

Speciation of organic selenium compounds using reversed-phase liquid chromatography and inductively coupled plasma mass spectrometry

Part III.† Application of a sector field instrument with low and high mass resolution for selenium speciation in herring gull eggs.‡

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A HPLC procedure based on reversed phase chromatography with methanol as the eluent for the speciation of organic Se compounds has been developed to study the appearance of Se species in herring gull eggs collected from mud flats of the North Sea. The extraction of Se species by using hot water was limited to a recovery of 8% only. The multielement capability of ICP-MS was exploited to obtain elemental correlation, and for Se compounds (selenocystine, selenocystamine, selenomethionine, selenoethionine) were used as standards. Among these only the presence of selenocystamine was evident, and correlations of this fraction with Cu, As and S became obvious.

Introduction

In the aquatic environment, Se is part of a complex biogeochemical cycle involving organic as well as inorganic selenium compounds. Knowledge of the species in the marine ecosystem, however, is still restricted at present. It has been observed for some types of mussels that the intake is significantly higher for Se(IV) than for Se(VI).¹ Selenium accumulation in marine fish may amount up to a factor of 400,² and for fish cultivated in cooling water reservoirs of coal fired power plants a Se concentration up to the 100-fold of that in fish from unpolluted water has been observed.³

A necessary requirement to obtain a more detailed insight into the Se cycle in organisms and the contributing bio-transformation processes in the nutritional chain is the development of procedures for trace- and micro-analysis of Se species in biomaterials. Recently considerable progress has been made by applying different techniques of atomic spectrometry, among which atomic emission spectrometry and mass spectrometry with an inductively coupled plasma as the excitation/ionization source excel in providing low detection limits, in particular ICP-MS.⁴⁻⁶

Concerning ICP-MS, a special impetus into research in the field of Se speciation is actually coming from the life sciences, not only from basic biochemistry, but also from medicine, biology, and environmental and food science. Recent studies have been made of the elucidation of the role of albumin in the metabolism of selenite⁷ and in the interaction of Hg²⁺ ions with Se and the seleno-protein P by application of ICP-MS coupled to HPLC.⁸ Michalke and Schramel have applied

different separation techniques in combination with ICP-MS and electrospray ionisation (ESI)-MS for Se speciation in blood serum and milk.⁹ Four species were identified in milk (Se-carrying glutathione, selenocystamine, selenocystine and selenomethionine) by application of standards for comparison, and in serum more than ten unknown species were observed. Further attempts towards identification by ESI-MS will require special enrichment techniques. Uden *et al.* studied the accumulation of Se in plants and yeasts using different separation techniques in combination with ICP-AES and ICP-MS.¹⁰ Only a few out of ten different organic Se compounds could be identified. Some unknown species were observed in human urine by LaFuente *et al.*; however identification was impeded by the insufficient sensitivity of organic MS.¹¹ The latter was also the limitation in investigations of food by Crews *et al.*¹² and of fish by Larsen.¹³ Therefore ICP-MS with its high detection power is increasingly considered as a promising screening technique for seleno-organic compounds.

Based on these experiences in Part I¹⁴ and II¹⁵ we have now applied ICP-sector field-MS (ICP-SFMS) in low and high resolution mode for speciation of Se compounds in herring gull eggs. These are among a set of environmental bioindicators which are collected annually and prepared for long time storage by the German Environmental Specimen Bank.¹⁶ The specimens include parts of plants, animal organs and other biological samples from typical ecosystems in the marine, limnic and terrestrial environment. Within the frame of this long-term project, herring gull eggs are collected from mud flats of the North Sea, and unexpectedly high total Se levels in the mg kg⁻¹ range were observed in these eggs.¹⁷ It is well known that the food reservoir for the herring gulls in this area is contaminated with heavy metals, such as Cd and Hg, and with

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‡Dedicated to Prof. Dr. K. Heumann on occasion of his 60th birthday.

organic compounds originating mainly from the rivers Weser and Elbe.^{18–20} Accumulation of Se and heavy metals has already been observed for this region in brown algae and common mussels, and Cu, Hg and Se appear particularly enriched in mussels as compared to algae and fish, with mean concentration values of 1–10 mg kg⁻¹. In comparison, the mean concentration of As is more drastically increased by a factor of 2–3.²¹ Applying the procedure developed in our previous work, speciation of Se in herring gull eggs was therefore investigated in order to obtain a more detailed insight into the processes contributing to species formation.

Experimental

The experimental details of the instrumentation as well as of chemicals and standards used were presented in detail in Part I of this series. Operational conditions applied to the present investigations are compiled in Table 1.

An important aspect of the present work is the preparation of the environmental samples for speciation. The herring gull eggs were collected in an area of the German mud flats on the island of Trischen in the estuary of the River Elbe, which over many years has been highly polluted. The sampling strategy of the environmental specimen bank and the sample preparation for storage of the herring gull eggs are described in more detail elsewhere.²²

Analytical samples from the herring gull eggs were prepared as follows. After collection, up to 2.5 kg of the material was immediately deep-frozen in liquid nitrogen to avoid any risk of chemical alterations. A mean Se concentration of 706 ± 7 ng g⁻¹ was determined for fresh samples (corresponding to about 3.1 µg g⁻¹ Se dry mass) by applying high pressure ashing and hydride generation atomic absorption spectrometry. From the deep-frozen samples, a homogeneous powder was prepared by cryogenic grinding, which was then divided into subsamples each one corresponding to 10 g of fresh mass. Only subsamples from material collected in 1996 were taken into account for this investigation.

For speciation, an aliquot of approximately 5 g of a subsample was extracted by shaking with 20 ml of a methanol–water mixture (9:1 v/v) overnight. After ultracentrifugation at 17000g and decanting of the solution, the residue was washed three times with 20 ml of a methanol–water

mixture (9:1 v/v) and then discarded. Afterwards the total of the resulting solutions was centrifuged for 30 min. Then methanol was carefully removed by evaporation at reduced pressure and the resulting residue dissolved in 10 ml of doubly distilled water and stored at 4 °C in the dark. The extract from the herring gull eggs was not clear and had a yellowish colour. To prevent particles from reaching the analytical column, the stored solution was filtered through cellulose–acetate filters with a pore diameter of 0.45 µm. Alternatively, a low molecular weight fraction was prepared by use of ultrafiltration at 8000g for 3 h with a cut-off of 30 kDa. An aliquot of 200 µl was taken for chromatography.

For determination of the recoveries in each step of the procedure, 1.5 g of fresh sample material was digested using 2 ml of concentrated sub-boiled HNO₃ in a high pressure asher (HPA, Parr, Graz, Austria) followed by analysis using hydride generation ASS (PerkinElmer 4100, Überlingen, Germany). For the deep frozen material, 1 g sample was digested with 3 ml of the extraction solution.

Results and discussion

Recovery

For checking the recovery of the HPLC step in the speciation procedure, four organoselenium standard compounds (selenocystine, selenocystamine, selenomethionine, selenoethionine) were used in concentrations of 100 ng ml⁻¹ each. By measuring the isotope ⁸²Se the mean recovery for total selenium was determined as $106 \pm 5\%$ ($n=5$), which was considered satisfactory. The excess is probably due to memory effects of the column and the whole HPLC system including the HHPN²³ unit with its rather high dead volume.

Extraction is the most crucial step in the whole procedure. It must be done without changing the species form in the deep-frozen material. The efficiency should be as high as possible, and the extract should represent the true composition of the sample. Using a methanol–water mixture (9:1 v/v), the recovery of the extraction procedure was determined by measuring the total Se in the extract (10 ml) using a five point calibration. From three replicates of 1.5 ml of the extract each, a Se concentration of 56.9 ± 1.9 ng g⁻¹ was determined in the extract. In comparison with 706 ± 7 ng g⁻¹ of the initial deep frozen material as obtained with atomic absorption

Table 1 Instrumental components, operating conditions and chromatographic parameters

<i>ICP-MS system</i>	Prototype of the ELEMENT (Finnigan MAT, Bremen, Germany)
Generator type	ICP-20, Rf Plasma Products
Frequency	27 MHz
Power	1350 W
Resolution	$R=400; 3000$
Sampler	Nickel, 1.0 mm orifice diameter
Skimmer	Nickel, 0.7 mm orifice diameter
<i>Argon flow rates</i>	
Outer	13.0 l min ⁻¹
Auxiliary	1.60 l min ⁻¹
Nebulizer	1.06 l min ⁻¹
<i>Sample introduction system</i>	
Nebulizer	Hydraulic high pressure nebulization (Knauer, Berlin, Germany)
Sample uptake rate	1.20 ml min ⁻¹
Pump	HPLC pump (Knauer)
<i>Desolvation system</i>	
Heating temperature	140 °C
Cooling temperature	First stage -5 °C, second stage -10 °C
<i>Reversed-phase chromatography</i>	
Analytical column	Eurospher 100 Å (Knauer); Nucleosil 120 Å, C18, 5 µm (Knauer)
Mobile phase	30 mM ammonium formate buffer, methanol–water 5:95 (v/v)
pH	3.0
Flow rate	1.20 ml min ⁻¹
Sample injection volume	200 µl

spectrometry, the extraction recovery can be estimated to be about 8% only. Though unsatisfactory, this is in agreement with the findings of Casiot *et al.*²⁴ who investigated different extraction procedures for speciation of Se in yeast and found similar recoveries with methanol–water mixtures. For further improvement they proposed a sequential leaching procedure including the use of enzymes.

Choice of isotopes and mass resolution

In speciation by means of ICP-MS as an element detection technique with high detection power and true multielement capabilities, correlations between fractions of the main element under investigation and chromatographic signals of selected elements with indicator functions for special types of compounds can be a valuable tool for identification of the species.²⁵ For the selection of suitable elements, a survey analysis with semiquantitative determination of the elements present in the extracts was made after microwave digestion. The results for extracts from the herring gull eggs are presented in Table 2. In comparison with Se, high concentrations of Mg and Ca and also of As, Ca and Br are noticeable. In general the determination of Se using the isotope of 82 u may be disturbed by an interference from ⁸¹Br¹H⁺, however, application of high mass resolution showed that this was not the case here. Furthermore high concentrations of Rb and I in the extracts become evident, while the concentrations of other heavy metals such as Co, Ni, Cd, Sn, Sb and Pb are not significant.

According to these results, the isotopes ³⁴S, ⁸⁸Sr, ⁸⁵Rb, ⁷⁵As, ⁶³Cu, and ⁶⁵Cu were selected to measure their correlation to the Se isotopes. In Part II it was shown that a higher mass resolution (*R*=1400) can be applied to overcome solvent-induced interferences and simultaneously improve the limits of detection by a factor of 4 at least.¹⁵ Since, in the present investigation, ultimate detection limits were not a primary aim, the resolution could generally be restricted to a low value (*R*=400). Only in the case of ³⁴S⁺ was a resolution of *R*=3000 necessary for separation of the signal from the interfering ¹⁶O¹⁸O⁺ signal. As already discussed in Part II in more detail, both Se isotopes, ⁷⁷Se and ⁸²Se, were measured to check for any interferences from co-eluted substances. For the latter isotope the interference from ⁸²Kr⁺ had also to be considered, but was usually negligible when commercial argon was used as the plasma gas. A possible interference from ⁶⁴Zn¹⁸O can be ignored because Zn is not a major component in the extract, as shown by the data from the semiquantitative analysis. If not mentioned otherwise, results are presented for ⁸²Se, since this isotope showed a higher signal-to-background ratio in comparison to ⁷⁷Se.¹⁵

Rh was not detected in the samples and did not show any retention on the column. Therefore this element was used as an internal standard even for the HPLC separations. It generates a continuous chromatographic signal suitable for correction of drift effects.

Table 2 Semiquantitative determination of major and trace elements in extracts of herring gull eggs after microwave digestion

Element	Isotope	Concentration/ng ml ⁻¹
Mg	24	4350
Ca	44	6383
Cu	65	48
As	75	45
Br	79	51000
Rb	85	400
Sr	88	7
I	127	950

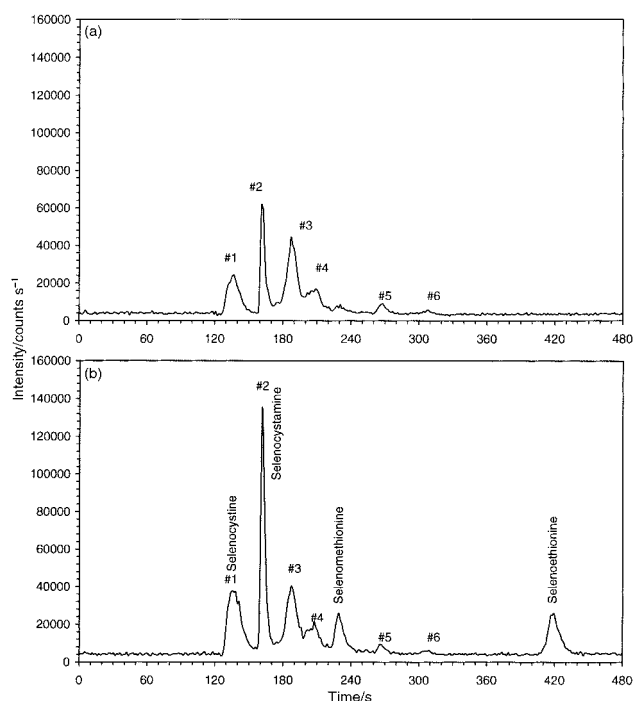


Fig. 1 (a) Chromatogram measured at mass 82 of herring gull egg extract (1 : 5 diluted) and (b) herring gull egg extract spiked with 2 ng ml⁻¹ of each of the organoselenium standard compounds; column, Eurospher; injected volume 200 µl.

Chromatograms

The procedure described in Part I was applied to the speciation of Se in samples from herring gull eggs which were prepared as outlined above.¹⁴ Results are shown in Fig. 1(a) for a 200 µl injection, representing a chromatogram of the 1 : 5 diluted extract measured at 82 u. Six fractions containing Se species are observed. Fraction 1 is eluted close to the column void volume; fractions 2 and 3 contained more than 60% of the total Se. After additional ultrafiltration with a cut-off of 30 kDa the chromatogram appears unchanged, showing that only small molecules are present in the extract.

In Fig. 1(b) an additional chromatogram is shown that was measured after addition of 2 ng ml⁻¹ of each of the four organic Se standard compounds with otherwise unchanged conditions. It is concluded from this measurement that, within the detection limits, the sample does not contain selenomethionine and selenoethionine, because, in comparison to the spiked extract, the corresponding signals do not appear in the chromatogram of the non-spiked one.

Selenocystine is eluted in the first peak; however, the conclusion that this compound is present in the sample is questionable. This is because it is only weakly retained on the column and other Se compounds such as selenite and selenate may be co-eluted, although the application of cation- and anion-exchange chromatography (*cf.* Part I) has indicated that inorganic Se is not present in this sample.

Transformation processes of Se species

In order to check the stability of Se species in the extracts from herring gull eggs, changes in the peak areas and their ratios in the chromatograms were monitored. As indicated by change of smell and colour, such transformations are probably due to microbial decomposition of the species in spite of storage in a refrigerator; additionally hydrolysis may take place.

Generally, however, the qualitative information was identical for all samples under investigation, and fractions 2, 3, 4 and 5 could always be detected. Only the peak area ratios changed to a certain extent (<25%), in particular for 2 and 3.

Due to its low intensity, the peak from fraction 6 was excluded from further investigations. The significance of fraction 1 could not be determined, because the chromatographic signal was not clearly resolved in all chromatograms.

Identification of Se species

Several strategies, such as variation of the pH value, standard additions or application of different separation techniques are normally applied for species identification using standard compounds. They were required in this work to verify the presence of selenocystamine in the second fraction. In reversed-phase (RP) chromatography, strong changes in the pH value of the eluent result in deterioration of the chromatographic resolution. Therefore RP chromatography could not be applied here. Among alternative separation techniques, ion exchange chromatography looks promising, because in the pH range 3–8 most organic Se compounds—especially selenoaminoacids—are present as zwitter ions. They become positively charged at lower and negatively charged at higher pH values depending on their pK_a . Therefore anion- as well as cation-exchange chromatography was applied additionally, but for the eluents used, the detection limits were not good enough for detection of the compounds under discussion. Therefore future work will be focused on complementary separation techniques among which the combination of RP chromatography with ion pair chromatography or size exclusion chromatography (SEC) looks especially promising.^{10,26,27}

This was the reason why standard additions was used as the only strategy for verification. For this experiment addition of selenocystamine in concentrations of 1, 2, 5 and 10 ng ml⁻¹ was employed for calibration as well as for identification. From the curve obtained, the Se concentration in the extract was calculated to be 7.4 ng ml⁻¹, which is equivalent to 14.1 ng g⁻¹. Therefore it can be concluded that 25% of the Se in the extract is present as selenocystamine. No peak splitting appeared, and therefore it was concluded that this fraction is indeed made up of selenocystamine. The presence of selenocystamine in biological samples is not unlikely, because this compound has also been identified in other biomaterials such as human milk,⁹ so that the presence of selenocystamine may be interpreted as the result of metabolization of selenoaminoacids, perhaps *via* decarboxylation.

A further attempt towards identification was made by application of electrospray ionisation mass spectrometry (ESI-MS) in combination with the separation technique. This, however, failed because the detection limits were up to three orders of magnitude higher than with ICP-MS. This is in agreement with the findings of Michalke and Schramel,⁹ Crews *et al.*¹² and Larsen,¹³ who tried to use ESI-MS for identification of organic Se or As compounds, but also failed due to insufficient sensitivity. This demonstrates the advantage of ICP-MS as an extremely sensitive screening technique for organic Se compounds such as aminoacids and proteins.

At least four fractions remain unidentified up to date, and for selenocystamine further experiments are necessary for verification. The main problem with respect to the unknown fractions is that no other standard compounds were available for our experiments and therefore alternative methods providing information are needed for further identification.

Correlation with co-eluted elements

Owing to the higher concentration levels of some trace and heavy metal elements in the extracts, the signals of the elements S, Sr, Rb, As and Cu in the chromatographic peaks were additionally measured to check for correlations with Se, and the results are presented in Fig. 2 (a)–(g). No correlation was able to be established for fraction 1, because the corresponding compounds are eluted immediately after the column void

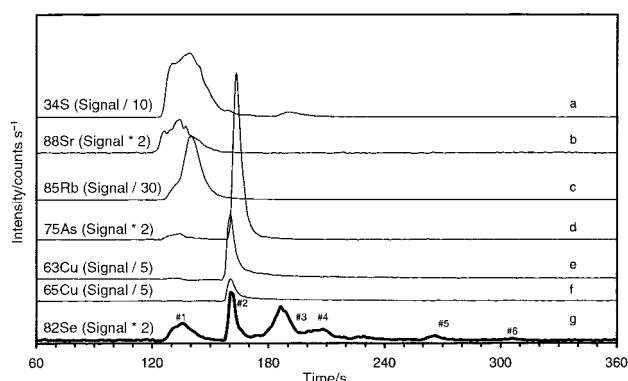


Fig. 2 Chromatograms of different isotopes of a herring gull egg extract (1:5 diluted) measured at low mass resolution (column: Nucleosil): (a) S measured at mass 34; signal intensity divided by 10; (b) Sr measured at mass 88; signal intensity multiplied by 2; (c) Rb measured at mass 85; signal intensity divided by 30; (d) As measured at mass 75; signal intensity multiplied by 2; (e) Cu measured at mass 63; signal intensity divided by 5; (f) Cu measured at mass 65; signal intensity divided by 5; and (g) Se measured at mass 82; signal intensity multiplied by 2.

volume, and do not show significant interaction with the column material. This holds true in particular for Rb and Sr for which a broad unresolved chromatographic signal appears. In the case of As, the first of the two appearing signals shows a certain correlation with fraction 1 of the Se chromatogram, probably because it contains arsenite and arsenate which, as ionic species, (as in the case of Se) cannot be resolved with RP chromatography.

The main signal of As appears close to the dominant Se signal (fraction 2) at a retention time of 170 s. By using comparison with standard compounds, this component could be identified as arsenobetaine, which was also identified as the main As species in herring gull eggs from the North Sea coast by speciation analysis with HPLC-ICP-MS as described recently.²⁸ This demonstrates that the analytical procedure presented here, though not optimised for the detection of As, is capable of giving multielement and multispecies information on organic compounds of metals and semi-metals. The correlation of Se and As is surprising, because an antagonistic behaviour of both elements has been described earlier.²⁹

For fraction 2 identified already as selenocystamine, a remarkable correlation with Cu is obvious. This element was measured using both isotopes, the presence of which was confirmed by the correct abundance ratio. Like Se, Cu is a biologically essential metal and a component of a variety of metallo-enzymes. The biological correlation of Se and Cu has already been the subject of many animal experiments.³⁰ It is, for instance, well known that Cu impedes the accumulation of Se and thus its availability for glutathione-peroxidase by absorption or formation of insoluble intracellular Se compounds. Due to contradictions in these investigations, no unequivocal conclusions can be drawn as to the relationship between Se and Cu to date, but it should be mentioned that accumulation in brown algae and mussels of Se, Cu and As was observed in the region where the eggs had been sampled. The element pattern of mussels essentially corresponds to that of herring gull eggs, so that, in future work, it should be possible to prove whether the species pattern in the eggs is influenced by the species pattern of the food chain.

Since the selenoaminoacids are analogues of thioaminoacids,³¹ the chromatograms of sulfur were also measured using the less abundant but also less interfered isotope ³⁴S. A broad signal of high intensity appeared immediately after the solvent front. Another weak signal was observed at a retention time of about 160 s, obviously not sensitive to variations in the first one. To obtain more detailed information on the broad sulfur

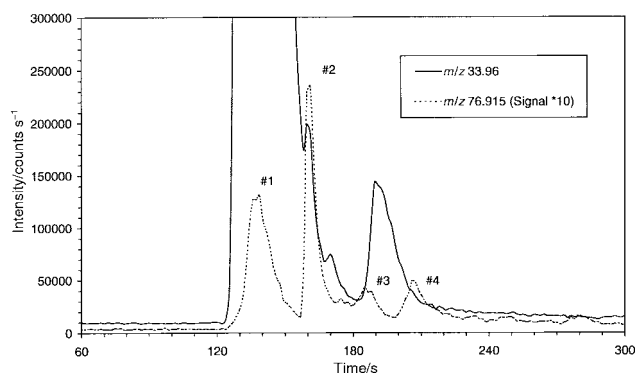


Fig. 3 Chromatograms of S(34) and Se(77) of a herring gull egg extract (1:5 diluted) measured at high mass resolution ($R=3000$) (column, Nucleosil).

signal, the measurement was repeated with higher mass resolution ($R=3000$), and the signal of ^{77}Se was registered in addition to that of ^{34}S . The resulting chromatograms are presented in Fig. 3 with magnification of the Se signal by a factor of 10. Correlations of S and Se are now observed. Fraction 1 of Se coincides with the dominating S signal, and—what is of particular interest—the second signal attributed to selenocystamine shows a correlation with a S peak in the tailing of the first broad S signal. The peak at 195 s in the S signal covers the region of fractions 3 and 4 in the Se signal. Thus all Se-containing fractions show a correlation to S, which might hint at a certain exchange mechanism in some amino-acids and proteins.

Conclusion

RP chromatography coupled to an ICP-SFMS instrument has been applied in order to obtain a more detailed insight into the Se species present in herring gull eggs, which are used as a biomonitor to trace the heavy metal status of a polluted marine ecosystem. Six selenium-containing fractions were observed. By use of high mass resolution for all Se-containing fractions, a correlation between Se and S became obvious, which might indicate that S was exchanged by Se, present in elevated amounts in the ecosystem under investigation. In one fraction a correlation of Se with arsenobetaine and with a Cu-containing fraction was observed.

In summary the present investigation demonstrates that ICP-SFMS is a high performance tool for the screening of organic Se compounds in biomaterials. The multielement capability of ICP-MS emphasizes the role of indicator (fingerprint) elements as information sources for interpretation, which has been demonstrated here by the sulfur measurements. High mass resolution instruments display the potential of becoming an important tool for speciation of elements with specific biochemical relevance, such as Fe, S and P.²⁵ For some of these elements ICP-MS instrumentation equipped with a collision and reaction cell appears to be also very promising for Se speciation, as is shown in a forthcoming paper.²⁶

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References

- 1 P. V. Hodson and J. W. Hilton in *Environ. Biogeochem. (Ecol. Bull.)*, ed. R. Hallber, Ecological Bulletins, Stockholm, 1983, vol. 35, p. 335.
- 2 W. J. Adams and H. E. Johnson in *Aquatic Toxicology Hazard Assessment: Fourth Conference*, ed. D. R. Branson and K. L. Dickson, American Society For Testing and Materials, Philadelphia, 1981, p. 124.
- 3 P. C. Baumann and R. B. Gillespie, *Environ. Toxicol. Chem.*, 1986, 5, 695.
- 4 H. Crews, *Spectrochim. Acta, Part B*, 1998, 53, 213.
- 5 R. Munoz Olivas, O. F. X. Donard, C. Cámara and P. Quevauviller, *Anal. Chim. Acta*, 1994, 286, 357.
- 6 G. Kölbl, *Mar. Chem.*, 1995, 48, 185.
- 7 Y. Shiobara and K. T. Suzuki, *J. Chromatogr. B*, 1998, 710, 49.
- 8 K. T. Suzuki, *Anal. Chem.*, 1998, 70, M57.
- 9 B. Michalke and P. Schramel, *Biol. Trace Element Res.*, 1997, 59, 45.
- 10 P. C. Uden, S. M. Bird, M. Kotrebai, P. Nolibos, J. F. Tyson, E. Block and E. Denoyer, *Fresenius' J. Anal. Chem.*, 1998, 362, 447.
- 11 J. M. González La Fuente, M. Dlaska, M. L. Fernández Sánchez and A. Sanz-Medel, *J. Anal. At. Spectrom.*, 1998, 13, 423.
- 12 H. M. Crews, P. A. Clarke, D. J. Lewis, L. M. Owen, P. R. Strutt and A. Izquierdo, *J. Anal. At. Spectrom.*, 1996, 11, 1177.
- 13 E. H. Larsen, *Spectrochim. Acta, Part B*, 1998, 53, 253.
- 14 C. Thomas, N. Jakubowski, D. Stuewer, D. Klockow and H. Emons, *J. Anal. At. Spectrom.*, 1998, 13, 1221.
- 15 I. Feldmann, N. Jakubowski, D. Stuewer and C. Thomas, *J. Anal. At. Spectrom.*, 2000, 15, 371.
- 16 H. Emons, J. D. Schlodt and M. J. Schwuger, *Chemosphere*, 1997, 34, 1875.
- 17 Umweltprobenbank, Umweltprobenbank des Bundes. Ergebnisse aus den Jahren 1996 und 1997, UBA Texte 61/99, Berlin, 1999 (ISSN 0722-186x).
- 18 H. Amer, P. Ostapczuk and H. Emons, *Environ. Monit.*, 1999, 1, 907.
- 19 P. Ostapczuk, J. D. Schlodt, H. Emons, K. Oxyne, K.-W. Schramm, G. Grimmer and J. Jacob, *Chemosphere*, 1997, 34, 2143.
- 20 K. Hopstock and H. Emons, *Chemie i. U. Zeit.*, 1999, 33, 95.
- 21 P. Ostapczuk, M. Burow, K. May, C. Mohl, M. Froning, B. Süßenbach, E. Waidmann and H. Emons, *Chemosphere*, 1998, 34, 2049.
- 22 H. Emons, *Nachr. Chem. Tech. Lab.*, 1997, 45, 897.
- 23 N. Jakubowski, I. Feldmann, D. Stuewer and H. Berndt, *Spectrochim. Acta, Part B*, 1992, 47, 119.
- 24 C. Casiot, J. Szpunar, R. Łobiński and M. Potin-Gautier, *J. Anal. At. Spectrom.*, 1999, 14, 645.
- 25 N. Jakubowski, C. Thomas, D. Klueppel and D. Stuewer, *Anal. Chem.*, 1998, 70, M37.
- 26 J. M. Marchante-Gayón, C. Thomas, I. Feldmann and N. Jakubowski, *J. Anal. At. Spectrom.*, 2000, 15, 1093.
- 27 N. Jakubowski, C. Thomas, D. Stuewer, I. Dettlaff and J. Schram, *J. Anal. At. Spectrom.*, 1996, 11, 1023.
- 28 K. Falk and H. Emons, *J. Anal. At. Spectrom.*, 2000, 15, 643.
- 29 A. L. Moxon, *Science*, 1938, 88, 81.
- 30 R. J. Shamberger, in *Biochemistry of the elements*, ed. E. Frieden, Plenum Press, New York, 1983, vol. 2, p.1.
- 31 R. J. P. Williams, *New Trends in Bioinorganic Chemistry*, Academic Press, New York, 1978.