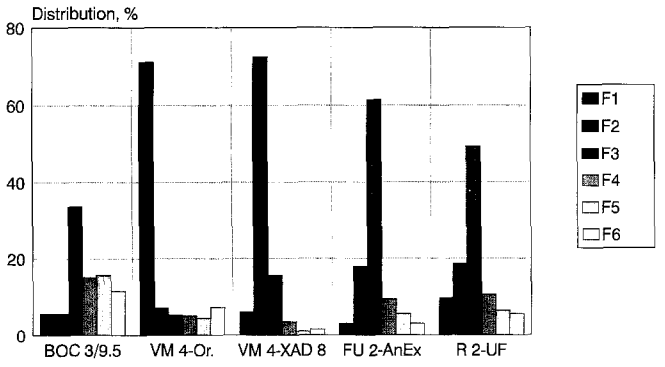

Fig. 7. Molecular distribution pattern of different aquatic HS (1.0 mg/ml HS, each, pH 6.0, HS description in Table 1). Fractions: $F1 > 100$; $F2 = 50-100$; $F3 = 10-50$; $F4 = 5-10$; $F5 = 1-5$; $F6 < 1$ (kDalton)

HS reveal quite a pattern of macromolecules. In three cases (BOC 3/9.5, FU 2-AnEx, R 2-UF) the main mass of HS (30–55%) is to be attributed to middle fraction F 3 (10–50 kD), whereas in the case of VM 4 (bog water) the high-molecular weight fractions (F 1 and F 2) are dominant. Moreover, in this case a clear influence of the HS isolation procedure (XAD 8) shifting the maximal fraction of HS from >100 kD to lower molecular-weights can be detected, whereas considerable amounts of the low-molecular weight fractions (F 5, F 6) are missing. Both effects might be caused by strong pH-changes necessary for HS isolation by XAD sorbents (pH 2.0 for sorption, pH 13.0 for elution of HS).

From typical fractionation patterns as obtained above for a series of aquatic HS by multistage UF, the question arises whether the low-molecular and the high-molecular weight fractions of such a complex HS mixture reveal comparable properties (e.g., partial structures, complexing capacity) or not. The answer to this question is presented in the following for a well-characterized reference HS (BOC 3/9.5). Table 2 summarizes the fractionation pattern (F 1 to F 6) of this HS (7.5 mg/ml BOC 3/9.5, pH 6.0) obtained under experimental conditions as already described. The main fractions are to be attributed to F 3 to F 6 (about 18, 11, 22 and 18% resp.). Moreover, the UV-VIS absorption ratio E_{350}/E_{450} depending roughly on the humification degree and the molecular weight of HS molecules [22] continuously decreases from the low-molecular weight fractions (e.g., F 6) to high-molecular ones (e.g., F 2, F 1). A similar trend can be evaluated for the complexing capacity CC determined by Cu(II) loading of the attained HS fractions. The highest CC is exhibited by the low-molecular weight fraction F 6 (1.28 mmol Cu(II)/g DOC), the lowest one (0.93 mmol Cu(II)/g DOC) for big HS molecules (F 1).

Table 2. On-line ultrafiltration (UF) of aquatic humic substances: distribution of the extinction ratio E_{350}/E_{450} and the complexing capacity (CC).
Distribution of E_{350}/E_{450} and $CC_{Cu(II)}$

Fraction	Amount (%)	E_{350}/E_{450}	CC (mmol Cu(II)/g DOC)
BOC 3/9.5	–	6.45[a]	1.15 ± 0.12[b]
F1 (>100 kD)	2.3	3.31	0.93
F2 (50–100 kD)	5.9	3.75	1.08
F3 (10–50 kD)	18.0	4.85	1.10
F4 (5–10 kD)	11.3	5.71	1.17
F5 (1–5 kD)	22.0	6.22	1.15
F6 (<1 kD)	17.9	8.90	1.28

[a] 1.0 mg/ml BOC 3/9.5, pH 6.0
[b] Standard deviation s from 5 determinations (ISE)
HS: 7.5 mg/ml BOC 3/9.5, 10 ml sample, pH 6.0; UF: On-line fractionation (nominal cut-off: 100, 50, 10, 5 and 1 kD); E_{350}/E_{450}: Ratio of UV/VIS extinctions at 350 and 450 nm; $CC_{Cu(II)}$: Evaluation by ion-sensitive electrode (ISE for Cu(II))

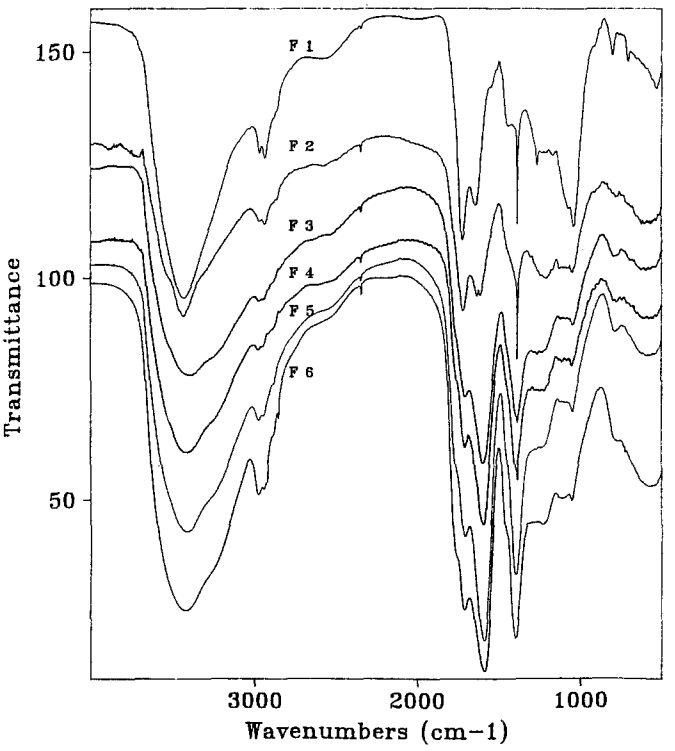

Fig. 8. FTIR spectra of fractionated aquatic HS (HS sample: 7.5 mg/ml BOC 3/9.5, pH 6.0)

The latter findings are in good agreement with the data of FTIR spectra of the HS fractions F 1 to F 6 in the case of BOC 3/9.5 as revealed in Fig. 8 and Table 3. Accordingly, the IR signals at 1630/1720 cm^{-1} being typical for carboxylic groups in HS [22] systematically increase from F 1 for F 6. Other characteristics of this series of IR spectra are the increase of carbohydrate substructures (attributed to 950–1170 cm^{-1}) in the high-molecular weight fractions (F 1, F 2) and, supposedly, the increase of aromatic structures (derived from phenolic groups at 1380 cm^{-1}) in the low molecular ones (F 5, F 6). It is to be realized, however, that the possibility of qualitative and quantitative evaluation of typical IR spectra of HS is still limited [5].

In Fig. 9 structural differences for the same UF fractions (F 1 to F 6) of BOC 3/9.5 are revealed by means of ^{1}H-NMR spectrometry. In general, ^{1}H-NMR spectrometry allows to differentiate roughly and qualitatively aliphatic, carbohydrate and aromatic substructures in HS [23] (as assigned in Table 4). Such HS spectra typically

Table 3. FTIR spectra (BOC 3/9.5): Signals and their assignment in the fractions (F1 – F6)

Absorption (cm^{-1})	Assignment
3400	– OH of alcohols, phenols and carbon acids
2950	– CH in methyl and methylene groups
1720 and 1630	– C = O in carboxyl, keto and ester groups
1380	– OH, C – O (phenolic) and CH (aliphatic)
950 – 1170	Polysaccharides

Fraction	Absorption bands (cm^{-1})				
	3400	2950	1720/1630	1380	1170 – 950
F1	+ + +	+ + +	+ +	+	+ + +
F2	+ + +	+ +	+ +	+	+ +
F3	+ + +	+	+ + +	+ +	+
F4	+ + +	+	+ + +	+ +	+
F5	+ + +	+ +	+ + +	+ + +	+
F6	+ + +	+ + +	+ + +	+ + +	+

Absorption: + + +, very strong; + +, strong; +, fair

suffer from strong interferences by H_2O (2.2 ppm) and HDO signals (4.8 ppm). Nevertheless, the signal at 3 – 4 ppm predominantly found in the fractions F 1 to F 3 exhibits the presence of carbohydrates in the high-molecular weight fractions, but not in the low-molecular weight ones comparable to the IR findings. From this fact the supposition might be drawn that carbohydrates as substructures in large HS molecules are more resistant against chemical and microbiotic attack, than in small

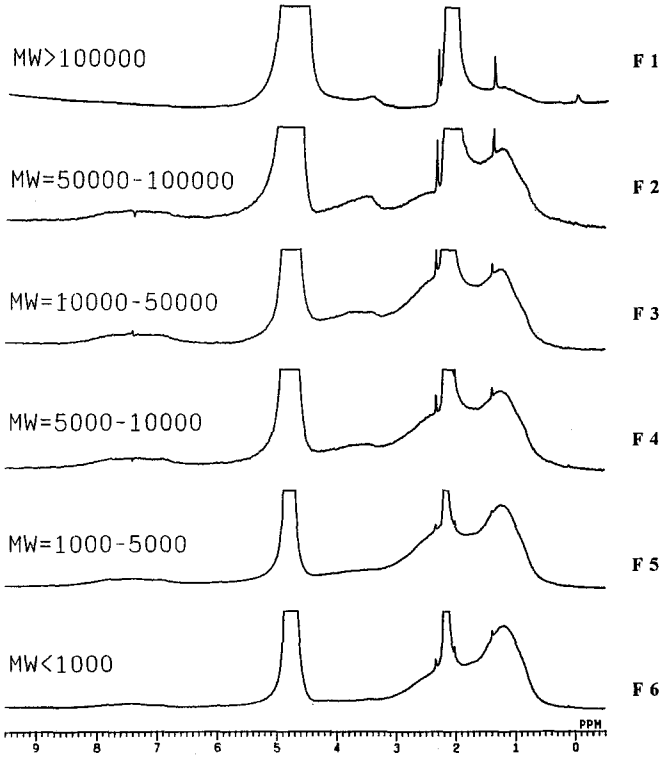

Fig. 9. ^{1}H-NMR spectra of fractionated aquatic HS (HS sample: 7.5 mg/ml BOC 3/9.5, experimental conditions as in Fig. 8)

Table 4. ^{1}H-NMR (BOC 3/9.5): Assignment and intensity of the signals of the fractions (F1 – F6)

Range (ppm)	0.8 – 1.5	2.5 – 3	3 – 4	6.5 – 8
Assignment:	Aliphates	Aliphatic CH	Sugars	Aromates
Fraction	Intensity			
F1	+	– –	+ +	– –
F2	+ +	–	+ +	+
F3	+ +	+	+	+ +
F4	+ +	+	+	+
F5	+ +	+	–	+
F6	+ +	–	– –	+

Intensity: + +, very strong; +, strong; –, weak; – –, very weak
Interferences: 2.2 ppm (by H_2O), 4.8 ppm (by HDO)

ones. Moreover, broad signals at 0.8 – 1.5 ppm and 6.5 – 8 ppm, clearly indicate the presence of aliphates and aromates, preferably in F 4 to F 6, but this tendency may be caused by enhanced sensitivities of these substructure signals in relatively small HS molecules.

Altogether both the IR and the ^{1}H-NMR spectra hint considerable structural differences between the low- and high-molecular weight fractions of the on-line ultrafiltrated HS sample BOC 3/9.5. Due to difficult quantification of these methods applied to HS, however, reliable assertions concerning their structure are difficult.

Another interesting point to be discussed in the case of multistage UF of dissolved HS is the distribution of trace metals associated with the HS fractions in the different stages (F 1 to F 6). The pattern of HS molecules and metals of a typical aquatic HS (VM 4, 96.0 mg/l DOC), which contains its original metal loading (e.g., Al, Cu, Fe, Mn, Zn) at its natural pH-value (pH 3.7), after a fivestage UF (under standardized experimental conditions as described above) is summarized in Fig. 10. At a slightly acidic pH-value (3.7) only Fe (total content: 17.2 µg/mg C) exhibits a pattern comparable to the distribution of HS molecules, which are preferably found in the high-molecular weight fraction (about 70% in F 1). The Al and Cu distribution in various size fractions (about 20% in F 1) appear similar, whereas Mn and Zn

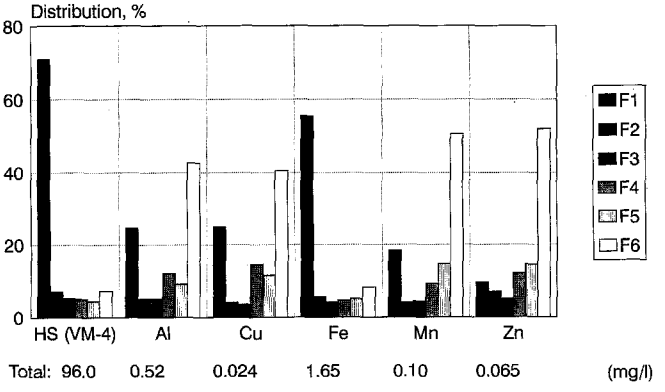

Fig. 10. Distribution of the original metal loading (Al, Cu, Fe, Mn, Zn) in a natural aquatic HS sample (HS: VM 4; DOC: 48 mg/l; pH: 3.7; fractions *F 1 – F 6* as in Fig. 4)

preferably forming relatively weak complexes with aquatic HS [24] highly penetrate, presumably as dissociated ions, all membranes and thus they are enriched in the low-molecular weight fraction F 6 (< 1 kDalton). A considerable fraction of the latter metals, however, being complexed with high-molecular weight HS molecules still remains in F 1. Unfavourably, in the case of multistage ultrafiltration the mathematical treatment of the concerning dissociation equilibria of HS/metal complexes is much more complicated than for one-stage ultrafiltration [14].

4 Conclusions

In general, aquatic HS are complex mixtures of natural polyelectrolytes exhibiting strongly varying molecular weight distributions [2]. Besides their solubility, other relevant properties of HS molecules (for instance their complexing capacity and partial structures) in such mixtures might depend on their molecular weight. Therefore, considerable demand has arisen for the characterization of molecular-size distributions in HS samples usually evaluated by conventional size-exclusion chromatography [25] or by fractionation using convenient UF techniques [14].

For size-fractionation of dissolved HS molecules, UF consisting of a series of sequential separation stages equipped with adequate membranes can be an effective tool, as it has been proved in recent studies [14, 19]. Conventional (stirred) UF cells, however, suffer from high volumes, tedious handling and a relatively small number of separation stages coupled together.

Compared to this state, the presented multistage UF device consisting of a variable small-size cascade of five (or more) separation stages offers a considerable number of advantages for HS fractionation. Due to the tangential-flow principle used for the solution transport over the installed membranes, a relatively high penetrate flow can be obtained even by means of a low-pressure peristaltic pump (2 bar). Up to six (or more) HS fractions in the molecularweight range < 1 to > 100 kDalton being relevant for the size distributions of aquatic HS are obtainable by only one filtration run. The separation efficiency, however, is strongly dependent on the quality of the applied membranes. Accordingly, advanced membranes of the polyethersulfone-type offering a convenient series of molecular weight cut-offs are to be preferred for HS fractionation.

Fast separations using merely small washing volumes, however, cause incomplete fractionation of HS molecules penetrating one of the membranes with small penetration coefficients P_m of < 0.5. Due to practical feasibility of the multi-stage ultrafiltration, the washing step is restricted to a volume of 10 ml. Accordingly, for nearly quantitative fractionation ($P_m < 0.1$ for retained HS molecules) the washing volume is to be increased by the factor of ten.

The fractionation pattern of ultrafiltrated HS shown in the case of a reference HS (BOC 3/9.5) as a function of relevant solution parameters particularly depends on the pH-value and the salt concentration. Thus, such HS patterns are only comparable together on the basis of comparable pH-values, low salt concentrations and washing volumes. Moreover, owing to increased HS sorption on the UF membranes, pH-values of < 4 are to be avoided. According to these findings, fractionation patterns being different as exhibited for a series of aquatic HS can be merely compared and discussed under defined experimental conditions. In addition, they are to be confirmed by an independent size-distribution method, for instance conventional size-exclusion chromatography [24].

Some relevant properties (e.g., E_{350}/E_{450}, complexing capacity, partial structures) of the obtained HS fractions (e.g., BOC 3/9.5) reveal (un)expected trends. Expectedly, the ratio E_{350}/E_{450} roughly depending on the humification degree and the molecular size of HS molecules [22], exhibits relatively high values for small-size fraction F_6 (< 1 kDalton). A similar trend is observed in the case of the complexing capacity (towards Cu(II)). Thus, the small-size fraction F_6 of BOC 3/9.5 reveals a complexing capacity being 10% higher than the average (1.15 mmol Cu(II)/g DOC), the large-size fraction F_1 being 20% lower. Surprisingly, molecular spectroscopy methods (FTIR, ^{1}H-NMR) prove considerable differences of substructures in the obtained HS fractions, indicating an enrichment of carbohydrates in the large HS molecules and an increase of refractory aromatic structures in the small ones of BOC 3/9.5.

Altogether, multistage UF of aquatic HS on-line performed in a single run by means of an advanced filtration device is a simple and effective technique for their molecular-size fractionation. This multistage technique might also be useful for the differentiated characterization of HS/metal and HS/pesticide interactions, as it is already known from one-stage UF [25].

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