

Tandem size-exclusion chromatography-polyacrylamide gel electrophoresis fractionation of aquatic humic substances - characterization of size-separates with TMAH treatment and pyrolysis-GC/MS

Tero Lehtonen,^{a*} Juhani Peuravuori,^a Kalevi Pihlaja,^a Olga Trubetskaya,^b and Oleg Trubetskoj^c

^a*Department of Chemistry, University of Turku, FI-20014 Turku, Finland*

^b*Branch of Shenmyakin and Ovchinnikov Institute of Bioorganic Chemistry*

^c*Institute of Basic Biological Problems, Russian Academy of Sciences, 142290 Puschino, Moscow region, Russia*

E-mail: kpihlaja@utu.fi

Dedicated to the 60th birthday of Professor Harri Lönnerberg

Abstract

Aquatic humic (HA) and fulvic acids (FA) from two different origins were fractionated by the tandem size-exclusion chromatography and polyacrylamide gel electrophoresis (tandem SEC-PAGE). Three HA and two FA fractions exhibited different molecular sizes, electrophoretic mobilities and structural compositions based on elemental analyses and pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) after tetramethyl-ammonium hydroxide (TMAH) treatment. Based on comparison of the size-separates and unfractionated bulk samples, the highest molecular size fractions of aquatic humic substances (HS) appear to be enriched in fatty acids (mainly C₁₆ and C₁₈), which are strictly linked to the humic core. This and the presence of low chain fatty acids and unsaturated and di-tri acids may suggest a microbial rather than a vascular plant origin. Even if the majority of lignin derived components in aquatic HS represent relatively high state of biodegradation (oxidation), a certain variation between different size-fractions also exists, supporting a high heterogeneity and composite nature of aquatic HS. Although SEC-PAGE fractionation appears to produce fairly homogeneous HS fractions, the absorption of some urea into of the separates cannot be completely avoided in this method, which limits its suitability for evaluating, especially the presence of N-containing constituents.

Keywords: Humic and fulvic acids, molecular size fractions, pyrolysis, structural elucidation

Introduction

Humic substances (HS), operationally divided into humic (HA insoluble in water at low pH) and fulvic acids (FA, soluble in water under all pH conditions), are widely distributed in soils, natural waters and sediments being an important component of biospheric organic carbon. Largely due to their complex chemical nature, wide array of properties and varying molecular size, the question about the genesis and structural organization of HS is still not fully understood.

SEC and electrophoresis are among the most extensively applied methods to separate HS into sub-fractions of more homogeneous composition and molecular size.¹ Recently, Trubetskoj et al.^{2,3} adopted a reproducible method for the fractionation of bulk HS samples based on a combination of SEC and polyacrylamide gel electrophoresis (PAGE), named tandem SEC-PAGE. Denaturing agents such as 7 M urea are used to break up associations between different humic aggregates and also to eliminate adsorptive hydrogen bonding interactions with the fractionating media. Therefore, this method was suggested to separate disaggregated primary humic structures rather than larger HS aggregates, as it is usual in many attempts to fractionate HS.⁴ In this way, three mutually uniform sub-fractions from both soil and aquatic HS (labelled A, B and C+D), differing in their electrophoretic mobility (EM) and molecular size were obtained by tandem SEC-PAGE.⁵⁻⁷ A further optimization of the method confirmed the disaggregating effect of strong urea solution and also allowed the production of preparative quantities of sub-fractions amenable to structural analyses by more sophisticated methods.

Several SEC-PAGE studies performed with soil HA fractions from different origins have already revealed clear differences in their structural compositions (aromatic, aliphatic and N-containing components) and chemical properties in terms of UV-visible spectra, fluorescence and photochemical activity.⁸⁻¹¹ Analytical pyrolysis (Py) is an appropriate technique to characterize such hardly isolable and heterogeneous organic materials; useful structural information can be produced with a good reproducibility and extremely low sample consumption. However, conventional Py-GC/MS of HS have limitations derived from the polar nature of compounds like poor chromatographic separation as well as from the pyrolysis process itself able to produce artifacts by thermal reactions. In order to overcome said side effects a modified pyrolysis method in the presence of tetramethylammonium hydroxide (TMAH) was introduced by Challinor.¹² The addition of TMAH avoids thermal decarboxylation and dehydroxylation reactions and improves GC separation by converting carboxylic acids and phenols into more volatile methyl derivatives. Despite of some uncertainty concerning possible oxidative and secondary effects of this strongly basic reagent,^{13,14} it is well demonstrated that TMAH considerably assists thermal degradative analysis of aquatic HS and thus provides valuable detailed information about their complex molecular compositions.¹⁵

The present study intends to complement previous extensive tandem SEC-PAGE studies⁶ and analytical pyrolysis characterisations¹⁵ of unfractionated bulk isolates of aquatic HS from freshwater environments. The major aim is to use tandem SEC-PAGE to fractionate aquatic HS

into a limited number of more homogeneous size-fractions and to study their compositional features by TMAH-treatment and Py-GC/MS.

Results and Discussion

Fractionation procedure

The electrophoregrams of M3HA, M3FA, NoHA and NoFA has been published previously.⁶ As a summary, the PAGE of aquatic HS exhibited four narrow colored electrophoretic zones: a starting zone A that did not move into the gel; zone B in the middle part of the gel; and rather poorly separated zones C and D at the bottom of the gel. Identically marked zones from all HA and FA samples had similar electrophoretic mobilities (EM) and the zones C and D were finally combined in the preparative step due to their incomplete separation. Finally, altogether three distinct size-fractions from HA (A, B and C+D) and two size-fractions from FA (B and C+D) samples were collected and analyzed. The weight distributions of the fractions (Table 1) show that the relative amounts of fractions were increased in the order C+D > B > A, while the molecular sizes of the fractions decreased in the reversed order (A > B > C+D) independent of the sample source.

Table 1. Weight distribution of different HS fractions obtained by SEC-PAGE

Sample	A(%)	B(%)	C+D(%)
M3HA	7.1	12.5	32.3
M3FA	-	6.6	54.3
NoHA	5.4	16.5	29.8
NoFA	-	11.3	48.4

Elemental compositions

The results for elemental analysis of the bulk aquatic HS and their SEC-PAGE fractions are shown in Table 2.¹⁹ As a summary, elemental compositions for different size fractions of aquatic HA and FA showed some clear trends: (a) the C content increased with the decreasing molecular size and increasing EM except NoHA; (b) the N content decreased with the decreasing molecular size and increasing EM of all aquatic HS fractions; (c) the highest H content was obtained for the highest molecular weight fraction A, independent of the HS source.

Pyrolysis products

The total ion current (TIC) chromatograms of the pyrolysates of M3HA, M3FA and their subfractions are displayed in Figures 1 and 2. The corresponding chromatograms of NoHA and NoFA are not shown, but their overall profiles were similar to the TIC patterns displayed. For clarifying the vast number of pyrolysis products, they were divided in more specific structural groups (Table 3) and some conclusions could be drawn as follows.

A first notable feature in the pyrolysis products of all fractionated size-separates was their higher yields of nitrogen compounds with respect to the corresponding bulk HS samples. Such N-compounds like methylurea, N,N'-dimethylurea, dimethylacetamide, pyridine, alkylpyrroles, pyrrolidinediones and pyrimidinediones conform to the high N-contents in Table 2 and also confirm that the absorption of urea into the SEC-PAGE fractions cannot be thoroughly avoided in spite of the intensive purification by dialysis. The observed decrease of the partially methylated urea peak towards lower molecular size fractions (Figures 1 and 2) supports the idea that high molecular size fractions of aquatic HS may have better absorption abilities for nitrogen than lower molecular size fractions. However, it is also clear, that during pyrolysis this contaminant material may also undergo thermal alterations (e.g. cyclization) to produce artifacts as secondary products, making it difficult to reliably evaluate the original N signature in the size-separate fractions. For this reason, all N-compounds were excluded from the pyrolysis product yield calculations in Table 2 and only not-N-containing pyrolysis products were considered for further structural comparisons between HS fractions.

Table 2. Elemental compositions (% ash-free) of different HS and their SEC-PAGE fractions

Sample	Fraction	Ash (%)	C (%)	H (%)	N (%)	O (%)
M3HA	Bulk	5.8	59.0	4.6	1.2	34.1
	A	10.2	44.0	5.2	6.4	44.4
	B	12.0	47.9	4.8	3.9	43.4
	C+D	12.6	51.0	4.9	2.9	41.3
M3FA	Bulk	3.8	56.9	4.3	0.6	37.2
	B	10.2	40.4	4.3	4.3	51.0
	C+D	13.4	48.0	4.9	2.4	44.8
NoHA	Bulk	2.2	54.6	4.5	1.0	39.0
	A	8.6	53.7	5.5	5.0	35.8
	B	11.7	53.7	4.8	4.4	37.1
	C+D	8.2	53.2	5.1	3.0	38.8
NoFA	Bulk	1.3	53.1	4.6	0.8	40.7
	B	10.3	48.1	4.6	4.1	43.2
	C+D	10.6	49.1	4.7	2.7	43.5

Among the aromatic pyrolysis products produced by aquatic HS and their fractions, phenolic acids, usually considered as biodegraded lignin components, were identified as the most

dominant class of compounds. They constituted ca. 50% of all aromatics and most prevailing individual compounds were methyl esters of 3,4-dimethoxybenzoic and 3,4,5-trimethoxybenzoic acids (peaks 3 and 4 in Figures 1 and 2). Other major aromatic products detected were methylated benzenecarboxylic acids, aromatic aldehydes and ketones, and phenols.

Correspondingly, the most abundant aliphatic pyrolysis products were methyl esters of moderately short chain (C4-C5) mono- and dicarboxylic acids and also those of longer chain fatty acids dominated by the methyl esters of tetradecanoic (C14), hexadecanoic (C16) and octadecanoic (C18) acids (peaks 5 and 6 in Figures 1 and 2). Also their unsaturated homologues, particularly hexadecenoic and octadecenoic acids, were quite common in the pyrolysates, while the most dominant methyl esters of dicarboxylic acids were those of butanedioic and butenedioic acids (peak 3 in Figures 1 and 2).

The compiled structural results in Table 3 show some clear irregularities between different structural constituents of aquatic HS samples and their molecular size fractions. Despite the fact that in all four bulk HS samples aromatic pyrolysis products dominated over aliphatic (75% for HA and ca. 65% for FA), a much higher contents of aliphatics were regularly found in the highest molecular size fractions (A for HA and B for FA). This increased aliphatic character was especially caused by the abundance of C14 to C18 fatty acids and this is also apparent in Figures 1-2, where the peaks of hexadecanoic acids (nr. 5) and octadecanoic acid (nr. 6) in the chromatograms of M3HA-A and M3FA-B are clearly enhanced as compared to the lower molecular size fractions. A similar trend was also observed for the fraction NoHA-A, but not for NoFA-B.

Whereas the pyrolysates of high molecular size fractions of aquatic HS showed an enrichment in long chain fatty acids, the lower molecular size fractions showed an increased abundance of methyl esters of aliphatic di- and tricarboxylic acids. However, after fractionation, all HS pyrograms showed a lower portion of these polycarboxylic constituents than the original bulk samples. This difference can be explained, at least partially, by a mixture character of aquatic HS based in the idea that whereas even similar structural constituents can be associated into HS structures in various ways, it is generally assumed that carboxylic acid units can be bound in the macromolecular humic matrix via ester bonds¹⁹ or hydrogen bonds.²⁰ In TMAH/pyrolysis of the bulk samples, both of the above mentioned constituents are released as methyl esters, but after SEC-PAGE procedure, only the former will be still present in the pyrolysates due to breakage of hydrogen bonds exerted by the urea solution. Due to this kind of disaggregation all the released low molecular size acids were possibly eluted in the C+D fraction and dialyzed out during the final purification of the fraction.

Table 3. Relative proportions (%) of pyrolytic structural product groups obtained from different HS and their SEC-PAGE fractions (values for N-compounds in parentheses were not included in the final calculations)

Structural group	M3HA	M3HA	M3HA	M3HA	M3FA	M3FA	M3FA	NoHA	NoHA	NoHA	NoHA	NoFA	NoFA	NoFA
	Bulk	A	B	C+D	Bulk	B	C+D	Bulk	A	B	C+D	Bulk	B	C+D
alkylbenzenes and styrenes	0.7	0.8	1.4	1.5	0.2	2.2	1.5	1.2	3.6	0.9	2.0	1.1	0.5	0.4
phenols	3.9	2.5	5.9	3.2	4.6	5.3	7.3	5.6	5.2	8.2	11.2	10.7	10.1	4.7
alkylphenols	4.8	0.6	5.8	3.0	0.9	2.6	5.2	3.7	4.2	5.7	10.3	4.0	1.8	2.0
aromatic aldehydes and ketones	7.8	4.1	5.7	6.1	4.0	5.4	3.4	7.5	7.0	12.1	9.2	4.5	6.6	6.2
benzenecarboxylic acids	3.2	2.9	1.9	3.8	4.7	1.9	3.0	6.6	1.5	0.5	0.7	5.2	2.2	3.8
phenolic acid (α)	39.9	16.7	40.2	30.2	34.8	19.8	30.9	37.0	20.9	50.2	42.4	27.2	38.0	23.6
phenolic acids (β,γ)	5.4	0.4	10.5	7.1	3.4	2.6	3.8	6.8	3.3	4.5	2.0	4.2	1.2	5.1
other aromatics	9.9	2.4	1.1	7.9	12.7	2.6	5.1	9.8	2.9	3.2	2.6	8.6	5.1	10.5
Total aromatics	75.6	30.4	72.5	62.8	65.3	42.4	60.2	78.1	48.6	85.3	80.4	65.5	65.5	56.3
alkanes and alkenes	0.7	5.0	-	-	0.5	-	-	1.9	1.7	0.6	0.6	1.0	-	0.1
monocarboxylic acids (<C ₁₄)	2.3	11.0	2.0	2.9	4.1	3.1	3.3	3.2	2.6	1.7	8.9	4.2	3.0	0.5
monocarboxylic acids (C ₁₄₋₁₈)	6.0	41.9	11.0	10.2	2.8	34.2	10.1	2.9	39.9	3.8	4.1	3.6	6.6	4.1
dicarboxylic acids	5.0	2.9	2.3	6.0	12.1	4.9	8.9	5.2	0.6	2.4	1.2	12.2	2.9	5.2
tricarboxylic acids	1.6	-	0.9	1.6	2.6	-	1.5	0.3	-	-	-	2.0	-	2.0
other aliphatics	0.3	2.4	1.6	2.2	0.8	2.1	2.3	0.2	2.1	0.5	0.5	0.2	2.0	0.9
Total aliphatics	15.9	63.2	17.8	22.9	22.9	44.3	26.1	13.7	46.9	9.0	15.3	23.2	14.5	12.8
furans	0.7	-	1.0	1.6	1.3	0.5	1.4	0.7	0.3	0.4	0.2	2.3	0.2	0.3
other cyclic compounds	0.2	-	0.2	1.3	1.3	1.8	2.4	0.3	0.1	0.1	1.2	1.4	0.3	0.9
carbohydrate derivatives	0.4	1.7	1.5	1.2	-	1.5	1.3	-	-	-	-	-	-	-
nitrogen compounds	(3.4)	(20.7)	(22.2)	(18.9)	(3.7)	(31.7)	(21.7)	(3.8)	(18.6)	(19.6)	(18.2)	(6.8)	(38.5)	(10.0)

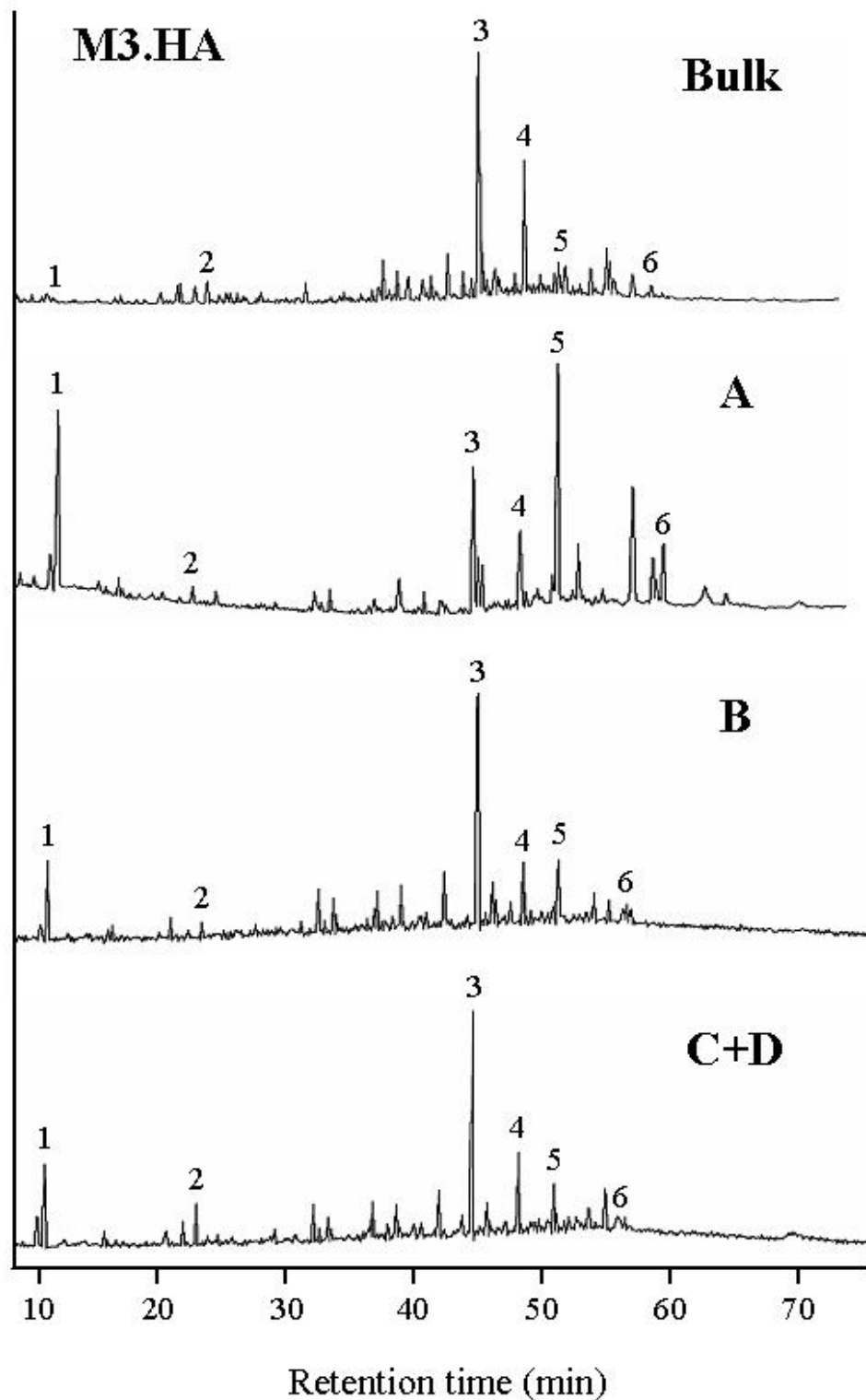


Figure 1. TIC chromatograms of M3HA and its SEC-PAGE fractions. Labelled peaks (acids as methyl esters): (1) N,N'-dimethylurea, (2) butanedioic acid, (3) 3,4-dimethoxybenzoic acid, (4) 3,4,5-trimethoxybenzoic acid, (5) hexadecanoic acid, (6) octadecanoic acid.

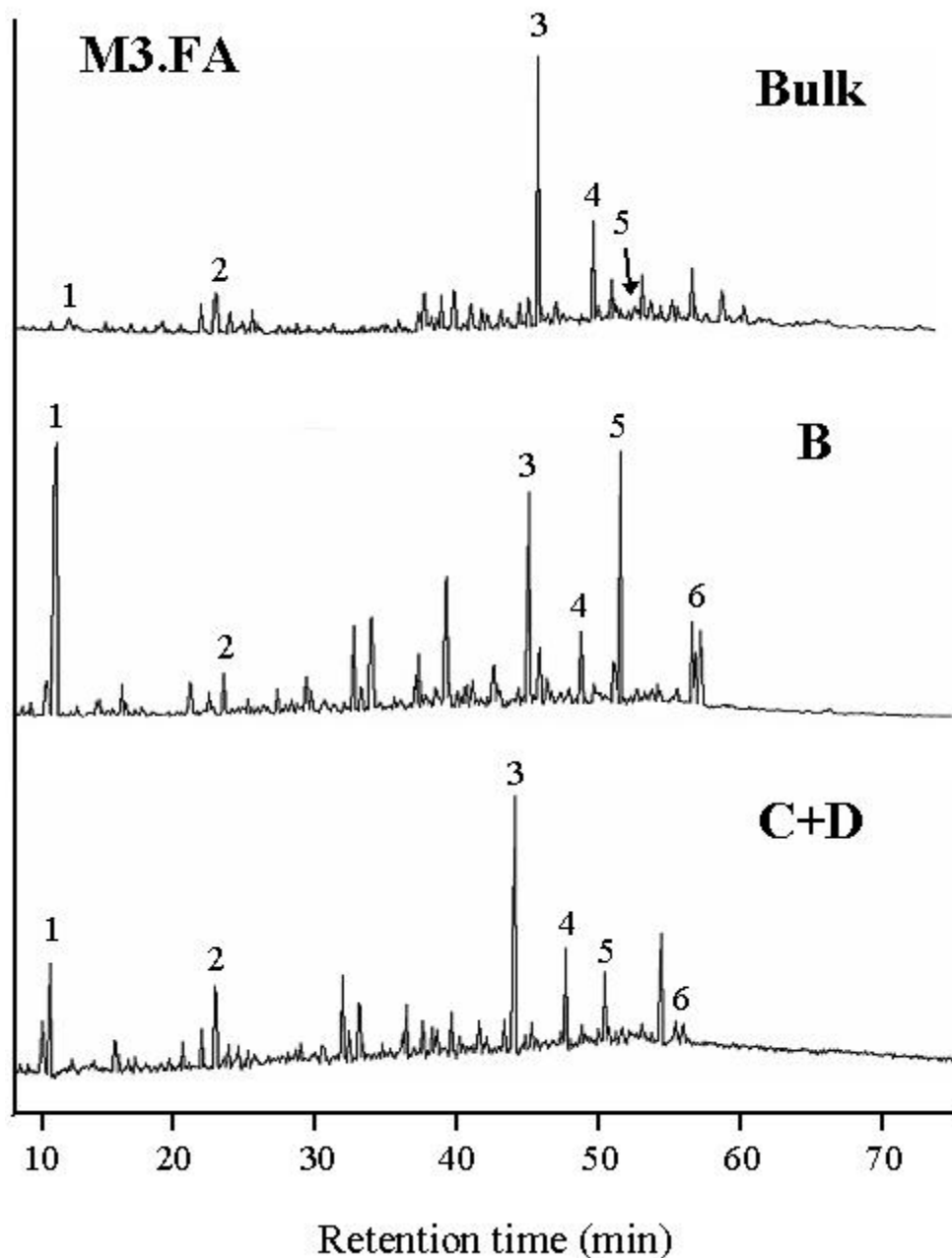


Figure 2. TIC chromatograms of M3FA and its SEC-PAGE fractions. Labelled peaks (acids as methyl esters): (1) N,N'-dimethylurea, (2) butanedioic acid, (3) 3,4-dimethoxybenzoic acid, (4) 3,4,5-trimethoxybenzoic acid, (5) hexadecanoic acid, (6) octadecanoic acid.

The structural results also show some differences in the distribution of the aromatic pyrolysis products detected in the bulk aquatic HS and their SEC-PAGE fractions. For instance, the slight decrease in the benzenecarboxylic acid content after fractionation also corroborates the hypothesis that some aromatic acids are partially bound in the HS structure via hydrogen bonds. The most conspicuous variation in the aromatic pyrolysis products was found in the distribution

of phenolic acids, which in general seem to conform an important role of fractions B and C+D when compared to the highest molecular size HA-A fractions. Another interesting observation is the different behavior in B fractions of two FA samples; M3FA showed the lowest content in phenolic acids whereas that fraction of NoFA the highest one. This indicates that even similarly obtained SEC-PAGE fractions from different aquatic sources have distinct structural compositions in spite of their similar electrophoretic mobilities. A clear dominance of the α -carboxylic phenolic acids, as compared to the corresponding β - and γ -carboxylic derivatives (Table 3), suggest the occurrence of an advanced biodegradation (oxidation) status of aromatic precursors, including probably lignin derived constituents through all the molecular size HS fractions studied.

Conclusions

The results of TMAH/pyrolysis show that tandem SEC-PAGE procedure allows the fractionation of the complex aquatic HS into a limited number of more homogeneous separates enriched in different structural constituents. According to our results, aquatic HS seems to have a rather uniform molecular size and electrophoretic behavior. Minor structures with more aliphatic nature including large molecular size components and some hydrogen bonded low molecular weight acids are also observed. Whereas the bulk aquatic HS together with its major size-fractions are characterized by an abundance of phenolic acids and rather short chain aliphatic polycarboxylic constituents, the high molecular size fraction shows an enrichment in long chain fatty acids that include unsaturated and di-tri acids which suggest a probable microbial source. This high molecular size fraction is obtained only from aquatic HA, which also correlates with its generally larger molecular size and increased polydispersity as compared to aquatic FA.

Experimental Section

General Procedures. The Nordic aquatic humic acid (NoHA, code IR105H) and fulvic acid (NoFA, code IR105F) standards of the IHSS (International Humic Substances Society) were isolated by the XAD technique during the summer of 1986 from the runoff water (colour 230 mg Pt/l, DOC 22 mg C/l, pH 4.4) of a Norwegian mire. The lake water humic-sample (M3) was collected from Lake Mekkojärvi (1989), a small forest lake situated in the Evo district of Lammi in Southern Finland (colour 180 mg Pt/l, DOC 21 mg C/l, pH 5.5). The water sample M3 was treated with XAD-8 resin to obtain the integrated wholes of fulvic (FA) and humic (HA) acid fractions (M3HA and M3FA). The characteristic properties, pre-treatment and isolation procedures of the Nordic reference and lake water samples are thoroughly reported elsewhere.¹⁶

Size-exclusion chromatography (SEC)

In brief, the bulk HA or FA sample (10 mg) was dissolved in 7 M urea and loaded onto a Sephadex G-75 column (Pharmacia, Sweden, 1.5x100 cm) equilibrated with the same solution. The total column volume (V_t) was 160 ml and the void column volume (V_o) 47 ml, determined using Dextran Blue 2000. The flow rate of 7 M urea eluent was 15 ml h⁻¹. The elution curves were detected at 280 nm using a UA-5 detector (ISCO, USA). Fractionation ranges for Sephadex G-75 were 80000 to 3000 for proteins and 50000 to 1000 for polysaccharides. Column effluents were collected as 2 ml aliquots for further PAGE analysis. The low-pressure SEC profiles obtained for NoHA, NoFA, M3HA and M3FA fractions have been reported earlier.^{17, 18}

Electrophoresis on polyacrylamide gel (PAGE)

In brief, 9.7% acrylamide and 0.3% N,N'-methylenebisacrylamide (Bis, a cross-linking agent for polymer networks of the gel) 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 were used as the electrode buffer. Electrophoresis was performed for 1 h at a 25 mA current intensity. The HS 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 PAGE procedure gave three fractions from the HA (A, B and C+D), and two from the FA samples (B and C+D). Finally, the fractions were dialysed for seven days against distilled water and lyophilized. In order to obtain enough sample for further characterisation, the whole fractionation procedure was repeated 10-20 times for each HA and FA sample. The electrophoregrams obtained for NoHA, NoFA, M3HA and M3FA fractions have been reported earlier.^{17,18}

Elemental analysis

Elemental analyses (carbon, hydrogen and nitrogen; the sum of oxygen and sulphur ($\leq 1\%$) was taken as a difference from 100%) were performed by various combustion techniques on a PerkinElmer Series II CHNS/O Analyzer 2400 for the bulk aquatic HS materials and their SEC-PAGE fractions. The values for the elemental analyses were corrected for the water and ash contents of the sample. The content of inorganic constituents (ash) was measured by thermogravimetric analysis [TA Instruments 2200 Thermal Analysis, temperature program: 2°C min⁻¹ from room temperature to 105 °C (water content) and 5 °C min⁻¹ from 105 to 605 °C (ash content)].

TMAH treatment and Pyrolysis-GC/MS

Analytical pyrolysis experiments of HA, FA samples and their different molecular size fractions were performed as previously described.¹⁵ The HS sample (ca. 300 µg) was weighed in a quartz sample tube, moistened with 1.5 µl of a 25% (w/v) aqueous solution of TMAH and dried for 10 min at 100°C. After this off-line methylation procedure, the sample was pyrolysed

(600°C/10s) with a resistively heated Pt-filament pyrolyzer (CDS Pyroprobe 1000). The quartz-lined interface (180°C) was purged continually with helium (31 ml min⁻¹) and connected directly to the GC injector (270°C). The pyrolysis products were collected into a steel cold trap submerged in liquid nitrogen. For GC/MS analysis the trap was attached to a DB-1701 capillary column (length 30 m, i.d. 0.32 mm, film thickness 1 µm) and the gas chromatograph (HP 5890) was programmed from 30 to 220°C at a rate of 4°C min⁻¹. The carrier gas was helium at a flow rate of 1.0 ml min⁻¹. Mass spectral analyses were carried out on a VG Trio-1 quadrupole mass spectrometer using electron impact ionization (70 eV) and a m/z range of 45-600. The identification of the pyrolysis products was based on a NIST spectral library searches, self-collected spectral bank and manual interpretation. The relative proportions of the products were estimated using the normalized peak areas of TIC (total ion current) chromatograms and all the experiments were duplicated to confirm their repeatability. The precision of different TIC chromatogram peaks was on the average ± 18% of the mean, which is reasonably good for the relative inter-sample comparison aimed at.

Acknowledgements

The authors wish to thank both the Russian Academy of Sciences and the Academy of Finland for financial support (project 12). The Russian Foundation for Basic Research has also partly supported this research (project 04-05-64687-a).

References

1. Janoš, P. *J. Chromatogr. A* **2003**, 983, 1.
2. Trubetskoj, O. A.; Trubetskaya, O. E.; Afanaseva, G. V.; Reznikova, O. I.; Saiz-Jimenez, C. *J. Chromatogr. A* **1997**, 767, 285.
3. Trubetskoj, O. A.; Trubetskaya, O. E.; Afanaseva, G. V.; Reznikova, O. I.; Hermosin, B.; Saiz-Jimenez, C. *Z. Pflanzenernähr. Bodenk.* **1998**, 161, 619.
4. de Nobili, M.; Chen, Y. *Soil Science* **1999**, 164, 825.
5. Trubetskoj, O. A.; Trubetskaya, O. E.; Reznikova, O. I.; Afanaseva, G. V. *Geoderma* **1999**, 93, 277
6. (a) Peuravuori, J.; Pihlaja, K.; Trubetskaya, O. E.; Trubetskoj, O. A. *Int. J. Env. Anal. Chem.* **2001a**, 79, 217. (b) Peuravuori, J.; Pihlaja, K.; Trubetskaya, O. E.; Reznikova, O. I.; Afanaseva, G. V.; Trubetskoj, O. A. *Int. J. Env. Anal. Chem.* **2001b**, 80, 141.
7. (a) Trubetskaya, O. E.; Trubetskoj, O. A.; Saiz-Jimenez, C. *Fres. Environ. Bull.* **2001**, 10, 635. (b) Trubetskaya, O. E.; Trubetskoj, O. A.; Ciavatta, C. *Bioresource Technol.* **2001**, 77, 51.
8. Trubetskaya, O. E.; Afanaseva, G. V.; Reznikova, O. I.; Markova, L. F.; Muranova, T. A.; Trubetskoj, O. A. *Environ. Int.* **1998**, 24, 573.

9. Hermosin, B.; Trubetskoj, O. A.; Trubetskaya, O. E.; Saiz-Jimenez, C. *J. Anal. Applied Pyrol.* **2001**, 58-59, 341.
10. Trubetskaya, O. E.; Trubetskoj, O. A.; Guyot, G.; Andreux, F.; Richard, C. *Org. Geochem.* **2002**, 33, 213.
11. Richard, C.; Trubetskaya, O. E. Trubetskoj O. A.; Reznikova O. I.; Afanaseva G. V.; Aguer, J. P.; Guyot, G. *Env. Sci. Technol.* **2004**, 38, 2052.
12. Challinor, J. M. *J. Anal. Appl. Pyrol.* **1989**, 16, 323.
13. Hatcher, P. G.; Minard, R. D. *Org. Geochem.* **1995**, 23, 991.
14. Tanczos, I.; Schöflinger, M.; Schmidt, H.; Balla, J. *J. Anal. Appl. Pyrol.* **1997**, 42, 21.
15. Lehtonen, T.; Peuravuori, J.; Pihlaja, K. *Anal. Chim. Acta* **2000**, 424, 91.
16. Peuravuori, J. *Anal. Chim. Acta* **2001**, 429, 75.
17. Peuravuori, J.; Pihlaja, K.; Trubetskaya, O.; Trubetskoj, O. *Int. J. Env. Anal. Chem.* **2001**, 79, 217.
18. Peuravuori, J.; Pihlaja, K.; Trubetskaya, O.; Reznikova, O.; Afanaseva, G.; Trubetskoj, O. *Int. J. Env. Anal. Chem.* **2001**, 80, 141.
19. de Leeuw, J. W.; Baas, M. *J. Anal. Appl. Pyrol.* **1993**, 26, 175.
20. Schnitzer, M.; Khan, S. U. *Humic Substances in the Environment*, Marcel Dekker, New York, 1972.