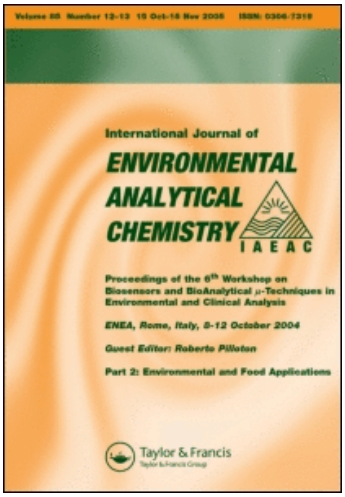

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Sec-Page Characterization of Lake Aquatic Humic Matter Isolated with Xad-Resin and Tangential Membrane Ultrafiltration

Juhani Peuravuori[a]; Kalevi Pihlaja[a]; Olga Trubetskaya[b]; Olga Reznikova[b]; Gaida Afanas'eva[b]; Oleg Trubetskoj[c]

[a] Department of Chemistry, Laboratory of Physical Chemistry, University of Turku, Turku, Finland [b] Branch of the Institute of the Bioorganic Chemistry, Russian Academy of Sciences, Moscow region, Russia [c] Institute of Basic Biological Problems, Russian Academy of Sciences, Moscow region, Russia

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SEC-PAGE CHARACTERIZATION OF LAKE AQUATIC HUMIC MATTER ISOLATED WITH XAD-RESIN AND TANGENTIAL MEMBRANE ULTRAFILTRATION

JUHANI PEURAVUORI[a*], KALEVI PIHLAJA[a], OLGA TRUBETSKAYA[b], OLGA REZNIKOVA[b], GAIDA AFANAS'EVA[b] and OLEG TRUBETSKOJ[c]

[a]*Department of Chemistry, Laboratory of Physical Chemistry, University of Turku, FIN-20014 Turku, Finland,* [b]*Branch of the Institute of the Bioorganic Chemistry and* [c]*Institute of Basic Biological Problems, Russian Academy of Sciences, 142292, Pushchino, Moscow region, Russia*

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Several aquatic heterogeneous humic-solute fractions with known molecular-size ranges were fractionated further by coupled size-exclusion chromatography (SEC) and polyacrylamide gel electrophoresis (PAGE). The results of SEC-PAGE, obtained for several humic-solute fractions, corresponded reasonably well to the known molecular-size distributions of the dissolved organic matter (DOM). Despite the limited resolving power PAGE appears to offer an alternative coarse method, beside SEC, for fractionation of the heterogeneous humic matter (HM).

Keywords: Electrophoresis; size-exclusion chromatography; polyacrylamide gel; humic matter; molecular sieving

INTRODUCTION

Natural macromolecules such as humic matter (HM) of both terrestrial and aquatic origin play an integral role in the carbon cycle. In general, aquatic HM in fresh water ecosystems accounts for the major part of the dissolved organic carbon (DOC). However, separation of the heterogeneous aquatic HM matrix from

* Corresponding author. Fax: +358–2–3336700. E-mail: juhpeur@utu.fi

the total DOM, and ultimately its fractionation into separate, identifiable species is extremely labourious strongly depending on the definitions and methods applied. Besides, the classification of DOM into humic and non-humic solutes is merely operational based on a given method. Therefore, the generic term of HM, which is not strictly related to any specific isolation procedure, is preferably utilized.

SEC has been the most extensively applied technique to separate a sampled humic mixture into fractions of more homogeneous molecular sizes. In particular, the attempts to study humic solutes by means of low-pressure SEC with dextran gels became once such a widespread routine that this period has been named as "Sephadex Period".[1] Although the results obtained by Sephadex gel chromatography were finally proven[2] to be of limited usefulness and even inadequate in many applications, SEC with dextran gels still finds potential in certain predeterminations.

Likewise, electrophoresis is an old technique and the usefulness of which for the study of natural macromolecules was first recognized around 1930.[3] Numerous HM separations by means of a solid support such as polyacrylamide gel (PAGE) have been reported.[e.g.4–14] In these studies the HM samples have usually been resolved into two to six sub-components; the presence of disaggregating agents such as SDS (sodium dodecyl sulphate, an anionic detergent)[5] or, in particular, urea[7] leads to a higher number of sub-components.

Recently, a resuscitation of vertical electrophoresis in PAGE, where electrophoretic mobility is related to both size and charge of HM solutes, has been suggested, e.g. for fixing experimental conditions to determine the optimal interrelations between PAGE and SEC fractions of HMs.[15–18] In the above reports the electrophoretic analyses of SEC-PAGE profiles of HMs allowed to find out exactly those SEC eluates which were more homogeneous in respect of electrophoretic mobility containing less complex humic material in comparison with a bulk HM sample.

The present study is an integral part of a series of extensive tandem SEC-PAGE investigations for fractionation of the natural HM. The humic samples for the SEC-PAGE experiments were isolated-fractionated from two lake waters by means of different techniques. The main aim of the present study was to test the resolving power of the SEC-PAGE system by means of several aquatic humic-solute fractions (in all 17 examples) – especially by means of those having molecular-size ranges known in advance.

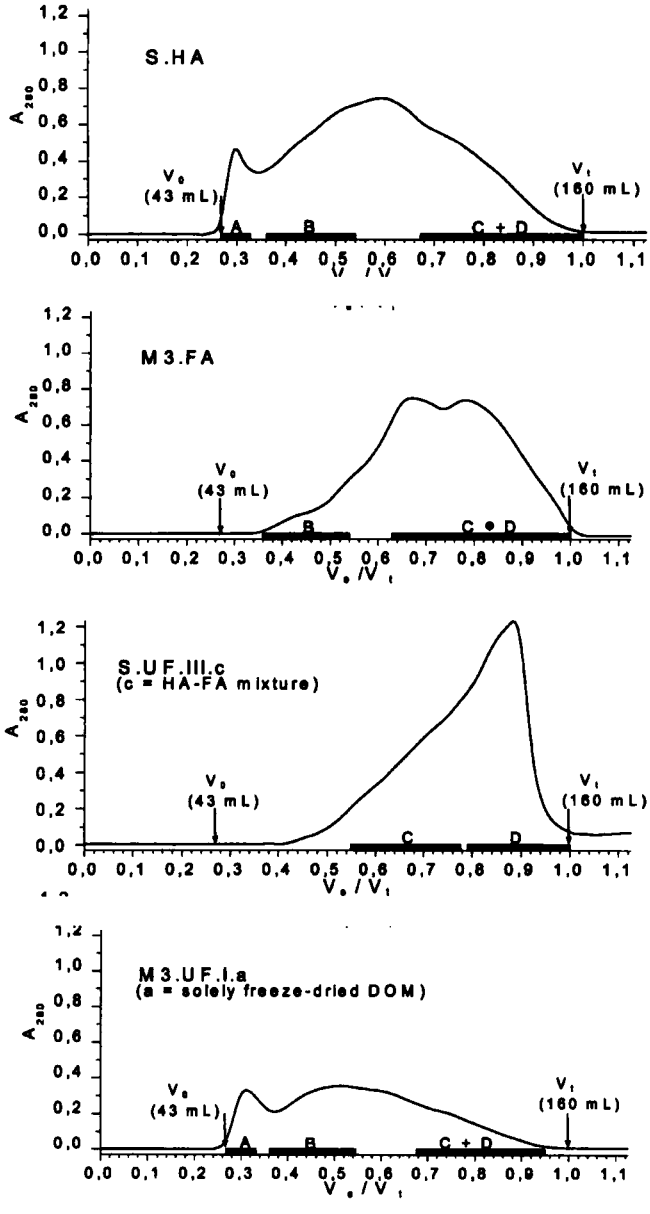

FIGURE 1 Most typical examples of the SEC elution profiles obtained for the various HM samples studied. Black boxes on the x-axis show the combined fractions A, B, C and D (or C+D) obtained on the basis of electrophoretic analyses of the chromatographic profiles. V_0 = void volume, V_t = total gel volume, V_e = elution volume. V_e/V_t = normalised elution volume. Abbreviations of samples are presented in Table I

TABLE I Abbreviations, environmental sources and isolation methods of HM samples studied in the SEC-PAGE experiments[a]

Sample	*Source/definition*	*Isolation method*
S.HA	Lake Savojärvi (1988), humic acid (HA)	XAD-8 resin
S.FA	Lake Savojärvi (1988), fulvic acid (FA)	XAD-8 resin
M3.HA	Lake Mekkojärvi (1989), humic acid (HA)	XAD-8 resin
M3.FA	Lake Mekkojärvi (1989), fulvic acid (FA)	XAD-8 resin
S.UF.I.c	Lake Savojärvi (1988), humic-fulvic acid mixture (HA-FA)	ultrafiltration, XAD-8 resin
S.UF.II.c	Lake Savojärvi (1988), humic-fulvic acid mixture (HA-FA)	ultrafiltration, XAD-8 resin
S.UF.III.c	Lake Savojärvi (1988), humic-fulvic acid mixture (HA-FA)	ultrafiltration, XAD-8 resin
S.UF.IV.FA	Lake Savojärvi (1988), fulvic acid (FA)	ultrafiltration, XAD-8 resin
M3.UF.I.a	Lake Mekkojärvi (1989), solely freeze-dried DOM	ultrafiltration, no further treatment
M3.UF.II.a	Lake Mekkojärvi (1989), solely freeze-dried DOM	ultrafiltration, no further treatment
M3.UF.III.a	Lake Mekkojärvi (1989), solely freeze-dried DOM	ultrafiltration, no further treatment
M3.UF.I.HA	Lake Mekkojärvi (1989), humic acid (HA)	ultrafiltration, XAD-8 resin
M3.UF.II.HA	Lake Mekkojärvi (1989), humic acid (HA)	ultrafiltration, XAD-8 resin
M3.UF.I.FA	Lake Mekkojärvi (1989), fulvic acid (FA)	ultrafiltration, XAD-8 resin
M3.UF.II.FA	Lake Mekkojärvi (1989), fulvic acid (FA)	ultrafiltration, XAD-8 resin
M3.UF.III.FA	Lake Mekkojärvi (1989), fulvic acid (FA)	ultrafiltration, XAD-8 resin
M3.UF.IV.FA	Lake Mekkojärvi (1989), fulvic acid (FA)	ultrafiltration, XAD-8 resin

a. $UF.I_i$, $UF.II_i$ and $UF.III_i$ denote retentates of tangential-flow membrane-ultrafiltration with different NMW limits (≥ 100 000, 100 000–10 000 and 10 000–1 000 g/mol, respectively) and $UF.IV_i$ remained filtrate (NMW ≤ 1000 g/mol). Sublabellings HA_i, FA_i and c_i denote methods carried out for different UF_i-fractions (e.g. c_i is the mixture of humic solutes (HA_i-FA_i) obtained by the XAD technique but not further divided at pH 1 into distinct HA_i and FA_i type fractions).

EXPERIMENTAL

Samples

Natural humic-water samples were collected from two lakes: Savojärvi (S, February 1988), situated in a marsh region in the southwestern part of Finland; and Mekkojärvi (M3, May 1989), a small, forest lake situated in the Evo district of Lammi in Southern Finland. Both lakes are very brown-water (colour as cobalt-platinum units ca. 200 mg Pt/L; DOC 22 mg C/L; pH 5.6). The basic characteristic properties and pre-treatments of the original water samples are reported previously.[19]

The water samples (S and M3) were treated both with the XAD-8 resin to obtain the integrated wholes of fulvic (FA) and humic (HA) acid type solute-fractions (S.FA, S.HA, M3.FA and M3.HA), and with an ultrafiltration (UF) procedure using a tangential flow membrane technique (4 GPM Pellicon Cassette Systems by Millipore) to obtain different DOM subfractions.[19] Three membranes with different nominal molecular-weight (NMW) limits were applied for concentrating the whole DOM and, accordingly, three retentates with NMW cutoffs ≥100 000 (UF.I), 100 000 – 10 000 (UF.II) and 10 000 – 1 000 g/mol (UF.III), and a filtrate ≤ 1 000 g/mol (UF.IV) were obtained. The UF retentates were further refined with the modified XAD technique (method c, producing HA-FA mixture) to separate humic and non-humic solutes. In the case of the water sample M3 the retentates UF.I. – UF.III were further divided at pH 1 into the distinct FA and HA type subfractions. One part of the original UF retentates was also freeze-dried without further treatment for obtaining unrefined mixed solutes (method a). The humic matter remained in the UF filtrates (UF.IV) was concentrated by the XAD technique. As a peculiarity of UF isolates, the UF.III retentates corresponded to the greatest part (ca. 55%) of DOC, and as to the UF retentates, the loss of DOM was the largest (ca. 32%) during the refinement with method c (the ratio of HA/FA was ca. 2:98). Likewise, the UF.IV filtrates consisted practically solely of FA type solutes (~100%). All HM samples were after their isolation hermetically sealed and stored in the dark at 4°C. The physical-chemical characterizations and structural classifications (e.g. molecular-size distributions, elemental analyses (C,H,N,S), functional groups and ^{1}H and ^{13}C NMR spectroscopy) of the different HM isolates are reported previously.[19–21] Table I shows the abbreviations of the seventeen (17) HM samples studied in the SEC-PAGE experiments.

Size-exclusion chromatography (SEC)

The various lake aquatic HM isolates were fractionated by SEC. The sample (5 mg) was dissolved in 1 mL of 7 M urea solution and loaded onto a Sephadex G-75 (Pharmacia, Sweden) column (1.5x100 cm) equilibrated with 7 M urea. Urea is a weak base with dissociation constant (K_b) of ca. $1.5 \cdot 10^{-14}$and the calculated pH of 7 M urea solution is thus ca. 7.5. The flow rate of 7 M urea eluent was 15 mL/h. The outer column volume (void volume, $V_0 = 43$ mL) was determined using Blue Dextran 2000. The total gel volume (V_t) was 160 mL. The elution curves were detected at 280 nm using a UA-5 detector (ISCO, USA). Elution volumes were normalised with V_e/V_t where V_e is the elution volume of the solute.[22] Figure 1 demonstrates the most typical examples of the SEC elution profiles obtained for the various HM samples studied (the intensities are normalised on the same scale). Table II describes the SEC elution behaviours for the whole sample set.

TABLE II SEC elution behaviours of the whole sample set[a]

Sample	V_e/V_t				
	A	***B***	***C***	***D***	***C+D***
S.HA	0.27–0.33	0.36–0.54			0.67–1.00
S.FA		0.36–0.54			0.63–1.00
M3.HA	0.27–0.33	0.36–0.48			0.67–1.00
M3.FA		0.36–0.54			0.63–1.00
S.UF.I.c	0.27–0.33	0.36–0.54			0.68–0.95
S.UF.II.c		0.31–0.54			0.68–0.95
S.UF.III.c			0.55–0.78	0.79–1.00	
S.UF.IV.FA			0.55–0.78	0.80–1.00	
M3.UF.I.a	0.27–0.33	0.36–0.55			0.68–0.95
M3.UF.II.a		0.36–0.55			0.68–1.00
M3.UF.III.a					0.51–1.00
M3.UF.I.HA	0.27–0.34	0.36–0.55			0.68–0.95
M3.UF.II.HA		0.35–0.54			0.68–1.00
M3.UF.I.FA		0.32–0.55			0.68–0.99
M3.UF.II.FA		0.35–0.54			0.67–1.00
M3.UF.III.FA					0.50–1.00
M3.UF.IV.FA					0.51–1.00

a. V_e / V_t = normalised elution volume. A, B, C and D (or C+D) denote combined SEC fractions obtained on the basis of electrophoretic analyses of the chromatographic profiles (cf. Figure 1).

Electrophoresis on polyacrylamide gel (PAGE)

The method used for electrophoretic fractionation of the HM has been reported previously in detail.[15–18] In brief, 9.7% acrylamide and 0.3% N,N′-methylenebisacrylamide (Bis, a cross-linking agent for polymer networks of the gel)[23] were dissolved in 89 mM Tris-borate (pH 8.3) with 1 mM EDTA and 7 M urea. The fractionation was carried out at room temperature on a vertical electrophoresis device (LKB 2001 Vertical Electrophoresis) with gel slab (20x20 cm). 89 mM Tris-borate and 1 mM EDTA solutions were used for the electrode buffers. Electrophoresis was performed for 1 h at a current intensity of 25 mA. The HM samples were dissolved in the buffer solution containing 89 mM Tris-borate, 7 M urea, 1% SDS and 1 mM EDTA and applied onto the gel. The concentration of all samples was 250 μg/50 μL. The electrophoregrams were run separately on the original HM samples and in tandem with the different eluates obtained by SEC (SEC-PAGE, subsequent testing of SEC elution profiles by PAGE). The electrophoregrams obtained for the whole sample set are shown in Figures 2 and 3.

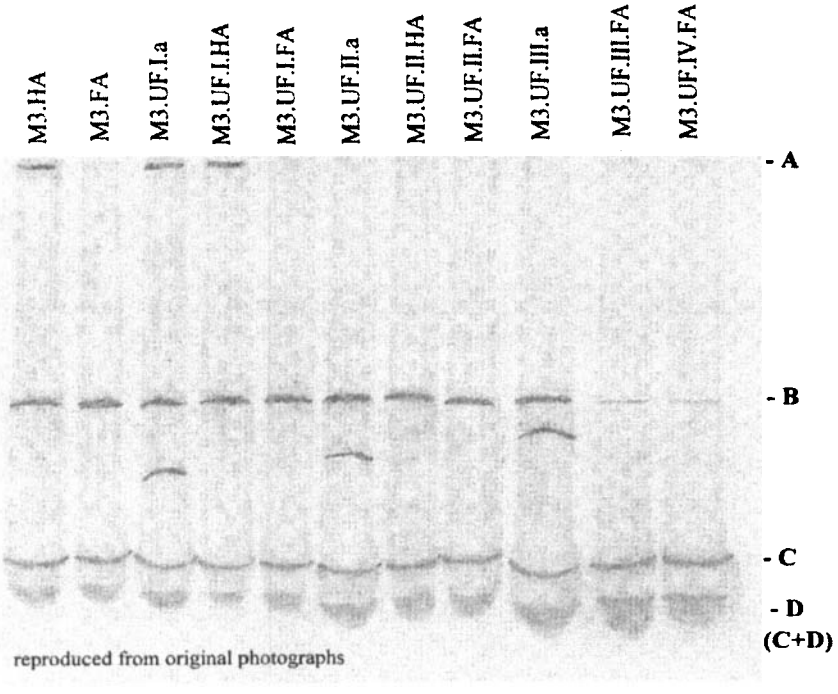

FIGURE 2 Electrophoregrams of different HA and FA type humic and unrefined DOM fractions with different molecular-size ranges. A, B, C and D (or C+D) are discrete coloured zones of fractionated HMs. Abbreviations, sources, definitions and isolation methods of the HMs are presented in Table I

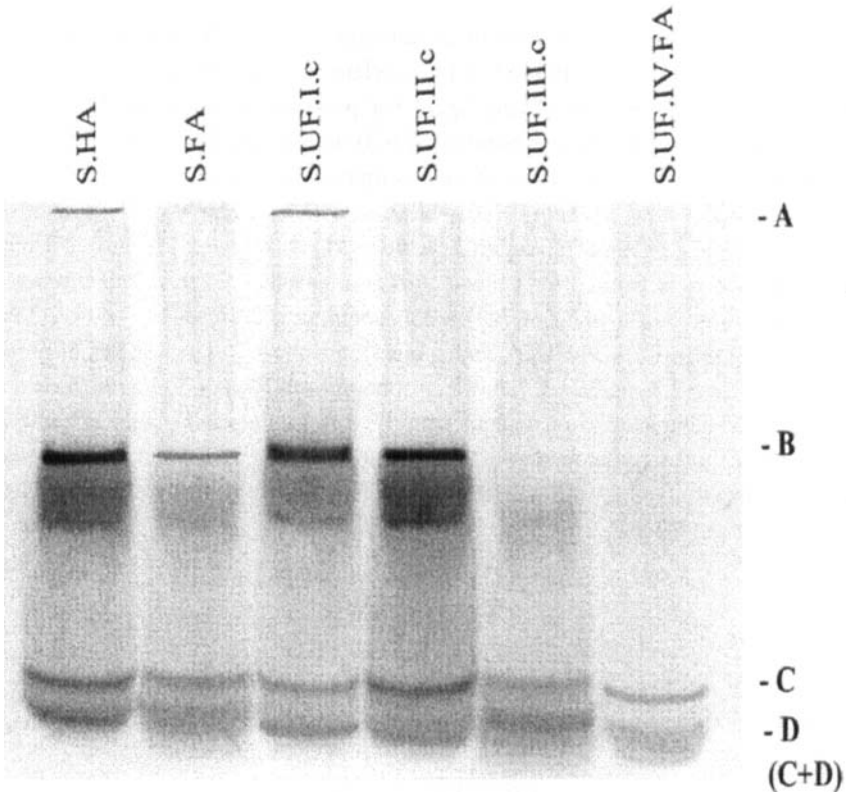

FIGURE 3 Electrophoregrams of different HA and FA type and partly refined (c = HA-FA mixture) humic fractions with different molecular-size ranges. A, B, C and D (or C+D) are discrete coloured zones of fractionated HMs. Abbreviations, sources, definitions and isolation methods of the HMs are presented in Table I

RESULTS AND DISCUSSION

PAGE analysis of SEC eluates of DOM

To evaluate the ultrafiltration of DOM and to estimate the relationship between ultrafiltration, XAD technique and SEC with dextran gels, all isolated HM samples were fractionated by SEC in 7 M urea on Sephadex G-75. As demonstrated in Figure 1, the molecular-sieving action of the Sephadex column for HM was fairly poor and only two main subfractions were at best visualized. However, regardless of the sample type all applied organic matter was eluted within the

total column volume. As shown in Table II, the integrated wholes of S.HA and M3.HA and most UF concentrates within the largest molecular-size cutoff (the HA-FA mixture of S.UF.I.c, the unrefined DOM of M3.UF.I.a and HA type solutes of M3.UF.I.HA) gave two different elution humps in the chromatograms (cf. Figure 1), the sharper one at the column void volume and a wide hump covering the rest of the fractionation range of the column. On the other hand, the integrated wholes of S.FA and M3.FA and all other UF concentrates (independent of their refinement methods), including also FA type solutes of M3.UF.I.FA, gave no SEC peak for the void volume but only the wide hump for the rest of the fractionation range. It is interesting to note (Table II) that although UF.I type solutes were retained by the membrane with NMW limit of ≥ 100 000 g/mol, the refined M3.UF.I.FA fraction did not show any peak around the SEC void volume (V_e/V_t=0.27) compared to corresponding fractions of M3.UF.I.a, M3.UF.I.HA and S.UF.I.c. This outcome conforms to the statement that compositional and structural alterations take place during the refinement of humic solutes and that the extremely high molecular-size constituents are solely connected to HA type fractions. Despite wave-like SEC elution profiles there occurred also at the end part of the elution hump some kind of "fine separation", as seen in Table II in the case of S.UF.III.c and S.UF.IV.FA.

The SEC analyses of the UF concentrates demonstrate that a clear fractionation has taken place during ultrafiltration and the elutions of different retentates shifted toward lower molecular sizes simultaneously with decreasing of membrane pore diameters. The V_e/V_t normalised elution volume ranges of 0.27 – 0.33, 0.35 – 0.55 and 0.64 – 1 in Table II corresponded to the nominal molecular-mass ranges of $198 \cdot 10^3$ – $114 \cdot 10^3$, $108 \cdot 10^3$ – $23 \cdot 10^3$ and $9.8 \cdot 10^3$ – $1 \cdot 10^3$ g/mol, respectively.[24]

Separation power of SEC for the whole sample set was tested by PAGE according to Trubetskoj et al.[15] The SEC eluates were divided into different fractions and assayed by PAGE. Those SEC eluate fractions which formed homogeneous electrophoretic zones A, B, C or D on PAGE have been indicated also in Table II (cf. also bold bars on the x-axis of Figure 1). Because of the poor resolution of SEC, the eluate fractions representing lower molecular sizes (i.e. C and D) have in common mostly labelled as C+D in Table II. Similarly assigned SEC eluate fractions had analogous PAGE properties independent of the sample nature which suggests that the molecular-size range of the eluate fraction A is greater than that of B which is greater than that of C+D for the whole sample set.

After PAGE analyses the electrophoregrams exhibited zones A, B and C+D for the refined UF retentate M3.UF.I.HA but only zones B and C+D for M3.UF.I.FA (Figure 2). The refined retentates M3.UF.II.HA and M3.UF.II.FA also gave only zones B and C+D. Finally, the refined FA type subfractions of UF retentates

M3.UF.III.FA and M3.UF.IV.FA, with the smallest molecular sizes, gave almost solely zones C and D (only some trace of zone B was visible in Figure 2). Accordingly, by the method c partly refined UF retentates, containing the HA-FA mixture, exhibited zones A, B and C+D for S.UF.I.c, zones B and C+D for S.UF.II.c, and almost solely zones C and D both for S.UF.III.c and for the FA type subfraction of S.UF.IV.FA (only some trace of zone B was visible in Figure 3). It was notable that the decreasing intensity of zone B, corresponding nominal molecular-mass range of ca. $108 \cdot 10^3$-$23 \cdot 10^3$ g/mol, was depending on the decreasing membrane pore sizes. Thus, the UF membrane PTGC (NMW limit 10 000 g/mol) was almost completely retained the larger size organic solutes (only some traces of B zone were obtained for UF.III fractions (i.e. NMW: 10 000 – 1 000 g/mol) in their PAGE separations). This result was not predictable without PAGE analyses because the SEC eluate fraction within the V_e/V_t normalised elution volume range of 0.35 – 0.55 did not form any distinctive resolving properties on the Sephadex chromatograms.

PAGE analyses revealed also in Figure 2 an additional electrophoretic zone between zones B and C+D for the UF retentates M3.UF.I.a, M3.UF.II.a and M3.UF.III.a, which represent unrefined DOM with different molecular sizes. The electrophoretic mobility of these additional zones decreased with the decreasing NMW limits of the membranes. The appearance of these additional electrophoretic zones speaks for the presence also of another kind material (not defined as HM) in these unrefined UF retentates. The results confirm the hypothesis that aquatic HM must, in certain way, play a role as definite entity in the original DOM, despite the difference between the non-humic and humic material is very marginal.

PAGE analyses confirmed that tangential flow membrane ultrafiltration resulted in molecular-size fractionation of DOM but some by-products of low molecular-weight solutes, however, still occurred also in the UF retentates obtained by the membranes with high nominal molecular-size cutoffs (e.g. zones C and D in the case of UF.I retentates). This finding does not prove the weak separative power of the tangential UF membranes but rather that the high molecular-size aggregates of the original DOM are not real macromolecular compounds, and that they contain also smaller, weakly bound solute units, which become loose during the conditions (e.g. disaggregating effect of urea) of further analysis.

PAGE analyses of the integrated wholes of HAs and FAs showed characteristic differences (Figures 2 and 3): HAs gave electrophoretic zones A, B and C+D but FAs only B and C+D. This finding verifies that the total fraction of humic solutes separated by the XAD technique and further divided at pH 1 into HA and FA type fractions show distinct physico-chemical properties. The same phenomenon

was also seen in the case of M3.UF.I.HA vs. M3.UF.I.FA (Figure 2) but not in the case of M3.UF.II.HA vs. M3.UF.II.FA indicating that the macromolecular properties of HA type subfractions with smaller molecular sizes begin to resemble those of the FA homologous.

SEC with dextran gels did not allow the estimation of the exact elution volumes of the separate, structurally different, HM solutes with definite properties, which have their own influence on the quality of DOM. For this purpose association of SEC with PAGE is recommended, e.g. to solve some peculiarities in the distribution of different organic aggregate fractions. Moreover, without this association it is difficult to determine the feasibility of the different fractionation techniques (SEC, UF, etc.) of HM from DOM. However, it is essential to realize that the resolving power of the SEC-PAGE system is rather limited since it produces predominantly only three different molecular-size fractions, as also it has been demonstrated[25] in the case of the SDS-PAGE system. On the other hand, PAGE needs considerably less material for analysis in comparison with SEC (0.1 – 0.2 mg vs. 5 – 10 mg). Therefore, in addition to economical reasons, if the quantity of humic or dissolved organic matter is limited, PAGE can serve as an alternative coarse method for fractionation of the complex natural material into limited number of more homogeneous isolates, which can then be subsequently further scrutinised, after effective removal of urea, by special analyses.

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