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Rapid quantification of humic and fulvic acids by HPLC in natural waters

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Abstract

A simple method based on high-performance size-exclusion chromatography (HPSEC) has been developed for rapid quantification of humic and fulvic acids (HA and FA) in stream waters. A Tsk-gel column was used to separate natural dissolved organic matter (DOM) into two components: peak A and B. In terms of HPSEC chromatograms and fluorescence patterns, peak A and B were similar to the corresponding XAD-extracted HA and FA, respectively. It is suggested that peak A fraction mainly consisted of HA, and peak B fraction FA. The similar separation of HA and FA using HPSEC and a conventional XAD method suggests the consistency of molecular size distribution and physical–chemical properties of DOM. HPSEC offers a simple and rapid method for the quantification of HA and FA instead of tedious extractions of humic substances. Analyses of natural water samples show that the calculation of HA/FA based on UV absorbance was under- or over-estimated, the calibration using the extracted HS allows a more accurate quantification. The fast screening of HA and FA provides useful quantitative and qualitative information that can be used in environmental or monitoring studies.

1. Introduction

Dissolved organic matter (DOM) plays a significant role in the biogeochemistry of metals, nutrients and organic pollutants in the aquatic and terrestrial environments, it is important to investigate the nature of this material (Frimmel et al., 1992; Breault et al., 1996; Xue and Sunda, 1997; Wakeham et al., 1997). However, DOM is a complex mixture of mostly unidentifiable compounds and structure, its separation and characterization are extremely difficult, and proper separation and characterization of DOM are the key to further understanding its role in natural environments. In spite of this, the fractionation and characterization of DOM using high-performance size-exclusion chromatography (HPSEC), reversed-phase HPLC and other methods has led to substantial information on the nature and role of DOM (Thurman and Malcolm, 1981; Susic and Boto, 1989; Chin et al., 1994; Peuravuori and Pihlaja, 1997; Wu and Tanoue, 2001). New methods and additional studies of separation and character-

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ization of DOM are needed before our understanding of its role is improved.

It is well known that humic substances (HS) are the major component of DOM in natural waters, and that HS can be operationally defined and separated into humic and fulvic acid (HA and FA). The conventional method for quantifying HA and FA requires an extraction or isolation procedure. The most often employed extraction technique is the conventional XAD method (Thurman and Malcolm, 1981; Mills, 2003). However, this method is usually tedious, time-consuming, and requires a large volume of water, thus severely limiting the number of samples that can be studied, and preventing systematic analyses of short-term variability. Furthermore, contamination and structural alteration may be easily introduced during the extraction processes. Therefore, there is considerable need for new approaches that perform direct analysis of HA and FA in natural matrices. A fast and direct quantification technique for HA and FA in natural water would provide a tool that can be used to improve the understanding of their biogeochemistry, monitoring and modeling in aquatic environments.

HPSEC is a technique frequently used to characterize and separate DOM without pre-extraction due to its rapidity, sensitivity, reproducibility and availability (Chin et al., 1994; Peuravuori and Pihlaja, 1997; Muller et al., 2000; Her et al., 2002; Alberts et al., 2002). HPSEC with UV absorbance detection has been used to calculate DOM molecular size and polydispersivity (Chin et al., 1994; Peuravuori and Pihlaja, 1997; Hoque et al., 2003). In addition, HPSEC with UV absorbance and/or online dissolved organic carbon (DOC) detection was employed to characterize DOM properties as a function of molecular size (Frimmel et al., 1992; Muller et al., 2000; Her et al., 2002; Alberts et al., 2002). Those studies mainly focused on the molecular size distribution, few quantitative analyses of HA and/or FA were performed.

The first objective of this research was to use HPSEC to separate DOM, and the XAD-isolated HA and FA, to compare their HPSEC chromatograms, and to characterize the HPSEC peak fractions using both on-line UV absorbance detection and three-dimensional excitation (Ex) emission (Em) matrix fluorescence (3DEEM) spectrophotometry. The second objective was to develop a calibration method to directly quantify HA and FA fractions in natural water samples.

2. Experimental

2.1. Apparatus

The HPLC system was composed of a Waters 600 s high-performance liquid chromatograph, a Waters 996 photodiode array detector (PDA), a 3DEEM spectrofluorometer (SPEX, Jobin Yvon, Instruments S. A. Inc.) and a Waters 717_{plus} autosampler. The spectrofluorometer was connected directly to the waste line of the PDA detector. Autosampler and PDA data acquisition were controlled by the Waters Millennium software™. The 3DEEM data over time were collected using Grams/32c™ software.

Due to its fast and sensitive 3DEEM acquisition, the spectrofluorometer allows for a full Ex/Em 300/500 nm spectra in 1 s or less and can be used as an on-line detector in the HPLC system. On-line 3DEEM detection can not only provide fluorescence intensities, but can simultaneously provide fluorescence patterns. Therefore, the use of HPSEC with sequential on-line UV–vis absorbance and 3DEEM detection can allow measurements of fluorescence properties of each HPSEC fraction. In this study, the 3DEEM data were automatically collected every 9 s. The PDA detector is a sensitive photometer, which can be operated over a wavelength range between 210 and 400 nm.

2.2. Columns

A pre-packed analytical Tsk-Gel® column (Tosohaas, G2500PWXL, 7.8 mm × 30 cm, and 5 μm particle size) was used for HPSEC separation. The column void volume (V_0, 5.45 mL) and total permeation volume ($V_0 + V_i$, 8.55 mL) of the column were determined using Blue Dextran (10.9 min) and acetone (17.1 min), respectively. Sodium polystyrene sulfonates were used as molecular weight calibration standards (1430, 1800, 7950, 34 700 Da).

2.3. Chemicals, sample collection and preparation

All mobile phases were made using Milli-Q water (Millipore). Phosphate buffer (pH 6.8, 0.1 M NaCl) was used as an eluent with a flow rate of 0.5 mL min^{-1}. A total of 50 μL of sample was injected onto the column.

Stream water samples were collected in Harp and Dickie Lake in 2001 (Kothawala, 2002). Harp 4 (HP4) river is one of the 6 watersheds of Harp Lake

(45°23′N, 79°08′W) on the Precambrian shield in South-central Ontario, Canada. Water samples were collected at 15 sites along the length of the main tributary for the application of HPSEC analyses. Sampling site HP-1 is located in a large wetland collecting headwaters from a primarily mixed deciduous and coniferous forest. An ephemeral stream, HP-2, drains the large wetland and enters a swamp. The outflow of the swamp is HP-4. HP-5 and HP-7 are located further downstream in a section which passes through a primarily coniferous forest for 120 m, prior to reaching the *Sphagnum*-surrounded site HP-9, HP-10 is the first of 3 consecutive beaver ponds, representing study sites HP-10, HP-11 and HP-12, respectively. HP-13 is situated at the outflow of beaver pond HP-12, while HP-15, HP-19, HP-23 and HP-27 are located at 40, 130, 250 and 370 m downstream, respectively. The final site was in the littoral zone of the lake, HP-LK (Kothawala, 2002). Samples were filtered through 0.45 μm nylon syringe filters (Life Sciences, Ontario) before analyses. Total dissolved DOC concentration was measured using a TOC analyzer (Shimadzu 5000, Japan).

The natural water samples were subjected to both HPSEC analysis and HS extraction. HA and FA were separated from water samples using XAD-8 resin (Thurman and Malcolm, 1981; Mills, 2003). All samples were adjusted to pH 8.2 with NaOH (Baker) or HNO_3 (Sigma) before subsequent spectral and chromatographic analyses. Two commercial HS standard was used for comparison with the natural HA and FA: Suwannee River Fulvic Acid (SRFA) and Aldrich Humic Acid (AHA, Aldrich Chemical Co. Inc.).

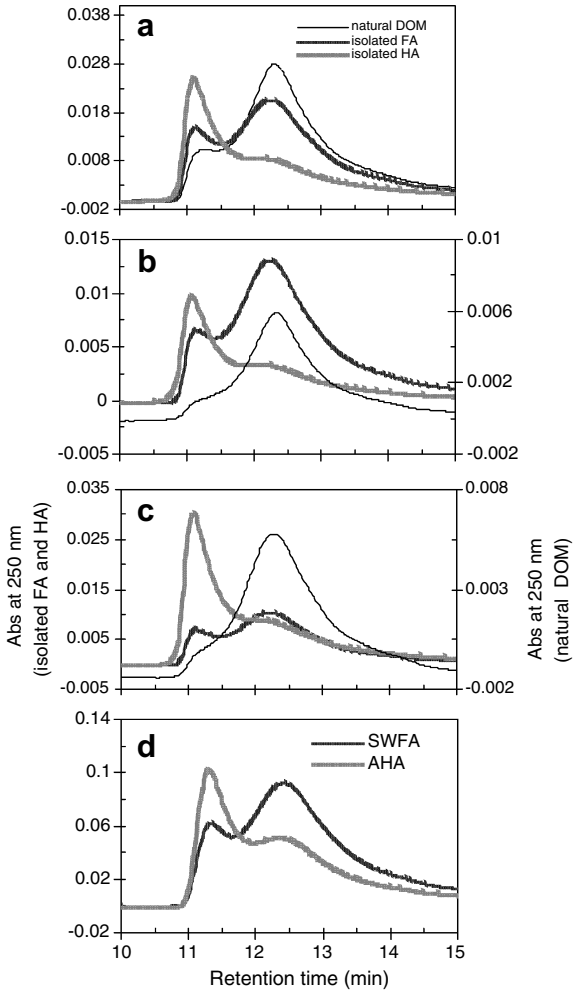

Fig. 1. HPSEC chromatograms of water samples, and the XAD-isolated HA and FA. (a) Dickie Lake-1, (b) Lake Harp-1, (c) Lake Harp-2 and (d) AHA (Aldrich Humic Acid) and SWFA (Suwannee River Fulvic Acid).

3. Results and discussion

3.1. HPSEC chromatograms and fluorescence patterns of DOM, and its XAD-extracted HA and FA

Fig. 1 shows the HPSEC chromatograms of the water samples, their XAD-extracted HA and FA, and SRFA and AHA. All natural water samples revealed two major incompletely-resolved peaks: peak A and B with retention times of 11.09–11.37 min and 12.22–12.33 min, respectively. Sometimes peak A was just a shoulder of peak B; this is because larger molecular size fractions have lower absorption efficiencies at the UV wavelengths than do smaller molecular size fractions (Frimmel et al., 1992; Artinger et al., 1999; Her et al., 2002). However, peak A and B fractions apparently had distinct UV–vis absorbance spectra (Fig. 2), possibly indicating their different physical–chemical properties. In addition, the major peak of the isolated HA corresponded to pre-dominant peak A, while that of the isolated FA corresponded to pre-dominant peak B. The major peaks of the HA and FA had retention times of 11.09–11.28 min and 12.15–12.23 min, respectively. These isolated HA and FAs also had similar HPSEC chromatograms to the AHA and SRFA; the retention time of the major peak was 11.31 min for AHA and 12.42 min for SRFA. Based on the molecular weight calibration, the molecular weight of peak A fractions was approximately higher than 4500 Da, and that of

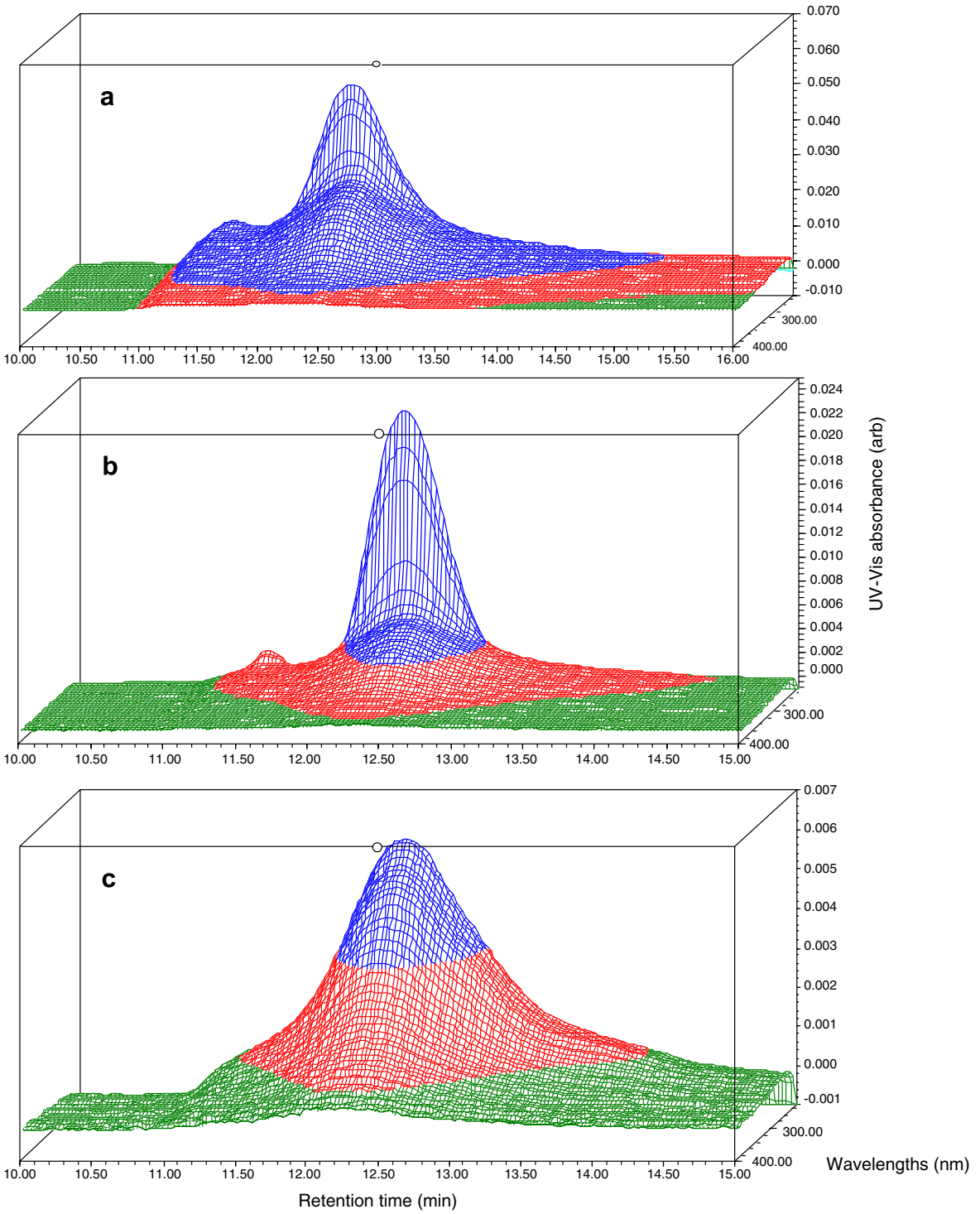

Fig. 2. UV–vis absorbance spectra of HPSEC chromatograms of water samples. (a) Lake Dickie-1, (b) Lake Harp-1 and (c) Harp Lake-2.

Peak B fractions was approximately lower than 4500 Da.

On-line specific Ex/Em fluorescence detection has often been used in the HPSEC separation and characterization of DOM (Susic and Boto, 1989; Frimmel et al., 1992; Artinger et al., 1999; Alberts et al., 2002), but it provides limited information and poor separation resolution compared to UV

absorbance detection. On-line 3DEEM fluorescence detection with HPSEC gives both intensities and fluorescence maximum patterns at the same time, thus providing qualitative chemical and structural information, in addition to molecular size, as compared to UV absorbance and specific Ex/Em fluorescence detection (Wu et al., 2003). Fig. 3 shows the Ex/Em wavelengths of the fluorescence maxima of the HPSEC peak fractions, and the isolated HA and FA. Peak A and B fractions had subtle differences in the fluorescence patterns, with peak A being evident at longer Ex and Em wavelengths, and peak B at shorter wavelengths. Furthermore, the fluorescence patterns of peak A and B were similar to those of the isolated HA and FA, respectively.

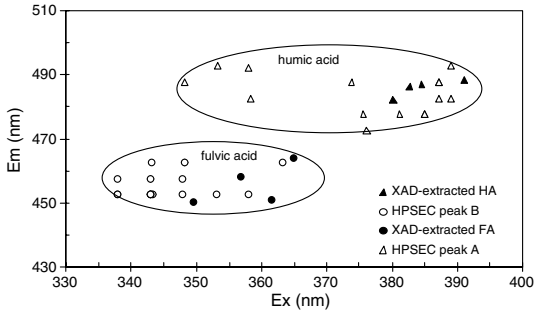

Fig. 3. Ex/Em wavelengths of fluorescence maxima of the isolated HA, FA and HPSEC peak fractions. (a) Lake Dickie-1, (b) Lake Harp-1 and (c) Harp Lake-2.

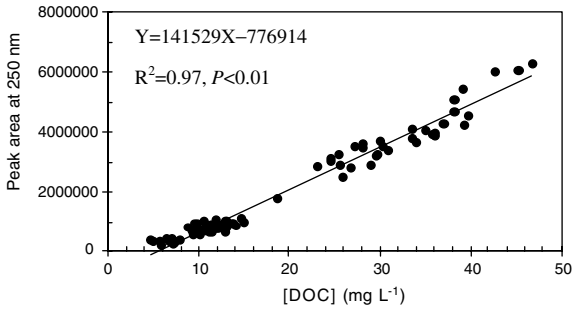

Fig. 4. The relationship between the peak area at 250 nm of HPSEC chromatograms and [DOC] for the water samples collected from Harp 4 inflow to Lake Harp.

Previous studies have shown that DOM can be separated into several peaks by size-exclusion chromatography or gel permeation chromatography with various columns (Frimmel et al., 1992; Chin et al., 1994; Peuravuori and Pihlaja, 1997; Muller et al., 2000; Her et al., 2002; Alberts et al., 2002; Wu et al., 2003). Her et al. (2002) reported that the first eluted peak in the HPSEC chromatogram was lacking in aromatic compounds, and suggested that it mainly consisted of HA (Her et al., 2002). This study demonstrated that DOM can be separated into two major fractions using the Tsk-gel column. Peak A and B fractions had differences in both molecular size, absorbance spectra and fluorescence patterns, and they were similar to their corresponding HA and FA isolates, suggesting that peak A and B fractions mainly consisted of pre-dominant HA and FA, respectively.

HPSEC separation is based on molecular size, and thus the separation of HA and FA in HPSEC is not surprising as there is a difference in molecular size distribution between HA and FA (Chin et al., 1994; Peuravuori and Pihlaja, 1997; Mills, 2003). The classic XAD isolation of HA and FA is based on their polar properties (Thurman and Malcolm, 1981). Several previous studies have demonstrated strong relationships between molecular size and physical–chemical properties of DOM and HS; larger molecular size fractions have been related to stronger hydrophobicity while smaller molecular

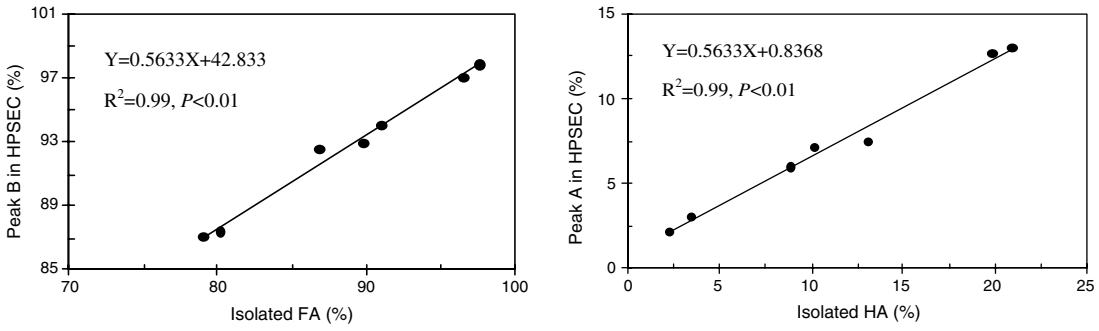

Fig. 5. The relationship between the relative abundance of the isolated HA and FA, and the relative abundance of peak A and B of HPSEC chromatograms.

size fractions have been related to higher hydrophilicity (Chin et al., 1994; Peuravuori and Pihlaja, 1997; Her et al., 2002; Trubetskaya et al., 2002; Wu et al., 2003). It appears that the HA and FA isolates not only had differences in molecular size, but also had differences in physical–chemical properties. The similarity of HA and FA separation between using HPSEC and classic XAD resin presented here provides further support for the consistency of molecular size and polar properties of HS in natural waters.

3.2. Calibration and quantification of HA and FA in natural waters

Since there was a strong relationship ($R^2 = 0.97$, $p < 0.001$, Fig. 4) between the total peak area (achieved with UV absorbance at 250 nm) of HPSEC chromatograms, and DOC of water samples, the total peak area can be used to calculate DOC of the water samples, and the relative abundance of HA and FA can be then predicted based on their peak areas. Peak A was less pronounced than peak B (Fig. 1); their peak areas can be obtained by a two-peak fitting of the HPSEC chromatograms using PeakFit™(AISN software Inc.).

Since different molecular size fractions have different UV absorbance per unit of C, the direct use of UV absorbance may not give an accurate estimate of HA and FA. In order to overcome this problem, the isolated HS was used to calibrate the relative abundance of HA and FA in the water samples. Fig. 5 shows significant correlations between relative abundance, in terms of UV absorbance at 250 nm, of peak A and B in the HPSEC chromatograms, and the relative abundance, in terms of DOC concentration, of HA and FA in the HS isolate. These strong correlations further confirm the similarity between peak A and peak B derived using HPSEC and pre-dominant HA and FA isolated using conventional the XAD method. This also shows that the use of UV absorbance would underestimate HA and consequently over-estimate FA.

Thus, assuming 100% recovery in HPSEC and HS consisting of 100% DOM, HPSEC can be used to rapidly quantify HA and FA in natural water samples based on their HPSEC chromatograms. The underestimate of HA and the over-estimate of FA due to their different UV absorbance efficiencies were strongly eliminated using the XAD-extracted HS from the study area. Due to the fact that the y-intercept is <0 (i.e. peak area at 250 nm is < 0 when [DOC] = 5.49 mg L^{-1}) in Fig. 4, water samples where [DOC] was below 5.49 mg L^{-1} would not be applicable, suggesting that this method is only for some dystrophic waters. In the case of a low resolution of peak A or a low level of HA in some water samples, pre-concentration procedures may be needed. Other than that, HPSEC can provide a fast

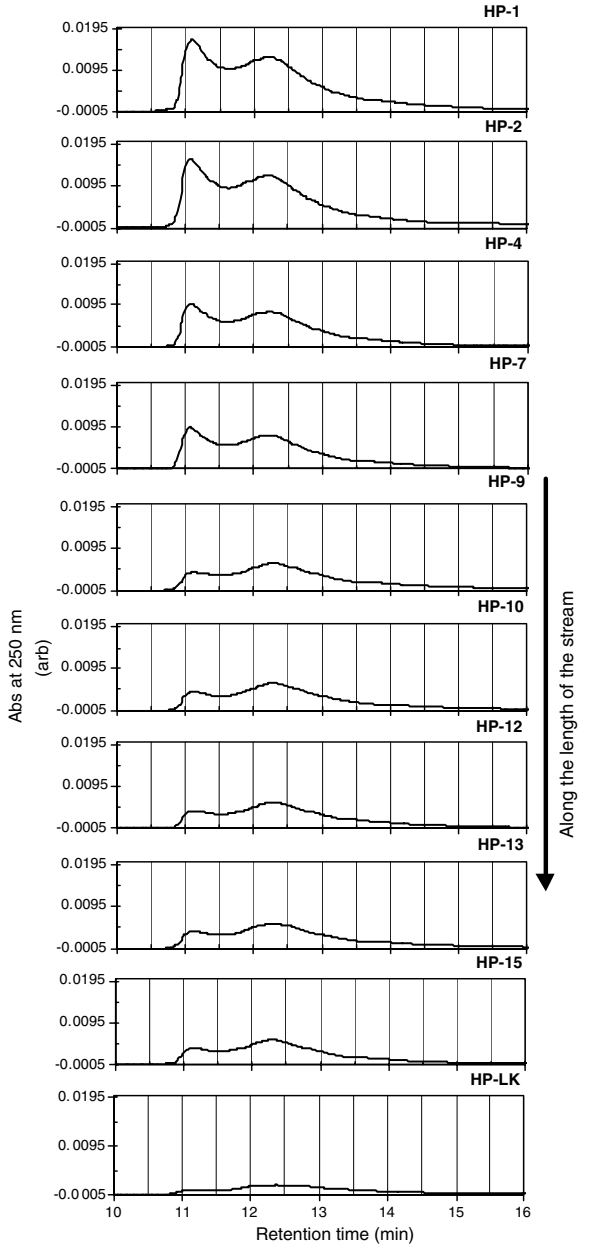

Fig. 6. HPSEC chromatograms of the water samples collected along the length (sampling sites) of the Harp 4 stream on October 31 2001. 1, 2, 4, 7, 9, 10, 12, 13, 15, 19, 23, 27 and LK were the sampling sites: HP-1, HP-2, HP-4, HP-7, HP-9, HP-10, HP-12, HP-13, HP-15, HP-19, HP-23, HP-27 and HP-LK.

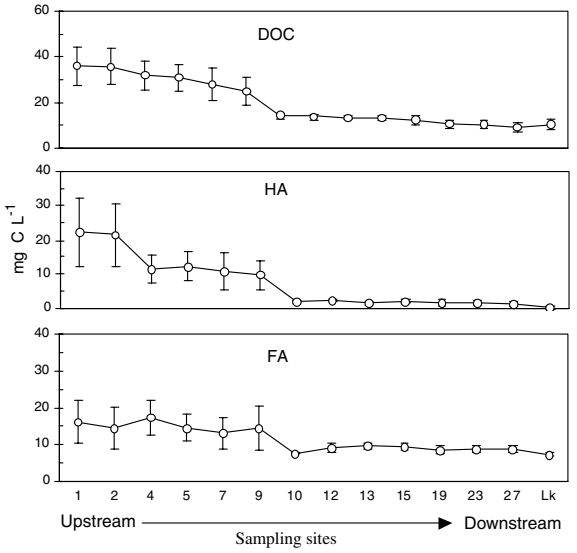

Fig. 7. Average [DOC], [HA], [FA] along the length (sampling sites) of the Harp 4 stream over the period Jan–Jul 2001. The sampling sites were the same as in Fig. 6.

estimate of HA and FA with high sensitivity and rapidity. Simply by using the calibration equations provided in Figs. 4 and 5, the HPSEC technique allows a straightforward estimate of HA and FA concentration and distribution without having to perform the extraction procedure.

Fig. 6 shows the results of the HPSEC application for the water samples collected along the length of the Harp 4 inlet to Lake Harp. The results reveal that the major retention time increased downstream (Fig. 6), indicating decreases in molecular size distribution. In addition to the decreases in DOC concentration, HA/FA composition was also greatly altered. HA and FA concentrations estimated ranged from 21.6 to 0.1 mg C L^{-1} and from 15.9 to 9.7 mg C L^{-1}, respectively, in the stream (Fig. 7). For HA, the relative contribution to the total [DOC] decreased downstream from 58.7 to 1.2%. But it increased from 45.1 to 99.7% for FA. This suggests the occurrence of DOM degradation and transformation by various possible factors including photodegradation, coagulation, sedimentation and selective adsorption of larger molecular size molecules to inorganic particles, metal oxides and bottom sediments along the length of the stream. This result would be significant in terms of understanding the fate and biogeochemical role of DOM in terrestrial environments.

Therefore, it appears that HPSEC can be used not only for molecular size estimate, but also for a rapid quantitative estimate of HS in stream waters.

4. Conclusions

DOM can be separated into two major fractions (peak A and B) using HPSEC with the Tsk-gel column. With the on-line absorbance and 3DEEM fluorescence detection in HPSEC, the comparison between natural water samples and the HA and FA isolates suggests that peak A and B mainly consisted of pre-dominant HA and FA, respectively. Based on the significant correlation between the relative abundance of the isolated HA and FA, and the peak area of peak A and B, and the correlation between total [DOC] and the total peak area of the HPSEC chromatogram, it is possible to eliminate the effect due to the difference in absorbance efficiency of different molecular size fractions, and to quantify the HS, HA and FA.

Previous studies have shown the applicability of HPSEC for the characterization of DOM, but no quantification of HA and FA has been reported. Therefore, the method presented here represents a new tool that can be used in geochemical studies for the rapid quantification of HA and FA in stream waters.

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