



Working Report 2007-23

# Humic and Fulvic Acids in Groundwater

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## HUMIC AND FULVIC ACIDS IN GROUNDWATER

### ABSTRACT

A non-ionic polymeric sorbent DAX-8 (XAD-8) was applied to the isolation of humic and fulvic acids from groundwater. This procedure was able to isolate approximately 40% of the DOC as humic solutes. For the investigation of the structure and molecular size distribution of the isolated humic solutes, the hyphenated SEC-ESI-MS system with a quadrupole mass spectrometer and SEC-UV and fluorescence detectors was utilized. For the higher-molecular-weight humic acids, the ESI-MS loses sensitivity compared with the parallel UV detection, because of the difficulty in getting the ionized humic compounds to fly efficiently through the mass spectrometer.

**Keywords:** Humic and fulvic acids, groundwater, XAD, DAX, SEC, mass spectrometry.

## HUMUS- JA FULVOHAPOT POHJAVEDESSÄ

### TIIVISTELMÄ

Humus- ja fulvohappojen eristämiseen pohjavedestä käytettiin adsorptiota polymeeri-sorbenttiin (DAX-8). Tällä menetelmällä saatiin eristettyä noin 40 % liuenneesta orgaanisesta aineksesta. Eristettyjen humus- ja fulvohappojen rakenteen ja molekyyli-kokojakauman tutkimiseen käytettiin SEC-ESI-MS laitteistoa kvadropolimassa-spektrometrillä sekä UV ja fluoresenssi detektoreita. Laitteisto osoittautui tehokkaaksi erityisesti fulvohappojen tutkimuksessa. Raskaamman molekyyli-painon omaavilla humushapoilla ESI-MS laitteiston herkkyys laskee koska ionisoidut humus aineet on vaikea saada lentämään tehokkaasti massaspektrometrin läpi.

**Avainsanat:** Humus- ja fulvohapot, pohjavesi, XAD, DAX, SEC, massaspektrometria.

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## 1. INTRODUCTION

Aquatic humic substances are polar brown-coloured organic acids, which are derived from soil humus and terrestrial and aquatic plants. They generally comprise one third to over half of the dissolved organic carbon (DOC) in natural surface waters.

Because of the dilute nature of aquatic humic substances, the material must first be isolated and concentrated for further studies. Dissolved humic matter contains a large number of diverse chemical functionalities, and no single analytical method is available to reveal the actual concentration of these macromolecular organic solutes in the sample. The main difficulty is that the aquatic humic solutes must be separated from the other organic and inorganic solutes in natural water. Different methods for concentrating and isolating the dissolved organic matter such as freeze drying, chemical precipitation, solvent extraction, reverse osmosis, ultra filtration and adsorption to solids have been presented. All of these methods have some disadvantages of not isolating the aquatic humic matter and, at the same time, keeping the chemical and structural composition unaffected as it occurs in its original state in an aquatic environment (Aiken et al. 1985).

The classification of dissolved organic matter into humic and non-humic solutes is based on the isolation method. The isolation method and the chemical conditions applied determine the degree of separation of humic from non-humic matter. The fraction of humic substances that are not soluble in water at a pH lower than 2, are defined as humic acids. The fraction of humic substances that are soluble under all pH conditions are referred to as fulvic acids (Malcolm 1990).

The most frequently used isolation procedure for humic substances is the column chromatographic method by non-ionic sorbents such as XAD resins (polymethyl methacrylate) or analogous materials (Leenheer 1981, Thurman et al. 1981 and Peuravuori et al. 1997). The XAD resin classifies organic solutes in an acidified water sample into artificial hydrophobic and hydrophilic fractions according to their random ability to adsorb onto non-ionic macroporous resin. This operational classification of aquatic humic substances is clear but, on the other hand, the XAD isolation technique under strongly acidic conditions might involve certain risks for uncontrolled fractionation or reactions.

The goal of this study was to determine the amount of humic substances and their properties such as the structure and molecular size distribution in the groundwater from a shallow depth (about -85 m b.s.l.) in the bedrock at Olkiluoto.

The isolation procedure applied was the adsorption of humic substances on non-ionic macroporous resin (polymethyl methacrylate, DAX-8). The hyphenated SEC-ESI-MS system with a quadrupole mass spectrometer and SEC with fluorescence and UV detection were applied to the investigation of the structure and molecular size distribution.

## 2. EXPERIMENTAL

### 2.1 Sampling

Groundwater samples were collected from groundwater station ONK-PVA3 (depth about –85 m b.s.l.) in ONKALO, Olkiluoto, Finland. The samples were on-line pressure filtered (Pall-Gelman) in situ, using Millipore HA 47 mm, 0.45µm filters. The tubing material used in the sampling was nylon. The glass containers (1-5 L) that were used to store the samples were washed with 6% HNO<sub>3</sub>, high-purity MQ water and ethanol. The samples were stored at 6° C and protected from the light by wrapping the glass containers in aluminium foil.

### 2.2 Chemicals and reference compounds

1.0 M HCl and NaOH (Reagecon, Shannon Free Zone, Shannon, Co. Clare, Ireland) were used by diluting to the desired concentration with MQ water.

n-Hexane (Fluka) and methanol (J. T. Baker) were of the “for residue analysis” or “Baker Analyzed” quality.

Standard solutions containing humic and fulvic acids were made by dissolving IHSS (International Humic Substances Society) Nordic Aquatic Humic Acid Reference (1R105H) and IHSS Nordic Aquatic Fulvic Acid Reference (1R105F) in an 80/20 (v/v) water/methanol mixture with 10 mM NH<sub>4</sub>HCO<sub>3</sub>.

PEO (polyethylene oxide) standards (Polymer Laboratories) were used for the investigation of the molecular size distribution. PEO standards were dissolved in an 80/20 (v/v) water/methanol mixture with 10 mM NH<sub>4</sub>HCO<sub>3</sub> using sonication at 40° C during several hours.

QCWW4 reference solution (Eurofins A/S) was used as a quality control sample for the TOC determinations.

DAX-8 resin (Supelite DAX-8, Supelco, a new substitute for XAD-8 resin) was used for the isolation of humic substances from groundwater.

### 2.3 Isolation

The resin was first cleaned according to Thurman et al. (1981) with slight modifications. The DAX-8 material was first cleaned by rinsing the resin in a beaker with 0.1 M NaOH and thereafter with methanol until the pH was neutral. Next, the resin was Soxhlet-extracted sequentially for 6 h with methanol and n-hexane. DAX-8 was stored in methanol at 6° C until used.

### **Isolation procedure with DAX-8**

The cleaned DAX-8 polymer in methanol was slurry-packed into a 160-mm I.D. class column, resulting in an 8-cm sorbent bed. The packed column was rinsed with MQ water until free of methanol. The groundwater sample, acidified to a pH just above 2 with 1.0 M HCl, was placed in a separatory funnel and treated on the polymer. The humic and fulvic substances adsorbed on the resin were eluted with 0.1 M NaOH.

## **2.4 TOC analysis**

The TOC-V CPH analyzer is able to analyse samples only in liquid state. In TOC analysis, both inorganic and organic forms of carbon (TC, total carbon) are converted into carbon dioxide (CO<sub>2</sub>) using a chemical oxidizing agent (platinum on alumina pellets) and a high temperature such as 680° C. The CO<sub>2</sub> formed is then detected using non-dispersive infrared adsorption (NDIR). When measuring the amount of inorganic carbon in the sample, it is first acidified with HCl. The CO<sub>2</sub> formed from the inorganic forms of carbon (IC) is purged from the sample with air and detected with an NDIR detector. The amount of total organic carbon (TOC) can then be calculated as a difference between total carbon and inorganic carbon. The TOC analyzer was calibrated in the range of 0.5 to 100 mg/l for organic and inorganic carbon. Quality control samples (2.0 and 20.0 mg/L, QC WW4) were analysed at the beginning and at the end of each run.

The TOC measurements were performed with the TOC-V CPH analyzer connected to an ASI-V autosampler (Shimadzu Corporation). The samples were neutralized with 1.0 M HCl before the measurements.

## **2.5 HPLC fluorescence and UV**

Size exclusion chromatography (SEC) with fluorescence detection was used for the investigation of the molecular size distribution of isolated humic substances. The HPLC instrument was a Shimadzu LC-6A pump coupled to a Shimadzu RF-551 fluorescence detector and an SPD-6A ultraviolet detector. The excitation and emission wavelengths of the fluorescence detector were 340 nm and 435 nm, respectively. The wavelength of the ultraviolet detector was set at 254 nm. A PL Aquagel-OH 30 column from Polymer Laboratories (Shropshire, U.K) of 250 × 4.6-mm i.d. and a particle size of 8 µm was used as the SEC column. The separation was performed at a flow rate of 1.0 ml/min with an eluent consisting of an 80/20 (v/v) water/methanol mixture with 10 mM NH<sub>4</sub>HCO<sub>3</sub> at ambient temperature. The injection was performed manually and the injection volume was 100 µl.

## **2.6 HPLC-MS**

Size exclusion chromatography (SEC) was performed on an HP 1100 (Hewlett-Packard) liquid chromatography system, which was coupled to a Quattro LC triple-quadrupole mass spectrometer (Micromass, Manchester, U.K) with a Z-spray



electrospray source. The drying gas ( $N_2$ ) flow rate was 650 l/h and the nebulizer gas ( $N_2$ ) flow rate was 65 l/min. The source temperature was 110° C and the desolvation temperature was 200° C. A capillary voltage of 3.26 kV was used and the cone voltage was set at 20 kV. The mass spectrometer was operating in the negative ion mode. The  $m/z$  range of 150-750 was scanned in 5 s. The samples were also screened by injecting the samples straight into the mass spectrometer. The injection was performed through a T piece with the eluent from the SEC column. For the analysis of the high-molecular-weight fraction (humic acids), two separate analyses were performed with the ranges  $m/z$  200-1200 and 1000-2000. The low-molecular-weight fraction (fulvic acids) was scanned in the range of  $m/z$  100-1100. The SEC column was the same as used in the investigation of the molecular size distribution. The separation was performed at a flow rate of 0.3 ml/min with an eluent consisting of an 80/20 (v/v) water/methanol mixture with 10 mM  $NH_4HCO_3$  at ambient temperature. The injection volume was 20  $\mu$ l.

### 3. RESULTS

#### 3.1 TOC results

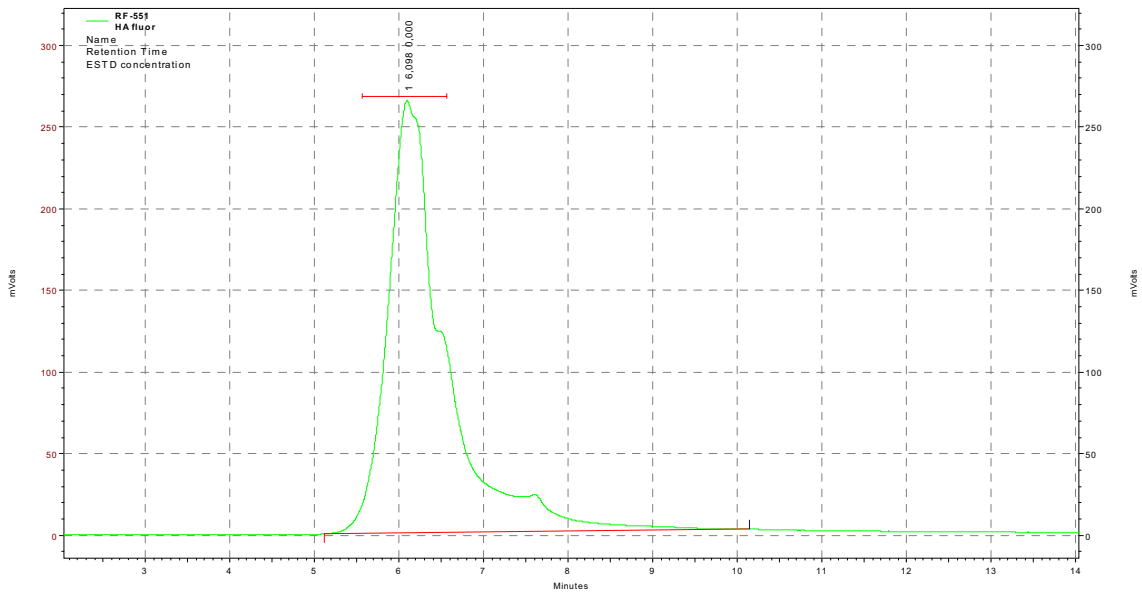
Three parallel 2.0-L samples of groundwater (ONK-PVA3) and one blank sample (MQ water) were preconcentrated with the DAX-8 procedure. The adsorbed humic and fulvic substances were eluted with 60 mL of 0.1 M NaOH. The results from the TOC measurements and the calculated recovery are shown in Table 1.

*Table 1. Recovery percentages of the total organic carbon (mg/isolated sample) using the XAD procedure.*

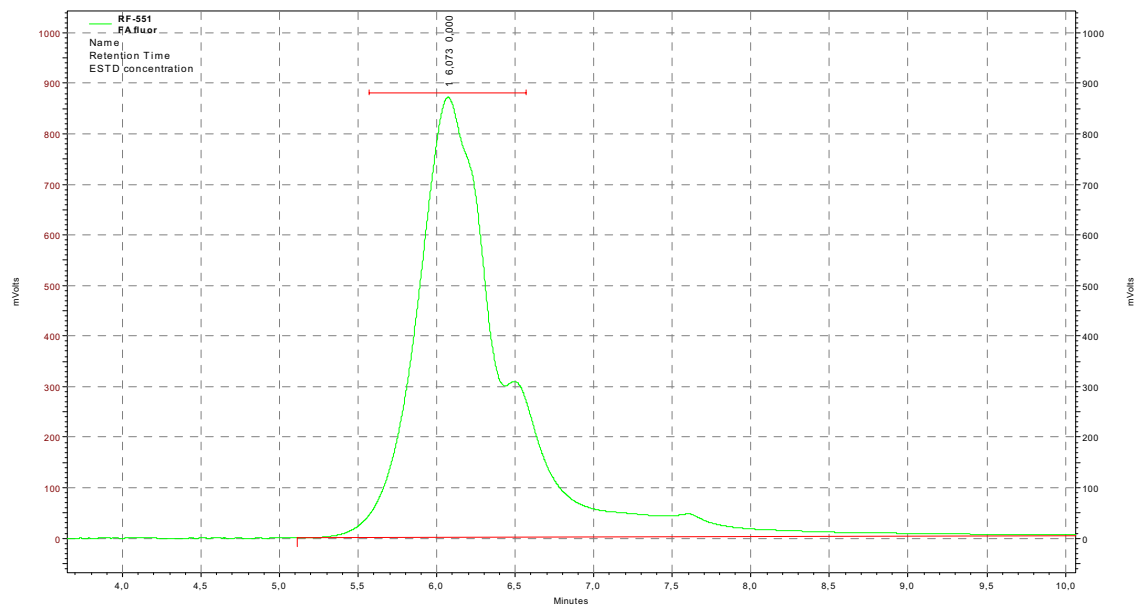
	<b>ONK-PVA3 (1)</b>	<b>ONK-PVA3 (2)</b>	<b>ONK-PVA3 (3)</b>
<b>TOC, original sample, mg</b>	12.95	11.06	17.15
<b>TOC, isolated sample, mg</b>	5.95	4.64	6.37
<b>% of original TOC</b>	45.9	42.0	37.1

#### 3.2 HPLC with a fluorescence detector

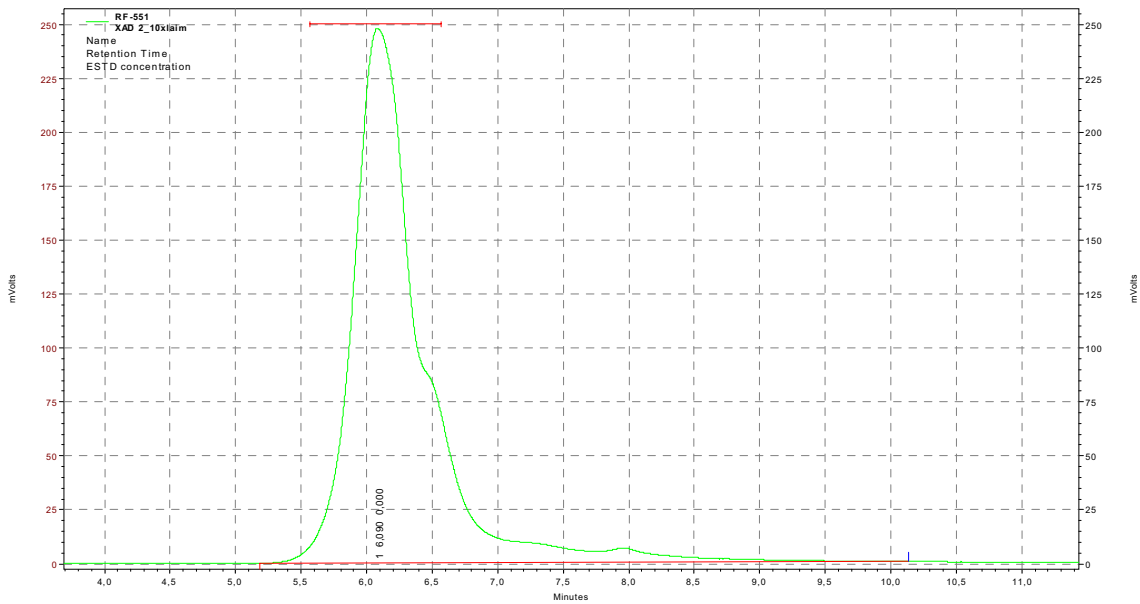
The fluorescence chromatograms of humic and fulvic acid standards are shown in Figures 1 and 2. The fluorescence chromatogram of the isolated groundwater sample is shown in Figure 3. In the humic acid standard, there is one peak with the highest intensity at 6.1 min and two peaks at 6.5 and 7.6 min. Similar peaks can be seen in the fulvic acid standard. Humic substances in the isolated groundwater sample exhibit one peak with high intensity at 6.1 min and two peaks with lower intensity at 6.5 and 8.0 min.



**Figure 1.** SEC-fluorescence chromatogram of the humic acid standard (80 mg/l).

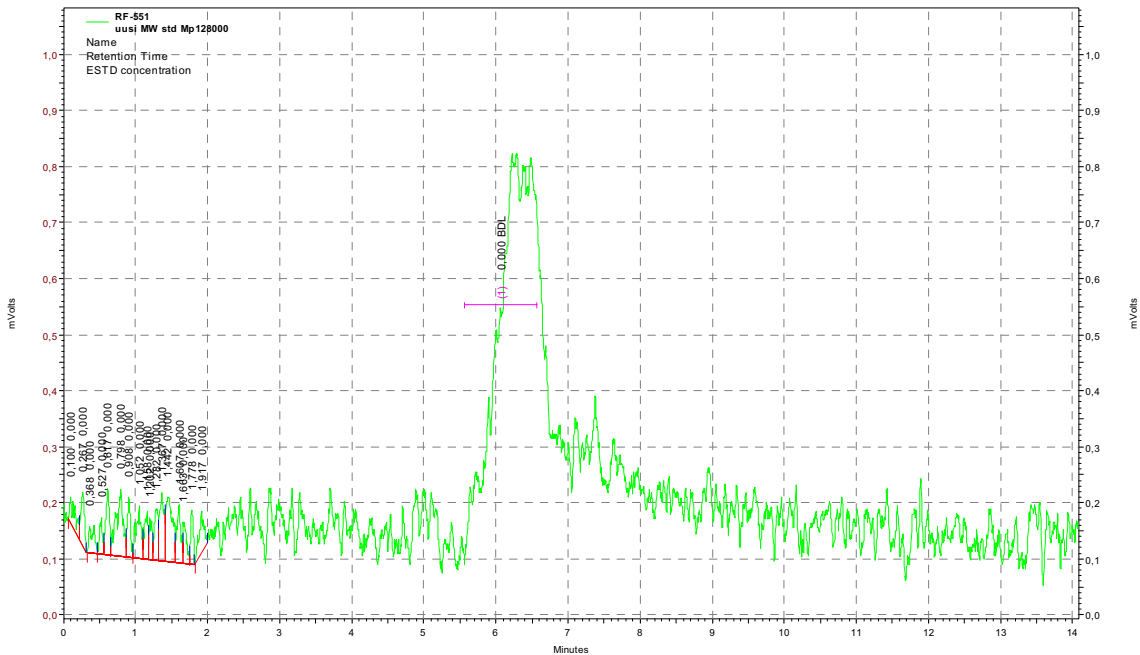


**Figure 2.** SEC-fluorescence chromatogram of the fulvic acid standard (100 mg/l).



**Figure 3.** Fluorescence chromatogram of the isolated groundwater sample (ONK-PVA3) diluted 10 fold.

PEO standards (Polyethylene oxide) with a molecular weight of 520500 amu, 128000 amu, 43580 amu and 20000 amu were used for the investigation of the molecular size distribution of the isolated humic substances. The concentration of standards was 100 mg/l. The fluorescence intensities of the PEO standards were found to be low and thus the determination of the exact retention times was not reliable. The fluorescence chromatogram of the 128000-amu PEO standard is shown in Figure 4 as an example.



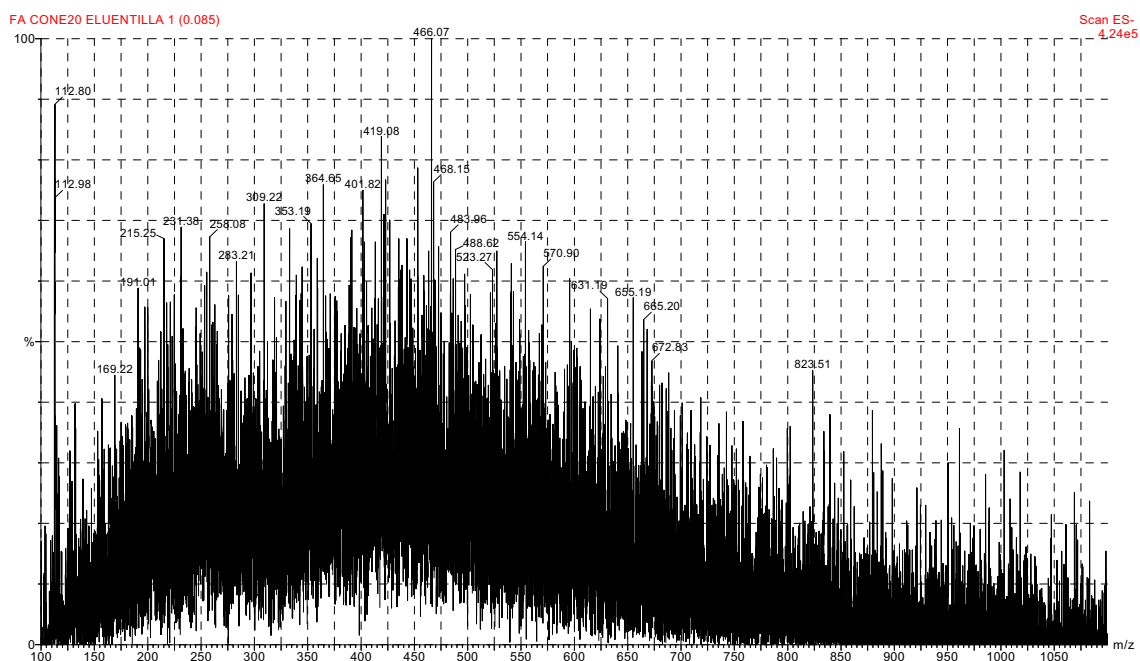
**Figure 4.** Fluorescence chromatogram of the 12800-amu PEO standard.

### 3.3 MS results

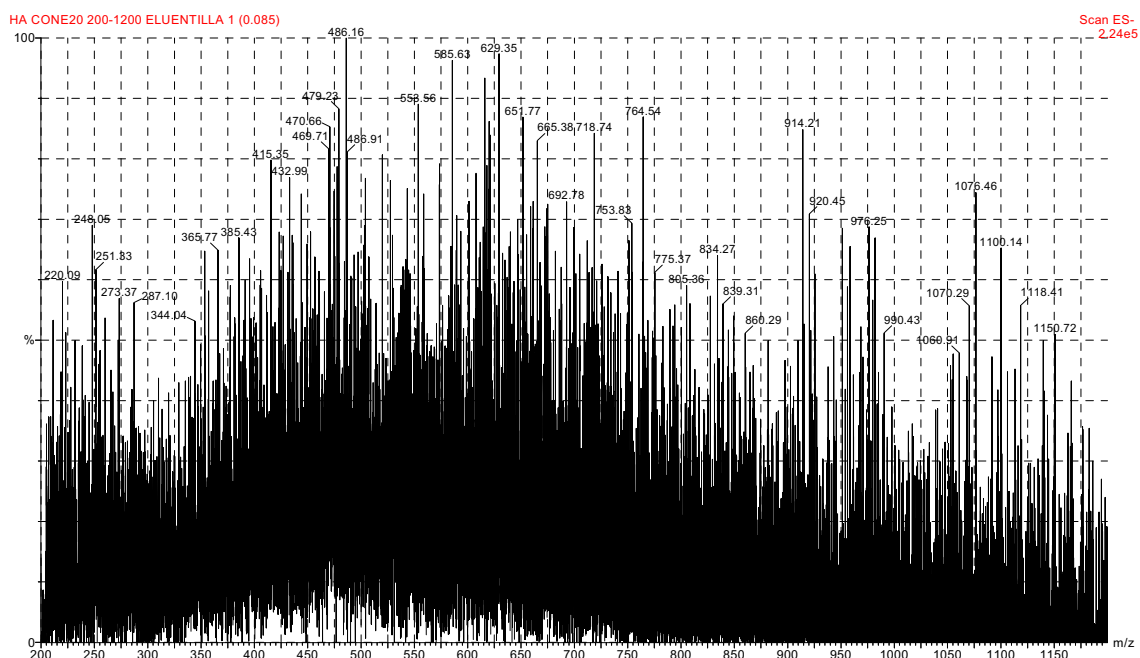
#### 3.3.1 Direct injection

Humic and fulvic acid standards (80 mg/l and 100mg/l) were first screened by injecting them directly into a mass spectrometer. This resulted in unreproducible results most likely due to pulsed flow of the direct injection pump. Because of this, the direct injection of the standards was performed straight into the eluent flow of the SEC column using a T piece.

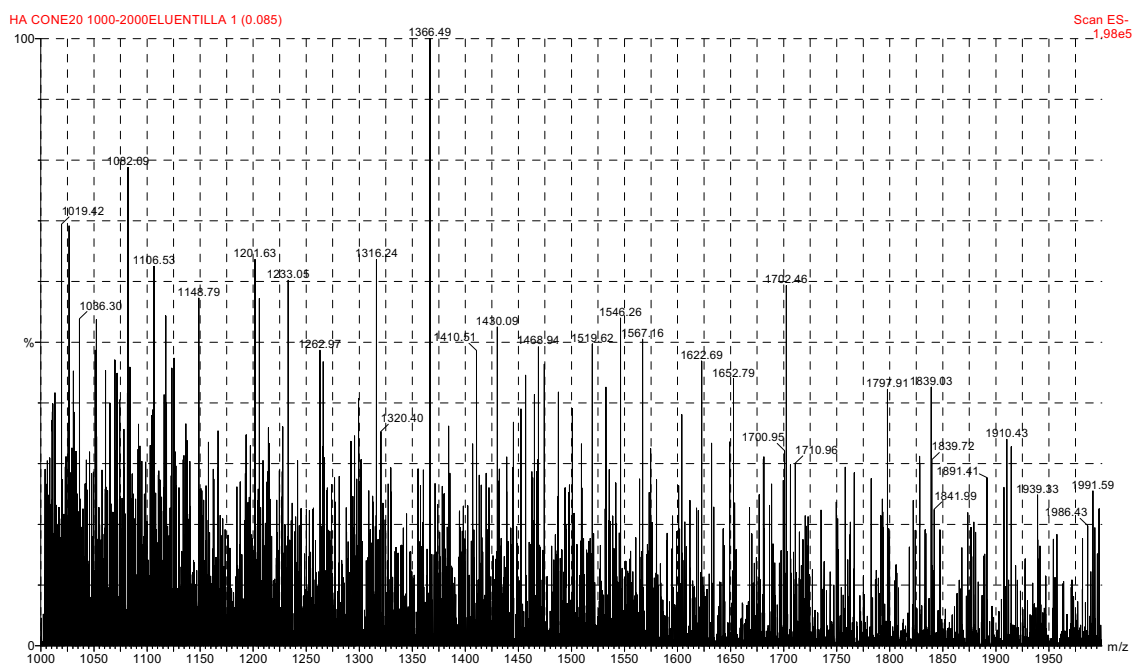
Directly injected fulvic acid (Figure 5) and humic acid (Figures 6 and 7) standards show the most intensive mass spectra in the mass range of  $m/z$  150-750. This mass range was selected for further studies.



**Figure 5.** A mass spectrum of the directly injected fulvic acid standard in the mass range of 100-1100  $m/z$ .



**Figure 6.** A mass spectrum of the directly injected humic acid standard in the mass range of 200-1200 m/z.



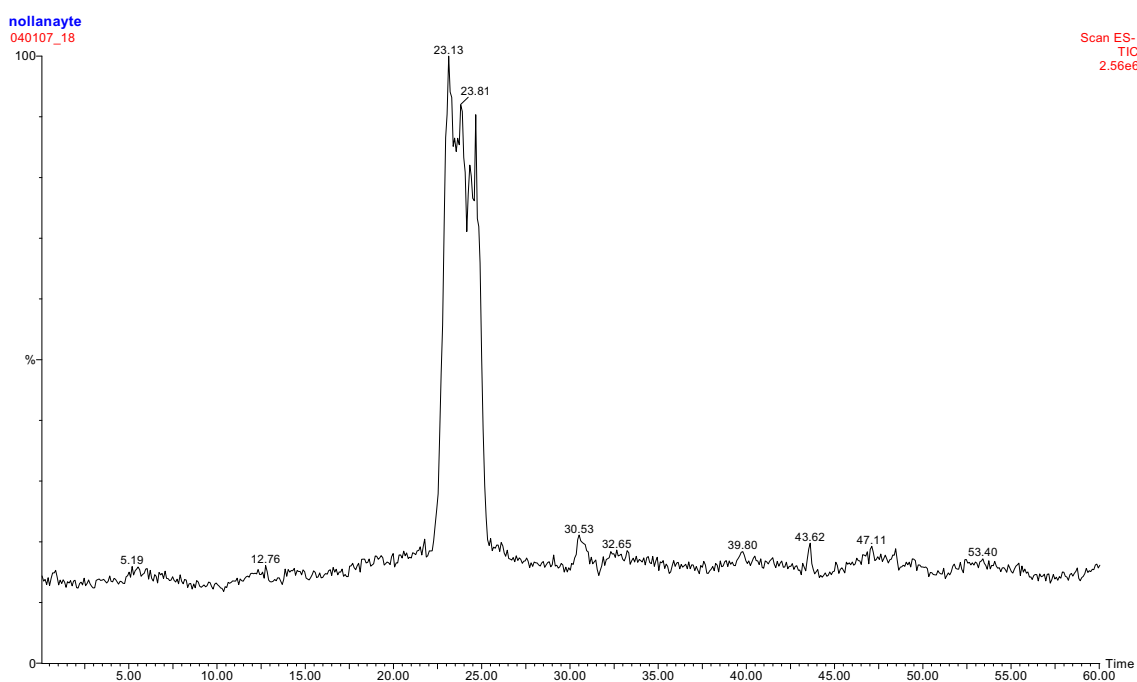
**Figure 7.** A mass spectrum of the directly injected humic acid standard in the mass range of 1000-2000 m/z.

### 3.3.2 SEC-MS

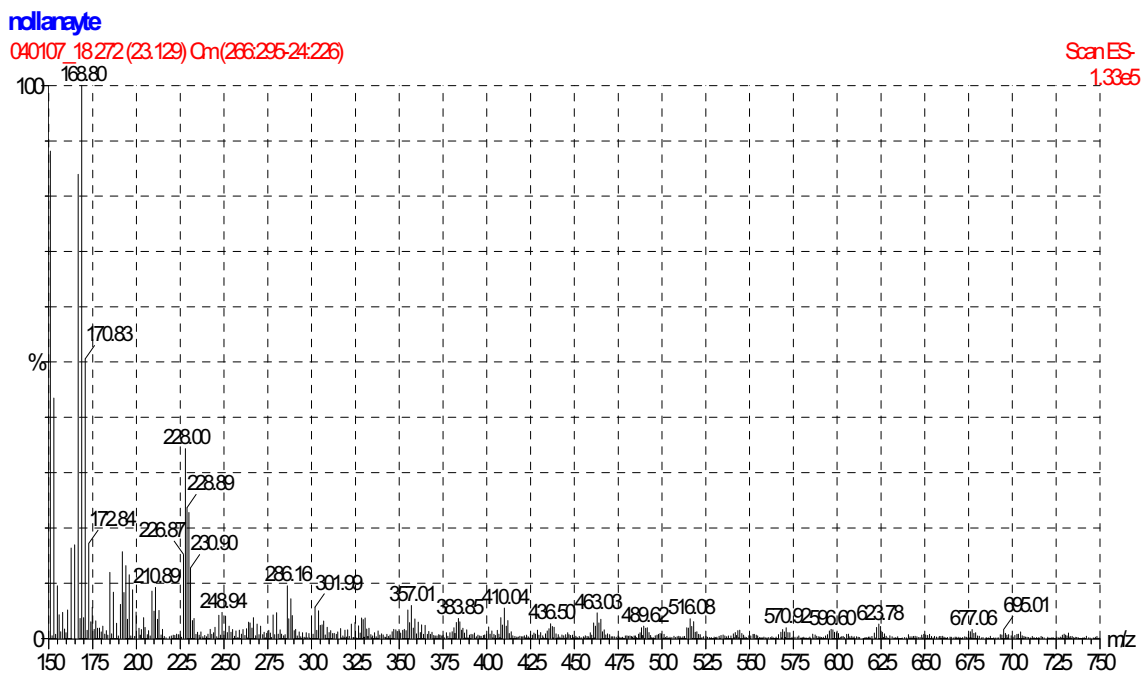
Figures 8 to 19 show the results of the size exclusion chromatography (SEC) with mass spectrometry detection of the blanks, standards and samples. Chromatographic data is presented as the total ion chromatogram of all masses together. A background-

subtracted mass spectrum, which was summed across the detected mass peak, is also shown.

Figures 8 and 9 show the total ion chromatogram and the mass spectrum of a blank sample. The peak in the total ion chromatogram consists probably of some compounds originating from the isolation procedure. The mass spectrum does not resemble the mass spectrum of humic substances. The same peak can also be found in the isolated groundwater samples but not in the humic and fulvic acid standards and in the elution solvent, which have not gone through the isolation procedure.

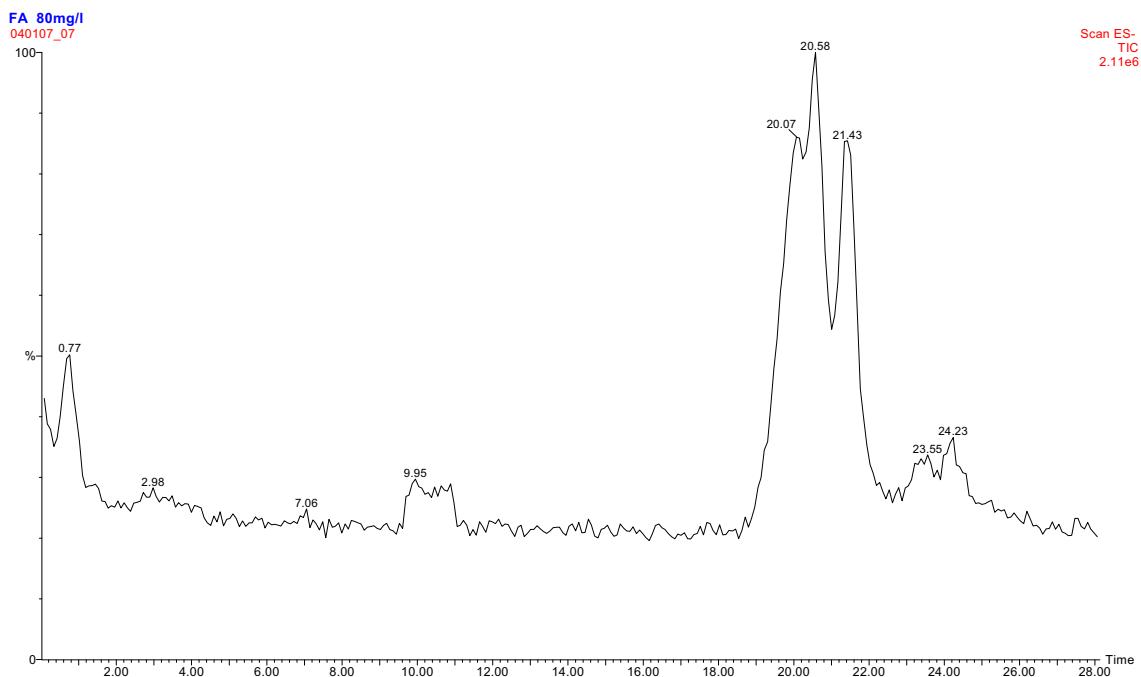


**Figure 8.** Total ion chromatogram of the blank sample.



**Figure 9.** A mass spectrum of the peak in the blank sample.

The total ion chromatogram of the fulvic acid standard (100 mg/L of fulvic acid in 20/80 (v/v) MeOH/MQ water) is shown in Figure 10. The main peaks in the total ion chromatogram are at about 20.5, 21.4 and 23.6 min. Mass spectra of these peaks are shown in Figures 11, 12 and 13. It can be seen that earlier eluting peaks contain higher mass compounds.



**Figure 10.** Fulvic acid standard (100 gm/l).



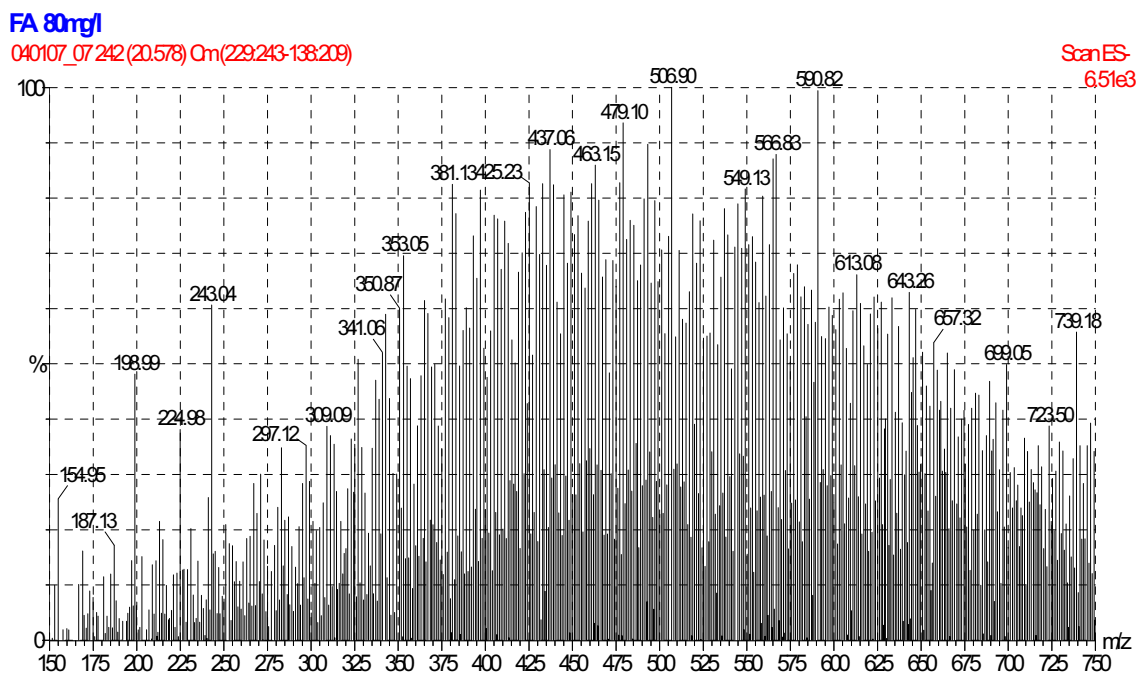


Figure 11. A mass spectrum of the first peak at 20.5 min in the fulvic acid standard.

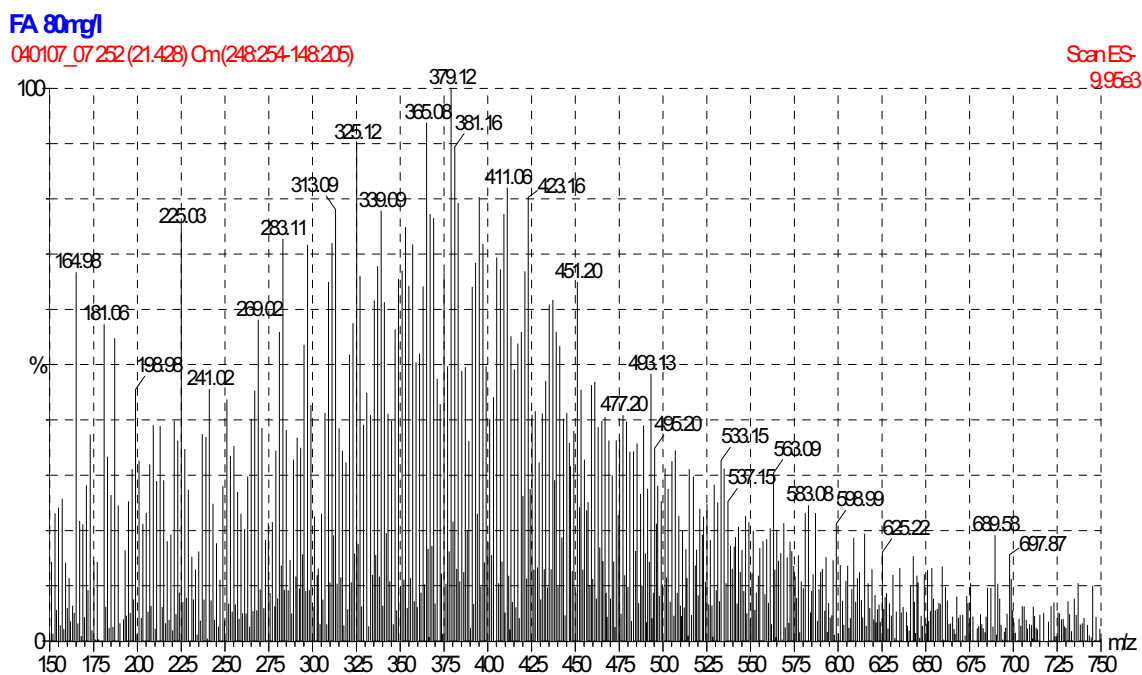
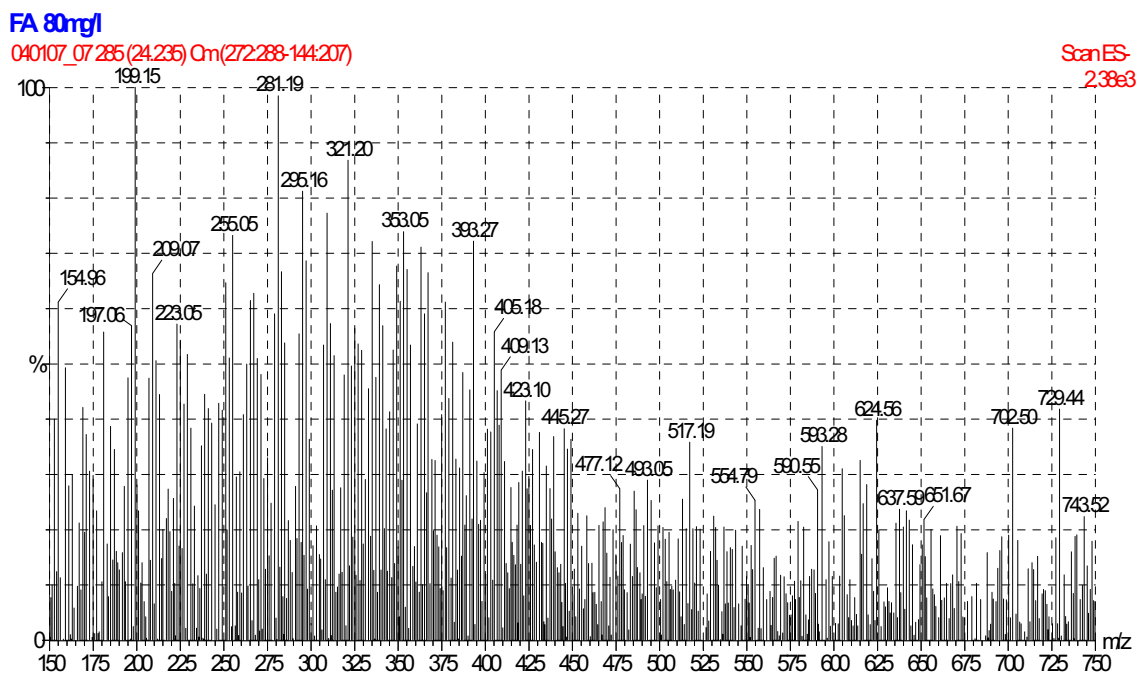
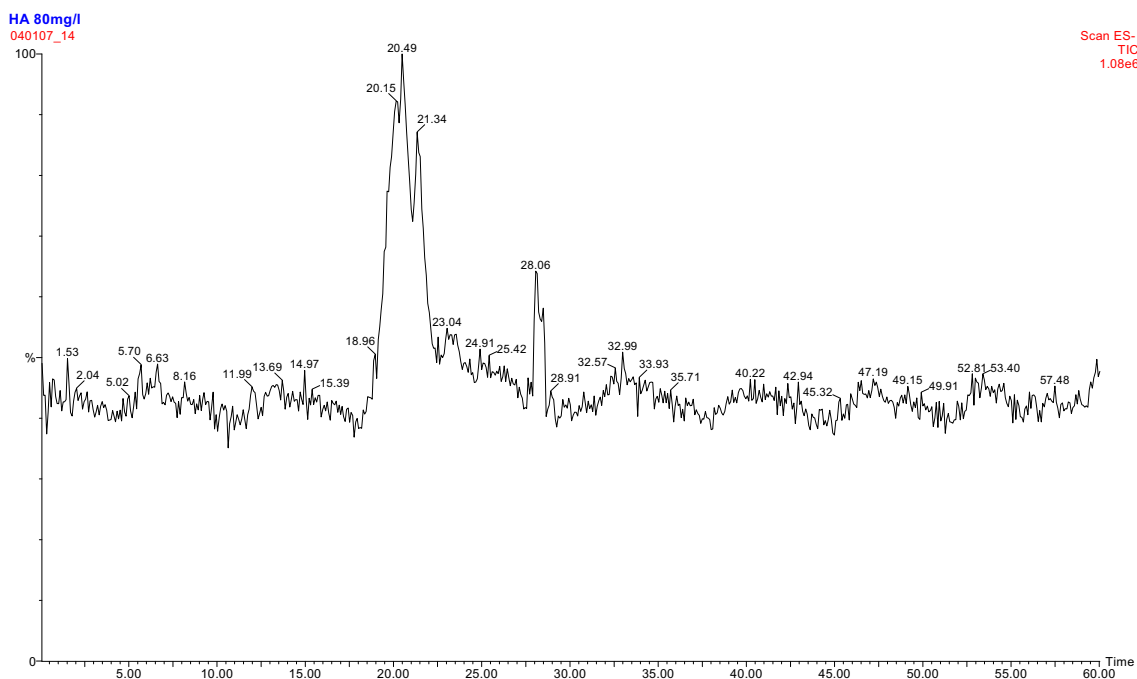


Figure 12. A mass spectrum of the second peak at 21.4 min in the fulvic acid standard.

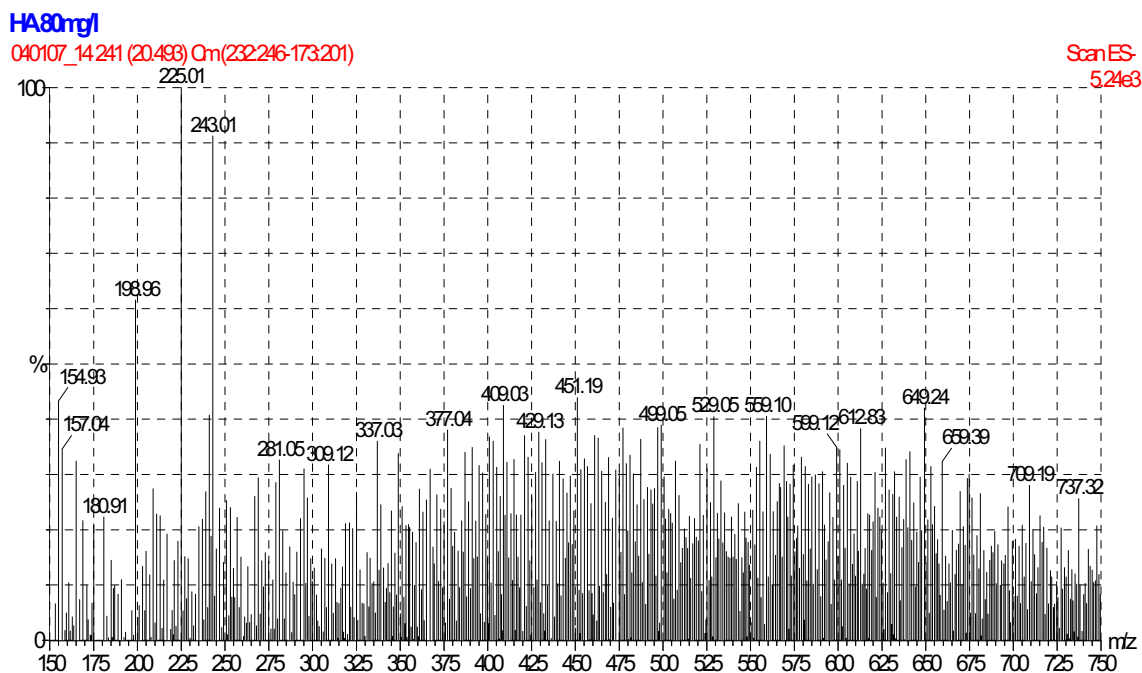


**Figure 13.** A mass spectrum of the third peak at 23.6 min in the fulvic acid standard.

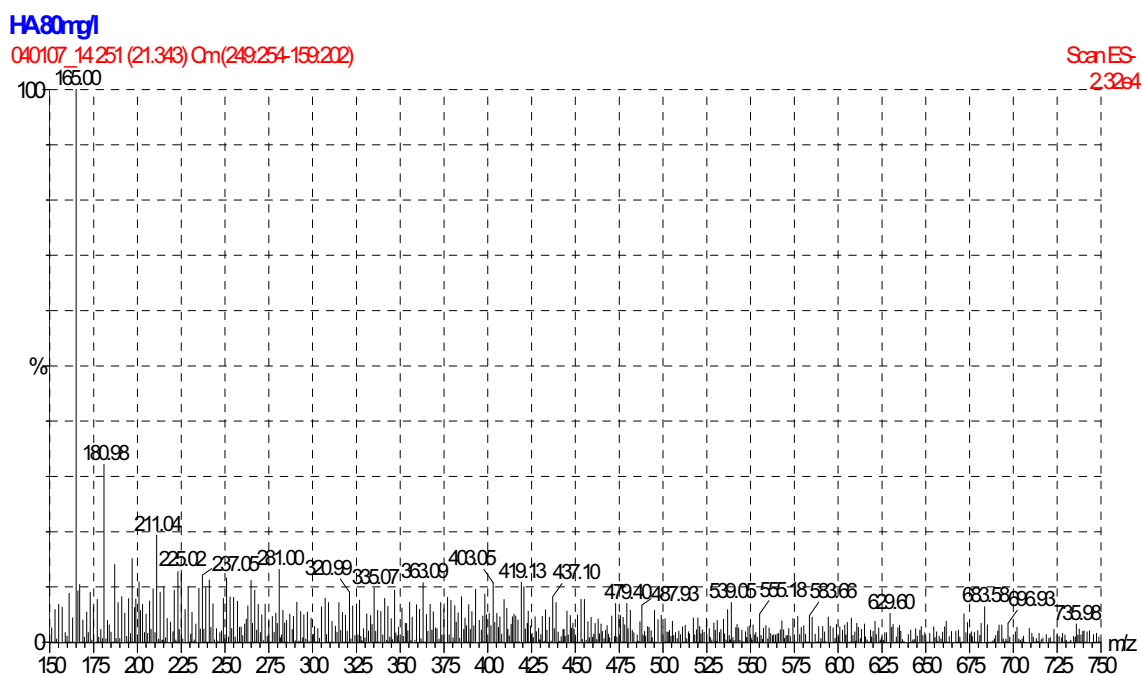
The total ion chromatogram of the humic acid standard (84 mg/L of humic acid in 20/80 (v/v) MeOH/MQ water) is shown in Figure 14. The main peaks in the total ion chromatogram are at about 20.6 and 21.4 min. Mass spectra of these peaks are shown in Figures 15 and 16. The mass spectrum is slightly broader and has fewer patterns than that of the fulvic acid standard.



**Figure 14.** Total ion chromatogram of the humic acid standard (84 mg/l).



**Figure 15.** Total ion chromatogram of the first peak at 20.6 min in the humic acid standard.



**Figure 16.** Total ion chromatogram of the second peak at 21.4 min in the humic acid standard.

The total ion chromatogram of the isolated groundwater sample is shown in Figure 17. The main peak in the total ion chromatogram is at about 21.0. The mass spectrum of this peak is shown in Figure 18. Most of the second peak in the sample consists of the impurities originating from the isolation procedure. The mass spectrum of this second peak is shown in Figure 19 and it shows similar patterns as the mass spectrum of the

peak in the blank sample. All the groundwater samples showed equal chromatograms and spectra.

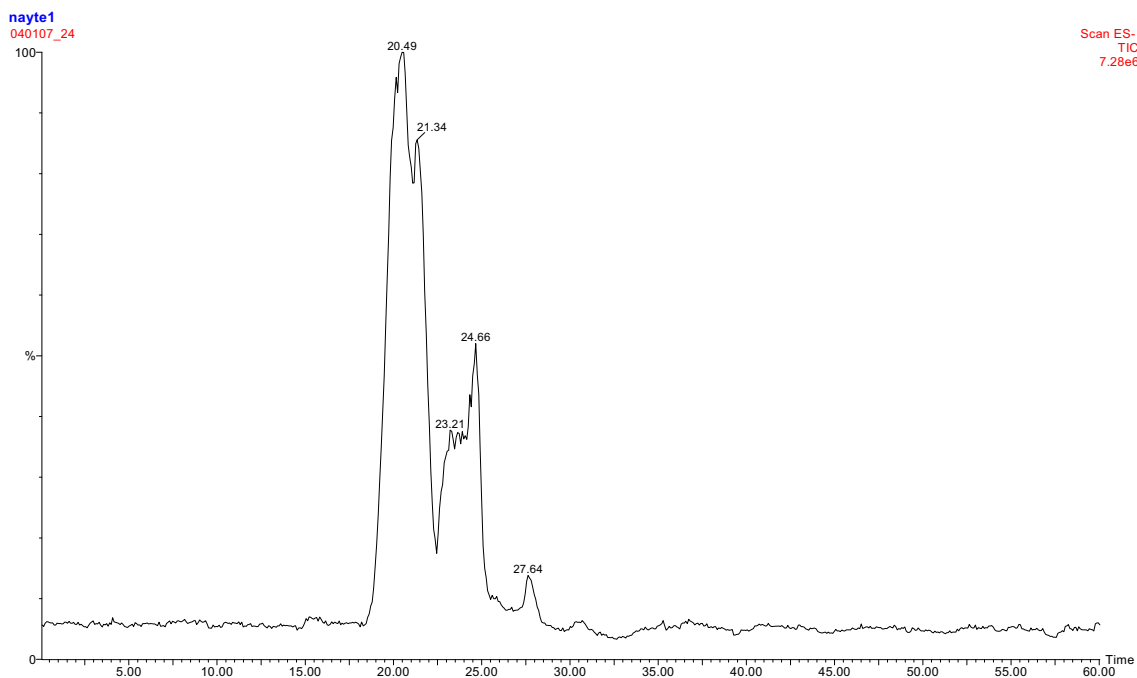


Figure 17. Total ion chromatogram of the isolated groundwater sample.

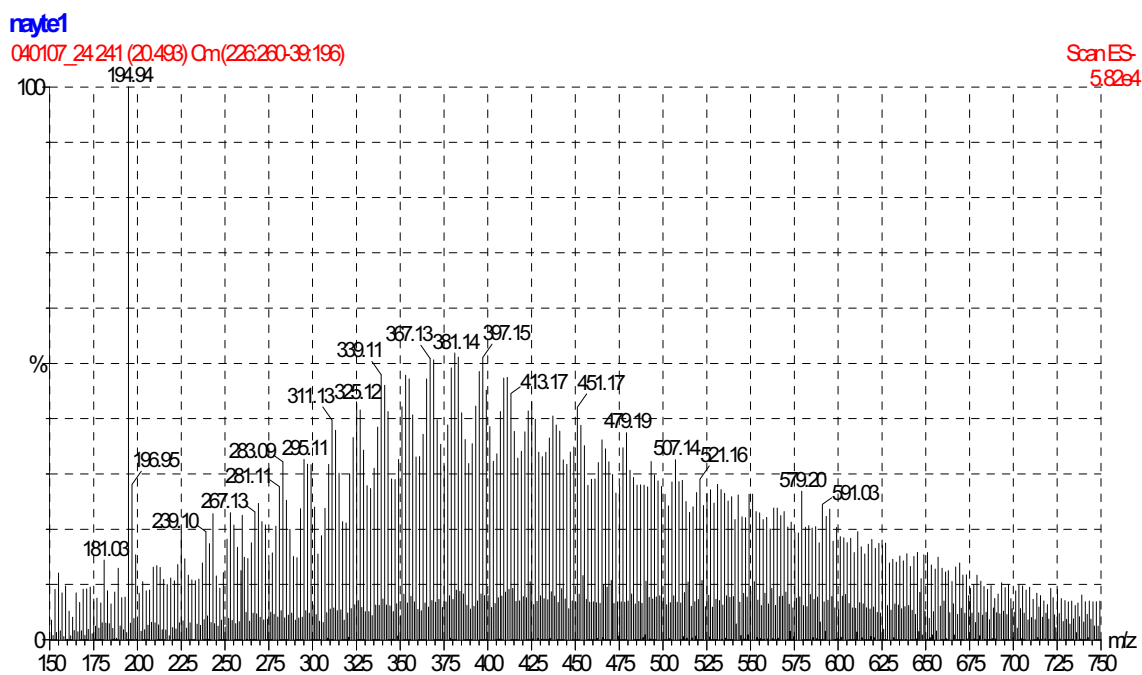
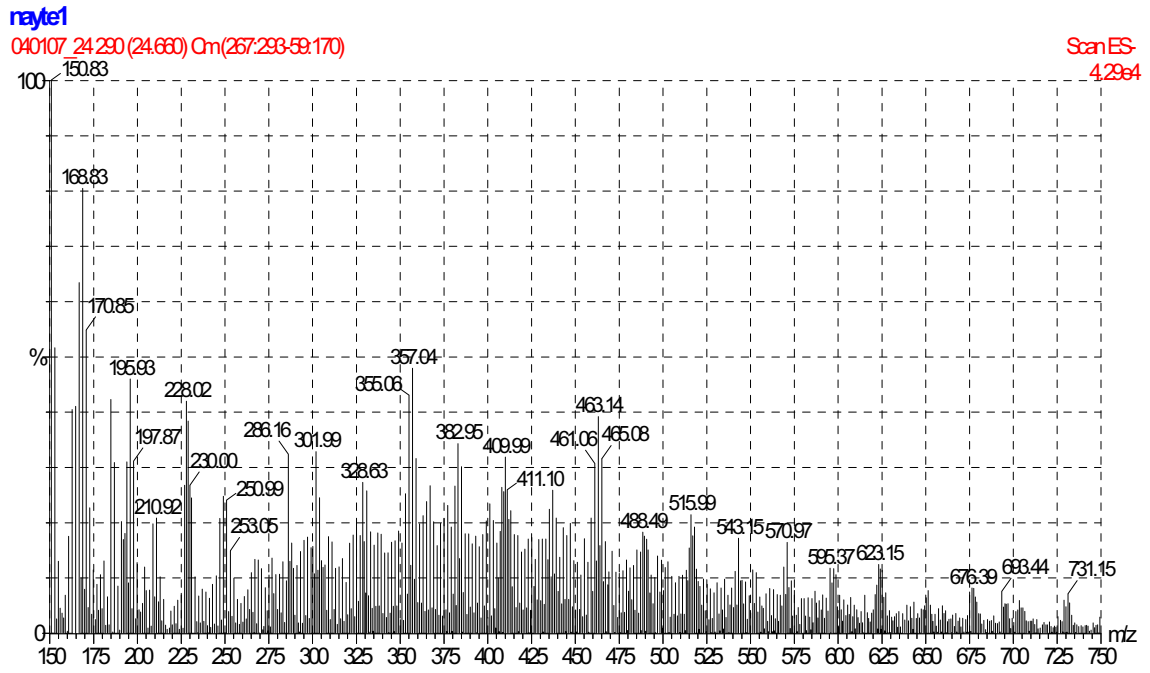


Figure 18. A mass spectrum of the peak at 21.0 min in the isolated groundwater sample (ONK-PVA3).



**Figure 19.** A mass spectrum of the peak at 24.0 min (impurities) in the isolated groundwater sample (ONK-PVA3).

#### 4. DISCUSSION

Table 1 shows the results of the TOC measurements of the groundwater samples isolated with the DAX-8 procedure. The average recovery as total organic carbon is 41.7%. These results are in good agreement with the results by Peuravuori et al. (1997), where 53% of the DOM (dissolved organic matter) was recovered as hydrophobic acids and with our previous results, where the average recovery was 39.3% (Manninen et al. 2006). The blank sample (MQ water) showed no significant amount of total organic carbon. The amount of humic substances in the groundwater at this depth in ONKALO seems to be the same as in the previous investigation (ONK-PVA1, depth about -20m b.s.l.).

In the size exclusion chromatography with fluorescence detection, the chromatograms of humic and fulvic acid standards resemble each other. However, the signal intensity of the peak in the fulvic acid standard is about 2.5 times higher than the intensity of the peak in the humic acid standard. When assuming that all of the measured TOC (mg/l) in the isolated groundwater sample consists of humic substances, the following calculations can be performed: the fluorescence intensity of the highest peak in the groundwater sample is about 8 times that of the humic acid standard and about 3 times that of the fulvic acid standard. This difference in fluorescence is probably due to the different functional groups and the organic compounds that make up the humic substances. According to this assumption, the humic substances isolated from the groundwater differ in structure from the IHSS Nordic aquatic fulvic and humic acid reference standards.

The determination of the molecular size of humic acids was difficult because of the low fluorescence intensity of the polyethylene oxide (PEO) standards. With small peaks it was difficult to determine the exact retention time for the standards, which is needed for the calibration curve. The PEO standards showed multiple peaks with UV detection and they were therefore found unsuitable for the molecular weight determination. The humic substances isolated from the groundwater can be estimated to have about the same molecular weight as the IHSS (International Humic Substances society) humic and fulvic acid standards. For the fulvic acid standard, the molecular weight has been estimated at around 7500 Da (Persson et al. 2000).

The repeatability of the SEC-MS runs was moderate and some instability of the system was observed. The sensitivity of the instrument seemed to decrease when running several samples, probably owing to the clogging of the multi-port valve system between the column and the ion source. The hyphenated SEC-ESI-MS system with the quadrupole mass spectrometer is a powerful instrument especially for lower-molecular-weight fulvic acid research, whereas for the higher-molecular-weight humic acids there seems to be problems to get ionized humic compounds to fly efficiently into and through the mass spectrometer.

In the fulvic acid total ion chromatogram, two distinctive peaks can be found. According to the separation mechanism of SEC, the first eluting peaks are compounds with a higher molecular weight. This can be clearly seen from the mass spectra from the different peaks; every peak has a different mass spectrum, and with the increasing retention times the maximum  $m/z$  ratios decrease. These figures demonstrate the benefits of the SEC separation when comparing them with the direct injection mass

spectra. Less complex spectra are obtained with the SEC separation preceding the MS detection. The most typical differences in mass patterns were -2 and -14 amu. The latter can be attributed to homologous series differing in their number of methylene groups, and the former originates probably from the double bonds of the carbon skeleton. With the high-molecular-weight humic acids, the mass spectrum is not so ordered and these mass patterns are not so obvious. In the isolated groundwater samples, the -2 and -14 amu spacings are the most distinctive in mass spectra. The groundwater sample mass spectrum clearly resembles the mass spectrum of fulvic acids rather than that of humic acids. The obtained results are comparable to those by Reemtsma et al. (2003). They indicate that fulvic acids are more “symmetrical” in structure than has been thought before.

## 5. CONCLUSIONS

The isolation method based on a non-ionic polymeric sorbent (DAX-8) has turned out to be applicable and reliable with groundwater samples. The developed method is shown to isolate approximately 40% of the total organic carbon as humic substances. With the DAX technique, adsorption is based on hydrophobic interactions and the salt concentration of the groundwater does not interfere with the isolation process. In contrast, the adsorption will increase with an increasing ionic strength.

The SEC-ESI-MS seems to be a powerful technique especially to investigate low-molecular-weight fulvic acids. With high-molecular-weight humic acids, there is the well-known problem of sensitivity loss with ESI-MS. When increasing the cone voltage, increasing signal intensity is obtained, but the average  $m/z$  values shift to lower values, indicating that mostly fragment ions are obtained. Thus, the range of the compounds suitable for ESI-MS is limited by the stability of higher-molecular-weight molecules of humic acids in the electrospray process. However, with lower-molecular-weight fulvic acids, SEC-ESI-MS can provide valuable information on the investigated compounds.



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