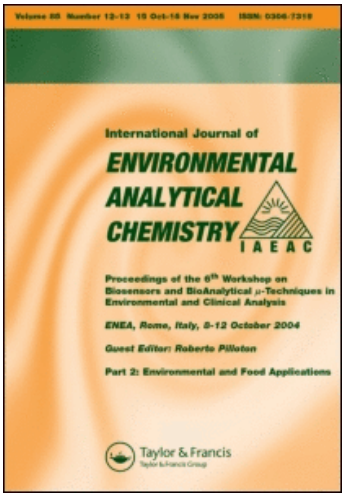

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

The Effects of a Strong Disaggregating Agent on Sec-Page of Aquatic and Soil Humic Matter

Juhani Peuravuori[a]; Kalevi Pihlaja[a]; Olga Trubetskaya[b]; Oleg Trubetskoj[c]
[a] Department of Chemistry, Laboratory of Physical Chemistry, University of Turku, Turku, Finland [b] Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow region, Russia [c] Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, Moscow region, Russia

To cite this Article Peuravuori, Juhani , Pihlaja, Kalevi , Trubetskaya, Olga and Trubetskoj, Oleg(2001) 'The Effects of a Strong Disaggregating Agent on Sec-Page of Aquatic and Soil Humic Matter', International Journal of Environmental Analytical Chemistry, 79: 3, 217 — 228
To link to this Article: DOI: 10.1080/03067310108044400
URL: http://dx.doi.org/10.1080/03067310108044400

Intern. J. Environ. Anal. Chem., Vol. 79(3), pp. 217-228
Reprints available directly from the publisher
Photocopying permitted by license only

THE EFFECTS OF A STRONG DISAGGREGATING AGENT ON SEC-PAGE OF AQUATIC AND SOIL HUMIC MATTER

JUHANI PEURAVUORI[a*], KALEVI PIHLAJA[a], OLGA TRUBETSKAYA[b] and OLEG TRUBETSKOJ[c]

[a]*Department of Chemistry, Laboratory of Physical Chemistry, University of Turku, FIN-20014 Turku, Finland,* [b]*Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry and* [c]*Institute of Soil Science and Photosynthesis, Russian Academy of Sciences, 142292 Pushchino, Moscow region, Russia*

(Received 24 April 2000; In final form 4 September 2000)

Size-exclusion chromatographic (SEC) fractionation and electrophoretic separation of aquatic humic matter samples from a Finnish lake using Sephadex G-75 with 7 M urea solution as eluent and 10% polyacrylamide gel (PAGE) with urea and sodium dodecyl sulphate solution (SDS), respectively, were performed and compared to similar analyses performed on a Russian chernozem soil humic acid sample and Nordic reference fulvic and humic acid samples. The integrated whole of aquatic humic solutes and soil humic acids were found to exhibit similar SEC-PAGE behaviours. Humic matter was not excessively disaggregated by the 7 M urea and hence SEC-PAGE can with confidence be applied as a coarse, initial fractionation procedure or for certain predeterminations of the structural composition.

Keywords: electrophoresis; size-exclusion chromatography; polyacrylamide gel; humic matter; chaotropic agent

INTRODUCTION

Biological macromolecules such as humic matter (HM) of both terrestrial and aquatic origin play an integral role in the carbon cycle. The physical and chemical characteristics of HM and their interactions with other chemicals present in the environment have been widely investigated using various methodologies and have been neatly summarised elsewhere, e.g. by Klavins.[1] However, fractionation of the heterogeneous matrix, and ultimately isolation into separate, identifia-

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

ble species is extremely difficult and strongly dependant on the method applied. In fact, up to now, there is no one specific fractionation technique for HM that is considered to be overwhelmingly preferable and the determination of various basic macromolecular properties, such as molecular size or weight distribution, is not a simple task.[2]

SEC has been the most extensively applied technique to separate a sampled humic mixture into fractions of more homogeneous molecular size. In particular, the application of low-pressure SEC (LPSEC) to the study of humic solutes became such a widespread routine procedure in numerous laboratories that this period of humus chemistry (1964–1973) has been named the "Sephadex Period" by Malcolm.[3] However, the results obtained by Sephadex gel chromatography were finally proven to be of limited usefulness and even to be inadequate in many applications.[e.g. 4] According to many research groups, a molecular-sieving process of this kind, regardless of the nature of the gel material used or the composition of applied eluents, is not recommended for the characterisation of HM. Nonetheless, LPSEC still finds applications in certain predeterminations.

Electrophoretic separation, which can be based on either the isoelectric points of the components or on their molecular sizes/weights, can also be applied to HM due to the polyelectronic nature of HM. Several methods for carrying out electrophoresis on a solid support such as polyacrylamide gel (PAGE) have been described[5] and numerous HM separations by means of PAGE have been reported[e.g. 6–16] during the past 30 years. In these studies the HM samples have usually been resolved into between two to six sub-components; the number of sub-components increasing in the presence of disaggregating agents such as SDS (sodium dodecyl sulphate, an anionic detergent)[7] or, in particular, urea.[9] Duxbury[17] has also reviewed fundamental applications of electrophoretic separation based on electrophoretic mobility (EM) under different conditions. However, the question which is the most appropriate method – SEC, PAGE, ultrafiltration (UF), etc. – for the fractionation of HM, still remains open.

Recently, fixed experimental conditions to determine the optimal interrelationship between PAGE and SEC fractionations of HM for the characterisation of humic isolates have been reinvestigated.[18–22] The main aim of the present study was to compare the effectiveness of PAGE and SEC by adapting the fixed experimental conditions mentioned above for the fractionation of certain aquatic and soil HMs. The aquatic and soil HMs in question were isolated by totally different procedures and are representative of entirely different environments. The present study forms an integral part of the extensive tandem SEC-PAGE studies and furthermore confirms the disaggregating effect of strong urea solution.

EXPERIMENTAL

Samples

Finnish lake water samples were collected into glass containers from Lake Savojärvi in southwestern Finland, in February 1988 (S) and in September 1994 (SS). Lake Savojärvi, located in a marsh region, is a very brown-water lake (colour as cobalt-platinum units ca. 150 mg Pt/L; DOC 19 mg C/L; pH 5.8). The samples were filtered (0.2 μm; Nuclepore polycarbonate filter cartridge, no. 611101) as soon as possible after sampling and stored thereafter in the dark at 4°C prior to analysis and isolation procedures. The characteristic properties of the lake water studied have been previously reported.[2]

Briefly, the analytical procedure of the XAD technique for the isolation and fractionation of the DOM was as follows: the water sample acidified to pH 2 was eluted through three columns connected in the sequence: XAD resin (XAD-8) ⇒ cation exchanger (Dowex 50W X-8) ⇒ weakly basic anion exchanger (Amberlite IRA-67). This procedure led in the first step to "hydrophobic" humic (HA) and fulvic (FA) acids (generally specified as humic substances) followed by "hydrophobic" neutrals ([MeOH]) which were eluted in the second step with methanol from the XAD-8 resin. Finally, in the third step "hydrophilic" acids ([IRA]) were obtained from the effluent of the XAD-8 resin by the anion exchanger. The isolation procedures have been reported[2,23,24] in detail previously together with the physico-chemical characteristics of the different fractions obtained.

Organic HM was also isolated from the SS water sample by a weakly basic anion exchanger (Sigma: DEAE cellulose, fine mesh, capacity 0.99 meq/g) at the natural pH of the water and labelled [DEAE] to obtain practically all the macromolecular organic acids together corresponding to the integrated whole of different humic solutes. The DEAE procedure together with the physico-chemical characteristics of the isolate has also been reported in detail previously.[2,23,24]

The Nordic reference samples of Nordic aquatic humic acid (No.HA, code IR105H) and fulvic acid (No.FA, code IR105F) of the IHSS (International Humic Substances Society) were isolated by the XAD technique during the summer of 1986 from the runoff water (colour ca. 230 mg Pt/L; DOC 22 mg C/L; pH 4.4) of a Norwegian mire.[25]

The isolation procedure and basic characteristics of the Russsian soil HM (chernozem Soil.HA) have been reported previously.[22] Briefly, the soil sample representing typical chernozem (Kursk region, central part of Russia) was extracted with 0.1 M pyrophosphate and 0.1 M NaOH solution under nitrogen gas with subsequent precipitation by HCl (pH 2.0) for obtaining HA type acids

from the HM extract. After isolation, all the samples were hermetically sealed and stored in the dark at 4°C.

Size-exclusion chromatography (SEC)

The different lake aquatic humic matter samples of SS.FA, SS.HA, SS.[MeOH], SS.[DEAE], No.FA and No.HA (with the exception of SS.[IRA] due to insufficient sample) and the chernozem soil humic acid (Soil.HA) were fractionated by SEC. The sample (5 mg) was dissolved in 1 mL of 7 M urea solution. 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. 6.5. The sample was loaded onto a Sephadex G-75 (Pharmacia, Sweden) column (1.5x100 cm) equilibrated with 7 M urea. The flow rate of 7 M urea eluent was 15 mL/h. The outer column volume (void volume, V_0 = 40 mL) was determined using Blue Dextran Blue 2000. The total gel volume (V_t) was 152 mL. The elution curves were detected at 254 nm using an UA-5 detector (ISCO, USA). Column effluent was collected as 2 mL aliquots for further PAGE analyses. Elution volumes were normalised either with V_e/V_t or $K_{av} = (V_e-V_0)/(V_t-V_0)$ where V_e is the elution volume of the solute.[26]

Previous reports have highlighted[21] three important points regarding SEC of HM, namely: i) that SEC fractionation, in the presence of 7 M urea as a disaggregating agent is based solely on molecular-size differences, ii) the whole HM sample is eluted within the total gel volume, and iii) there is no interaction between the gel matrix and the HM macromolecules.

Electrophoresis on polyacrylamide gel (PAGE)

The method used for electrophoretic fractionation of the HM has been reported previously in detail.[18] In brief, 9.7% acrylamide and 0.3% N,N'-methylenebisacrylamide (Bis, a cross-linking agent for polymer networks of the gel)[27] 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 solution as the electrode buffer were used. 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, with the exception of the chernozem Soil.HA sample which was 150 μg/50 μL. The electrophoregrams were run separately on the

original HM samples and in tandem with the different aliquots obtained by SEC (SEC-PAGE, subsequent testing of SEC profiles by PAGE).

RESULTS AND DISCUSSION

Chromatographic behaviour of humic solutes on dextran gels with different eluents

The elution profiles shown in Figure 1 for SS.HA and SS.[MeOH] samples (column Sephadex G-75, eluent 7 M urea, pH ca. 6.5) resembled somewhat those recorded earlier for S.HA and S.[MeOH] isolates of the same lake water and fractionated also by LPSEC but with Sephadex G-100, G-75 and G-25 columns connected in series and using 0.02% NaN_3 solution (pH 7.8, ionic strength nearly equal to the original water sample) as the eluent.[25] Corresponding similarities were also found for SS.FA, S.FA, No.FA and No.HA samples under these two different SEC conditions. The basic characteristic properties of the natural-water samples S and SS taken at different times from the same lake, the spectroscopic properties and the molecular-size distributions of the original organic solutes as well as those obtained for FA, HA and [MeOH] type isolates at constant measuring conditions were practically analogous as were their structural compositions.[2,25,28] In both cases, the gel matrix for the SEC was the same (Sephadex) with the gels differing only in their molecular-sieving ability. Thus, it can be inferred that the variation between the chromatograms shown in Figure 1 (the chromatograms being scaled to the same dimensions) is a result of the interactions between the gel matrix and the organic solutes of the HM. Because the effects of the acidity and ionic strength in both SEC cases is essentially insignificant, the most likely physical explanation for the variation is the chaotropic effect of urea.

It is well known that the elution profile of a sample of HM will depend on the combined effects of gel chromatography, charge density and adsorption. The acidity primarily controls the size and shape of the humus molecule whilst adjustment of the adsorption and repulsion between the gel matrix and humus molecule has mainly been attributed to the ionic strength of the sample.[e.g. 4] Dextran gels, like other gels, e.g. polyacrylamide, contain residual carboxyl groups whose ionisation creates negative charges on the gel polymer chains; thus the matrix can repulse negatively charged molecules during separation, resulting in increased exclusion and consequently a smaller consequent retention volume (c.f. the main fraction of S.HA to that of SS.HA in Figure 1). This effect is well known – humic solutes with their strong polyanionic character are therefore sus-

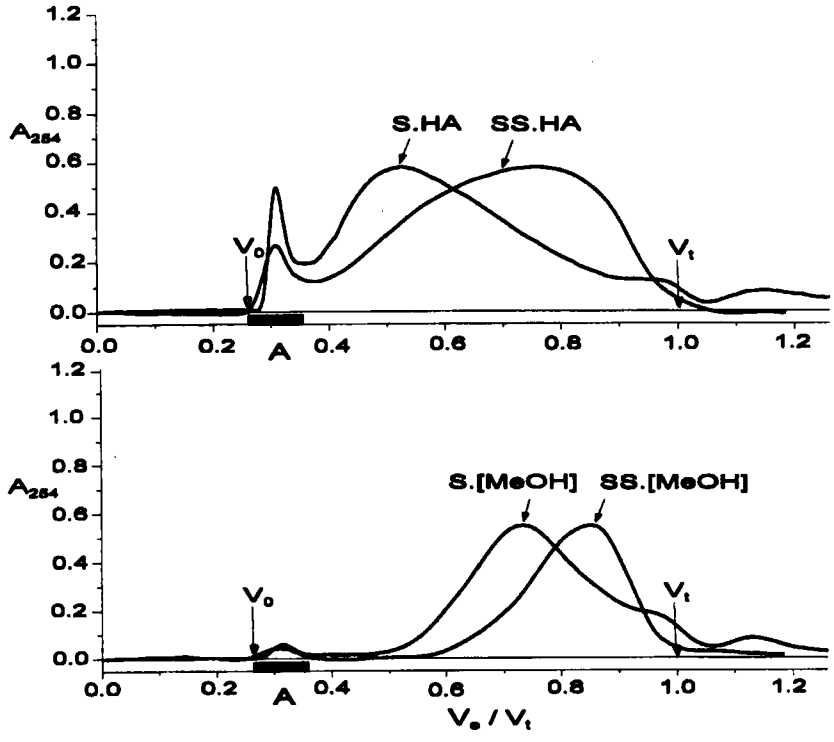

FIGURE 1 SEC profiles of analogous lake aquatic humic isolates obtained by a Sephadex G-75 column using 7 M urea as eluent (SS) and Sephadex G-100, G-75 and G-25 columns using 0.02% NaN_3 solution (S). V_0 = void volume, V_t= total gel volume, V_e = elution volume

ceptible to this kind of interaction and generally this repulsive effect is nullified by increasing the ionic strength of the eluent. On the other hand, it has been reported[29] that a significant increase in the ionic strength can cause an inordinate retardation of the HM on Sephadex columns resulting in an underestimation of their molecular size and/or shape.

In Figure 1, within the same normalised elution volume limits (V_e/V_t) for SS.HA, S.HA (both with 7 M urea solution as the eluent), SS.[MeOH] and S.[MeOH] solutes (both with 0.02% NaN_3 solution as the eluent appears a subfraction labelled A. Since urea is a fairly powerful disaggregant which breaks up intermolecular hydrogen-bonds and since the subfraction A is present when both 7 M urea and 0.02% NaN_3 are used as eluents it is inferred that this subfraction is some type of strongly-linked macropolymer unable to form hydrogen-bond based aggregates. Subfraction A though, yielded six components in the case of S.[MeOH] solutes but only one main component in the case of S.HA solutes by

high-performance size-exclusion chromatography (HPSEC) using 10 mmol sodium acetate solution (pH 7.0) as the eluent and a macroporous silica-based TSK column. Therefore, the structural compositions of these two subfractions, A from S.[MeOH] and from of S.HA) are in fact dissimilar irrespective of the LPSEC implications.

Figure 1 shows that the SEC combination of G-100 ⇒ G-75 ⇒ G-25 (0.02% NaN_3) columns separated the humic solutes into four subfractions of different molecular sizes. However, not all the organic matter was eluted from the column system within the V_t volume implying that interactions between the gel matrix and the HM are responsible for these retardations. For the humic solutes that did elute below the $V_e/V_t = 1$ limit a realistic profile of the compounds in order of their molecular size was obtained as evidenced by HPSEC. The relatively coarse molecular-sieving action of the G-75 column when using 7 M urea solution as eluent separated the humic solutes nicely into two main subfractions. The elution of all the organic matter in this case occurred within the V_t volume. This chromatographic behaviour supports the observation[21] that the use of an eluent containing strong urea solution, which prevents adsorption of HM on Sephadex, is recommended. The 7 M urea solution did not result in either an aggregating or a completely disaggregating effect on the HM leading toward an extraordinarily larger or smaller molecular size or profile, respectively. The results appear in line with the common utilisation of urea in protein chemistry where this chaotropic agent has been used for disrupting the intermolecular peptide-peptide hydrogen-bonds without significant side-reactions at very high concentrations (up to 8 M).

Polyacrylamide gel electrophoresis (PAGE)

As early as 1978 it was demonstrated that it is possible to increase the number of electrophoretic subfractions of soil HA type matter by PAGE disc electrophoresis by increasing the urea concentration (0.0 ⇒ 6.0 M urea).[9] Up to four different electrophoretic zones could be obtained including, independent of the urea concentration, an immobile zone at the top of the gel. It was subsequently confirmed that the different electrophoretic subfractions of HM produced by PAGE did indeed truly represent molecular-size fractions supporting the notion that all soil humic acids are not simply statistical polymers but that they in fact contain constant and uniform molecular-size fractions.[16] According to references [9, 16] and several other earlier publications dealing with PAGE, it has been stated that a more accurate view on the humic macromolecule can only be obtained if the hydrogen bonds of the aggregates are ruptured.

Figure 2 shows the PAGE of the lake water HM and soil HA isolates carried out according to Trubetskoj et al.[18] The aquatic SS.HA and No.HA as well

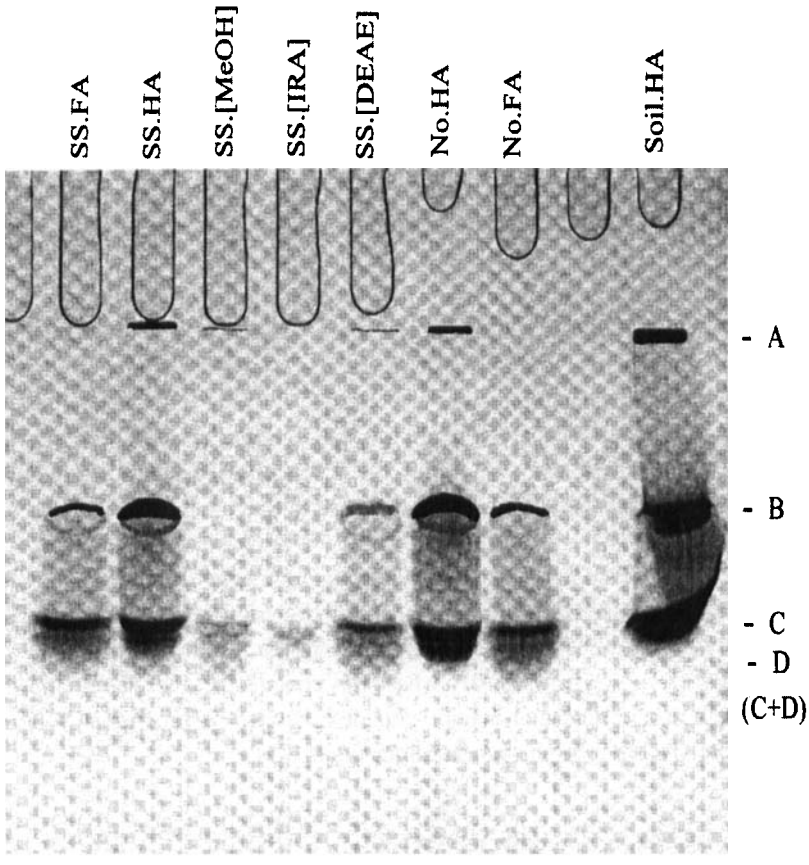

FIGURE 2 Electrophoregrams of 250 μg/50 μL of SS.FA, SS.HA, SS.[MeOH], SS.[IRA], SS.[DEAE], No.HA, No.FA and 150 μg/50 μL of chernozem Soil.HA

Soil.HA gave electrophoretic patterns with starting zones A, which did not move into the gel; zone B in the middle part of the gel; zones C and D at the bottom of the gel combined into zone C+D due to the relative close EM. The SS.[MeOH] showed a weak intensity at zone A and a weak combined zone C+D (zone B was absent). The SS.[IRA] exhibited only a very pale zone C+D. The SS.[DEAE] produced similar electrophoretic profiles to those of HA type samples originating from aquatic as well as soil environments (zones A, B and C+D), although the intensities of all the zones were much weaker. The aquatic humic fractions of SS.FA and No.FA gave only intensely coloured zones B and C+D. It had been shown earlier that the PAGE of river FA, ground-water FA and grey forest soil FA also consisted of zones B and C+D, but not zone A.[20]

Coupled SEC-PAGE

Figure 3 shows the SEC profiles of all lake water HM samples (with the exception of SS.[IRA] due to insufficient sample) and soil HA. Despite the inherent low resolution, it is clear that every chromatogram has its own individual character. This dissimilarity indicates that both the aquatic FA and HA type solutes posses their own characteristic nature though certain HM mixtures may contain variable amounts of components common to each sample. The SEC column effluents were divided into chromatographic aliquots and every third aliquot was assayed by PAGE. The aliquots, each of which formed a homogeneous electrophoretic zone on PAGE, consisted of HM fractions A, B and C+D. Similarly--assigned fractions had analogous elution volumes independent of the sample origin which suggests that, for all HM samples, the molecular size of fraction A is greater than that of B which is greater than that of C+D.

The SEC profiles of all HAs investigated (Figure 3) covered the whole fractionation range of the column. The normalised elution volumes of the SEC were for fraction A: V_e/V_t: 0.26 – 0.35, K_{av}: 0 – 0.12; for fraction B: V_e/V_t: 0.37 – 0.57, K_{av}: 0.14 – 0.41; and for fraction C+D: V_e/V_t: 0.70 – 1, K_{av}: 0.60 – 1. By means of these normalised elution volumes, using a previously developed equation,[25] the nominal molecular-weight limits (NMW) for different PAGE fractions were calculated: A: $206 \cdot 10^3 - 108 \cdot 10^3$; B: $93 \cdot 10^3 - 22 \cdot 10^3$ and C+D: $9 \cdot 10^3 - 1 \cdot 10^3$. The NMW values calculated in the present study correspond quite well to those estimated earlier for PAGE fractions A and B obtained from chernozem soil HA (>$100 \cdot 10^3$ and $100 \cdot 10^3 - 30 \cdot 10^3$, respectively) but somewhat less than those estimates for C+D ($30 \cdot 10^3 - 5 \cdot 10^3$).[21] From these results, it can be concluded that lake water and soil HM exhibit similar electrophoretic behaviour. Therefore further investigations of the fractions, A, B and C+D, obtained by coupled SEC-PAGE appears to be relevant from their genetic point of view.

The SEC of SS.[MeOH] showed only one main peak at the end and one quite small peak at the very beginning of the elution range of the column resulting in electrophoretic zones C+D and A on PAGE. Regarding the comparison of the SEC of SS.FA and No.FA to those of the corresponding HA samples, the elution profile covers the second part of the fractionation range of the column and when tested by PAGE it produced electrophoretic zones B and C+D.

Recently, it has been demonstrated that aquatic organic matter separated by DEAE cellulose (SS.[DEAE]) consists of an average combination of HM isolated by XAD-8 (SS.FA, SS.HA and SS.[MeOH]) and Amberlite IRA-67 resins (SS.[IRA]) in a ratio of about 51, 9, 5 and 35%, respectively.[23] Based on the fact that SS.[MeOH] and SS.[IRA] type matter accounts for 40% of the bulk of SS.[DEAE], the intensity of zones B and C+D on the SS.[DEAE] electrophore-

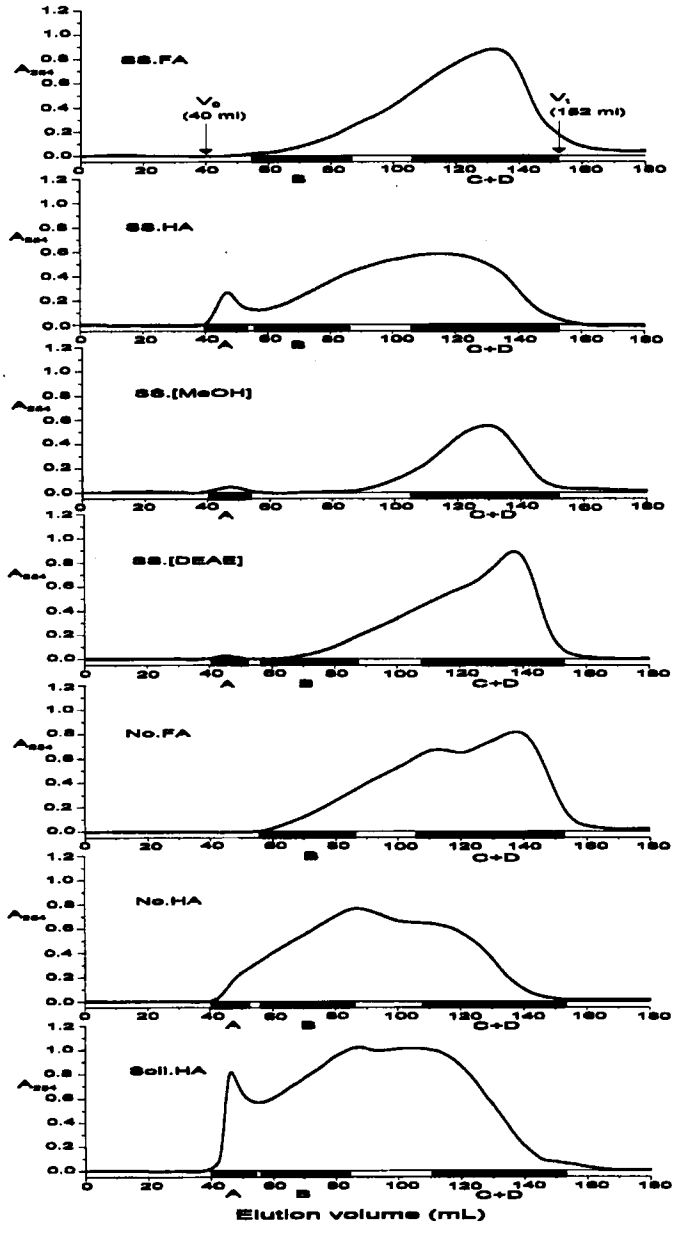

FIGURE 3 SEC of 5 mg lake aquatic SS.FA, SS.HA, SS.[MeOH], SS.[DEAE], No.FA, No.HA and chernozem Soil.HA (Sephadex G-75, 7 M urea). Black boxes on the x-axis show the combined fractions A, B and C+D, obtained on the basis of electrophoretic analyses of the chromatographic profiles. V_o = void volume, V_t = total gel volume

gram should be weaker than that of SS.FA and SS.HA, and this indeed is the case as shown by the electrophoretic patterns in Figure 3. Moreover, zone A is practically missing from the SS.[DEAE] electrophoregram, which is consistent with the fact that it only accounted for about 9% of the SS.HA type matter.

Although SEC-PAGE provides only modest analytical information, the results presented here support previous conclusions[24,28,30] based on comprehensive analyses; namely that the bulk of humic solutes obtained at the natural pH by a "soft" sorption method (DEAE procedure) in all likelihood is a combination of so-called "hydrophobic" and "hydrophilic" acid solutes which can be isolated by a multi-stage approach under very acidic conditions (XAD technique). Accordingly, the disaggregating effect of 7 M urea is not particularly strong for humic solutes and under carefully-adjusted conditions a similarity between SEC and PAGE results.

The SEC-PAGE results also appear to reaffirm an old supposition[9] that the strong immobile electrophoretic band at the top of the PAGE obtained from soil HA type matter most probably consists of a strongly linked macropolymer unable to form hydrogen-bond based aggregates. It is interesting to note that this immobile electrophoretic band also occurred in an aquatic HM sample which represented a conglomeration of different humic solutes in addition to the refined aquatic HA type samples. It was surprising to also find this immobile electrophoretic band in the strongly refined, so-called hydrophobic neutral fraction, which is often not even classified as a real humic substance. The results also indicate a certain similarity in the molecular size profiles between aquatic and soil humic matter, although upon closer scrutiny their structural compositions are considerably different. Despite the modest molecular-sieving ability of PAGE, it does offer a coarse option for the fractionation of HM into a limited number of subfraction which can then be subsequently further scrutinised by special analyses.

Acknowledgements

The authors wish to thank Dr. Karel D. Klika for improving the English of the manuscript and both the Russian Academy of Sciences and the Academy of Finland for financial support (project 12).

References

[1] M. Klavins, *Aquatic Humic Substances, Characterization, Structure and Genesis* (ISBN 9984–516–52–0, Riga, Latvia, 1997), 234 pp.

[2] J. Peuravuori and K. Pihlaja, *Anal. Chim. Acta*, **337**, 133–149 (1997).

[3] R.L. Malcolm, In: *Humic Substances in Soil, Sediment and Water – Geochemistry, Isolation, and Characterization* (G.R. Aiken, D.M. McKnight, R.L. Wershaw and P. MacCarthy eds., Wiley, New York, 1985), pp. 181–209.

[4] P.T. Hine and D.B. Bursill, *Water Res.*, **18**, 1461–1465, (1984).
[5] O. Vesterberg, *J. Chromatogr.*, **480**, 3–19, (1989).
[6] V.V. Stepanov and A.N. Pakhonov, *Sov. Soil. Sci.*, **1**, 742–749, (1969).
[7] R. Klöcking, *J. Chromatogr.*, **78**, 409–416, (1973).
[8] N.R. Curvetto, N.A. Balmaceda and G.A. Orioli, *J. Chromatogr.*, **93**, 248–250, (1974).
[9] M. Castagnola, R.G. de las Heras, G.B. Marini-Bettòlo and C. Nigro, *J. Chromatogr.*, **147**, 438–442, (1978).
[10] M. Castagnola, C. Nigro, G.B. Marini-Bettolo, A. Malina and R.G. de las Heras, *J. Chromatogr.*, **177**, 130–134, (1979).
[11] S.V. Kasparov, F.A. Tikhomirov and A.D. Fless, *Sov. Soil. Sci.*, **36**, 21–28, (1981).
[12] N.M. Gonzales, M. de Castagnola and D. Rossetti, *J. Chromatogr.*, **209**, 421–431, (1981).
[13] B. Ceccanti, J.M. Alcaniz-Baldellou, M. Gispert-Negrell and M. Gasiot-Matas, *Soil. Sci.*, **142**, 83–90, (1986).
[14] M. De Nobili, *J. Soil. Sci.*, **39**, 437–445, (1988).
[15] M. De Nobili, G. Bragato, J.M. Alcaniz, A. Puigbo and L. Comellas, *Soil. Sci.*, **150**, 763–770, (1990).
[16] R.M. Baxter and J. Malysz, *Chemosphere*, **24**, 1745–1753, (1992).
[17] J.M. Duxbury, In: *Humic Substances in Soil, Sediment and Water – Geochemistry, Isolation, and Characterization*(G.R. Aiken, D.M. McKnight, R.L. Wershaw and P. MacCarthy eds., Wiley, New York, 1985), pp. 593–620.
[18] O.A. Trubetskoj, L.Y. Kudryavceva and L.T. Shirshova, *Soil. Biol. Biochem.*, **23**, 1179–1181, (1991).
[19] O.A. Trubetskoj, O.E. Trubetskaya and T.E. Khomutova, *Soil Biol. Biochem.*, **24**, 893–896, (1992).
[20] O.A. Trubetskoj, O.E. Trubetskaya, L.F. Markova and T.A. Muranova, *Environ. Int.*, **20**, 387–390, (1994).
[21] O.A. Trubetskoj, O.E. Trubetskaya, G.V. Afanas'eva, O.I. Reznikova and C. Saiz-Jimenez, *J. Chromatogr. A*, **767**, 285–292, (1997).
[22] O.E. Trubetskaya, O.I. Reznikova, O.I, G.V. Afanas'eva, L.F. Markova, T.A. Muranova and O.A. Trubetskoj, *Environ. Int.*, **24**, 573–581, (1998).
[23] J. Peuravuori, K. Pihlaja and N. Välimäki, N, *Environ. Int.*, **23**, 453–464, (1997).
[24] J. Peuravuori, N. Paaso and K. Pihlaja, *Thermochim. Acta*, **325**, 181–193, (1999).
[25] J. Peuravuori, *Isolation, Fractionation and Characterization of Aquatic Humic Substances: Does a Distinct Humic Molecule Exist*? (Thesis, ISNN 0786–7050, University of Turku, FIN, 1992), 99 pp.
[26] H. Determann, *Gel Chromatography, Gel Filtration, Gel permeation, Molecular Sieves* (Springer-Verlag, New York, NY, 1968), 195 pp.
[27] P.G. Righetti and C. Gelfi, *J. Chromatogr. B*, **699**, 63–75, (1996).
[28] J. Peuravuori and K. Pihlaja, *Anal. Chim. Acta*, **363**, 235–247, (1998).
[29] H. De Haan, R.I. Jones and K. Salonen, *Freshwater Biology*, **17**, 453–459, (1987).
[30] J. Peuravuori, N. Paaso and K. Pihlaja, *Anal. Chim. Acta*, **391**, 331–344, (1999).