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Distribution of elements binding to molecules with different molecular weights in aqueous extract of Antarctic krill by size-exclusion chromatography coupled with inductively coupled plasma mass spectrometry

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Abstract

The distribution of silver, arsenic, cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, selenium and zinc binding to species with different molecular weight in aqueous extract of krill was studied by on-line size-exclusion chromatography (SEC)/inductively coupled plasma mass spectrometry (ICP-MS). The extract was fractionated in three fractions with different molecular weight (MW) ranges (>20,000 relative molecular mass (rel. mol. mass), 2000–20,000 rel. mol. mass and <2000 rel. mol. mass), which were further analyzed by SEC with columns having different optimum fractionation ranges in order to obtain more detailed information about the MW distribution of the elements. Various distribution profiles for the target elements among different MW ranges were observed. The results obtained indicated that manganese, zinc, silver, cadmium and lead species were mostly distributed in the higher MW range (>20,000 rel. mol. mass). In the case of chromium, iron, cobalt, arsenic and selenium, most of them bind to species with lower MW (<2000 rel. mol. mass). Only copper and nickel species was predominantly present in middle MW range (2000–20,000 rel. mol. mass). Further speciation of arsenic compounds in the small MW fraction was carried out with anion exchange chromatography (AEC) coupled with ICP-MS. The results showed that the dominant arsenic species in this fraction is As(III) (63% of extractable arsenic), while As(V) (13%) and two unknown arsenic species (19% and 5%, respectively) are present in lower amounts.
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Keywords: Fractionation; Elements; Antarctic krill; Size-exclusion chromatography; Inductively coupled plasma-mass spectrometry

1. Introduction

Antarctic krill is a small marine crustacean, which is used as feed in aquaculture farms. Generally, the Antarctic is a zone of low contamination, with natural background levels of heavy metal concentration [1], however, due to the industrial use of krill, it is desirable to obtain detailed information of its contamination status. The toxicity, environmental mobility and accumulation of trace elements in living organisms usually depend on the particular chemical species, for this reason, the speciation analysis of trace elements in food and other biological organisms is increasingly getting attention [2–8].

Speciation procedures in biological samples mainly consist of separation, detection and identification of individual compounds of elements. Undoubtedly, hyphenated techniques are the most suitable tools for the speciation analysis of trace elements in biological matrices [9–11], which include efficient separation methods like high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) and element-specific detection methods, such as atomic absorption spectrometry (AAS) [12], inductively coupled plasma atomic emission (ICP-AES) [13] or mass spec-

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trometry (ICP-MS) [14,15]. Size-exclusion chromatography (SEC) [16–18] is often applied as a first step in the separation of element species in complex matrices. Advantages of SEC are the high capacity of SEC columns and the limited interaction of the analytes with the stationary phase, which may be important when dealing with labile species. However, identification of unknown compounds based on the comparison of retention times (or elution volumes) with reference material cannot be achieved by SEC and more effective chromatographic techniques such as reversed phase-HPLC (RP-HPLC) and IEC are necessary for proper speciation analysis [19]. This two-dimensional speciation approach is exemplified in this work for arsenic compounds.

Trace element speciation studies in marine biological samples mainly deal with arsenic and have been reviewed recently [20]. Other elements targeted in species analysis in marine organisms include selenium [21–23], copper, zinc and cadmium [24]. The distribution of selenium between fractions with different MW has been presented in different fish species [25,26], while some investigations of soluble element species in food (such as soybean flour, pea and lentil seeds and white bean seeds) were reported by Koplík et al. [27,28], in which data were presented on the fractionation of Mn, Fe, Co, Ni, Cu, Zn, Se, Mo and P species in different MW ranges.

In this work, the total contents of Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn in Antarctic krill were determined, as well as the distribution of the elements binding to substances with different molecular weight. Different size-exclusion analytical columns with different optimum fractionation ranges were used in order to get detailed information. Profiles for target elements distributed among nine different MW ranges were obtained. Further identification of arsenic species in the small MW fraction (<2000 rel. mol. mass) was carried out by anion exchange chromatography (AEC) coupled with ICP-MS.

2. Experimental

2.1. Instrumentation

For chromatography, an Agilent Series 1100 (Agilent Technologies, Waldbronn, Germany) HPLC system was used, which included an online vacuum degasser, a binary pump, an auto sampler, a thermostatted column compartment and a diode array detector. The krill extract was fractionated using a HiLoad 16/60 Superdex 75 preparation grade column (bed volume: 120 ml) and an ÄktaPrime LC system equipped with a 2 ml sample loop (Amersham Biosciences, Uppsala, Sweden). Three different size-exclusion chromatographic columns (dimensions 300 mm × 10 mm for each) were used to analyze the fractions, namely Superdex 200 10/300 GL (optimum fractionation range 10,000–600,000 rel. mol. mass), Superdex 75 HR 10/30 (optimum fractionation range 3000–70,000 rel. mol. mass) and Superdex peptide HR 10/30 (optimum fractionation range 100–7000 rel. mol. mass) (Amersham Biosciences, Uppsala, Sweden). For the speciation of Arsenic, an IonPak AS14 column (250 mm × 4 mm, Dionex) was employed.

An Agilent 7500 s inductively coupled plasma mass spectrometer (ICP-MS) was employed as an element-specific detection system. The signals were monitored in the time-resolved analysis mode when ICP-MS was coupled with HPLC. The instrumental operation conditions for ICP-MS are given in Table 1.

Lyophilization of fractions collected was carried out using a gamma 1–20 lyophilisator (Christ, Osterode, Germany). The closed vessel microwave system used for the digestion of krill powder was an MLS-1200 MEGA high performance microwave digestion unit (MLS, Leutkirch, Germany).

2.2. Chemicals and samples

All reagents used in this work were at least of analytical-reagent grade. Multi element standard stock solution for Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn (10580 ICP Multi elemental standard VI) was purchased from Merck (Darmstadt, Germany). Nitric acid (65%, Suprapur grade, Merck) and hydrogen peroxide (30%, Suprapur grade, Merck) were used in the digestion of krill. All arsenic species standard solutions (1000 mg l^{-1}) including As(III), As(V), MMA, DMA, AsB and AsC were from Merck (Darmstadt, Germany) or Sigma–Aldrich (Steinheim, Germany). Low concentration standards were freshly prepared from the stock solutions by dilution with ultra-pure water, which was produced by a Milli-Q Synthesis A10 system (Millipore, Bedford, MA, USA).

Table 1
Instrumental operation conditions for ICP-MS detection

	ICP-MS for the determination of total contents of elements	SEC/ICP-MS for fractionation of element species	AEC/ICP-MS for the speciation of arsenic
Forward power	1450 W	1450 W	1450 W
Carrier gas	1.24 l min^{-1}	1.26 l min^{-1}	1.26 l min^{-1}
Makeup gas	0 l min^{-1}	0 l min^{-1}	0 l min^{-1}
Nebulizer	PFA MicroFlow 100	PFA MicroFlow 100	PFA MicroFlow 100
Isotopes monitored	^{107}Ag, ^{75}As, ^{111}Cd, ^{59}Co, ^{53}Cr, ^{63}Cu, ^{57}Fe, ^{55}Mn, ^{60}Ni, ^{208}Pb, ^{77}Se and ^{66}Zn, ^{103}Rh (internal standard element)	^{107}Ag, ^{75}As, ^{111}Cd, ^{59}Co, ^{53}Cr, ^{63}Cu, ^{57}Fe, ^{55}Mn, ^{60}Ni, ^{208}Pb, ^{77}Se and ^{66}Zn	^{37}Cl, ^{51}V(^{35}Cl^{16}O), ^{75}As

Ammonium acetate was purchased from Fluka (Steinheim, Germany). The proteins used for SEC column calibration were purchased from Sigma (St. Louis, MO, USA) or Amersham Pharmacia Biotech (Uppsala, Sweden), and included thyroglobulin (669,000 rel. mol. mass), catalase (232,000 rel. mol. mass), transferrin (81,000 rel. mol. mass), bovine serum albumin (BSA, 67,000 rel. mol. mass), ovalbumin (43,000 rel. mol. mass), chymotrypsinogen A (25,000 rel. mol. mass), myoglobin (17,000 rel. mol. mass), ribonuclease A (14,000 rel. mol. mass), aprotinin (6500 rel. mol. mass), cyanocobalamine (1300 rel. mol. mass), Ala-His (230 rel. mol. mass), $(Gly)_3$ (190 rel. mol. mass) and L-cysteine (120 rel. mol. mass).

Deep-frozen Antarctic krill was obtained from Marine BioProducts (Bremerhaven, Germany), and lyophilized and powdered before extraction.

The accuracy of the determination of total elements concentration was verified by analysis of the certified reference material Dorm-2 (dogfish muscle) (National Research Council, Ottawa, Canada).

2.3. Procedures

2.3.1. Determination of the total element contents in krill

Lyophilized krill was submitted to microwave-assisted acid digestion. An improved digestion programme based on literature [29] was developed in our work. Before the microwave digestion, a pre-digestion procedure for krill is necessary, due to high contents of organic matter, by soaking the sample (0.1 g) in 4 ml concentrated nitric acid overnight. The same procedure was also used for the digestion of the reference material Dorm-2. The element contents in the digests were determined by ICP-MS.

2.3.2. Extraction procedure for krill

Triplicate sub-samples (1.0 g) from krill powder were extracted with 10 ml water by shaking them at 190 rpm in a water bath at 38 °C for 24 h. After centrifugation at 14,000 × *g* for 20 min, 7 ml crude extract was obtained. The contents of elements in the extract were determined by ICP-MS.

2.3.3. Fractionation of the krill extract and analysis of the fractions

Twenty parallel sub-samples (1.0 g) from krill powder were separately extracted with 10 ml water using the extraction procedure described above. The supernatants were combined and filtered (0.45 μm pore size). About 110 ml crude extract was obtained, which was concentrated to 40 ml. A small part was analyzed on a Superdex 75 HR 10/30 column with ICP-MS detection. The remaining extract was fractionated and collected with a HiLoad 16/60 Superdex 75 preparative column. The mobile phase was 10×10^{-3} mol l^{-1} ammonium acetate at 1.5 ml min^{-1} and fractions were collected every 2 min. Fractions were combined according to the UV signal to give G1 (fraction 11–21, >20,000 rel. mol. mass), G2 (fraction 22–32, 2000–20,000 rel. mol. mass) and G3 (fraction 33–40, <2000 rel. mol. mass) (Fig. 1). G1, G2 and G3 were further analyzed with the analytical size exclusion columns indicated in the instrumentation section.

2.3.4. Analytical procedure for the identification of arsenic species in the low molecular weight fraction

Identification of arsenic species in G3 fraction was carried out by AEC coupled on-line with ICP-MS detection, based on the method established by Lindemann et al. [30]. Estimation of the content of single species was realized by spiking the fraction G3 with different concentrations (0 μg l^{-1}, 100 μg l^{-1}, 200 μg l^{-1} and 300 μg l^{-1}) of arsenic standard

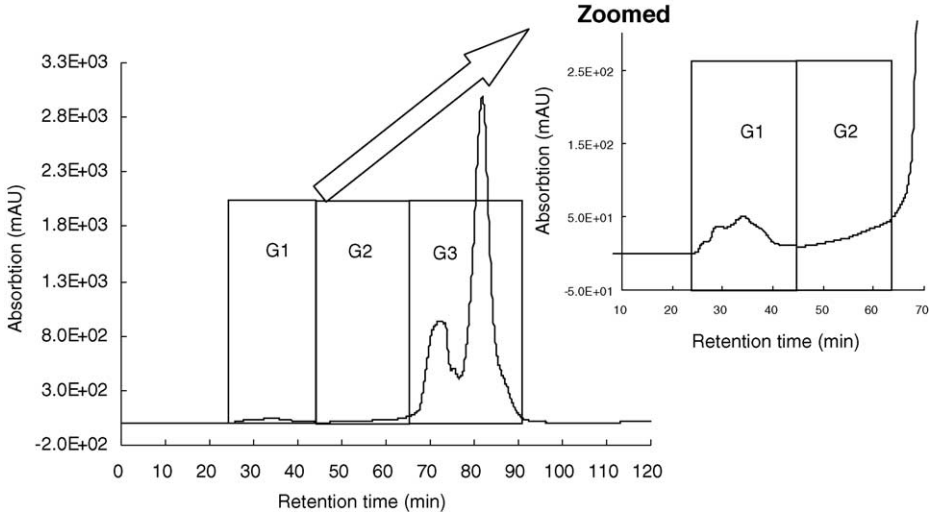

Fig. 1. Chromatogram of aqueous extract of krill on HiLoad 16/60 Superdex 75 preparation column with UV detection (λ = 254 nm) (mobile phase, 0.01 mol l^{-1} ammonium acetate; flow rate, 1.5 ml min^{-1}; injection volume, 2 ml). G1, fractions with MW >20,000 rel. mol. mass; G2, fractions with MW 2000–20,000 rel. mol. mass; G3, fractions with MW <2000 rel. mol. mass.

Table 2
Total contents of target elements in krill and dogfish muscle reference materials (Dorm-2) and the percentages extractable to water

Element	Krill					Dorm-2	
	Total content ($\mu g\,g^{-1}$)[a]	Value reported in literature [31]	Value reported in literature [32][b]	Tolerable limit ($\mu g\,g^{-1}$)[c]	Extractability (%)	Experimental value ($\mu g\,g^{-1}$)[a]	Certified value ($\mu g\,g^{-1}$)[a]
Cr	0.485 ± 0.08	0.1–0.26			41.6 ± 2.8	38.3 ± 3.11	34.7 ± 5.5
Mn	0.682 ± 0.067				5.9 ± 0.1	4.32 ± 0.34	3.66 ± 0.34
Fe	10.4 ± 1.813	11–26			5.9 ± 0.4	171 ± 14	142 ± 10
Co	0.019 ± 0.006				38.7 ± 0.7	0.211 ± 0.018	0.182 ± 0.031
Ni	0.318 ± 0.043				14.8 ± 1.2	19.1 ± 1.6	19.4 ± 3.1
Cu	7.4 ± 0.42	32–65	41.3		19.2 ± 2.6	2.58 ± 0.50	2.34 ± 0.16
Zn	42.3 ± 3.09		19.3		0.7 ± 0.1	20.6 ± 0.6	25.6 ± 2.3
As	1.99 ± 0.144	1.8–3.8			34.2 ± 0.8	14.8 ± 1.0	18.0 ± 1.1
Se	2.72 ± 0.227	2.9–3.5	3.3		14.8 ± 0.7	1.37 ± 0.10	1.40 ± 0.09
Ag	0.126 ± 0.008				5.3 ± 0.4	0.044 ± 0.001	0.041 ± 0.013
Cd	0.143 ± 0.008	2.2–2.7	0.8	0.05–0.1 (fish) 0.5 (crustaceans)	6.8 ± 0.1	0.044 ± 0.003	0.043 ± 0.008
Pb	0.030 ± 0.005	0.31–1.3		0.2–0.4 (fish) 1.5 (mussels)	21.7 ± 1.2	0.055 ± 0.013	0.065 ± 0.007

[a] Dry weight, mean ± S.D. of triplicate determination.
[b] Total content of elements detected in stomach content (mainly krill) of petrel.
[c] Tolerable limits established by European regulation (EG 221/2002, 6 February 2002).

compounds. The interference from chloride was traced by monitoring *m/z* 37 (^{37}Cl) and *m/z* 51 ($^{35}Cl^{16}O$) simultaneously.

3. Results and discussion

3.1. Total contents of target elements in krill

Three different acid media (HNO_3, $HNO_3/HClO_4$ and HNO_3/H_2O_2) were tested using the digestion programme developed in this work. The experimental results indicated that HNO_3/H_2O_2 is the best one among them in terms of digestion effect as well as spectrometric interference to ICP-MS caused from $^{40}Ar^{35}Cl$, which was verified by the analysis of the reference material (Dorm-2).

Target elements in this work were Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn. The results obtained for total element concentrations in krill and Dorm-2 are listed in Table 2. It can be seen from the results, that the total contents of elements are generally in accordance with values previously reported for Antarctic krill [31,32]. In the case of cadmium, the value are considerably lower, but still above the tolerable limits established by EU regulations for fish destined for human consumption. The accumulation of Cd in fish via the food chain may be a matter of concern when using krill as feed.

3.2. Analysis of the species of target elements in different MW ranges in the aqueous extract of Antarctic krill

3.2.1. Extractability of target elements

In order to choose a moderate and high-efficient extraction solution, several buffers were tested including Tris–HCl buffer (pH 8), phosphate buffer (pH 8), ammonium acetate buffer (pH 5) and water. The results showed that there was no difference between the extraction solutions, so water was chosen as extraction solvent.

As can be seen in Table 2, the extractability for all elements investigated is below 50%. Önning and Bergdahl [26] found that the extractabilities for selenium were 47% from plaice and 23% from cod, using Tris–acetate buffer as extraction solution. These results indicated that the extractability of elements is related to the solubility of animal protein, which in turn depends of the organism. Similar results have been reported for the extraction of elements from plant food, where element extractability was dependent of the origin and maturity of the plant [33,34].

3.2.2. Analysis of the extract with SEC/UV and SEC/ICP-MS

Preliminary analysis of aqueous krill extract was carried out on a Superdex 75 HR 10/30 analytical column with sequential UV and ICP-MS detection. Several buffers (phosphate, Tris–HCl buffer, ammonium hydrogen carbonate and ammonium acetate) with different pH values (pH 6–8) were tested as mobile phase, from which the best results were obtained with 0.1 mol l^{-1} ammonium acetate (pH 7).

Information about the distribution of compounds in different MW ranges was obtained from the UV absorbance chromatogram at 254 nm (Fig. 1). The results indicated, that most of the water-soluble compounds were present in the low MW range (<2000 rel. mol. mass), some existing in the high MW range (>20,000 rel. mol. mass) and very little amount in the middle MW range (2000–20,000 rel. mol. mass).

ICP-MS detection revealed the distribution of the elements bound to compounds with different molecular weight (Fig. 2). Mn, Zn, Ag, Cd and Pb are being found mainly in the high

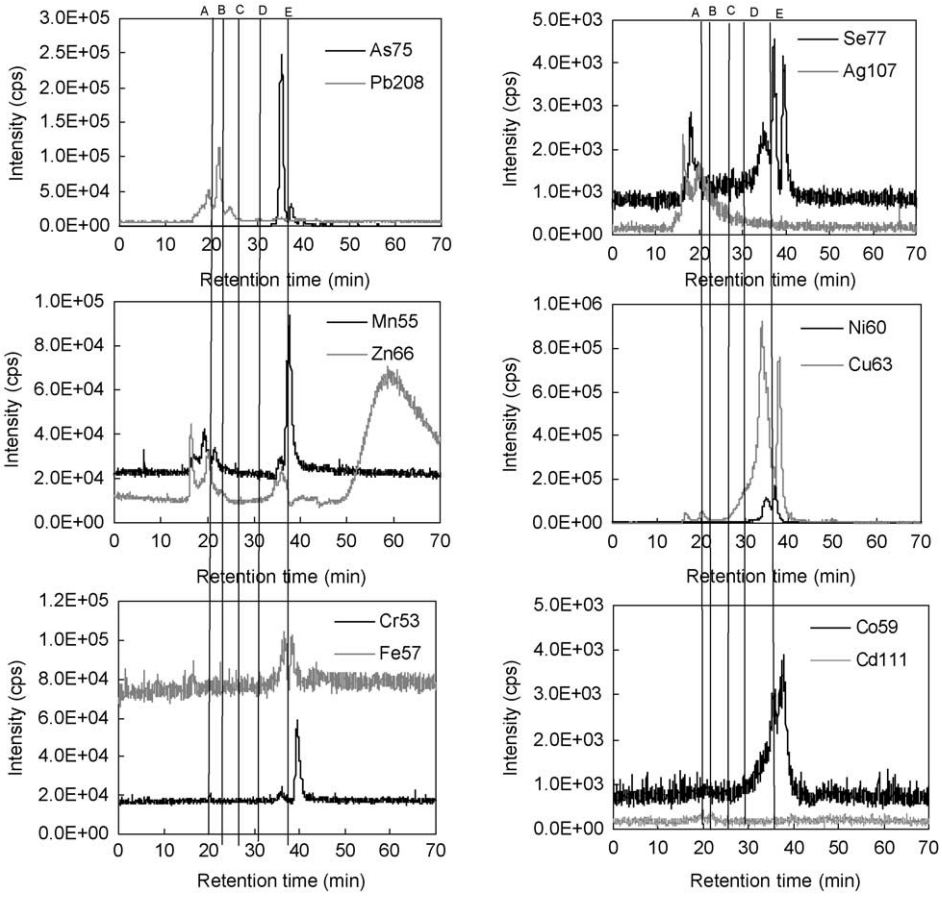

Fig. 2. Chromatograms of As, Pb, Co, Cr, Fe, Se, Ag, Mn, Zn, Ni, Cu and Cd compounds in aqueous extract of Krill on Superdex 75 HR 10/30 column with SEC/ICP-MS: (A) bovine serum albumin (67,000 rel. mol. mass); (B) ovalbumin (43,000 rel. mol. mass); (C) chymotrypsiogen A (25,000 rel. mol. mass); (D) aprotinin (6500 rel. mol. mass); (E) cyanocobalamin (1300 rel. mol. mass).

MW range (>20,000 rel. mol. mass) (Table 3), most notably for Pb, nearly all of which (87%) was associated with high molecular weight compounds. As for Cr, Fe, Co, As and Se, the dominant percentages of them were found combined with low MW species (<2000 rel. mol. mass). Among the 12 target elements, only Ni and Cu species dominated in the middle MW range (2000–20,000 rel. mol. mass). The recoveries of the elements after size exclusion chromatography were determined to be between 88% and 115%.

3.2.3. Fractionation in different MW ranges and analysis of the fractions by SEC/ICP-MS with different analytical size exclusion columns

In order to obtain more detailed molecular mass information about the species binding to the target elements, the fractions G1 (>20,000 rel. mol. mass), G2 (2000–20,000 rel. mol. mass) and G3 (<2000 rel. mol. mass) were collected as described in the experimental section and submitted to further analysis by size-exclusion chromatography. As the total amounts were quite low, fractions G1 and G2 were concentrated ca. 500-fold by lyophilization and resolubilization prior to analysis. The results are summarized in Tables 3 and 4.

Before discussing the results in detail, some general points concerning the estimation of molecular weights by size-exclusion chromatography have to be considered. The elution volume of a compound does not merely depend on its molecular weight, but rather on its hydrodynamic volume, i. e. on the shape during the separation process [35]. The calculation of the approximate MW of an unknown molecule via a formerly established calibration curve is therefore based on the assumption that calibrators and analyte possess the same shape, which in case of proteins usually is believed to be globular. Another aspect is the mono- or polydispersity of a sample [36]. From a peak obtained during an SEC/UV or SEC/ICP-MS run, the accurate MW can only be calculated when the eluting compound is monodisperse, i.e. consists of a single type of molecule with defined MW. When dealing with a polydisperse sample, compounds with molecular weights of roughly the same range may elute in a single peak. Calculation of MW using the elution volume read at the peak top then gives the average MW of these compounds. Undoubtedly, the aqueous extract of lyophilized krill contains a multitude of biopolymers in varying concentrations. The determination of MWs by SEC/UV will, therefore, not lead to reasonable results due to the polydispersity of the sample.

Table 3
Distribution of target elements in krill, G1, G2 and G3 (%) (average value ± S.D., $n = 4$)[a]

	Rel. mol. mass	Cr	Mn	Fe	Co	Ni	Cu	Zn	As	Se	Ag	Cd	Pb
Krill[b]	> 20000	8	59 ± 1	24 ± 4	8	2	5	72 ± 3	1	27 ± 1	79 ± 1	76 ± 5	87 ± 1
	2000 ± 20000	10 ± 1	2	25 ± 1	33 ± 0	65 ± 1	59 ± 1	11 ± 2	33 ± 1	30 ± 0	17 ± 0	11 ± 2	9
	< 2000	82 ± 2	39 ± 2	51 ± 3	59 ± 1	33 ± 1	36 ± 1	17 ± 1	66 ± 1	43 ± 1	4	13 ± 7	4
G1[b]	> 200000	ND	5	35 ± 2	27 ± 7	ND	12 ± 3	36 ± 2	ND	4	25 ± 1	15 ± 2	6
	100000 ± 200000	ND	15 ± 2	13 ± 2	5	ND	16 ± 1	11 ± 4	ND	39 ± 1	21 ± 1	24 ± 2	14 ± 1
	20000 ± 100000	ND	80 ± 1	52 ± 3	68 ± 8	ND	72 ± 2	53 ± 7	ND	57 ± 1	54 ± 1	61 ± 1	80 ± 2
G2[b]	10000 ± 20000	19 ± 1	ND	22 ± 2	16 ± 1	15 ± 1	9	29 ± 1	7	39 ± 2	69 ± 6	ND	61 ± 5
	5000 ± 10000	37 ± 5	ND	32 ± 3	41 ± 1	40 ± 4	32 ± 1	38 ± 1	27 ± 1	32 ± 1	18 ± 2	ND	20 ± 2
	2000 ± 5000	44 ± 6	ND	46 ± 2	43 ± 0	45 ± 4	59 ± 1	33 ± 1	66 ± 2	29 ± 1	13 ± 4	ND	19 ± 3
G3[b]	1000 ± 2000	3	10 ± 2	7	2	1	7	4	ND	3	ND	ND	1
	500 ± 1000	4	10 ± 2	14 ± 1	14 ± 2	3	17 ± 1	8	3	5	ND	ND	9
	< 500	93 ± 3	80 ± 3	79 ± 4	84 ± 3	96 ± 1	76 ± 1	88 ± 2	97 ± 1	92 ± 2	ND	ND	90 ± 2

[a] Results evaluated as the percentage of the peak area referred to the total area, under the chromatogram of Krill, G1, G2 and G3 separately with different size-exclusive analysis columns. The software for the analysis of chromatography was ICP-MS chromatographic software (No. G1834A, Agilent Technologies). Percentages below 10% are listed as the average value without S.D.

[b] Krill, aqueous extract of krill; G1, G2 and G3, fractions in different MW ranges collected from aqueous extract of krill with size-exclusive preparation column.

Table 4
Summary of SEC/ICP-MS analysis results for G1 and G2

Element	G1 Molecular weight (MW × 10^3) of compounds (rel. mol. mass unit)	G2 Molecular weight (MW × 10^3) of compounds (rel. mol. mass unit)
Cr	–	–
Mn	79	–
Fe	370 (major), 45	–
Co	45	4.4 (major), 2.0
Ni	–	6.5 (major), 3.7, 2.2, 1.4
Cu	370, 127, 60 (major)	4.4 (major), 3.1
Zn	370 (major), 60	3.0, 2.0 (major)
As	–	4.4 (major), 2.0
Se	139 (major), 68, 41, 29, 17	20, 11 (major), 4.5
Ag	870, 180, 68 (major), 30	20
Cd	68	–
Pb	370, 120, 68 (major), 41, 26	24 (major), 15

However, ICP-MS detects selectively the element-containing compounds and it is much more safe to assume that these represent single compounds when eluting from the SEC column.

Elements which had their major percentage in G1 (>20,000 rel. mol. mass), were Mn, Zn, Ag, Cd and Pb. Analysis with a Superdex 200 column showed, that most species of these elements are present in the 20,000–100,000 rel. mol. mass range. Considerable amounts of Zn are also found at very high molecular weight (>200,000 rel. mol. mass), with a major peak representing a compound of approx. 370,000 rel. mol. mass. A similar profile of very high MW compounds is also found associated with iron, the major compound also showing an apparent MW of 370,000 rel. mol. mass. This substance falls into the same MW range as ferritin, which in mammals has a MW of approx. 440,000 rel. mol. mass. Ferritin has been identified from crustaceans and other marine invertebrates and was shown to bind not only iron, but also other metals like Zn, Pb, Cu or Po [37,38]. In this work, Cu and, to a smaller extent, Pb were also found in the approx. 370,000 rel. mol. mass compound. Pb was mainly binding to an approx. 68,000 rel. mol. mass-ligand, together with the thiophilic metals Cd and Ag, which may indicate that this protein is rich in sulfur-containing residues. A manganese-binding protein showed an apparent MW of approx. 79,000 rel. mol. mass.

For G2, a Superdex 75 HR 10/30 column with optimum fractionation range of 3000–70,000 rel. mol. mass was employed. According to the preliminary distribution results of elements in krill extract, Ni and Cu are primarily present in this fraction. Cu is known to bind to cysteine-rich metallothioneins or metallothionein-like proteins, which in mammals and other vertebrates usually have a MW of 6000–7000 rel. mol. mass. In krill, Cu is bound to an approx. 4400 rel. mol. mass compound, which might correspond to the 4400 rel. mol. mass-metallothionein identified from the shore crab, *Carcinus maenas* [39]. Additionally, small amounts of Co were found associated with this compound in krill. Ni was found to bind mainly to a compound of approx. 6500 rel. mol. mass, but was also associated with some smaller polypeptides of approx. 3700 rel. mol. mass,

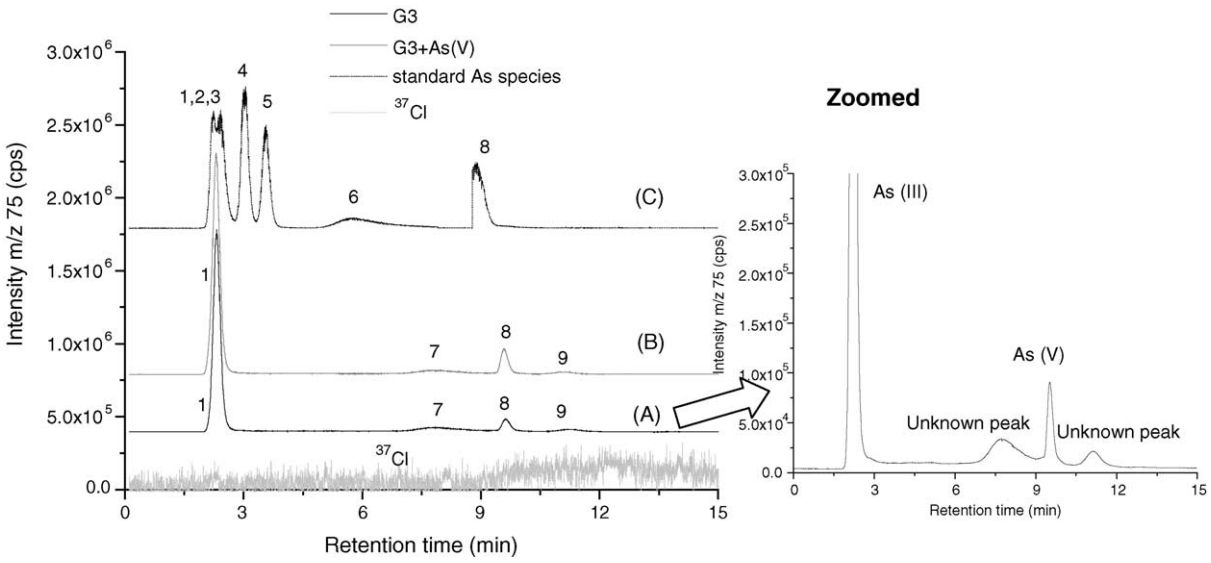

Fig. 3. Chromatograms with AEC coupled with ICP-MS for the speciation of arsenic in G3 fraction (A), G3 fraction spiking with standard As (V) (B) and seven kinds of standard arsenic species (C). (A) Peak 1, As (III); peak 7, unknown arsenic species; peak 8, As (V); peak 9, unknown arsenic species. (B) Peak 1, As (III); peak 7, unknown arsenic species; peak 8, As (V) from G3 sample and standard As (V); peak 9, unknown arsenic species. (C) Peak 1–3, mixture of As (III), AsB and TMAO; peak 4, DMA; peak 5, MMA; peak 6, AsC; peak 8, As (V) (concentration, 100 $\mu g\,l^{-1}$).

2200 rel. mol. mass, and 1400 rel. mol. mass, respectively. Furthermore, selenium showed an interesting pattern in this fraction, with a major compound of approx. 11,000 rel. mol. mass, and others with an approx. MW of 20,000 rel. mol. mass and 4500 rel. mol. mass.

The major part of Cr, Fe, Co, As and Se are found in fraction G3, which was analyzed using a Superdex peptide column (optimum range of 100–7000 rel. mol. mass). All of these elements appeared to be bound to small molecules (<500 rel. mol. mass). However, the limitations discussed above concerning the determination of MWs have to be considered particularly for the Superdex peptide column. Interactions of the molecules with the stationary phase that might lead to an inaccurate determination of the MW (e.g. binding to the stationary phase via dipol–dipol or ionic interactions) are much more likely to occur when dealing with small molecules. Strictly speaking, appropriate MW determination can be achieved only if analytes and calibrators belong to a homologous series of compounds. Co appeared as a single peak representing a compound of approx. 500 rel. mol. mass, while the nature of Fe in this fraction could not be determined due to bad chromatographic behavior. Cr is likely to be present as inorganic Cr(III) or Cr(VI), while Se and As may be of inorganic or organic nature. Selenomethionine had already been identified in this fraction [21], while a more detailed analysis of arsenic species is presented in the following section.

3.3. Identification of arsenic species in G3 fraction

For the speciation of arsenic, anion exchange chromatography coupled to ICP-MS was employed [30]. As shown in Fig. 3C , the standard compounds DMA, MMA, AsC and As(V) could be separated very well, while As(III), AsB and TMAO co-eluted near the void volume. Analysis of G3 revealed four peaks, one near the void volume, and three with retention time of 7.7 min, 9.2 min and 11.1 min, respectively (Fig. 3A). The peak at 9.2 min was shown to represent As(V) upon spiking the sample (Fig. 3B), while at the same time, it could be concluded, that no DMA, MMA and AsC was present. In order to clarify the nature of the compound eluting near the void volume, another method [40] with better separation for As(III), AsB and TMAO was employed. From these results (shown in Fig. 4), it was concluded, that peak 1 represents As(III), and no AsB or TMAO is present. The identity of the two compounds eluting at 7.7 min and 11.1 min remains unclear, as no more standard species were available. Here, speciation patterns of arsenic in G3 fraction of krill had some difference from that found in other marine organisms, such as pike, cod and mussel [41–43], where the most abundant and stable arsenic compound is AsB, while in algae inorganic arsenic and arsenosugars prevail [44]. In the present investigations, no organic species such as MMA, DMA, AsB, AsC and TMAO were detected in krill, but inorganic arsenic species prevailed. The most possible reason for the difference is the analysis of aqueous extracts, while other extraction procedures included the used of methanol–water mixtures. The analytical performance data of the AEC/ICP-MS method are summarized in Table 5. Detection limits varied between 1.4 $\mu g\,l^{-1}$ (for DMA) and 3.8 $\mu g\,l^{-1}$ (for TMAO).

The content of the arsenic species was estimated by adding three different levels of standard arsenic species (As(III) and As(V)) to the G3 fraction, as described in the experimental part ($R^2 = 0.998$ for As(III), $R^2 = 0.996$ for As(V)). In case of the unknown species, the calibration equation of As(V) was applied. Based on the results, it was calculated that

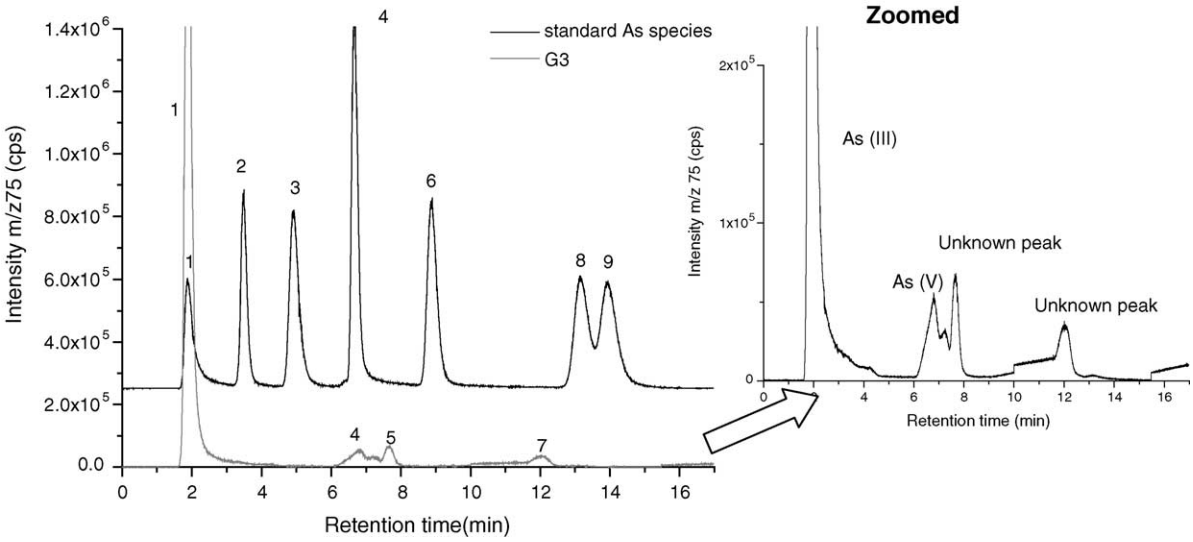

Fig. 4. Chromatograms with AEC coupled with ICP-MS for the speciation of arsenic in G3 fraction (A) and standard arsenic species (B). Chromatographic conditions: column, IonPak AS7 (250 mm × 4 mm, Dionex); mobile phase A, 5×10^{-4} mol l^{-1} nitric acid, 5×10^{-5} mol l^{-1} benzene-1,2 disulfonic acid dipotassium salt, 0.5% methanol; mobile phase B, 5×10^{-2} mol l^{-1} nitric acid, 5×10^{-5} mol l^{-1} benzene-1,2 disulfonic acid dipotassium salt, 0.5% methanol; flow rate, 1 ml min^{-1} [38]. (A) Peak 1, As (III); peak 4, As (V); peak 5, unknown arsenic species; peak 7, unknown arsenic species. (B) Peak 1, As (III); peak 2, MMA; peak 3, DMA; peak 4, As (V); peak 6, AsB; peak 8, TMAO; peak 9, AsC (100 μg l^{-1}).

Table 5
Analytical performance characteristics of the AEC/ICP-MS method for speciation of arsenic

	Detection limits[a]		R.S.D. (%)		
	Relative (μg l^{-1} as As)	Absolute (pg as As)	Retention time ($n = 7$)	Peak area ($n = 7$)	Peak height ($n = 7$)
As(III)	2.1	11	0.7	4.5	5.8
DMA	1.4	7.0	1.6	4.6	8.0
MMA	1.8	9.0	0.3	5.7	10
As(V)	2.4	12	2.4	5.1	6.5
AsB[b]	1.8	9.0	1.0	6.7	7.2
AsC[b]	1.6	8.0	2.1	5.9	8.0
TMAO[b]	3.8	19	1.9	4.8	9.4

[a] Calculated as three times the standard deviation of the background signal divided by the slope of the calibration curve.
[b] Data obtained with the method established in literature [38].

the dominant species of arsenic in G3 fraction of krill is As(III) (1.5 μg g^{-1}). As(V) (0.32 μg g^{-1}) and the other two unknown arsenic species (0.44 μg g^{-1} and 0.12 μg g^{-1}) are present in lower amounts.

4. Conclusion

Detailed information about the distribution of the target elements (Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se and Zn) in nine molecular size ranges was obtained with three size-exclusion analytical columns with different optimum fractionation ranges. Although the chromatographic profile obtained by SEC/ICP-MS cannot provide the accurate speciation information for the target elements, an approximation of their distribution among different molecular mass fractions led us to some suggestions about the identity of element-containing proteins, e.g. a metallothionein-like protein or a ferritin-like protein, and their element-binding properties. Further separation and identification of these and other species by more effective separation techniques (such as RP-HPLC and IEC) combined with ICP-MS or ESI-MS/MS is required to confirm these suggestions. The general usefulness of this two-dimensional approach was demonstrated by applying it to the speciation of arsenic in the low-molecular weight fraction. As(III) was found as a dominant species in G3 fraction, whereas As(V) and two unknown arsenic species were found in lower contents.

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