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Characterisation of humic substances using atmospheric pressure chemical ionisation and electrospray ionisation mass spectrometry combined with size-exclusion chromatography

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Abstract

Humic substances were analysed by atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) mass spectrometry in positive and negative modes. Using APCI the average m/z range of humic substances was reduced 5-fold compared to ESI. High-resolution time-of-flight mass spectrometry revealed the formation of multiply charged molecules in the ESI mode. Moreover, it was possible to obtain daughter ion mass spectra of humic substances by nanospray tandem mass spectrometry. The size-exclusion chromatography elution profile of humic substances was highly influenced by the pH of the analyte solution. By contrast, the pH had no significant influence on the observed mass spectra of humic substances.

Keywords: Mass spectrometry; Humic substances; Fulvic acids

1. Introduction

Humic substances are heterogeneous organic compounds that constitute the major organic matrix in surface water, soil and other environmental compartments. They are negatively charged and tend to bind heavy metals and various organic compounds. Therefore, it is of great interest to obtain more information about this class of compounds. Humic substances play an important role in the transport and immobilisation of xenobiotics. The analysis of xenobiotics bound to humic substances is a major issue in ecotoxicology [1–6]. Moreover, soluble bound residues are present in aquatic systems. Enhanced leaching resulting from the binding of xenobiotics to dissolved organic matter (DOM) in soil is of environmental concern [7,8]. Therefore, a profound knowledge of humic substances is necessary to understand the behaviour of bound residues in soil, sediment and water.

The analysis of humic substances and bound residues is a challenging and interesting field in environmental analysis. Unfortunately, little is known about the exact chemical structure of humic substances. In order to improve this situation it is important to investigate the molecular mass distribution in general and the chemical structure in detail. Earlier mass spectrometry (MS) applications such as pyrolysis–MS and gas chromatography (GC)–MS were only able to identify volatile, degraded fragments resulting from humic material [9–12]. Matrix-assisted laser desorption/ionisation (MALDI) combined with time-of-flight mass spec-

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trometry [13], fast atom bombardment [14,15], laser desorption and electrospray ionisation (ESI) combined with Fourier-transform ion cyclotron resonance mass spectrometry [16–19] and electrospray ionisation combined with quadrupole mass spectrometry [20,21] have been used to study the molecular mass distribution of humic substances to characterise this class of compounds. These measurements resulted in very complex spectra which were influenced to a greater or lesser extent by experimental parameters. The degree of fragmentation could often not be specified. Solouki et al. [22] described an interesting approach to count acidic hydrogens of fulvic acids by gas-phase hydrogen/deuterium exchange reactions by means of Fourier-transform ion cyclotron resonance mass spectrometry. The average number of exchangeable hydrogens at m/z 700–1000 was 7 to 9. During the present work, the applicability of ESI and atmospheric pressure chemical ionistation (APCI) mass spectrometry and the combination of mass spectrometry with size-exclusion chromatography was tested to characterise humic substances. The molecular size of humic substances was found to be responsible for different binding and transport of nonpolar [23] and polar organic pollutants [24].

2. Experimental

2.1. Humic substances

Humic acids and fulvic acids were isolated from water collected from different parts of Lake Hohloh (Black Forest, Germany) following the international humic substances society (IHSS) XAD-8 standard procedure [25–27]. Humic acids are soluble in alkaline solutions and fulvic acids are soluble in both alkaline and acidic solutions. This difference is utilised to separate fulvic and humic acids [28]. The samples were labelled as follows: the first letters describe the origin (HO=Hohlohsee) followed by the sample number. The last letters describe the type of sample (FA=fulvic acid, HA=humic acid, UF=ultrafiltrated). The ultrafiltered samples were dialysed. Original samples of HO 16 UF were filtered using a 0.45 μm tangential-flow membrane and concentrated by ultrafiltration with a 1000 g mol^{-1} cut-off tangential-flow membrane [8,29]. The concentrates were lyophilised and redissolved in demineralised water. Excess salt was removed by dialysis to demineralised water for 3 days.

Soil humic acids were extracted by shaking 10 g of a silt soil (from Burscheid, Germany, C_{org}: 2.6%, <2 μm: 10.2%, 50–2 μm: 81.3%, 2000–50 μm: 8.5%, $CaCO_{3\ total}$: 0.5%) with 0.1 *M* NaOH for 24 h under argon atmosphere to prevent oxidation. After centrifugation at 10 000 rev. min^{-1} for 10 min, the supernatant was separated by decanting and adjusted to pH 1.0 with conc. HCl. The resulting solution was heated for 1 h at 60°C in order to induce coagulation of humic acids. After 24 h the humic acids were separated by centrifugation at 10 000 rev. min^{-1} for 10 min, subsequently washed with demineralised water and lyophilised.

2.2. Flow injection analysis combined with mass spectrometry

Mass spectra of humic substances were obtained using a TSQ 7000 mass spectrometer (Finnigan MAT, Bremen, Germany) equipped with an ESI or an APCI source. Sample injection (100 μl) was accomplished with a GINA 50 autosampler (Gynkotek, Idstein, Germany) and a Gynkotek P 580 HDG HPLC pump. The flow-rate was 1.0 ml min^{-1} with Milli-Q water as eluent. The samples were dissolved in Milli-Q water with a concentration of 1.0 g l^{-1}. The pH values of aqueous solutions of fulvic acid and humic acid samples were about 5. For ESI, the ionisation voltage was set at 5 kV and the transfer capillary temperature at 220°C. A vaporiser temperature of 450°C and a transfer capillary temperature of 200°C were used for the APCI source. The ionisation current was set at 5 μA and the detector voltage to 1.6 kV. The mass range of the spectrometer was set to 100–4000 u at a scan speed of 2 s in centroid mode. The potential difference between the capillary and the tube lens was held at 60–120 V, depending on the mass range. Number-average (M_n) and weight-average molecular masses (M_w) were calculated by weighted summation of averaged (whole 100 μl peak) and background subtracted MS spectra assuming singly charged ions. The relative intensities of the base peaks in ESI–MS spectra were between $2–6\cdot10^4$ (negative mode) and $1–3\cdot10^3$ (positive mode) and in APCI–MS spectra

$0.5–1.0 \cdot 10^3$ (negative mode) and $4.0–7.0 \cdot 10^3$ (positive mode).

Quadrupole time-of-flight (Qq-TOF) mass spectra were obtained on a QSTAR Q_q-TOF hybrid mass spectrometer (Applied Biosystems, Weiterstadt, Germany) equipped with a nanospray source (Protana, Odense, Denmark). The potential at the nanospray needle was 800 V. The orifice potential was −80 V and the curtain gas was set at 1.3 ml/min. Argon was present in the collision cell for both full scan and product ion measurements. Product ion spectra were acquired using a collision energy of 30 eV/charge and a collision cell pressure of 5 p.s.i. (1 p.s.i.=6894.76 Pa). The pressure in the main TOF chamber was $2 \cdot 10^{-7}$ Torr, a pulse frequency of 20 kHz was utilised and the effective flight path was 2.5 m (1 Torr=133.322 Pa). A two-point external calibration was performed.

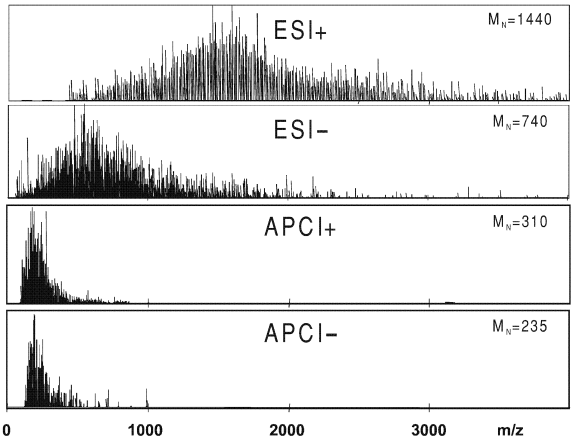

Fig. 1. Mass spectra of a fulvic acid fraction using different ionisation methods (ESI and APCI) with detection of positive or negative ions.

2.2.1. Size-exclusion chromatography (SEC) of humic substances

A TSK gel G 3000 PWXL gel permeation column from TosoHaas (30 cm×7.8 mm I.D., particle size 6 μm) was used with a 0.025 mol l^{-1} ammonium acetate solution (pH 7.0) with 1% (v/v) methanol at 25°C. The flow-rate was set at 0.4 ml min^{-1}. Fifty microliters of a 1.0 g l^{-1} humic or fulvic acid solution was injected. The eluting fractions were analysed by ESI–MS. Fulvic acid solutions for pH dependent experiments were adjusted to pH 3 or 10 and equilibrated in aqueous solution at 10°C overnight.

3. Results and discussion

3.1. Mass spectra of humic substances

Mass spectra of a variety of humic substances could be recorded with ESI and APCI in positive and negative ionisation mode. This is shown for an aquatic fulvic acid fraction (HO 10 FA) in Fig. 1. Significant differences are evident when the mass spectra obtained are compared. The ESI–MS spectra of positive ions exhibit the highest molecular mass distribution (m/z 500–3000). The masses recorded in the negative mode are significantly lower (m/z 100–1500). This effect is the result of different ionisation of various molecules [18], e.g. carboxylic acids are best ionised in negative mode by abstraction of a proton, whereas amines are preferentially ionised in positive mode by addition of a proton.

Comparing the molecular mass distribution of positive and negative APCI modes, the differences are not as obvious as in the ESI mode. Compared with ESI, the masses are in the range of m/z 100–500 and, therefore, considerably lower. APCI is a harder ionisation method than ESI and weak fragmentation of labile compounds cannot be excluded. Moreover, APCI is useful for ionising less polar molecules. By contrast, humic substances are considered to represent highly polar species, because of the large content of phenolic and carboxylic acid groups [30,31]. This fact could explain the low total ion current (TIC) compared to ESI (see Experimental section). It is possible that humic acids of different origin are ionised better by ESI or APCI due to different ionisation mechanisms. Because humic substances are very polar compounds, ESI is the more suitable ionisation method for this type of substances.

In order to test the efficiency of different types of ESI mode (positive and negative) to characterise different humic substances, several humic fractions and DOM were investigated (Fig. 2). The patterns of the mass spectra of aquatic humic substance fractions obtained with the same ionisation technique look

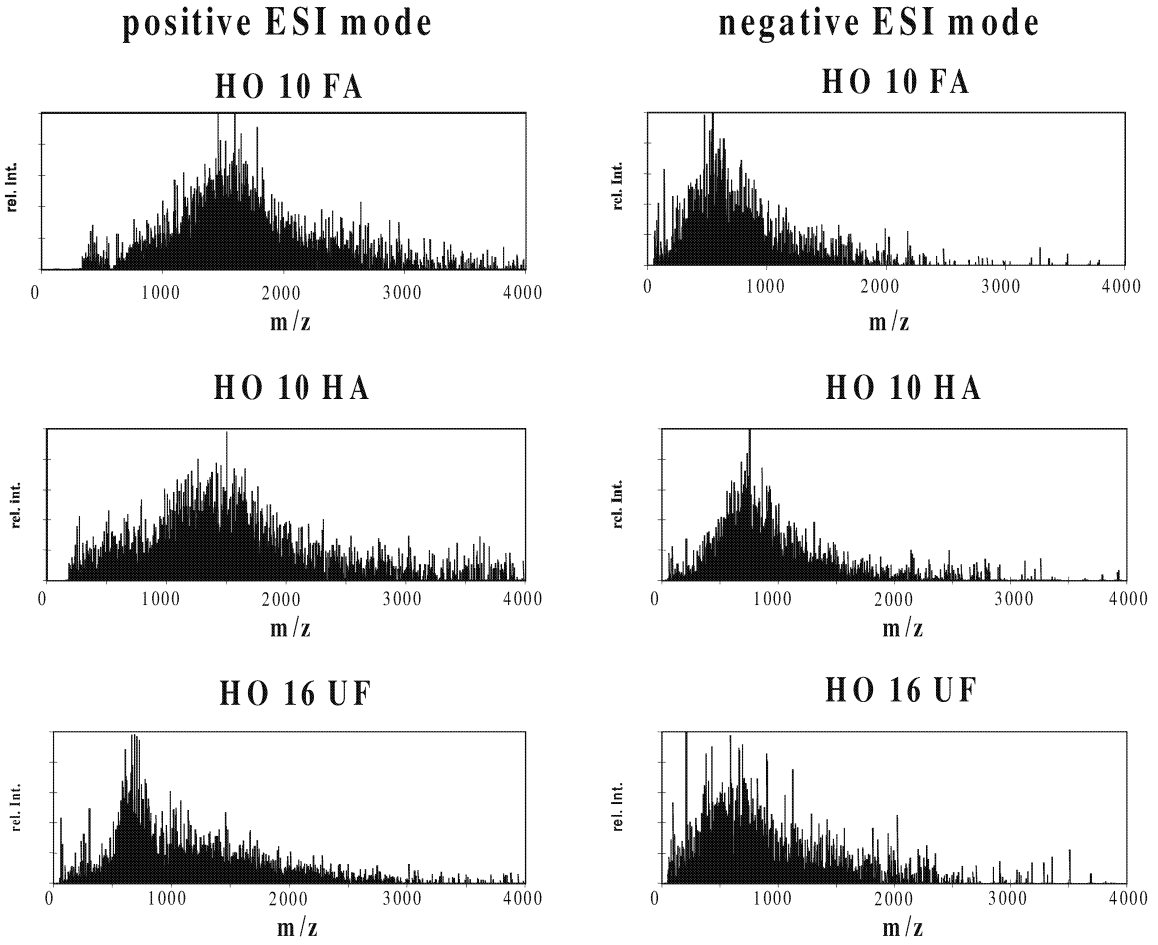

Fig. 2. Mass spectra of selected humic substances analysed by ESI in positive and negative mode.

similar, but on closer examination there are some differences. The ESI–MS spectra of humic acids and fulvic acids isolated by XAD-8 resin were comparable except for the higher average molecular masses of humic acids. The ESI–MS spectrum of HO 16 UF, prepared by ultrafiltration and desalted by dialysis, contained a higher proportion of molecules with lower m/z. This pattern might result from a later sampling date (Autumn instead of Summer) of the natural dystrophic brown water lake. During a longer period, chemical substances may react with other molecules forming substances with a higher molecular mass. Average molecular masses (M_n and M_w) were calculated for these samples by weighted summation of averaged scans (Table 1).

The most plausible explanation of the differences between positive and negative ion modes could be that they represent various components of humic substances [18]. Compounds containing a basic nitrogen are better ionised in the positive mode while

Table 1
Average masses of aquatic humic substances determined by ESI–MS assuming singly charged molecules

Humic substances	ESI positive		ESI negative	
	M_n	M_w	M_n	M_w
HO 10 FA	1440	1800	740	1000
HO 10 HA	1520	1920	900	1150
HO 16 UF	1120	1430	860	1230

compounds such as the majority of humic substances which contain acidic carboxyl and phenolic groups are better ionised in the negative mode. A higher total ion current in negative ion mode of all humic substances investigated (at least one order of magnitude) compared with the total ion current in the positive ion mode indicated a predominance of negatively charged molecules. Therefore, spectra obtained in the negative ion mode better represent the composition of humic fractions in solution.

The use of the very soft ESI for the determination of humic substance molecular masses has a great potential because the exact mass of the molecules in the mixture of humic substances is obtained. However, there are some questions concerning the influence of pH and organic solvents on the m/z pattern and the formation of multiple charged molecules under ESI conditions. The influence of the pH value on the m/z pattern was investigated with two fulvic and humic acids. A weak mass shift of about 50 u to lower masses was observed for all substances from pH 3 to 9 (not shown). At pH 3, the ionisation of carboxylic acids is favoured over ionisation of phenolic compounds without carboxyl groups. At pH 9, phenols are better ionised. It is possible that these phenols possess a lower average mass and, therefore, induce a shift to lower m/z ranges. The influence of methanol and acetonitrile on the m/z pattern was investigated using an organic solvent content of 0 to 50% (v/v) in the negative mode. No significant mass shifts were observed under these conditions. In order to exclude the formation of multiple charges in the gas phase, which is typical of large biopolymers, fulvic and humic acid fractions were investigated by a high resolving Qq-TOF hybrid tandem mass spectrometer (Fig. 3). An ion signal is observed at each nominal mass and there is a regular change of more intense peaks and less intense peaks with a distance of 1 u between these signals. For instance, the ion signal at m/z 336 is less intense than the signal at m/z 337 and the signal at m/z 338 is less intense than the signal at m/z 339, etc. An explanation for this phenomenon is the predominance of organic molecules without a nitrogen atom (or an even number of nitrogen atoms) because small molecules have an odd mass in the low m/z range if they are deprotonated ($M–H^+$). Furthermore, multiply charged ions are evident. The difference between the

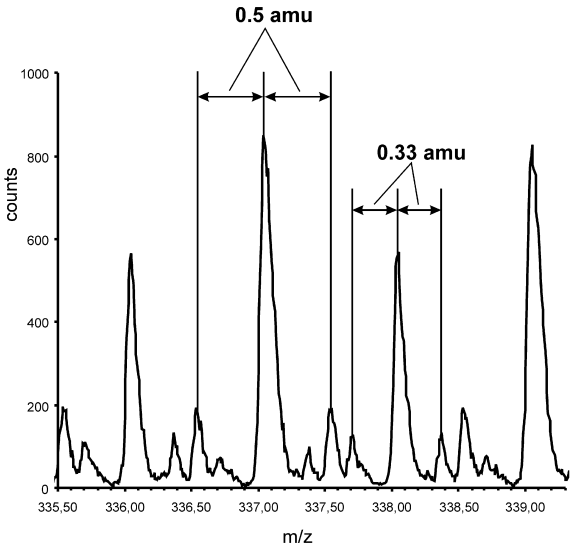

Fig. 3. High-resolution full scan mass spectrum (detail) of fulvic acid HO 10 FA in negative ion mode revealing the presence of multiply charged ions.

signals is 0.5 and 0.33 u, indicating the presence of doubly and triply charged ions. There is an interference between these multiply charged ions and singly charged ions. Multiply charged ions are usually observed with ESI for molecular substances with a higher mass, depending on the functional groups present. Thus the calculated average masses of fulvic and humic acids based on singly charged ions possess a significant error (Table 1) with a too high emphasis on the low m/z range. Multiply charged ions were observed with a very similar pattern as in Fig. 3 almost completely over the full mass range. There was a reduced abundance of these multiply charged ions in the low mass range below m/z 300. Signals of these ions were never higher than 25% of the base peak. The low resolution of all these signals (approximately 3000 formal weight/half height measurement (FWHM) compared with 7000–9000 FWHM resolution for standards) indicated an interference of molecules with similar masses.

Further evidence for the presence of multiply charged parent ions was obtained by the daughter ion mass spectrum of ions within the selected mass range of m/z 334.6–335.4 (Fig. 4). Elimination of CO_2 took place from a doubly charged parent ion resulting in formation of a doubly charged fragment

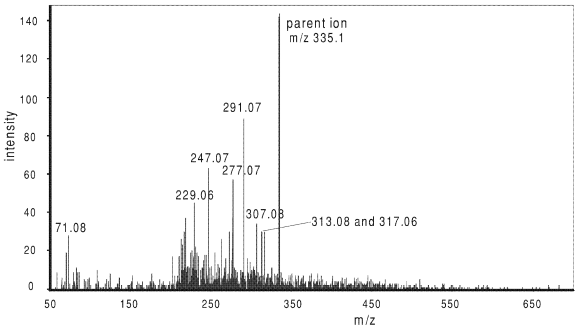

Fig. 4. Daughter ion mass spectrum of fulvic acid HO 10 FA (parent mass range m/z 334.6–335.4) at 30 eV collision energy/charge and 5 p.s.i. collision cell pressure. Possible fragments: m/z 317 ($-H_2O$), 313 (-22), 291 ($-CO_2$ or $2x-22$), 247 ($2x-CO_2$), 229 ($247-H_2O$).

ion with m/z 313 (m/z −22). There were also some nonspecific ions with very low intensity above the selected m/z range resulting from multiply charged ions forming fragment ions with a lower charge. Predominant fragment ions originated from neutral loss of CO_2 (m/z −44 for singly charged and m/z −22 for doubly charged ions) and loss of water (−18) or CO from the parent ion. The elemental composition of this sample could not be determined because the MS–MS spectrum must be internally calibrated for this purpose with the parent ion mass, the resolution of which was too low (Fig. 3). Loss of CO_2 was also observed over a wide mass range during a neutral loss experiment with a triple quadrupole instrument (not shown). Full scan spectra and neutral loss scan spectra (m/z −44) were very similar. Therefore, this neutral loss scan is indicative of these classes of compounds.

3.2. Size-exclusion chromatography of humic substances

Since mass spectra of humic fractions showed a high degree of complexity, a SEC approach was tested to separate different humic substances by molecular mass and to simplify interpretation. Mass determination by SEC alone is difficult because there are no appropriate standards for humic substances [32]. There are many non-specific ionic interactions and adsorption phenomena during SEC [33,34]. Both polymer and silica based gel interact strongly with aromatic compounds [35]. Non-ideal mechanisms like ion exclusion, hydrophobic interaction and ion-exchange [35–37] have a great influence on the gel permeation profile. Hydrophobic interactions prevail at high ionic strength and electrostatic interactions dominate at low ionic strength [35]. Chin et al. [38] used a phosphate buffer at pH 6.8 and NaCl with a ionic strength of 0.1 mol l^{-1} to suppress the ion-exclusion phenomenon. Unfortunately, this high buffer concentration is not compatible with electrospray ionisation mass spectrometry because of signal suppression. Perminova [39] and Piccolo et al. [40] described the strong influence of the pH on the elution behaviour of humic substances during SEC. Fig. 5 illustrates the influence of the pH on the SEC of fulvic acid HO 16 FA with MS detection in positive mode. The fulvic acid fraction was injected at pH 10 (adjusted with 25% NH_3 solution) and pH 3 (adjusted with conc. CH_3COOH). At pH 10, only one eluting fraction is observed at the void volume of the SEC column. Humic acids possess a high negative charge at alkaline pH and probably cannot

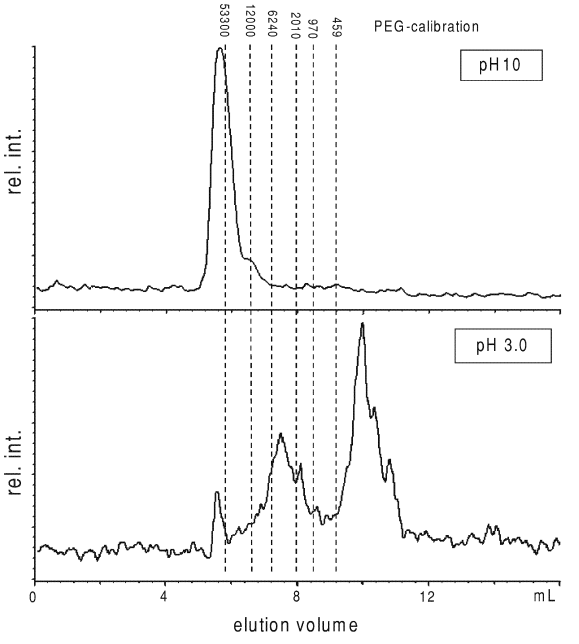

Fig. 5. Influence of the pH on the size-exclusion chromatography of fulvic acid HO 16 FA analysed with positive ESI in the full scan mode and (a) pH 10 or (b) pH 3. PEG = Poly(ethylene glycol).

diffuse into the pores of the gel matrix (ion-exclusion phenomenon) at this pH range. In the case of highly charged compounds the elution volume can be equal to the void volume of the column [39]. The high apparent molecular mass is only valid at alkaline pH. At pH 3, the self association of the majority of humic substances is broken. As a consequence, three distinct eluting fractions appeared with a shift to higher molecular masses at pH 3.

The TIC chromatogram obtained with a quadrupole MS and detection of positive ions in full scan mode is also shown in Fig. 6. Three different ranges (a, b and c) were observed. Averaged and baseline subtracted scans over the different ranges resulted in different mass spectra (Fig. 6a–c). SEC separates by molecular volume and only to a certain extent by molecular mass. Molecules with equal or similar masses but different chemical structures have different molecular volumes and on account of the special functional groups they differ in their interactions with the column material. Therefore, the mass range of single eluting fractions is still broad. Nevertheless, a trend from high to low molecular mass in the positive mode was observed.

SEC combined with quadrupole MS and detection of negative ions resulted in a similar chromatogram (Fig. 7). Averaged and baseline subtracted scans over the different ranges again produced different mass spectra (Fig. 7a–c). There was also an obvious trend for molecules with higher mass eluting first (a) and molecules with lower mass eluting later (b and c). It is known that the used TSK gels (like many other chromatographic materials) have some problems with irreversible or not reproducible adsorption of humic substances. This may influence the observed mass distribution. However, in experiments which compared the ESI mass spectra of the complete fraction of humic substances before SEC and the added spectra of single eluting fractions of humic substances no significant differences were found.

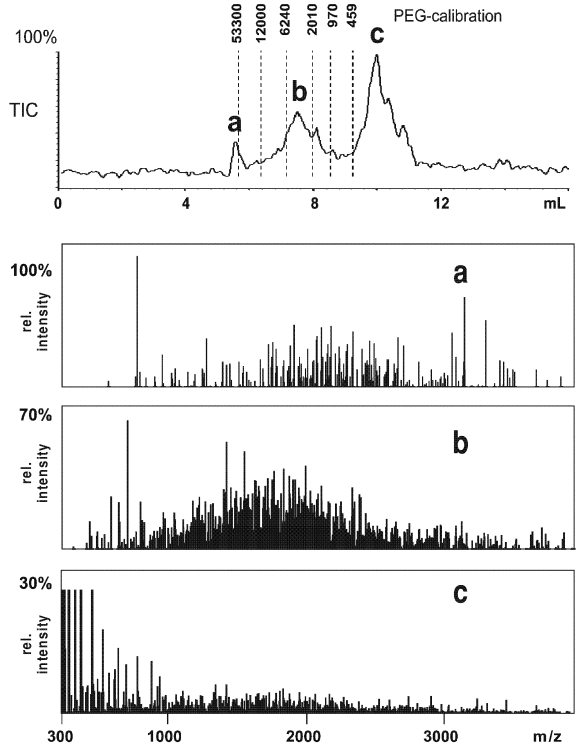

Fig. 6. SEC of fulvic acid fraction HO 16 FA with detection of positive ions. TIC (top) and averaged and baseline subtracted full scan mass spectra of fractions a–c.

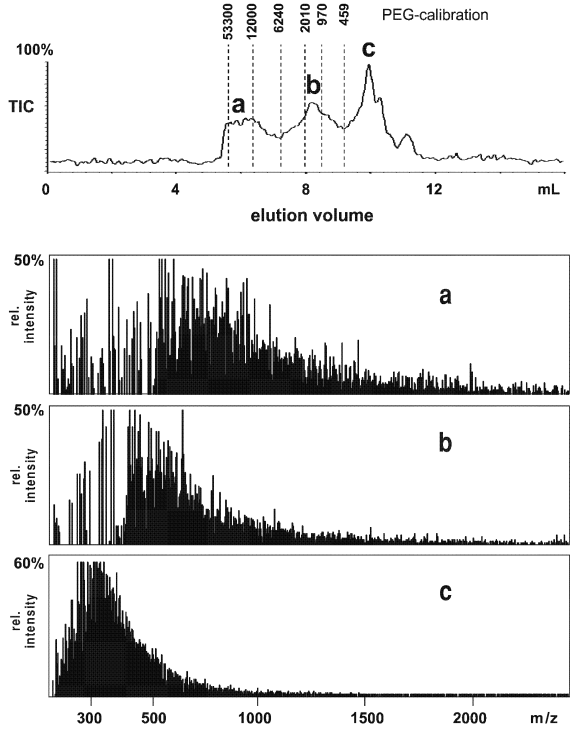

Fig. 7. SEC of fulvic acid fraction HO 16 FA with detection of negative ions. TIC (top) and averaged and baseline subtracted full scan mass spectra of fractions a–c.

Lepane [41] investigated the recoveries of humic substances after size exclusion chromatography on different XAD resins and obtained recoveries of humic substances between 35 and 89%. Some humic substances may be trapped onto the resin pores [42]. If the sample is unstable, the recovery of humic substances can also decrease after reinjection or longer storage.

4. Conclusion

A detailed characterisation of humic substances by chromatographic and spectroscopic methods has always been hampered by the chemical heterogeneity of this class of substances. Mass spectrometry is a powerful technique and offers the possibility of improving the characterisation of humic substances. Mass spectra of both various humic substances and dissolved organic matter were obtained in positive and negative ionisation modes using electrospray ionisation. The use of a time-of-flight analyser revealed the formation of multiply charged molecules in the negative electrospray ionisation mode. Size-exclusion chromatography combined with mass spectrometry resulted in different mass spectra for different eluting fractions. Moreover, the self association of humic substances can be broken by adding an organic acid. By contrast, only one eluting fraction was observed at alkaline pH. The presence of only one fraction at alkaline pH during size-exclusion chromatography is more likely due to self association of smaller humic substances to larger aggregates than to the presence of humic macromolecules [43]. This is an important aspect for a better understanding of the complex behaviour of humic substances in the environment.

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