Evaluation of tangential flow ultrafiltration procedures to assess trace metals bound to marine dissolved organic matter

Natalia García-Otero, Paloma Herbello-Hermelo, Raquel Domínguez-Gonzalez, Manuel Aboal-Somoza, Antonio Moreda Piñeiro, Pilar Bermejo-Barrera∗

Department of Analytical Chemistry, Nutrition and Bromatology. Faculty of Chemistry. University of Santiago de Compostela. Avenida das Ciencias, s/n. 15782 – Santiago de Compostela. Spain.

Abstract

A procedure based on two-dimensional size exclusion chromatography (SEC) and anion exchange chromatography (AEC) with UV (205 nm) and ICP-MS detection was used to assess dissolved organic matter (DOM) and trace metals associated to DOM in surface seawater. Marine DOM was isolated by tangential ultrafiltration (UF) using two different polyethersulfone membranes exhibiting different molecular weight cut-off (MWCO), 3 and 10 kDa. The procedures require a volume of seawater sample of 100 L, and marine DOM of molecular weight higher than 3 and 10 kDa (UF membranes of 3 and 10 kDa MWCO) was pre-concentrated in 0.5 L (retentate), which implies a pre-concentration factor of 200. Retentate fractions obtained after the different UF procedures were further desalted by using HI Trap desalting mini-columns before two-dimensional SEC/AEC. Apparent molecular weights of isolated compounds after SEC with UV detection ranged from 1.5 kDa (close to the permeable volume of the SEC column fixed by injecting vitamin B12) to 16 and 22 kDa (UF membranes of 3 and 10 kDa MWCO, respectively). Further AEC/UV characterization of SEC fractions showed a large group of macromolecules eluted at 4.5 min, and small signals at shorter retention times (2.5 and 3.5 min). In addition, AEC experiments of the isolated SEC fractions when using 10 kDa MWCO UF membranes showed a group of substances eluted at high retention times (13 min). SEC hyphenation with ICP-MS proved the existence of several trace elements (Ni, Co, Cu, Zn, Mn, Mo and Sr) bound to the isolated marine DOM. Mass balance studies after analyzing the retentate and permeate fractions for trace elements indicate good recoveries (close to 100 %) for elements such as Mo, Sr, Ba and Zn when performing the UF with both 3 and 10 kDa MWCO membranes. However, recoveries from 36 to 81 % were obtained for the remaining studied elements after either UF procedure. SEC-ICP-MS experiments showed percentages of metals bound to the isolated marine DOM ranging from 0.055 and 0.077 % (Zn) to 4.1 10^{-4} and 1.4 10^{-4} % (Sr).

Keywords:

Metal bound to marine dissolved organic matter, tangential flow ultrafiltration, size exclusion chromatography, anion exchange chromatography, inductively coupled plasma mass spectrometry

1. Introduction

Marine dissolved organic matter (DOM) is a complex mixture of very different organic molecules from natural and anthropogenic sources. Marine DOM is one of the planet's most important reservoirs of carbon (approximately 700 × 10^{15} g), an amount similar to the carbon content in atmospheric carbon dioxide (750 × 10^{15} g) [1,2]. The ocean is an important sink for anthropogenic carbon dioxide because it can absorb one third of the carbon dioxide emissions from combustion of fossil fuels and from tropical deforestation by fire. Marine DOM presents, therefore, a considerable

∗ Corresponding author.
E-mail address: pilar.bermejo@usc.es (P. Bermejo-Barrera)

contribution to the global carbon cycle [3,4]. On the other hand, the presence of various highly reactive functional groups in marine DOM is responsible for the binding properties of many different types of contaminants, such as organic compounds and trace metals. Marine DOM plays, therefore, an important role for controlling the transport of toxic contaminants and nutrients and influencing toxicity and bioavailability of those compounds in the marine environment [5–7]. Among the different compounds contained in marine DOM, proteins are important components of high molecular weight. The study of marine proteins has increased because they are the most reactive substances present in marine DOM [8]. Trace metals can be bound to marine proteins and thus affect metal toxicity and availability.

The assessment of marine DOM of high molecular weight is difficult because this fraction occurs at low concentrations, while a large amount of inorganic salts are present in seawater. Some solid phase extraction (SPE) procedures have been applied for pre-concentrating marine DOM. These procedures have been shown to be successful when separating DOM of low molecular weight [9], a fraction which can account for 65–75 % of DOM in surface seawater [4]. On the contrary, DOM of high molecular weight, such as marine proteins, is more easily isolated by tangential flow ultrafiltration (UF) procedures [5,8]. This technique offers the advantage of high pre-concentration factors and the possibility of using large sample volumes. UF procedures usually require membranes with a nominal molecular weight cut-off (MWCO) of 10 kDa, which guarantees the isolation of marine DOM of high molecular weight (marine proteins included) [10–20]. Nevertheless, marine DOM can be lost by adsorption onto the UF membranes [14,15], and this problem is especially important when dealing with marine proteins due to their highly adsorptive nature. Different reagents such as sodium azide and sodium dodecylsulphate (SDS) are commonly added to the filtered seawater before UF to prevent protein adsorption onto the ultrafiltration membrane [15–20]. On other occasions, UF procedures based on membranes with nominal MWCO of b 10 kDa have been proposed, and 1 kDa MWCO membranes have been used in several studies [11,21–25]. UF procedures by using 5 kDa MWCO membranes have also been reported [20].

The aim of this work is the development of pre-concentration procedures for marine DOM of high molecular weight using tangential UF procedures. Isolation was performed by using two membranes with different MWCO (3 and 10 kDa). Differences of the isolated marine DOM were established by two-dimensional size exclusion chromatography (SEC) and anion exchange chromatography (AEC) with UV detection. Inductively coupled plasma-mass spectrometry (ICP-MS) was also used as a detector for assessing trace elements bound to the isolated marine DOM.

2. Material and methods

2.1. Apparatus

The tangential UF system consisted of a Masterflex I/P pump (Millipore, Bedford, MA, USA), a Prep/Scale-TFF Cartridge (Millipore) with two polyethersulfone membranes (nominal MWCO 10 kDa and 3 kDa), and a Pre/Scale-TFF Holder (Millipore) equipped with a pressure gauge.

A Sievers Innovox Laboratory TOC analyzer from General Electric Analytical Instruments (Boulder, CO, USA) was used for DOM quantification. The analyzer uses a super critical water oxidation (SCWO) technique to achieve superior wet DOM oxidation (persulfate in acid medium), and a Non-Dispersive Infra Red (NDIR) for CO_2 detection.

A Dionex P680 HPLC pump (Dionex, Sunnyvale, CA, USA) equipped with a Rheodyne 4097 injector (Cotati, CA, USA) with a 50 μL injection loop, and a Dionex UVD170U absorbance detector were used for HPLC-UV determinations. A 250 μL Hamilton Gastight 1725 syringe (Bonaduz, Switzerland) was used for manual injection.

A Dionex UltiMateO 3000 LC HPLC (Dionex), equipped with a GP50 gradient pump (Dionex), an AS50 thermal compartment (Dionex) and an AS50 autosampler (Dionex) was coupled to an ICP-MS

Thermo Finnigan X Series (Thermo Fisher Scientific Inc., Waltham, MA, USA) for assessing metals bound to DOM. DOM fractions were separated with a TSK-G4000 SW_{XL} SEC column (30 cm × 7,8 mm I.D., 8 μm particle size, optimum separation range for Polyethylene Glycol (PEGs) between 2 and 250 kDa, and for proteins between 20 and 10,000 kDa) from Tosoh Bioscience (Tokyo, Japan) coupled to a 10 cm × 8 mm i.d. TSK-gel SW glass guard column (TosoHaas, Tokyo, Japan). A PRP-X100 anion-exchange column (250 mm × 4.1 mm i.d. × 10 μm) from Hamilton (Reno, NV, USA) coupled to a 25 × 2.3 mm I.D. PRP-X100 guard column (Hamilton) was also used.

Hi-Trap Desalting 5.0 mL columns containing Sephadex G-25 Superfine, cross-linked dextran (fractionation range between 1 and 5 kDa) from GE Healthcare (Buks, UK) were used for ultrafiltrate desalting. A Harvard Pump 11 Plus Single Syringe (Harvard Apparatus, Holliston, MA, USA) was used for ultrafiltrate loading into the Hi-Trap Desalting columns by using 5 mL BD Discardit™ II syringes (Becton, Dickinson and Company, Fraga, Huesca, Spain).

Other materials were an ORION 720A plus pH-meter with a glass-calomel electrode (ORION, Cambridge, UK), HAWP14250 Millipore 0.45 μm mixed esters of cellulose membrane filters (140 mm diameter), and Albet®LabScience 0.20 μm cellulose acetate syringe filters (25 mm diameter) from Albet–Hahnemuehle (Dassel, Germany).

2.2. Reagents

Ultrapure water, resistance 18 MΩ cm, was obtained from a Milli-Q water-purification system (Millipore). Potassium hydrogen phthalate stock standard solution (1000 mg L^{-1}) was prepared from 99.5 % potassium hydrogen phthalate supplied by Panreac (Barcelona, Spain). Potassium peroxodisulphate solution (30 %(m/v)) and phosphoric acid solution (6.0 M) were prepared from 99 % potassium peroxodisulphate and from 85 % phosphoric acid (Panreac), respectively. Ready Calkit PEO/PEG (PSS) containing polyethylene oxides (molecular weights between 6.7 and 478 kDa) were from PSS Polymer Standard Services GmbH (Mainz, Germany). Blue dextran 2000 (molecular weight 2000 kDa) was from Pharmacia Biotech (Piscataway, NJ, USA). Carbonic anhydrase (29 kDa MW), ribonuclease A (13.7 kDa MW), ovalbumin (43 kDa MW), conalbumin (75 kDa MW), aldolase (158 kDa MW) and ferritin (440 kDa MW) were from GE Healthcare.

Mixed exchange AG 501-X8 resin was from Bio-Rad (Richmond, CA, USA). Other reagents were high purity 69 % nitric acid (Panreac), high purity 25 % ammonia (Merck, Darmstad, Germany), sodium hydroxide (Merck), diammonium sulphate (Panreac), and diammonium hydrogenphosphate (BDH, Poole, UK). Multi-element standard solutions were prepared by combining stock standard solutions (1.000 g L^{-1}) supplied by Merck (Poole, Dorset, UK).

2.3. Procedures

2.3.1. Seawater sample collection procedure

Surface seawater samples (100 L) were collected from the Ría de Arousa estuary (north-western Spain) in pre-cleaned 12 L non-metallic free-flushing Niskin bottles attached to a 1015 rosette multibottle array (General Oceanics, Miami, FL, USA). After collection, seawater samples were filtered (0.45 μm) and immediately subjected to the ultrafiltration procedure.

2.3.2. Seawater tangential flow ultrafiltration procedure

According to manufacturer's instructions, the UF system was cleaned before use by re-circulating 2 L of 0.1 M NaOH at 45 ± 5 °C for 60 min; and rinsing with 9 L of Milli-Q water, also at 45 ± 5 °C. The seawater (100 L) was then concentrated by tangential flow UF through two different polyethersulfone membranes (size 0.6 m^2, nominal MWCO of 10 kDa and 3 kDa) until obtaining a volume of retentate (ultrafiltrate containing substances of molecular weight higher than 10 and 3

kDa, respectively) of approximately 500 mL. The remaining sample (permeate, ~99.5 L) contained substances of molecular weight lower than 10 and 3 kDa (membranes of nominal MWCO of 10 kDa and 3 kDa, respectively), and it was reserved for further mass balance studies. No preservatives for avoiding marine DOM adsorption onto the UF membrane were added, because mass balance studies for marine DOM were performed through TOC determinations.

2.3.3. Retentate desalting procedure by using HI Trap desalting columns procedure

Hi Trap Desalting columns were used for removing the salts from the retentate. The Sephadex G-25 Superfine contained in the columns offer a fractionation range for globular proteins between 1 and 5 kDa, with an exclusion limit of approximately 5 kDa. This ensures the separation of biomolecules exhibiting a molecular weight higher than 5 kDa from those molecules with molecular weights less than 1 kDa. The mobile phase used for desalting consisted of a 25 mM/25 mM ammonium sulphate/diammonium hydrogen phosphate buffer solution at pH 6.5. The column was connected to the chromatographic system with UV detection, and was first equilibrated by passing 25 mL of a buffer solution at a flow rate of 2 mL min^{-1}. Once equilibrated, 1.5 mL of re-dissolved retentate was loaded into the column using a 5 mL disposable syringe connected to a Harvard Pump 11 Plus Single Syringe for sample infusion, which worked at a flow rate of 2 mL min^{-1}. After loading, the column was again connected to the chromatographic system for sample elution (pH 6.5 buffer solution at a flow rate of 2 mL min^{-1}). The excluded fraction was finally collected for further SEC-HPLC characterization. The Hi Trap column was equilibrated with approximately 10 mL of the pH 6.5 buffer solution before desalting the following 1.5 mL retentate sample.

2.3.4. Seawater and permeate desalting procedure

A mixed exchange resin (AG 501-X8) was used for salt removal from seawater samples and for the permeate fractions. According with manufacturer's instructions, 5 g of fresh resin for every 100 mL of seawater sample/permeate was used (batch mode). The mixture was shaken for 1 h to achieve deionization. Finally, the desalted seawater sample/permeate was separated from the resin by filtration.

2.3.5. TOC measurement procedure

Concentrations of dissolved organic carbon in the untreated seawater, retentate and permeate were assessed as TOC by supercritical water oxidation (30 %(m/v) potassium peroxodisulphate in 6.0 M phosphoric acid), and CO_2 NDIR detection. The TOC analyzer was operated in the non-purgeable organic carbon (NPOC) measurement mode by mixing the acid (set at 1 %) and the oxidant (set at 15 %) with the sample in the sparger for 0.8 min (inorganic carbon removal). The solution is then pumped from the sparger by the peristaltic pump in the reactor module, where the mixture is subjected to conditions above the critical point of water (375 °C, 22.1 Mpa) for organic carbon oxidation to CO_2, and subsequent NDIR detection. The analyzer was calibrated using standard solutions of potassium hydrogen phthalate ranging from 0.1 to 1000 mg L^{-1} (concentrations referred to carbon). Each sample (seawater, retentate and permeate), as well as different blanks, was analyzed in quadruplicate. LOD (3 SD criterion, SD standard deviation of eleven measurements of a reagent blank) of the procedure was 0.13 mg L^{-1}; whereas, the LOQ (10 SD criterion) was 0.43 mg L^{-1}.

2.3.6. ICP-MS measurement procedure

Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Sr, Mo, Cd, Ba, and Pb were assessed in desalted seawater, permeate, retentate, and also in the SEC fractions ICP-MS under the operating conditions listed in Table 1. The standard addition technique (8.5 mL of desalted seawater, permeate or retentate diluted

to 10 mL) ranging in metal concentrations from 0 to 200 μg L^{-1} for all elements except for Sr (within the 0–20 μg L^{-1} range) was used. A mixture of different internal standards (Sc, Ge, Y, and In) was added to the standards and samples at constant concentrations of 50 μg L^{-1} for Sc, 10 μg L^{-1} for Ge, and 5.0 μg L^{-1} for Y and In. Metals bound to marine DOM (SEC fraction) were assessed by using an aqueous calibration matched with the mobile phase (25 mM/25 mM diammonium sulphate/diammonium hydrogen phosphate, pH 6.5), using trace metal concentrations ranging from 0 to 200 μg L^{-1}, except for Sr (from 0 to 20 μg L^{-1}). The SEC fractions (8.5 mL) were diluted to 10 mL with the mobile phase, and the same internal standards (at the same concentrations listed above) were used for calibration. For all cases, analysis were performed in triplicate. Different blanks were also analyzed for contamination control. Table 2 lists the limits of detection (LODs) and quantification (LOQs) taking into account the pre-concentration factor of 200 when analyzing the retentate and 150 when assessing trace elements bound to SEC fractions.

2.3.7. SEC-HPLC-UV/ICP-MS measurement procedure

SEC-HPLC separation (TSK-G4000 SW_{XL} column, 30 cm × 8 mm i.d.) was performed under isocratic elution conditions (flow rate set at 1.0 mL min^{-1}) with a mobile phase consisting of a 25 mM/25 mM ammonium sulphate/diammonium hydrogen phosphate buffer solution at pH 6.5. UV detection was performed at 205 nm, and the injection volume was set at 50 μL. Column calibration was performed with globular proteins of different molecular weight such as ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), conalbumin (75 kDa), and aldolase (158 kDa). In addition, calibration was also obtained by using PEO/PEG standards with molecular weights within the 6.7 – 478 kDa range. When calibrating with globular proteins, the exclusion volume (V_0) was determined using blue dextran (2000 kDa) and the permeation volume (V_p) was fixed by injecting vitamin B12 (1.9 kDa). When using PEO/PEG standards, V_0 and V_p were fixed with polymers of molecular weight of 1015 and 0.232 kDa, respectively. For ICP-MS detection (operating conditions in Table 3), the outlet of the chromatographic column was directly coupled to the nebulizer of the ICP-MS.

2.3.8. AEC-HPLC-UV/ICP-MS measurement procedure

DOM fractions collected by SEC were then subjected to Anion Exchange Chromatography (AEC) with UV and ICP-MS detection. Separation was performed with a PRP-X100 column under isocratic conditions (25 mM/25 mM ammonium sulphate/diammonium hydrogen phosphate buffer solution, pH 6.5, as a mobile phase, flow rate of 1 mL min^{-1}). Since the composition of the mobile phase when using AEC was the same as that used for SEC, collected SEC fractions were neither pre-treated nor pre-concentrated before AEC. When using UV detection, absorbance was monitored at 205 nm. When coupling the AEC-HPLC with ICP-MS for metal detection, the operating ICP-MS conditions listed in Table 3 were used.

A diagram showing the complete procedure is provided in Fig. 1.

3. Results and discussion

3.1. Marine DOM isolation by UF procedures

UF by using membranes with MWCO of 3 and 10 kDa was performed as described in Section 2.3.2. Filtered seawater samples (100 mL) were subjected to the UF procedure until obtaining a volume of retentate of approximately 0.5 L. The procedure implied ultrafiltration times of 2 h when using the membrane of 10 kDa MWCO; whereas, a time of approximately 12 h was required when performing UF with the membrane of 3 kDa MWCO.

The total organic carbon (TOC) concentration was previously assessed in the unultrafiltrated

seawater sample, and also in the permeate fraction (99.5 L), which contains marine DOM of molecular unultrafiltrated seawater sample (five replicates). Regarding UF with 3 kDa MWCO membranes, TOC concentration in the retentate (preconcentration factor of 200) was 0.17 ± 0.02 mg L^{-1}; whereas, TOC concentration in the permeate fraction was 0.65 ± 0.03 mg L^{-1}. TOC concentrations as a sum of TOC concentrations in the permeate and in the retentate (0.82 ± 0.04 mg L^{-1}) imply a marine DOM retention on the 3 kDa MWCO membrane of 2.4 ± 0.26 %. Similarly, the retentate and permeate fractions after UF with 10 kDa MWCO membrane showed TOC values of 0.004 ± 0.0003 mg L^{-1} and 0.80 ± 0.06 mg L^{-1}, respectively.

A mass balance study shows a TOC value of 0.804 ± 0.060 mg L^{-1} as a sum of TOC concentrations in the permeate and retentate fractions, which implies marine DOM losses by adsorption onto the UF membrane accounting for 4.3 ± 0.52 %. Therefore, similar marine DOM losses onto the UF membranes are observed when using either membranes of low and high MWCO. Losses by adsorption onto the UF membrane of 5 % have been reported by Schmitt et al. [26] when assessing NOM. However, marine DOM retention onto the UF membranes is also probably dependent on the nature of the dissolved DOM because other authors have reported marine DOM losses within the 11—16 % range for seawater UF with a 10 kDa MWCO membrane [14,15]. In addition, higher loss rates have been reported when combining a diafiltration stage before UF for salt removal. Hertkorn et al. [21] have therefore reported marine DOM losses within the 20–26 % range when using 1 kDa MWCO UF membranes. Similarly, losses close to 50 % have also been observed by Aluwihare et al. [24].

3.2. Separation of the preconcentrate marine DOM by size exclusion chromatography

As previously mentioned, large DOM losses by retention onto the UF membranes are observed when using the tangential UF system for diafiltrating the retentate. Low DOM losses and efficient salt removal can be obtained using preparative HI Trap columns for retentate desalting (Section 2.3.3) [14]. The delivering solution used for desalting consists of a 25 mM/25 mM ammonium sulphate/diammonium hydrogen phosphate buffer solution at pH 6.5, which is the solution chosen as a mobile phase for the TSK4000SW_{XL} SEC. Therefore, desalted retentates (UF with 3 and 10 kDa MWCO membranes) were characterized by SEC with UV detection under operating conditions listed in Table 3.

Fig. 2 shows typical chromatograms for retentates obtained after UF with 3 kDa (dashed line) and 10 kDa (bold line) MWCO membranes. In both cases an important peak was observed at a retention time of 12.6 min; whereas, elution of pre-concentrate marine DOM by 3 kDa UF membrane shows other small chromatographic peaks at a shorter retention time (12 min). As previously reported for SEC experiments when analyzing natural DOM [26] and marine DOM [14], sharp peaks (retention time of 12 min for UF preconcentration with 3 kDa MWCO membranes) can be attributed to a salt boundary peak produced by a gradient in the ionic strength between the sample and the mobile phase.

To obtain the molecular weight of the isolated fractions, the TSK4000SW_{XL} column was calibrated using two different sets of standards: a mixture of polyethylene oxides (PEOs) and polyethylene glycols (PEGs) standards (molecular weights within the 6.7 – 478 kDa range), and a mixture of globular proteins (ribonuclease A, 13.7 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 43 kDa; conalbumin, 75 kDa; and aldolase, 158 kDa).

3.2.1. Calibration with PEOs

In the first case, the calibration equation obtained was Log MW = − 0.302 tr + 7.5679 (R^2 = 0.9845). The exclusion volume (V_0) and the permeation volume (V_p) were assessed using two polymers with molecular weights of 1015 and 0.232 kDa, respectively. Assessed V_0 was 6.2 mL (retention time of 6.2 min), while V_p was 12.7 mL (retention time of 12.7 min).

3.2.2. Calibration with globular proteins

Regarding calibration with globular proteins, the equation was Log MW = − 0.6317 tr + 11.725 (R^2 = 0.9601). In this case V_0 was determined by injecting blue dextran (2000 kDa), and V_0 was fixed at 6.2 mL (retention time of 6.2 min). Similarly, V_p was fixed by using vitamin B12 as a standard (1.9 kDa) at a volume of 12.7 mL (retention time of 12.7 min).

As shown, part of the isolated marine DOM contained in the large fraction (retention time of 12.6 min), obtained for retentate isolation by both 3 and 10 kDa UF membranes fell out of the range of molecular weights fixed by either vitamin B12 or PEO of 0.232 kDa (12.7 min). Similarly, the small chromatographic signal at a retention time of 6.0 min (3 kDa UF membrane) is out of the exclusion volume of the column (6.2 min). However, it must be pointed out that conclusions from SEC experiments can only be used to determine molecular weight distribution profiles rather than the exact molecular weight of the substances [27]. Table 4 lists therefore the apparent molecular weights obtained for the different SEC fractions when analyzing the 3 and 10 kDa retentates. It can be observed that the molecular weight range obtained is quite different when using the PEOs/PEGs or the globular proteins standard sets for column calibration. However, molecular weights of the isolated compounds obtained when using globular proteins as standards for SEC calibration (within the 1.6 – 16.1 kDa and 1.2 – 21.6 kDa ranges when using 10 and 3 kDa MWCO membranes for UF, respectively) are more realistic than those offered when calibrating with PEOs/PEGs mixtures (molecular weights lower than 10 kDa for all cases).

3.3. Separation of the preconcentrate marine DOM by anion exchange chromatography

AEC with UV detection (25 mM/25 mM diammonium hydrogen phosphate/diammonium sulphate, pH 6.5; 1 mL min^{-1} flow rate) was performed by injecting the isolated SEC fractions after UF with 3 and 10 kDa MWCO membranes. Regarding SEC fractions obtained when pre-concentrating with 3 kDa MWCO membranes, both fractions (eluted compounds from 11.7 to 12.3 min, and from 12.3 to 13.7 min) were chromatographed. Chromatograms in Fig. 3(a) show the same AEC pattern for both SEC peaks (a large chromatographic peak at 4.5 min, and small signals at 3.5 and 9.0 min). This fact suggests that the two chromatographic SEC signals observed in Fig. 2 can be attributed to the same group of organic substances, and as previously reported [26], the sharp peak (retention time of 12 min) can be therefore attributed to the ionic strength gradient between the sample and the mobile phase. AEC analysis of the SEC fraction obtained when using UF membranes of 10 kDa MWCO (Fig. 3(b)) shows two different chromatographic signals at retention times of 4.5 and 13 min, and small chromatographic peaks at 2.5 and 3.5 min.

3.4. Trace metal study using size exclusion chromatography/anion exchange chromatography-inductively coupled plasma-mass spectrometry

Trace metal patterns (Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Sr, Mo, Cd, Ba, and Pb) were obtained after preparative SEC, and also after AEC of the SEC fractions, by coupling both chromatographic modes with ICP-MS. Fig. 4 shows the SEC-ICP-MS chromatograms where the maximum m/z signals were found to be close to the maximum of the UV signals for trace elements such as Ni, Zn, Mn and Sr when isolating marine DOM by UF with 3 and 10 kDa MWCO membranes. Significant chromatographic signals can also be observed for Cu and Mo for SEC of the isolated material by 3 kDa MWCO UF; whereas, the signals are less intense when analyzing marine DOM after UF with 10 kDa MWCO membranes. Finally, small signals were obtained when monitoring Co, and no significant differences in the elution volumes of trace elements such as Al, V, Fe, As Cd, Ba and Pb were observed.

AEC and ICP-MS coupling shows negligible signals for all trace metals found to be associated to

SEC fractions, except for Sr (Fig. 5); although no specific coelution with marine DOM monitored with UV detection was observed. As shown in Fig. 5, broad Sr signals encompass the small AE chromatographic peaks at 2.5 and 3.5 min, and part of the large AE signal at 4.5 min. These findings agree with data previously reported for Ni in natural DOM [27], and for Ni and Cd in marine DOM [14].

3.5. Mass balance studies for trace elements bound to marine DOM

A mass balance study was performed for those trace elements found to be associated to marine DOM after SEC (Cu, Mn, Mo, Sr, and Zn), and also for As, Ba, Rb and V. First, total concentrations of trace elements were determined in the seawater sample and also in the permeate and retentate fractions after the AG-501-X8 desalting procedure (Section 2.3.4). The retentate fraction was also desalted by using HI-Trap desalting as described in Section 2.3.3. Measurements were performed by using the standard addition technique plus the addition of a mixture of internal standards according to Section 2.3.6.

Selected trace elements were also measured in the SEC fractions (from 11.7 to 13.7 min, and from 11.9 to 13.5 min for desalted retentates, by UF with 3 and 10 kDa MWCO membranes), after combining collected fractions from four runs. This implies eluted volumes of 2 and 1.6 mL (3 and 10 kDa MWCO membranes, respectively) for each run, and total collected volumes of 8 and 6.4 mL for 3 and 10 kDa MWCO membranes, respectively. Direct ICP-MS analysis of the combined SEC fractions was performed after dilution to 10 mL with the mobile phase and the addition of internal standards as shown in Section 2.3.6. For all cases, analysis was performed in triplicate and also different blanks were analyzed for contamination control.

Table 5 lists the found metal concentrations in the desalted surface seawater, and in the desalted permeate and retentate fractions after both UF procedures (3 and 10 kDa MWCO membranes). Regarding metal concentrations in the retentate fraction, both desalting procedures (AG 501-X8 resin and HI Trap) offer similar results for Zn and Ba. However, higher values were found in the desalted retentate with AG 501-X8 resin for the remaining elements. This can be attributed to the fact that the mixed exchange resin only retains monovalent ions, while monovalent and divalent ions are separated by size exclusion when performing HI Trap desalting. It must be pointed out that the use of the mixed exchange resin for retentate desalting before SEC characterization was not performed due to organic matter contamination from the resin.

A mass balance study was therefore established by assessing trace elements in the surface seawater and in the retentate and permeate fractions after AG 501-X8 resin desalting. Percentages were calculated as a sum of the metal concentration in the permeate and the metal concentration in the retentate with respect to the metal concentration found in the untreated seawater sample. It can be seen that percentages vary from 34 % for Mn to 95 % for Sr and Rb when performing UF with membranes of 3 kDa MWCO; whereas, percentages vary within the 36–105 % range after UF with membranes of 10 kDa MWCO. This implies quantitative mass balances for some elements such as Mo, Sr, Ba and Rb. It can also be observed that recovered percentages are quite similar after both UF procedures for all studied elements except for Zn when using the 10 kDa MWCO membrane, or for Mn when using the 3 kDa MWCO membrane. Conclusions could not be made for other trace elements such as Al, Fe and Ni due to the existence of contamination throughout the procedure. The source of this contamination can be attributed to the UF membranes because high Al, Fe and Ni concentrations were found in the retentate and permeate fractions when using both 3 and 10 kDa MWCO membranes. Other trace elements such as Cd, Cr, Pb, Sb, Se and Sn were found at concentrations lower than the LODs of the method.

Table 6 lists the metal concentrations found in the SEC fractions as well as the percentages of metals bound to marine DOM, taking into account the total concentrations of the studied metals in the desalted surface seawater. Similar values were found after UF with both membranes. Regarding 3 kDa MWCO membranes, percentages account for 0.022 and 0.057 % for Mn and Zn, respectively, and

lower values were found for the remaining elements associated to the isolated marine DOM (2.0 10^{-3}, 3.2 10^{-3} and 4.1 10^{-4} % for Cu, Mo, and Sr, respectively). UF isolation with 10 kDa MWCO membranes gave percentages of 0.019 % for Mn, and 0.077 % for Zn; whereas, Mo and Sr percentages were 1.8 10^{-3} and 1.4 10^{-4} %, respectively.

4. Conclusions

Studies regarding the use of UF membranes of 3 and 10 kDa MWCO during marine DOM pre-concentration showed that both membranes offer a similar behavior in terms of marine DOM losses by adsorption onto the membranes (2.4 and 4.3 % for 3 and 10 kDa MWCO membranes, respectively), and also for trace elements bound to the isolated marine DOM. Mass balance studies for trace elements such as Mo, Sr, Ba and Rb have revealed recoveries close to 100 % when using either UF membranes; whereas, recoveries within the 34–61 % range were found for Cu, Mn and Zn. Results from SEC-UV experiments showed a group of organic substances comprising a variety of molecular weights ranging from 16.1 to 1.6 kDa (isolated material with 10 kDa MWCO membranes), and from 21.6 to 1.2 kDa (3 kDa MWCO membranes). Direct SEC-ICP-MS hyphenation showed that metals such as Mn, Mo, Sr and Zn form complexes with the isolated marine DOM. However, the presence of Cu-DOM complexes was only verified when ultrafiltrating with 3 kDa MWCO membranes. Percentages of metals bound to marine DOM were low, and they ranged from 4.1 10^{-4} and 1.4 10^{-4} % for Sr to 0.055 and 0.77 % for Zn. A further SEC fractions characterization by AEC-UV showed two main groups of organic substances for the isolated material with 3 kDa MWCO membranes. In contrast, organic substances contained in the SEC fraction after UF with 10 kDa MWCO membranes were separated in four different groups of substances after AEC. Dilutions made when coupling AEC with ICP-MS only allowed us to observe the presence of Sr. However, no specific co-elution with marine DOM monitored with UV detection was observed.

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References

[1] J.L. Sarmiento, Oceanic uptake of anthropogenic CO2: The major uncertainties, Global Biogeochem. Cy. 5 (1991) 309–313.

[2] J.L. Sarmiento, Ocean carbon-cycle, Chem. Eng. News 71 (1993) 30–43.

[3] D.A. Hansell, C.A. Carlson, Marine dissolved organic matter and the carbon cycle, Oceanography 14 (2001) 41–49.

[4] L.I. Aluwihare, D.L. Repeta, R.F. Chen, A major biopolymetric component to dissolved organic carbon in surface sea water, Nature 387 (1997) 166–169.

[5] K. Mopper, A. Stubbins, J.D. Ritchie, H.M. Bialk, P.G. Hatcher, Advanced instrumental approaches for characterization of marine dissolved organic matter: extraction techniques, mass spectrometry, and nuclear magnetic resonance spectroscopy, Chem. Rev. 107 (2007) 419–442.

[6] H. Ogawa, E. Tanoue, Dissolved organic matter in ocean Waters, J. Oceanogr. 59 (2003) 129–147.

[7] J.H. Park, Spectroscopic characterization of dissolved organic matter and its interactions with metals in surface waters using size exclusion chromatography, Chemosphere 77 (2009) 485–494.

[8] B.L. Nunn, A.T. Timperman, Marine proteomics, Mar. Ecol. Prog. Ser. 332 (2007) 281–289.

[9] J. Sánchez-González, N. García-Otero, A. Moreda-Piñeiro, P. Bermejo-Barrera, Multi-walled carbon nanotubes-solid phase extraction for isolating marine dissolved organic matter before characterization by size exclusion chromatography, Microchem. J. 102 (2012) 75–82.

[10] O. Gustafsson, K.O. Buesseler, P.M. Gschwend, On the integrity of cross-flow filtration for collecting marine organic colloids, Mar. Chem. 55 (1996) 93–111.

[11] M.J. Powell, J.N. Sutton, C.E. del Castillo, A.T. Timperman, Estuarine/marine UDOM as characterized by size-exclusion chromatography and organic mass spectrometry, Mar. Chem. 78 (2002) 75–102.

[12] M.J. Powell, J.N. Sutton, C.E. del Castillo, A.T. Timperman, Marine proteomics: generation of sequence tags for dissolved proteins in seawater using tandem mass spectrometry, Mar. Chem. 95 (2005) 183–198.

[13] E. Tanoue, Detection of dissolved protein molecules in oceanic waters, Mar. Chem. 51 (1995) 239–252.

[14] N. García-Otero, P. Bermejo-Barrera, A. Moreda-Piñeiro, Size exclusion and anion exchange high performance liquid chromatography for characterizing metals bound to marine dissolved organic matter, Anal. Chim. Acta 760 (2013) 83–92.

[15] M.J. Powell, A.T.Timperman, Quantitative analysis of protein recovery from dilute, large volume samples by tangential flow ultrafiltration, J. Memb. Sci. 252 (2005) 227–236.

[16] N. Yamada, E. Tanoue, Detection and partial characterization of dissolved glycoproteins in oceanic waters, Limnol. Oceanogr. 48 (2003) 1037–1048.

[17] S. Saijo, E. Tanoue, Characterization of particulate proteins in Pacific surface waters, Limnol. Oceanogr. 49 (2004) 953–963.

[18] N. Yamada, E. Tanoue, The inventory and chemical characterization of dissolved proteins in oceanic waters, Prog. Oceanogr. 69 (2006) 1–18.

[19] N. Yamada, E. Tanoue, Similarity of electrophoretic dissolved protein spectra from coastal to pelagic seawaters, J. Oceanogr. 65 (2009) 223–233.

[20] Y. Yamashita, E. Tanoue, Chemical characteristics of amino acid-containing dissolved organic matter in seawater, Org. Geochem. 35 (2004) 679–692.

[21] N. Hertkorn, R. Benner, M. Frommberger, P. Schmitt-Kopplin, M. Witt, K. Kaiser, A. Kettrup, J.L. Hedges, Characterization of a major refractory component of marine dissolved organic matter, Geochimica 70 (2006) 2990–3010.

[22] L. Guo, D. M. White, C. Xu, P. H. Santschi, Chemical and isotopic composition of high-molecular-weight dissolved organic matter from the Mississippi River plume, Mar. Chem. 114 (2009) 63–71.

[23] D. J. Repeta, T. M. Quan, L. I. Aluwihare, A. Accardi, Chemical characterization of high molecular weight dissolved organic matter in fresh and marine waters, Geochim. Cosmochim. Ac. 66 (2002) 955–962.

[24] L. I. Aluwihare, D. J. Repeta, R. F. Chen, Chemical composition and cycling of dissolved organic matter in the Mid-Atlantic Bight, Deep-Sea Research II 49 (2002) 4421–4437.

[25] J.-P. Simjouw, E.C. Minor, K. Mopper, Isolation and characterization of estuarine dissolved organic matter: comparison of C18 solid phase extraction and ultrafiltration techniques, Mar. Chem. 96 (2005) 219–235.

[26] D. Schmitt, M. B. Müller, F. H. Frimmel, Metal distribution in different size fractions of natural organic matter, Acta Hydrochim. Hydrobiol. 28 (2000) 400–410.

[27] L. Rottmann, K.G. Heumann, Determination of heavy metal interactions with dissolved organic materials in natural aquatic systems by coupling a high-performance liquid chromatography system with an inductively coupled mass spectrometer, Anal. Chem. 66 (1994) 3709–3715.

Table 1. Operating ICP–MS conditions

General		
	Radiofrequency power / W	1400
	Sample uptake rate / r.p.m.	3.0
	Stabilization delay / s	35
	Number of replicates	3
Nebulizer type	Beat impact (cooled spray chamber)	
Gas flows / L min^{-1}	Plasma	13.0
	Auxiliary	0.80
	Nebulizer	0.90
Ion optics / V		
	Extraction	-125
	Lens 1	-1000
	Lens 2	-80
	Lens 3	-195.3
	Hexapole Bias	-4.0
	Pole Bias	0.2
	D1	-40.8
	D2	-140
	Hexapole Bias	-4.0
	Pole Bias	0.2
Torch alignment / mm		
	Horizontal	80
	Vertical	405
	Sampling death	150
Mass-to-ratio		
	^{51}V, ^{52}Cr, ^{55}Mn, ^{57}Fe, ^{58}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{77}Se, ^{88}Sr, ^{98}Mo, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{127}Al, ^{137}Ba	
	Internal standards	
	^{54}Sc, ^{72}Ge, ^{89}Y, ^{115}In	

Table 2. Limits of detection and quantification for the different studied samples

	SEC procedure (ng L^{-1})[a]		Seawater and permeate fraction (ng L^{-1})[b]		Retentate fraction (μg L^{-1})	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Cu	1.3	4.5	0.20	0.67	1.0	3.4
Mn	0.38	1.3	0.057	0.19	0.29	0.95
Mo	0.30	1.0	0.045	0.15	0.23	0.75
Sr	0.13	0.45	0.019	0.067	0.095	0.34
Zn	6.9	23	1.04	3.45	5.2	17
As	0.73	2.7	0.11	0.40	0.55	2.0
Ba	0.13	0.45	0.020	0.067	0.10	0.34
Rb	0.067	0.22	0.010	0.033	0.05	0.17
V	0.02	0.067	0.0030	0.010	0.015	0.050

(a) Pre-concentration factor 150; (b) pre-concentration factor 200

Table 3. SEC/AEC-UV/ICP-MS operating conditions

SEC	TSK-G4000 SW_{XL} (30 cm × 8 mm I.D.) coupled to a TSK-gel SW glass guard column (10 cm × 8 mm I.D.)	
	Injection volume (µL)	50
	Column temperature (°C)	25
	Mobile phases flow rate /mL min^{-1}	1.00
Mobile phase	25mM/25mM ammonium sulphate/diammonium hydrogen phosphate, pH 6.5	
	UV detection (nm)	205
AEC	PRP-X100 (250 mm × 4.1 mm I.D.) to a PRP-X100 guard column (25 × 2.3 mm I.D.)	
	Injection volume (µL)	50
	Column temperature (°C)	25
	Mobile phases flow rate /mL min^{-1}	1.00
Mobile phase	25mM/25mM ammonium sulphate/diammonium hydrogen phosphate, pH 6.5	
	UV detection (nm)	205
ICP-MS		
	Radiofrequency power / W	1400
	Peristaltic pump speed / r.p.m.	2.5
Nebulizer type	Beat impact (cooled spray chamber)	
Gas flows / L min^{-1}		
	Plasma	13.0
	Auxiliary	0.8
	Nebulizer	0.90
Ion optics / V		
	Extraction	-125
	Lens 1	-1000
	Lens 2	-80
	Lens 3	-195.3
	Focus	11
	D1	-40.8
	D2	-140
	Pole Bias	0.2
	Hexapole Bias	-4
Torch alignment / mm		
	Horizontal	80
	Vertical	405
	Sampling death	150
Mass-to-ratio		
	^{52}Cr, ^{55}Mn, ^{57}Fe, ^{58}Ni, ^{63}Cu, ^{66}Zn, ^{75}As, ^{77}Se, ^{88}Sr, ^{98}Mo, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{127}Al, ^{137}Ba	

Table 4. Molecular weights obtained for the chromatographic peaks separated by SEC for the retentate fractions after UF with 3 kDa (R3) and 10 kDa (R10) MWCO membranes.

		t_R (min)	PEGs (kDa)	Globular Proteins (kDa)
R3	FI	11.7-12.3	10.8-7.1	21.6-9.1
	FII	12.3-13.7	7.1-2.7	9.1-1.2
R10		11.9-13.5	9.4-3.1	16.1-1.6

Table 5. Metal concentrations (n = 3) in surface seawater and the permeate fraction after AG501-X8 resin desalting, and in the retentate fractions after AG501-X8 resin and HI Trap desalting procedures.

	Concentration (μg L^{-1})								
		UF 3 kDa MWCO			UF 10 kDa MWCO				
		Permeate fraction[a]	Retentate fraction[b]		Permeate fraction[a]	Retentate fraction[b]			
			AG 501-X8 exchange resin	HI Trap desalting		AG 501-X8 exchange resin	HI Trap desalting	Percentage (%)[c]	
	Surface seawater							3 kDa MWCO	10 kDa MWCO
Cu	239 ± 6	148 ± 4	1.3 ± 0.2	0.07 ± 0.002	134 ± 6	1.2 ± 0.09	48 ± 0.4[d]	53 ± 2	48 ± 1
Mn	1.6 ± 0.2	0.52 ± 0.02	4.4 ± 0.8[d]	19 ± 0.1 [d]	0.77 ± 0.12	10 ± 0.9 [d]	4.2 ± 0.1[d]	34 ± 2	50 ± 3
Mo	13 ± 0.2	12 ± 0.1	71 ± 1[d]	0.67 ± 0.2[d]	13 ± 0.2	59 ± 0.8 [d]	2.9 ± 0.01[d]	93 ± 1	101 ± 2
Sr	9261 ± 44	8707 ± 91	52 ± 0.5	2.7 ± 0.01	9535 ± 79	44 ± 0.2	1.4 ± 0.01	95 ± 3	103 ± 1
Zn	102 ± 4	62 ± 7	0.26 ± 0.02	0.40 ± 0.004	36 ± 1	0.42 ± 0.02	0.23 ± 0.004	61 ± 2	36 ± 2
As	3.9 ± 0.1	2.43 ± 0.17	21 ± 1[d]	7.4 ± 0.4[d]	2.99 ± 0.30	18 ± 1 [d]	6.0 ± 0.3[d]	64 ± 3	78 ± 3
Ba	4.2 ± 0.06	3.7 ± 0.27	22 ± 2[d]	37 ± 1[d]	4.1 ± 0.21	19 ± 1 [d]	33 ± 1[d]	88 ± 4	96 ± 4
Rb	103 ± 1	97 ± 2	0.55 ± 0.004	6.8 ± 0.05[d]	108 ± 2	0.49 ± 0.01	3.4 ± 0.02[d]	95 ± 2	105 ± 3
V	5.7 ± 0.3	4.1 ± 0.03	27 ± 2[d]	1.1 ± 0.1 [d]	4.6 ± 0.3	26 ± 2[d]	1.0 ± 0.1[d]	72 ± 3	81 ± 2

(a) dilution factor of 0.995; (b) pre-concentration factor of 200; (c) percentage calculated as the sum of metal concentrations in the permeate and retentate fractions (AG 501-X8 desalting procedure) with respect to the metal concentration in the untreated seawater; (d) expressed in ng L^{-1}

Table 6. Metal concentrations (n = 3) in surface seawater and the permeate fraction after AG501-X8 resin desalting, and in the retentate fractions after AG501-X8 resin and HI Trap desalting procedures.

		SEC fraction (ng L^{-1})[a]		Percentage of metal bound to marine DOM (%)[b]	
	Surface seawater (μg L^{-1})	UF 3 kDa MWCO	UF 10 kDa MWCO	UF 3 kDa MWCO	UF 10 kDa MWCO
Cu	239 ± 6	5.8 ± 0.1	< 1.3	$2.0\ 10^{-3}$	----
Mn	1.6 ± 0.2	0.35 ± 0.01	0.3 ± 0.01	0.022	0.019
Mo	13 ± 0.2	0.42 ± 0.03	3.1 ± 0.1	$3.2\ 10^{-3}$	$1.8\ 10^{-3}$
Sr	9261 ± 44	38 ± 0.1	13 ± 0.1	$4.1\ 10^{-4}$	$1.4\ 10^{-4}$
Zn	102 ± 4	57 ± 4	79 ± 1	0.057	0.077

(a) pre-concentration factor of 200; (b) percentage calculated as the metal concentrations in SEC fraction after desalting the retentate by the HI Trap desalting procedure respect to the metal concentration in the untreated seawater

Figure captions

Figure 1. Sampling and analytical scheme used for marine DOM characterization

Figure 2. SEC chromatograms (UV detection at 205 nm) of marine DOM isolated by tangential UF with 3 and 10 kDa MWCO membranes.

Figure 3. AEC chromatograms (UV detection at 205 nm) of SEC fractions isolated by tangential UF with 3 (a) and 10 kDa (b) MWCO membranes.

Figure 4. SEC chromatograms (UV and ICP-MS detection) of marine DOM isolated by tangential UF with 3 (R3 kDa) and 10 kDa (R10 kDa) MWCO membranes.

Figure 5. AEC chromatograms (UV and ICP-MS detection for Sr) of marine DOM fractionated by SEC and isolated by tangential UF with 3 (R3 FI and R3 FII) and 10 kDa (R10) MWCO membranes.

Figure 1

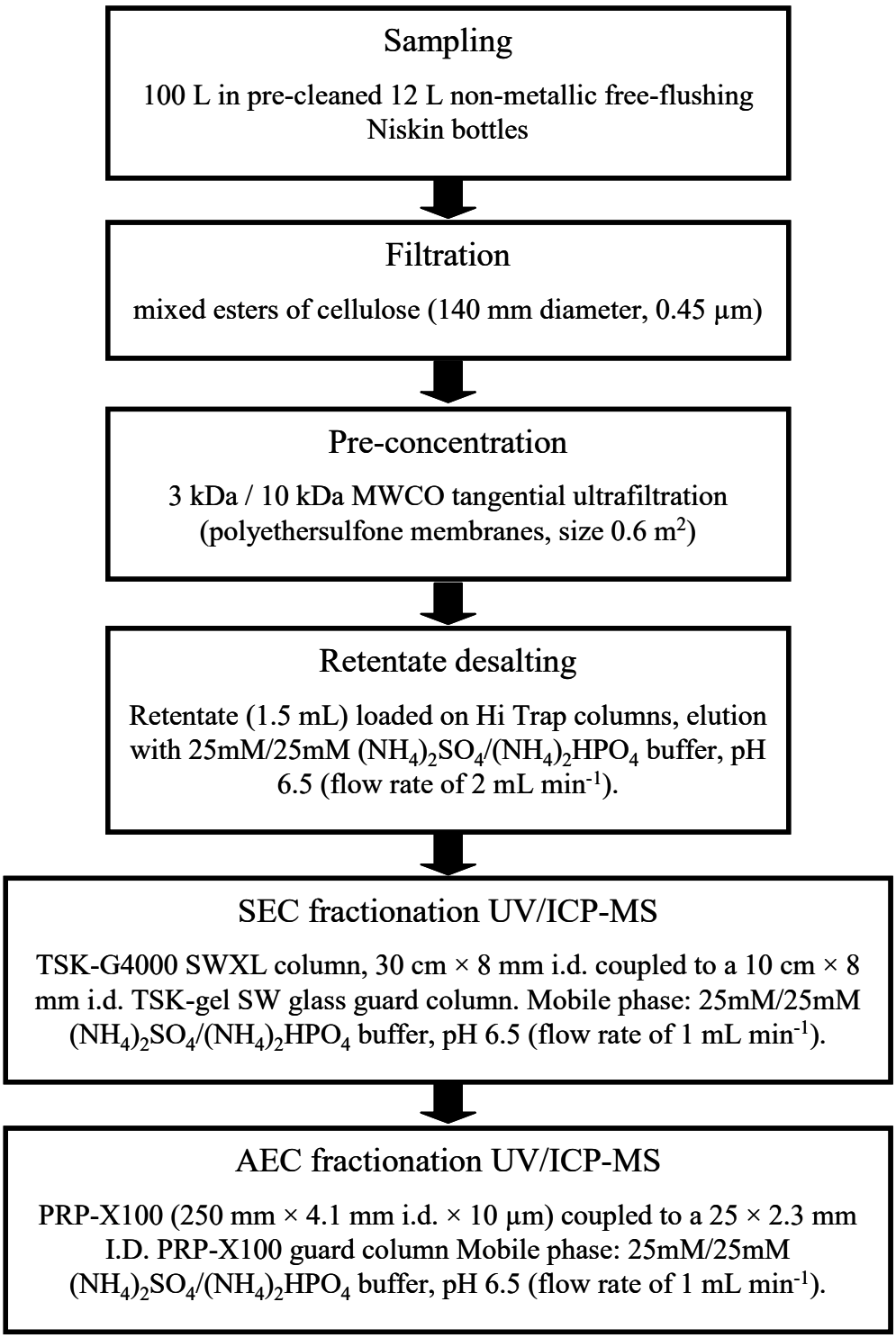

Sampling
100 L in pre-cleaned 12 L non-metallic free-flushing Niskin bottles
Filtration
mixed esters of cellulose (140 mm diameter, 0.45 µm)
Pre-concentration
3 kDa / 10 kDa MWCO tangential ultrafiltration (polyethersulfone membranes, size 0.6 m2)
Retentate desalting
Retentate (1.5 mL) loaded on Hi Trap columns, elution with 25mM/25mM (NH4)2SO4/(NH4)2HPO4 buffer, pH 6.5 (flow rate of 2 mL min-1).
SEC fractionation UV/ICP-MS
TSK-G4000 SWXL column, 30 cm × 8 mm i.d. coupled to a 10 cm × 8 mm i.d. TSK-gel SW glass guard column. Mobile phase: 25mM/25mM (NH4)2SO4/(NH4)2HPO4 buffer, pH 6.5 (flow rate of 1 mL min-1).
AEC fractionation UV/ICP-MS
PRP-X100 (250 mm × 4.1 mm i.d. × 10 µm) coupled to a 25 × 2.3 mm I.D. PRP-X100 guard column Mobile phase: 25mM/25mM (NH4)2SO4/(NH4)2HPO4 buffer, pH 6.5 (flow rate of 1 mL min-1).

Figure 2

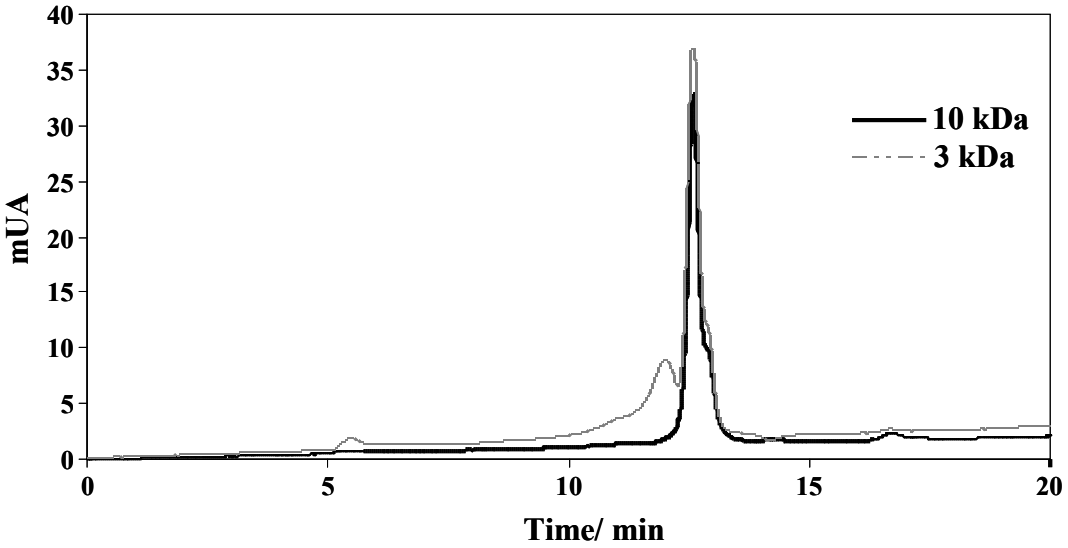


Figure 3

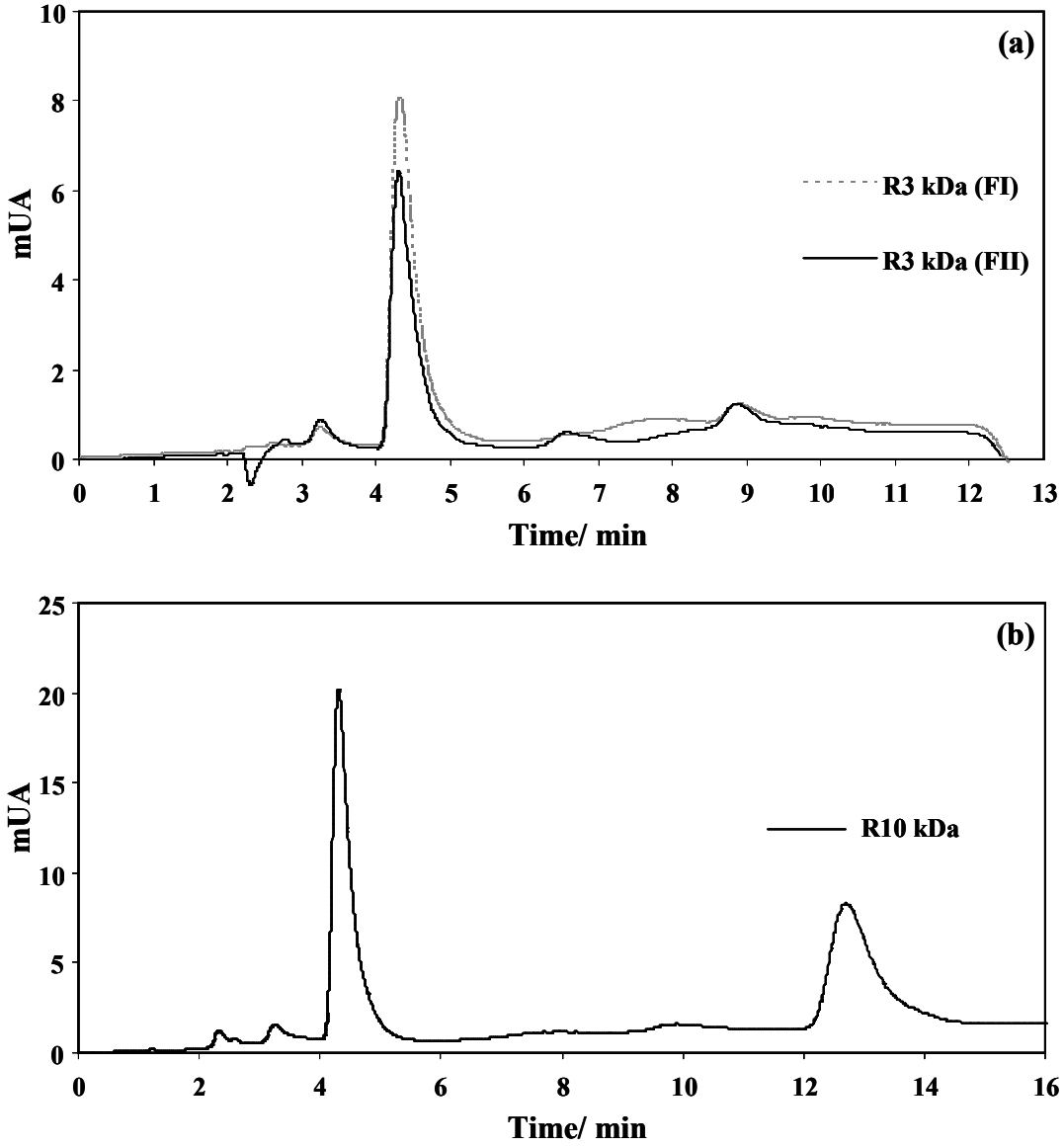
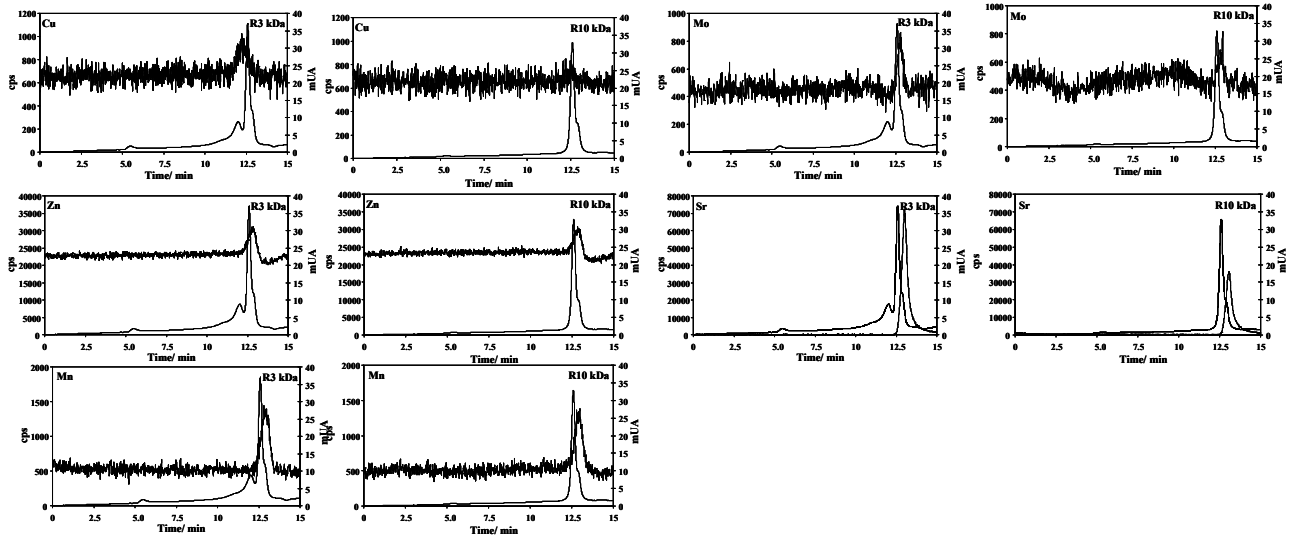


Figure 4


Cu
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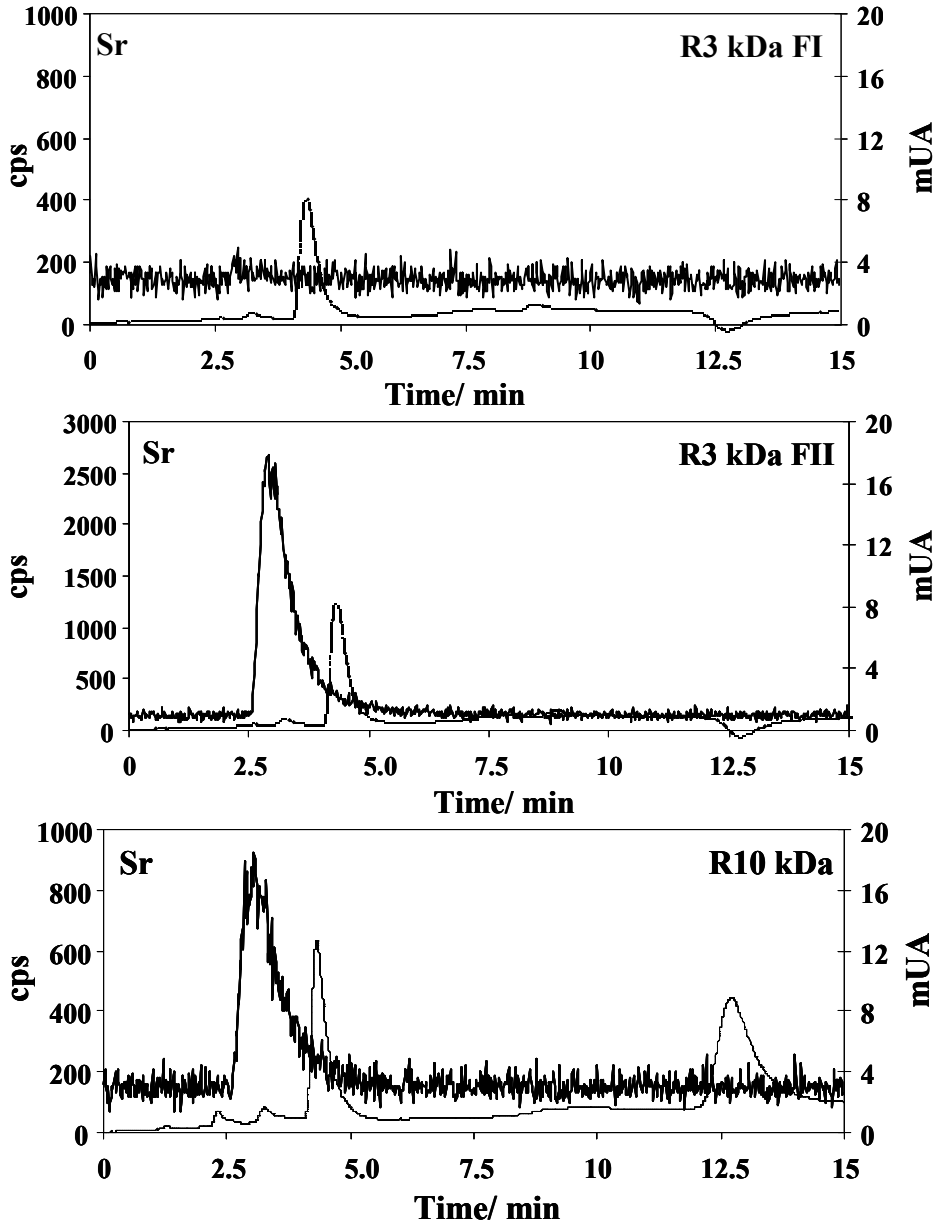
Cu
R10 kDa
Mo
R3 kDa
Mo
R10 kDa
Zn
R3 kDa
Zn
R10 kDa
Sr
R3 kDa
Sr
R10 kDa
Mn
R3 kDa
Mn
R10 kDa
cps
mUA
Time/ min

Figure 5


Sr
R3 kDa FI
cps
mUA
Time/ min
Sr
R3 kDa FII
cps
mUA
Time/ min
Sr
R10 kDa
cps
mUA
Time/ min