

# Quality control considerations for size exclusion chromatography with online ICP-MS: a powerful tool for evaluating the size dependence of metal–organic matter complexation

Erica R. McKenzie and Thomas M. Young

## ABSTRACT

Size exclusion chromatography (SEC), which separates molecules based on molecular volume, can be coupled with online inductively coupled plasma mass spectrometry (ICP-MS) to explore size-dependent metal–natural organic matter (NOM) complexation. To make effective use of this analytical dual detector system, the operator should be mindful of quality control measures. Al, Cr, Fe, Se, and Sn all exhibited columnless attenuation, which indicated unintended interactions with system components. Based on signal-to-noise ratio and peak reproducibility between duplicate analyses of environmental samples, consistent peak time and height were observed for Mg, Cl, Mn, Cu, Br, and Pb. Al, V, Fe, Co, Ni, Zn, Se, Cd, Sn, and Sb were less consistent overall, but produced consistent measurements in select samples. Ultrafiltering and centrifuging produced similar peak distributions, but glass fiber filtration produced more high molecular weight (MW) peaks. Storage in glass also produced more high MW peaks than did plastic bottles.

**Key words** | ICP-MS, molecular weight, organic matter, sample processing, size exclusion chromatography

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## INTRODUCTION

Size exclusion chromatography (SEC) is a liquid chromatography technique that separates molecules based on molecular volume, and is said to be ‘the premier method for determining the molar mass averages and distribution of polydisperse macromolecules’ (Striegel 2008). SEC has been employed to determine constituent size distribution in diverse fields including polymer sciences (e.g. Puskas *et al.* 2006; Castignolles *et al.* 2009), biochemistry (e.g. Lobinski *et al.* 2006; Ziegler & Zaia 2006), chemistry (e.g. Wang *et al.* 2009; Piel *et al.* 2011), and environmental science (e.g. Cabaniss *et al.* 2000). The application of SEC has included a broad range of constituent molecular weights (MW) from molecules of <1 kDa up to large macromolecules >10<sup>3</sup> kDa (Striegel 2008) and even engineered nanoparticles (Wang *et al.* 2009). In environmental sciences it has frequently been applied to analysis of natural organic matter (NOM; e.g. Cabaniss *et al.* 2000; Her *et al.* 2002a). NOM is a heterogeneous collection of macromolecules that range in size, composition, and

functional groups, and the combination affects the fate and transport of NOM, and its propensity to bind metals or hydrophobic organic pollutants. The heterogeneous nature of NOM makes its characterization a challenge but SEC offers the ability to measure a size continuum. SEC results have been found to be highly dependent on the chromatography system, the standards employed, and the data processing approach (Zhou *et al.* 2000); owing to these discrepancies, SEC is widely described as semi-quantitative. Though SEC analysis has often employed UV-visible absorbance detection ( $\lambda = 254$  nm; Zhou *et al.* 2000), analytical power has been increased via online coupling with other techniques including fluorescence (Huber & Frimmel 1992; Her *et al.* 2003), total organic carbon (Her *et al.* 2002b), and inductively coupled plasma mass spectrometry (ICP-MS; Schmitt *et al.* 2001; Casartelli & Miekeley 2003; Lobinski *et al.* 2006), where the latter (ICP-MS) provides information about the trace elements associated with the NOM.

The distribution of metals in the natural and engineered systems is an area of interest, as the distribution has implications for the fate, transport, and effects of the metals. Metals have been observed to negatively impact aquatic ecosystems (USEPA 2011). Dissolved metals and metals associated with low MW NOM are considered more bioavailable (Cabaniss *et al.* 2000; Di Toro *et al.* 2001), and therefore more likely to result in toxicity. Therefore, the MW distribution of metal associations has been studied in natural (Schmitt *et al.* 2001) and engineered systems (Worms *et al.* 2010).

Aromatic organic carbon and co-eluting metals can be detected with dual online detectors – UV-visible absorbance ( $\lambda = 254$  nm) and ICP-MS, respectively. Conventional sample collection and processing wisdom prescribes different practices for the analysis of organics compared with metals. Chief among these differences are that metal or glass instruments/containers are typically employed when trace organics/NOM are the constituents of interest, while plastic is typically used when metals are the constituents of interest. In each case, the material is selected to minimize sources and sinks for the constituents of interest. Additional challenges arise when both organics and metals are to be analyzed in a single sample.

The objective of this work is to investigate quality control factors in an SEC system with dual UV-visible absorbance and online ICP-MS detectors (SEC-ICP-MS), and to consider how sample processing will influence SEC-ICP-MS results.

## METHODS

Metal complexation dependence on NOM MW was investigated using SEC with dual online detectors: diode array detector (DAD; analysis of absorbing organics) and ICP-MS (analysis of metals). SEC analysis was conducted using an Agilent 1200 HPLC (high performance liquid chromatography) system and a Phenomenex BioSep s2000 column (300 × 4.6 mm). The mobile phase included a phosphate buffer (4 mmol/L  $\text{NaH}_2\text{PO}_4$ ) with 86 mmol/L  $\text{NH}_4\text{NO}_3$  for additional ionic strength (0.1 total ionic strength); the mobile phase pH was adjusted to 7.0 and it was pumped at 0.8 mL/min. DAD responses were recorded for  $\lambda = 210, 230, 254,$  and  $280$  nm; all presented DAD data are for response at 254 nm, as recommended by Zhou *et al.* (2000). Sodium polystyrene sulfonate standards (PSS; 18, 8, 4.6, and 1 kDa) as well as salicylic acid (138 Da), were included as MW standards with charge and coiling properties similar to NOM (Zhou *et al.* 2000). Zhou *et al.* (2000)

also advocated for the inclusion of acetone as a low MW standard; in this study, acetone's elution time was concentration dependent and it was therefore not included.

ICP-MS analysis was conducted on an Agilent 7500i, and the following elements were analyzed (m/z monitored): C (13), Mg (24), Al (27), Cl (35), V (51), Cr (52, 53), Mn (55), Fe (57), Co (59), Ni (60), Cu (63, 65), Zn (64, 66), As (75), Se (77, 82), Br (79), Cd (111), Sn (118), Sb (121), and Pb (208). Pd (105) and Pt (195) were previously investigated and no peaks were observed. The column eluent was divided after the DAD, prior to the ICP-MS, so that 0.4 mL/min was provided for direct online analysis by the ICP-MS analysis, and the other 0.4 mL/min was wasted. Prior to SEC analysis, SPEX 2A multi-element standard solutions (27 elements with 13 concentrations 0.01–100  $\mu\text{g/L}$ ) were ICP-MS analyzed to determine the standard curve slope. The SPEX 2A multi-element standard was selected because it included most environmentally relevant elements, but it did not include C, Cl, Br, Sn, and Sb. These five elements were not included in the comparison of detected concentration, but were retained for discussion of peak characteristics (columnless attenuation and peak signal:noise).

To avoid clogging the column, particulates were removed either via flow through centrifuge (FTC; calculated particle size cutoff 0.3  $\mu\text{m}$  assuming a density of 2.0  $\text{g cm}^{-3}$ ), ultrafiltration (UF; Pall, Microza Polysulfone, 10 kDa) or glass fiber filter (GFF; 0.7  $\mu\text{m}$ ). Conventional ICP-MS analysis requires sample acidification (e.g. EPA method 6020) for metals' extraction (solid dissolution or proton substitution). This is a less aggressive approach compared with sample digestion (e.g. hydrofluoric acid digestion). In this study, it was desired that metals remain complexed, so acidification wasn't warranted. Previous research has indicated that sample ionic strength and pH should be similar to the mobile phase (Her *et al.* 2002a). Therefore, following particle removal, sample ionic strength and solution chemistry were adjusted to closely match those of the mobile phase by adding the appropriate volume of 10x mobile phase stock; sample pH was adjusted to 7.0.

Samples were analyzed in duplicate; for each sample, 1,800 absorbance data points (per wavelength) and 330 elemental concentration data points (per element) were collected. A small solvent peak/dip was observed in the DAD data for MW ~1,500–2,400 Da, making this MW range less sensitive for the detection of organic matter (OM), however detected peaks were typically much larger than the solvent peak/dip; this region of the DAD chromatogram was linearly interpolated to overcome the solvent peak/dip. ICP-MS data were observed to be fairly noisy, especially for elements that

were present in low concentrations; a five-point moving average was used to smooth elemental chromatograms. The results were baseline corrected (least squares linear fit) to improve consistency (Zhou *et al.* 2000) and the results of the duplicate runs were averaged. ICP-MS counts per second data were converted to concentration ( $\mu\text{g/L}$ ) using slopes derived from the analysis of bulk multi-element standards. As per Zhou *et al.* (2000), peak lower MW cutoffs were set to 2% peak height or 100 Da, and high MW cutoffs were set to 1% peak height or 300 kDa.

## RESULTS AND DISCUSSION

### Sample collection and dissolved concentration

Sample collection, handling, and storage are detailed elsewhere (McKenzie 2012). In brief, eight storm-event samples were collected from four associated land uses as follows: unimpacted river ( $n = 1$ ), highway stormwater runoff ( $n = 3$ ), urban stream ( $n = 1$ ), and agricultural drainage ( $n = 3$ ). Anthropogenically impacted areas (save the unimpacted river) and larger storm events (total precipitation  $>6.4$  mm) were targeted; sample composition and constituent concentrations likely reflect these sampling decisions. Anthropogenic metal sources were indicated, particularly for highway and urban samples. Sample composition and constituent concentrations likely impact the performance of the SEC-ICP-MS system, particularly for constituents that were present at low concentrations. Centrifuged water OM concentrations ranged 12.0–36.7 mg OM/L (as measured by ‘loss on ignition’ method), of which 67–90% was included in the 10 kDa UF permeate. Elemental concentration ranges are presented in Table 1. Several of the elements listed in Table 1 have minimum detected concentrations that are below the limit of detection (LOD; seven blanks,  $\mu + 3\sigma$ ) including values listed as 0.00  $\mu\text{g/L}$ . SPEX 2A regression curves (minimum concentration 0.01  $\mu\text{g/L}$ ) were not forced through zero. Therefore, ‘negative concentrations,’ based on the fit regression, were possible and these were set to zero. Standards for C, Cl, Br, Sn, and Sb were not evaluated, so a quantitative evaluation of the bulk concentration of these elements could not be made and they are not included in Table 1.

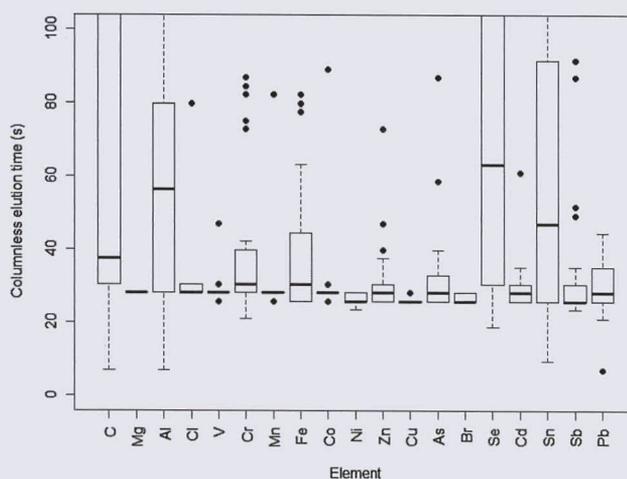
### Signal attenuation in a columnless system

In an ideal chromatography system, constituents only interact with the stationary phase, and not with any other system

**Table 1** | Constituent concentration of sample 0.45  $\mu\text{m}$  filtrate

Element	Concentration ( $\mu\text{g/L}$ )		
	LOD	Minimum	Maximum
Al	0.61	1.56	263.73
As	0.05	0.32	7.72
Cd	0.02	0.00	0.09
Co	0.12	0.14	17.82
Cr	0.44	0.00	2.62
Cu	0.74	0.33	14.06
Fe	3.17	0.00	155.42
Mg	0.43	0.00	34,160
Mn	0.06	2.63	58.01
Ni	0.14	0.76	4.63
Pb	0.03	0.03	91.54
Se	0.81	0.25	1.95
V	0.02	0.00	9.85
Zn	2.67	0.03	60.44

component. To determine whether unwanted interactions occurred in the system, samples were injected sans column, and their ‘elution’ was monitored. The distribution of the elemental columnless peak elution times is shown in Figure 1. Peak attenuation in a columnless configuration indicated that a constituent interacted with the non-column system components. The interactions appear to be at least in part reversible. In the case of SEC, this might bias the measured MW downward. C, Al, Cr, Fe, Se, and



**Figure 1** | Columnless elution distribution shown as a box and whisker plot ( $n = 32$ ), where the box delineates the interquartile range (IQR; 25<sup>th</sup> and 75<sup>th</sup> percentiles), the bolded bar delineates the median value, and whiskers extend to maximum or minimum values exclusive of extreme points which are defined as those that are more 1.5\*IQR from the IQR boundary.

Sn were all periodically retarded in the columnless system – with the SEC column in place, these elements may co-elute with smaller molecules. The current study was not able to determine what caused these elements to be inconsistently retarded, however the implications of this retardation should be kept in mind as the elemental–macromolecular size distribution is measured. Furthermore, the analysis reveals that environmental constituents exhibit non-specific interactions with the non-column components of a liquid chromatography system, which has implications for all NOM liquid chromatography analyses.

### Control and duplicate analysis

Buffer controls and ‘injectionless’ samples did not display any obvious elemental peaks. Duplicate sample analyses varied by the element. As shown in Table 2, all elements

**Table 2** | Evaluation of element quality control measures

Element	Signal:noise <sup>a</sup>	Duplicate analysis	
		Peak timing	Peak height
C	13	Poor	Poor
<b>Mg</b>	2394	Good	Good
Al	22	Mediocre	Mediocre
Cl	224	Good	Good
V	25	Good	Mediocre
Cr (52)	27	Poor	Poor
Cr (53)	15	Poor	Poor
<b>Mn</b>	49	Good	Good
Fe	16	Poor	Mediocre
Co	28	Mediocre	Mediocre
Ni	34	Mediocre	Mediocre
Cu (63)	20	Poor	Poor
Zn (64)	569	Poor	Poor
<b>Cu (65)</b>	47	Good	Good
Zn (66)	21	Mediocre	Mediocre
As	17	Poor	Poor
Se (77)	7	Poor	Mediocre
<b>Br</b>	38	Good	Good
Se (82)	6	Poor	Mediocre
Cd	18	Good	Mediocre
Sn	13	Poor	Mediocre
Sb	17	Mediocre	Mediocre
<b>Pb</b>	185	Good	Good

<sup>a</sup>Average P(95)/P(20).

Bold values indicate comparatively strong performance.

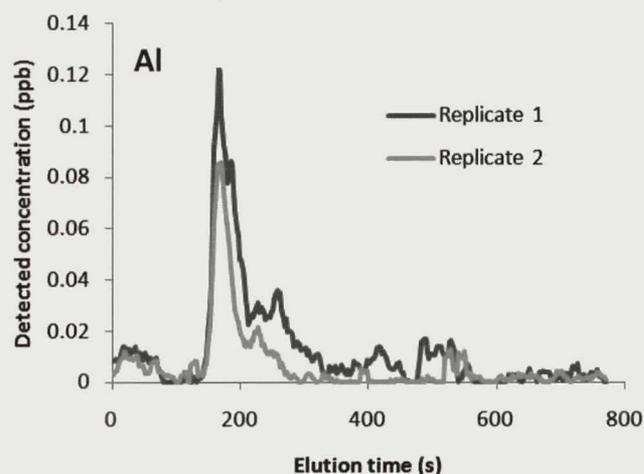
were evaluated based on three criteria: signal-to-noise ratio; reproducibility of peak chromatogram location; reproducibility of peak response (height). The latter two metrics were evaluated qualitatively compared to all elements included in the study. Peak placement was more reproducible than peak height.

Elements such as Mg, which had isolated clearly defined peaks with minimal noise, had very consistent peak elution times (typically identical), though peak height varied noticeably (~20%). A sample chromatogram of replicates is shown in Figure 2. Mg, Cl, and Br were the most consistent, followed by Mn, Cu, Pb, Al, V, Ni, and Cd. Some elements such as Al, Cd, and Ni were consistent in their elution timing, but less consistent in peak height.

Separation by SEC requires that down-column movement of smaller molecules be retarded due to interactions with the stationary phase, while larger molecules proceed down-column more quickly. As a result, larger molecules elute earlier, whereas smaller molecules spend more time interacting with the column, which introduces a great potential for variability and dilution. No obvious size effects on replicate analysis were observed. A strong and consistent peak is expected when an element is consistently bound by a group of molecules that are narrowly distributed in terms of composition, which reflects not only the molecular size but also likely the functional sites, charge density, and hydrophobicity.

### Total detected signal

To assess the ability of the system to produce quantitative results, identified peak concentration was compared to



**Figure 2** | Sample chromatogram for duplicates (Al from an urban creek; centrifuged and stored in plastic).

several other measures via linear regression. All concentrations were log transformed to prevent undue impact of higher concentration data points. The results of these regression analyses are shown in Table 3. Working backwards, the data processing program was validated by comparing total peak area (limited to areas of the chromatogram identified as peaks) with total area (all chromatogram areas), which produced strong linear relationships. Elements with low signal:noise ratio, most notably Cr, were less strongly related, which was to be expected. When the total area-detected peak area relationship was restricted to UF samples and elements with associated standards, the slope ( $m$ ) and  $R^2$  were both near unity ( $m = 1.01$  and  $R^2 = 0.8686$ ).

Further linear regression analyses included the following variables: identified SEC peak, columnless peak, and matching sample bulk concentration. In each of these comparisons, a perfect fit would be demonstrated by all points lying on the 1:1 line (i.e., slope  $m = 1$  and  $R^2 = 1$ ). In all cases, sample points were fairly broadly spread and were observed on both sides of the perfect fit line (e.g. Figure 3). While possible contributing factors have been identified in Table 3, it is likely that the low environmental concentrations (signal) contributed to the poor regression results reflected in Table 3. In nearly all cases the fit was improved by limiting the samples to UF permeate samples, indicating that the larger macromolecules/colloids are less regularly eluted, which is potentially due to either permanent sorption or mechanical screening. Additionally, in all cases, the fits were improved by excluding the 0-value bulk sample data points, indicating that the regression was compromised by the very low environmental concentrations observed in some cases.

The SEC identified peak was compared to the columnless injection, and moderate agreement was observed, but the relationship was improved when analysis was restricted to UF samples ( $m = 0.91$ ,  $R^2 = 0.71$ ) The results of this

regression indicate that the sample constituents are not only being reversibly sorbed by the non-column components, but they are also being screened or irreversibly bound by the column. SEC peak areas–columnless areas for individual elements were typically poorly related ( $R^2 < 0.5$ ), save Br and Mg, which demonstrated moderate agreement ( $R^2 \sim 0.75$ ).

SEC identified peak areas and columnless areas were also compared to bulk element concentration, and the results are shown in Figure 3; a 1:1 line is included for comparison. Columnless peak–bulk concentration resulted in a weak to moderate relationship ( $R^2 \sim 0.33$ – $0.6$ ), with similar slope values. In this case, the fit did not improve by restricting regression to UF permeate samples, indicating that molecular size did not play an obvious role in constituent adsorption to non-column components. As previously mentioned, calculated bulk concentrations resulted in 0-value concentrations, which are graphically displayed as 0.001  $\mu\text{g/L}$  (Figure 3). When the SEC-ICP-MS results were converted from counts per second (ICP-MS raw data) to concentration, only the regressed slope was used and the chromatogram was baseline corrected. Inclusion of the intercept for processing the SEC-ICP-MS data was explored, however this resulted in too many 0-value time points (or ‘signal-less’ chromatograms), even when obvious peaks were present in the raw data. The difference in the inclusion of an intercept is likely partially responsible for the discrepancies that are observable in Figure 3.

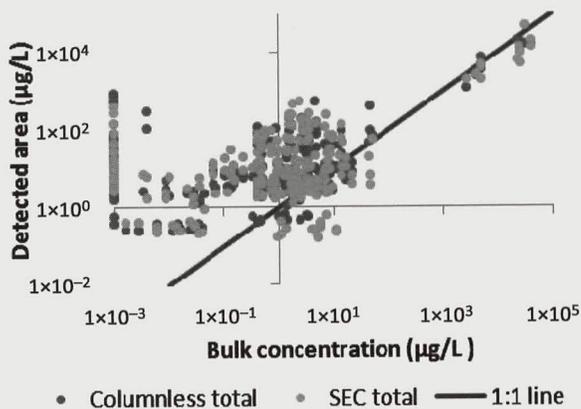
### Sample processing and storage

Storm event samples were processed with a FTC, UF, or GFF and were stored in polyethylene or glass containers and SEC-ICP-MS was used to evaluate the effect of sample processing and storage on the detected absorbance (i.e. OM) and elemental peaks. For SEC analysis purposes, all samples were transferred to glass autosampler vials. It

**Table 3** | Linear regression analyses for various sample and data processing steps

Independent variable (x) <sup>a</sup>	Dependent variable (y) <sup>a</sup>	Potential interference	All samples		UF samples	
			Slope	R <sup>2</sup>	Slope	R <sup>2</sup>
SEC total area	SEC identified peak	Poor peak identification	1.01	0.86	1.03	0.89
Columnless peak	SEC identified peak	Absorption/screening by column resin	0.81	0.60	0.91	0.71
Bulk concentration	Columnless peak	Absorption/screening by non-column elements	0.35	0.31	0.33	0.28
Bulk concentration. (no 0-values)	Columnless peak		0.58	0.57	0.57	0.56
Bulk concentration	SEC identified peak	Sinks/sources across entire analysis	0.28	0.18	0.34	0.25
Bulk concentration. (no 0-values)	SEC identified peak		0.52	0.40	0.60	0.50

<sup>a</sup>All variables are analyzed for log–log transformed linear regression (i.e.  $\log(y) \propto \log(x)$ ).



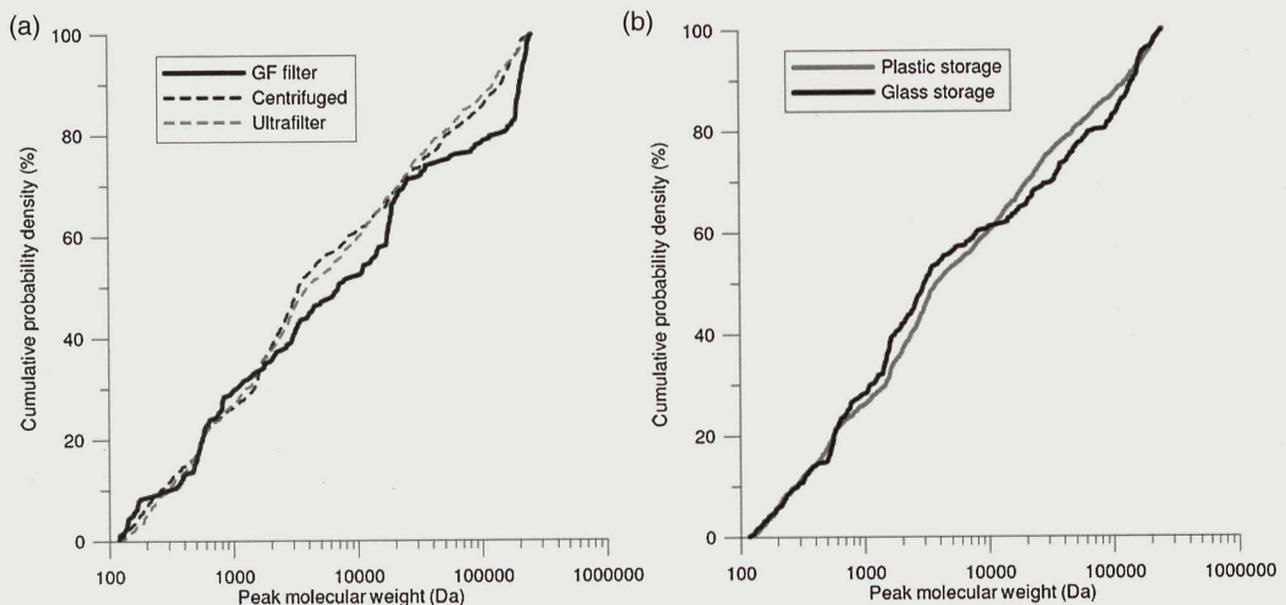
**Figure 3** | Relationship between bulk concentration and detected areas with/without SEC column.

is important to recognize that a given sample processing or storage approach could serve as a source or a sink for a constituent (OM or a metal), and that not all constituents will behave the same way.

Some sample pre-processing is required (removal of particulates); filters were assessed as potential sources of OM and metals by filtering double-deionized 18 MΩ water and then analyzing with SEC-ICP-MS. GFF filtered elemental chromatograms included a number of distinct elemental peaks (including Al, Cr, Fe, Ni, Cu, Zn, Cd, and Pb; generally observed ~1.4 kDa) indicating metal contamination. When the GFF was pre-washed with 0.1 mol/L acid, the metal contamination was virtually eliminated, save Ni.

Polyvinylidene difluoride (PVDF) (0.45 µm nominal pore size) filters were also tested, though were not used in this study, and were found to not produce metal contamination peaks. Based on these results, a PVDF filter should be considered superior to GFF filters for sample pre-processing for SEC-ICP-MS analysis.

To evaluate the effect of sample processing and storage, all identified sample peaks were plotted as a function of cumulative probability density (Figure 4). The expected distribution for UF samples would not include peaks above 10 kDa, however approximately 40% of UF samples' detected peaks (by number) were detected at MW > 10 kDa. Most of the detected peak area and the most prominent peaks were detected <10 kDa for most elements, regardless of sample handling and storage. Samples processed by centrifugation produced a peak distribution that was statistically similar to those observed in UF processed samples. Among the collected samples, only 10–30% of the OM in the centrifuge effluent was removed by the UF, so similar distributions were not surprising. Sample centrifugation and UF processing were pursued promptly following sample collection, however samples were not analyzed by SEC for several months, and OM aggregation, which has been previously observed (Schmitt *et al.* 2001), potentially led to similar distributions. Efforts should be made to conduct SEC analysis promptly. GFF had comparatively more high MW peaks. The expected removal cutoffs for centrifugation and GFF are 0.3 and 0.7 µm, respectively, which are



**Figure 4** | Cumulative probability density functions for peaks between 0.1 and 300 kDa: (a) separation method; (b) storage method.

both well above the column upper separation limit of 300 kDa (~3 nm). The high MW peaks detected in the GFF samples were either due to macromolecules/colloids that were not removed in the GFF but were removed in the other separation approaches, or were a result of GFF metal contamination that was then bound by high MW entities.

Since GFF displayed a different peak distribution, storage effects on size distribution analysis were restricted to centrifugation and UF samples. In spite of the fact that the glass and plastic cumulative distribution functions appear similar, storage in glass containers resulted in a statistically significant increase in high MW peaks, which may indicate that the high MW macromolecules/colloids are less polar and therefore are more susceptible to adsorption to the plastic.

Based on these results, native GFF filters should be avoided due to contamination concerns if both OM and metals are to be analyzed, and plastic storage may result in a loss of high MW components. Non-filtration separation methods (i.e. centrifugation) are a viable option, as is filtration with a PVDF filter or an acid-washed GFF. Samples should be stored in acid-washed glass containers.

## CONCLUSION

SEC-ICP-MS is a powerful tool for evaluating macromolecular metal complexation, which can be important in natural and engineered systems. Consistent sample processing and storage are important components of any study, but are particularly critical for trace analysis of multiple constituents. Replicate analysis is an important step in determining which elements produce strong and reproducible chromatograms. In this study of anthropogenically influenced natural systems, Mg, Cl, Mn, Cu, Br, and Pb were the most consistent, while Al, V, Fe, Co, Ni, Zn, Se, Cd, Sn, and Sb were moderately consistent.

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