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Capabilities of mixed-mode liquid chromatography coupled to inductively coupled plasma mass spectrometry for the simultaneous speciation analysis of inorganic and organically-bound selenium

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ABSTRACT

This work investigates for the first time the potential of mixed-mode (anion-exchange with reversed-phase) high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (ICP-MS) for the simultaneous retention and selective separation of a range of inorganic and organically-bound selenium (Se) species. Baseline separation and detection of selenocystine ($SeCys_2$), Se-methyl-selenocysteine (SeMC), selenomethionine (SeMet), methylseleninic acid (MSA), selenite, γ-glutamyl-methyl-selenocysteine (γ-glutamyl-SeMC), and selenate in a Se standard mixture by mixed-mode HPLC-ICP-MS was achieved by switching between two citrate mobile phases of different pH and ionic strength within a single chromatographic run of 20 min. Limits of detection obtained for these Se species ranged from 80 ng kg^{-1} (for SeMC) to 123 ng kg^{-1} (for selenate). Using this approach as developed for selenium speciation, an adequate separation of inorganic and organic As compounds was also achieved. These include arsenite, arsenate, arsenobetaine (AsB) and dimethylarsenic acid (DMA), which may coexist with Se species in biological samples. Application of the newly proposed methodology to the investigation of the elemental species distribution in watercress (used as the model sample) after enzymatic hydrolysis or leaching in water by accelerated solvent extraction (ASE) was addressed. Only SeMet, SeMC and selenate could be tentatively identified in watercress extracts by mixed-mode HPLC-ICP-MS and retention time matching with standards. Recoveries ($n = 3$) of these Se species from samples spiked with standards averaged 102% (for SeMC), 94.9% (for SeMet) and 98.3% (for selenate). Verification of the presence of SeMet and SeMC in an enzymatic watercress extract was achieved by on-line HPLC-ESI MS/MS in selected reaction monitoring (SRM) mode.

1. Introduction

Selenium, an essential nutrient to humans, has been the most widely investigated metalloid in food supplements and plants [1–6]. Whereas certain organic selenium compounds are linked to the beneficial effects of this mineral, inorganic forms of selenium can be toxic. Data on inorganic and organic Se species is therefore necessary to decide on the safety and marketability of selenium-fortified foodstuffs and the effect of Se interactions with toxic metals such as arsenic or mercury upon metal uptake by metal-accumulating plants [1,2,4,7–11].

The simultaneous speciation of inorganic and organically-bound Se poses some challenges. Reversed-phase (RP) ion-pairing HPLC has proven to be a powerful technique because of the possibility of simultaneous separation of ionic and neutral molecules [12–16]. However, using this separation mechanism with ICP-MS detection the speciation analysis of inorganic forms of Se is not possible due to the lack of retention of these compounds, which elute in the void volume. Ion-exchange HPLC has been reported to be the most suitable separation method for inorganic Se species such as selenite and selenate [14,17,18]. However, poor retention of target organic Se compounds (e.g. selenocystine and methyl-Se-cysteine) and co-elution of the oxidation product of selenomethionine (SeMet, form of Se found in most food/supplements and in biological samples) with relevant species have been observed using current anion-exchange based methodologies.

The use of parahydroxybenzoic acid as the mobile phase was recently reported to enable a fairly good separation of inorganic and relevant organic Se compounds on a Hamilton PRP-X100 anion-exchange column within a chromatographic run of 50 min [18]. However, a relatively low sensitivity for the ICP-MS detection of the more strongly retained Se amino acids (e.g. SeMet) was observed. This is likely due to the increasing content of organic modifier with

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increasing time, which affects the ICP conditions. Moreover, poor resolution between a key Se metabolite such as methylseleninic acid and the oxidation product of SeMet was observed using this method [18].

The on-line coupling of anion-exchange and reversed-phase HPLC columns was found to improve separation and retention of a range of Se compounds (e.g SeCys2) in comparison with the use of only one column (anion-exchange or reversed-phase). However, the simultaneous use of two columns resulted in an increased column back pressure, large dead volumes and did not lead to oxide peak separation [18,19]. Alternatively, mixed-mode high performance liquid chromatography that combines the aspects of ion-exchange chromatography and conventional RP chromatography in a single chromatographic column has demonstrated a great potential for separating a wide range of anionic and neutral compounds within a single chromatographic run. To the authors' knowledge, no application of this separation mechanism to the simultaneous speciation analysis of inorganic and organically-bound element species by HPLC-ICP-MS has been reported before.

This work aims at evaluating the potential of a mixed-mode liquid chromatographic method for the separation of a range of inorganic and organic Se compounds such as selenate, selenite, selenocystine (SeCys2), selenomethionine (SeMet), selenomethionine oxide (SeOMet), Se-methyl-selenocysteine (SeMC), methylseleninic acid (MSA) and gamma-glutamyl-Se-methyl-selenocysteine (γ-glutamyl-SeMC) using ICP-MS detection of Se. The influence of mobile phase ionic strength, pH, and content of organic modifier on the separation of such Se compounds onto a weak anion-exchange/reversed-phase mixed-mode HPLC column was investigated. The capability of the newly developed method was also studied for the simultaneous separation and detection of Se species with inorganic and organic As compounds such as arsenite, arsenate, arsenobetaine (AsB), dimethylarsenic acid (DMA), which coexist with Se species in biological samples, within a single chromatographic run. Application of the newly proposed methodology to the investigation of elemental species distribution in watercress, used as the model sample, after enzymatic hydrolysis or leaching in water by accelerated solvent extraction (ASE) was addressed. Verification of the major organic Se species detected by HPLC-ICP-MS was addressed by direct analysis of the watercress extracts using HPLC on-line coupled with ESI MS/MS in selected reaction monitoring (SRM) mode.

2. Materials and methods

2.1. Reagents, standards and samples

Selenium standards of Se-DL-methionine (SeMet), Se-DL-cystine (Se-Cys_2), Se-L-methlyselenocysteine (SeMC), sodium selenate and sodium selenite and arsenic standards of di-sodium hydrogen arsenate heptahydrate, sodium meta(arsenite) and dimethylarsenic acid (DMA) and other chemical substances were purchased from Sigma–Aldrich (Gillingham, Dorset, UK) unless stated otherwise. The standards of L-γ-glutamyl-Se-methylseleno-L-cysteine (γ-glutamyl-SeMC) and methylseleninic acid (MSA) were purchased from PharmaSe (Lubbock, TX, USA). Arsenobetaine (AsB) was obtained from IRMM (Geel, Belgium) as a solution of AsB in water (BCR 626) with a certified value of $1.03 \pm 0.07\ g\ kg^{-1}$. Individual stock solutions of $1\ mg\ g^{-1}$ were prepared by dissolving the Se standard substance in degassed ultra-pure water and stored in the dark at 4 °C. Hydrochloric acid (3% v/v) was used to dissolve $SeCys_2$. Selenomethionine Se-oxide (SeOMet) was prepared in-house by oxidation of SeMet with hydrogen peroxide following a procedure reported elsewhere [17].

Methanol (Fisher Scientific, Loughborough, UK) was of HPLC grade. All reagents used were of the highest available purity. Formic acid was purchased from Fisher Scientific. De-ionised water (18 MΩ cm) was obtained from an Elga water purification unit (Marlow, Buckinghamshire, UK). Super-purity concentrated nitric and hydrochloric acids and hydrogen peroxide were purchased from Romil (Cambridge, Cambridgeshire, UK).

A standard solution of $10\ \mu g\ kg^{-1}$ of Se in the corresponding mobile phase was prepared from a $1000\ mg\ kg^{-1}$ Se solution and used for the daily optimisation of the ICP-MS parameters. For quantification of the total Se content, working standard solutions were prepared daily by dilution of the $1000\ mg\ kg^{-1}$ Se stock solution with 3% (v/v) nitric acid aqueous solution.

Se-enriched watercress, grown in Se-enriched soil, was supplied by Nutrilaw (Narborough, Norfolk, UK). Watercress samples were freeze-dried, ground, thoroughly homogenised and stored at −20 °C prior to sample treatment.

2.2. Instrumentation

Enzymatic hydrolysis of watercress samples was performed in a hybridisation oven Model HB-2 (Techne, Duxford, UK). Extracts were centrifuged in a CENTAUR 2 centrifuge (Fisher Scientific, Loughborough, UK). Extraction of water-soluble selenium compounds from watercress was carried out by accelerated solvent extraction using a Dionex ASE 200 system (Sunnyvale, CA, USA). A Multiwave 3000 microwave oven (Anton Paar, Graz, Austria) was employed for acid digestion of the solid samples.

HPLC-ICP-MS measurements were carried out using an Agilent Technologies 1200 HPLC system (Palo Alto, CA, USA) for chromatographic separation and an Agilent 7500ce ICP-MS (using H_2 collision gas) for elemental specific detection. Mixed-mode (anion-exchange with reversed-phase) HPLC was performed on an Acclaim WAX-1 (250 mm × 4.6 mm id × 5 μm) column (Dionex Ltd, Surrey, UK) with an Acclaim organic acid (150 mm × 4 mm id × 5 μm) column (Dionex) connected in front of it. Reversed-phase (RP) HPLC was performed on an Agilent Zorbax Rx-C_8 column (250 mm × 4.6 mm id × 5 μm). The HPLC column was directly connected to a $100\ \mu L\ min^{-1}$ PFA microflow concentric nebuliser of the ICP-MS *via* PEEK tubing (0.1 mm id × 350 mm). The Agilent Technologies ICP-MS chromatographic software (G1824C Version C.01.00) was used for integration of the chromatographic signal. The chromatographic and instrumental parameters for on-line measurements with ICP-MS are summarised in Table 1.

For the RP HPLC-ESI MS/MS experiments, a 4000 QTRAP™ mass spectrometer (ABI/MDS Sciex) and an Agilent Technologies 1100 HPLC system (Palo Alto, CA, USA) were used. The effluent from the RP HPLC column ($0.5\ mL\ min^{-1}$) was fed directly into the electrospray source (operated in positive ion mode) using a PEEK connecting tube. Data acquisition and processing were performed using the ABI Analyst® software version 1.4.1. The chromatographic and instrumental parameters for on-line ESI-MS/MS measurements are summarised in Table 1.

2.3. Procedures

2.3.1. Determination of total Se

0.3 g of the watercress sample was digested with 6 mL of nitric acid–hydrogen peroxide (1 + 1, v/v) in a microwave oven following our procedure as reported elsewhere [7]. Digested samples were appropriately diluted with ultra-pure water prior to their analysis by ICP-MS. Quantification was performed by external calibration, monitoring the isotopes ^{77}Se and ^{78}Se and 2-fold diluting the sample by mixing it on-line with a solution of Ge used as the internal standard. A dogfish mussel CRM DORM-2 (National Research Council of Canada, Ottawa, Canada) with a certified Se concentration

Table 1
Optimum HPLC-ICP-MS and HPLC-ESI-MS/MS operating conditions.

ICP-MS parameters			
RF power: 1200 W			
Plasma Argon flow rate: 0.89 L min^{-1}			
Make-up gas flow rate: 0.30 L min^{-1}			
Sample/skimmer cones: Ni			
Spray chamber temperature: 2 °C			
Cell gas: H_2			
Cell gas flow rate: 2 mL min^{-1}			
Data acquisition			
Points per spectral peak: 1			
Isotopes monitored: ^{77}Se, ^{78}Se			
Integration time per mass: 300 ms			
HPLC conditions for ICPMS			
Analytical column:	Dionex Acclaim Organic Acid (150 mm × 4 mm id × 5 μm) with Dionex Acclaim Mixed-Mode WAX-1 (250 mm × 4.6 mm id × 5 μm)		
Mobile phase	0–7 min:	100% A	0.9 mL min^{-1}
	8–20 min:	100% B	0.9 mL min^{-1}
Gradient:	A: 4 mM NH_4 hydrogen citrate + 2% MeOH (v/v), pH 4.2		
	B: 65 mM NH_4 hydrogen citrate + 2% MeOH (v/v), pH 6.4		
Injection volume:	50 μL		
ESI-MS/MS parameters			
Source: Turbo V Ion spray			
Capillary voltage: 3.0 kV			
Desolvation temperature: 700 °C			
Dry gas: 70 psi			
Nebulisation gas: 70 psi			
Curtain gas: 20 psi			
Declustering potential: 40 V			
Collision energy (for MS/MS): 25 V			
Collision gas: N_2			
HPLC conditions for ESI-MS			
Column: Agilent Zorbax R_x-C_8 (4.6 × 250 mm, 5 μm)			
Mobile phase: (98 + 2)- H_2O: MeOH with 0.1% (v/v) formic acid			
Flow rate: 0.5 mL min^{-1}			
Column temperature: 25 °C			
Injection volume: 50 μL			

value of 1.4 ± 0.6 mg kg^{-1} for Se was used to evaluate the accuracy of the procedure. Please note that the use of a plant CRM would probably be a better choice. However, the dogfish mussel CRM was used for quality control because it was the only one available in our laboratory with a complex biological matrix and a certified Se concentration in the range of 1–10 μg g^{-1}. Precisions are given as standard deviations for $n = 3$ unless stated otherwise.

2.3.2. Leaching in water by accelerated solvent extraction (ASE)

0.3 g of watercress, accurately weighed, was extracted with water using an ASE system and the procedure described in our previous work [14]. For determination of total Se, the 1:10 diluted extract was analysed by ICP-MS using the procedure described above. For Se speciation analysis, the extract was filtered (0.45 μm acetate cellulose filter) and injected undiluted onto the HPLC column.

2.3.3. Extraction of protein-bound Se by enzymatic hydrolysis

Enzymatic hydrolysis was performed by adding 10 mL of a 30 mM Tris–HCl buffer solution (pH 7.5) containing 60 mg protease and 30 mg lipase to 0.3 g watercress sample and following the two-step procedure described elsewhere [15].

2.3.4. Se speciation analysis by mixed-mode HPLC-ICP-MS

For determination of SeMC, SeMet and selenate, a 50 μL aliquot of the 1:2 diluted extract was analysed by mixed-mode HPLC-ICP-MS at a flow rate of 0.9 mL min^{-1} switching between two citrate mobile phases of different pH and ionic strength (see conditions in Table 1). Preliminary identification of the Se compounds was performed on the basis of retention time matching with Se standards. Calibration was carried out by the standard addition calibration technique (at three spike levels) using peak area measurements of the chromatographic signals by monitoring the ^{78}Se signal. The Se standard solutions used for calibration were characterised for their total Se content by ICP-MS, using the same method as described above.

2.3.5. RP HPLC-ESI-MS/MS analysis

For verification of the presence of SeMet and SeMC detected by HPLC-ICP-MS in the watercress extract, 50 μL of an undiluted enzymatic extract was injected on to the reversed-phase column. The separation and elution of the Se compounds was achieved using the conditions summarised in Table 1. The parameters used for online ESI MS/MS measurements are also given in Table 1. For both, SeMet and SeMC, MS/MS spectra were acquired in the selected reaction monitoring (SRM) mode. The most abundant transitions m/z 198 > m/z 181 and m/z 198 > m/z 109 (for SeMet) and m/z 184 > m/z 167 and m/z 184 > m/z 95 (for SeMC) were monitored.

3. Results and discussion

3.1. Simultaneous HPLC-ICP-MS analysis of inorganic and organic elemental species

As a first step, the capability of a mixed-mode LC column (Acclaim® mixed-mode WAX-1) coupled with ICP-MS was investigated for the simultaneous separation and detection of a range of inorganic and organic Se standards. The column is packed with a silica based stationary phase that incorporates both hydrophobic and weak anion-exchange properties. Therefore, chromatographic

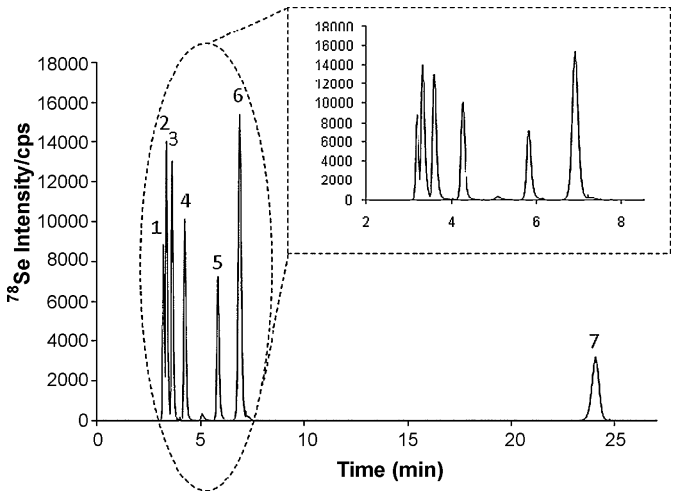

Fig. 1. Mixed-mode HPLC-ICP-MS profile of ^{78}Se obtained for a 10 ng g^{-1} Se standard mixture containing SeOMet (1), $SeCys_2$ (2), SeMC (3), SeMet (4), selenite (5), γ-glutamyl SeMC (6) and selenate (7). Isocratic elution with 4 mM ammonium hydrogen citrate (pH 5.4) in 2% (v/v) MeOH at 0.9 mL min^{-1}. Inset shows chromatogram with an expanded scale.

selectivity for a range of Se compounds including SeOMet, $SeCys_2$, SeMC, SeMet, MSA, selenite, γ-glutamyl-SeMC and selenate was investigated with ammonium hydrogen citrate or phosphate buffers by optimising parameters such as mobile phase ionic strength, pH and organic content.

The retention times of the investigated Se species using the two chromatographic buffers at equivalent pH and ionic strength were found to be similar with the exception of selenate, for which retention significantly increased with phosphate buffer. Increased pH and ionic strength of the phosphate mobile phase led to a decrease in the retention time of selenate but also to poor chromatographic selectivity for the other Se compounds. Therefore, ammonium hydrogen citrate buffer was selected for further work.

Fig. 1 shows a chromatogram obtained for a mixture of Se standards each containing 10 ng g^{-1} Se. The chromatographic conditions used are specified in the figure captions. Clearly, baseline resolution of SeMet, selenite, γ-glutamyl-SeMC and selenate was achieved under isocratic conditions. However, only partial resolution of Se compounds such as SeOMet, $SeCys_2$ and SeMC, with a shorter degree of retention on the mixed-mode stationary phase, was obtained. The small Se peak detected at 5 min was preliminarily identified as methylseleninic acid (known as the oxidation product of SeMC) [14] by HPLC-ICP-MS using retention time matching with a MSA standard.

From the above results, it can be concluded that the chromatographic selectivity achieved with this simple and straightforward method is fit for purpose, particularly for the quantitative analysis of methyl-Se compounds in samples, which do not contain $SeCys_2$. However, to extend the applicability of the methodology to a wider range of samples, an effort was made to improve resolution of SeOMet, $SeCys_2$ and SeMC by adding ion pair reagents such as trifluoroacetic acid (TFA) or pentafluoropropanoic acid (PFPA) to the mobile phase. Perfluorinated carboxylic acid ion-pairing reagents have been successfully used to increase retention of Se amino acids with reverse phase stationary phases. This occurs due to interaction of the protonated Se species with the fluorinated acid ions [13–19]. In this work, the addition of up to 1% TFA or PFPA to the citrate mobile phase did not seem to affect Se species resolution using the developed mixed-mode HPLC method. All these results suggest that the anion-exchange characteristics of the column seem to have the predominant effect on the Se species separation and retention. It should also be pointed out that the addition of 2% of methanol to the mobile phase helped improve Se detection by ICP-MS as previously reported elsewhere [7], without affecting retention of the Se species under investigation.

A further attempt to improve resolution of the organic Se species SeOMet, $SeCys_2$ and SeMC was made by connecting a short reversed-phase column [Acclaim® organic acid (150 mm × 4 mm id × 5 μm)] in front of the mixed-mode column. The Acclaim OA stationary phase has been reported [20] to offer consistency in the retention times, peak shape and efficiency for a range of Se species when using highly aqueous mobile phases such as the ammonium hydrogen citrate buffer used in this study. It is important to note that when using the on-line coupling of reversed-phase with mixed-mode HPLC, a significant increase in the column back pressure was not observed. Although an improved resolution between $SeCys_2$ and SeMC was achieved (see Fig. 2A), baseline resolution of SeOMet and $SeCys_2$ was not possible with the newly developed method. Moreover, the resolution between species such as MSA and SeMet appeared to be affected by the addition of the OA column and it was, therefore, necessary to re-optimise the mobile phase pH to overcome this problem. A decrease in the mobile phase pH from 5.4 to 4.2 resulted in an improved resolution between these Se species but also in an increased degree of retention of other Se species such as selenite, γ-glutamyl SeMC and, to a greater extent, selenate, which was found to elute at approximately 60 min under such conditions. The chromatographic conditions summarised in Table 1 were selected as optimal for the selective separation and detection of the investigated Se species, including selenate, within a reasonable time frame. As shown in Fig. 2B, baseline separation and detection of selenocystine ($SeCys_2$), Se-methyl-selenocysteine (SeMC), selenomethionine (SeMet), methylseleninic acid (MSA), selenite, γ-

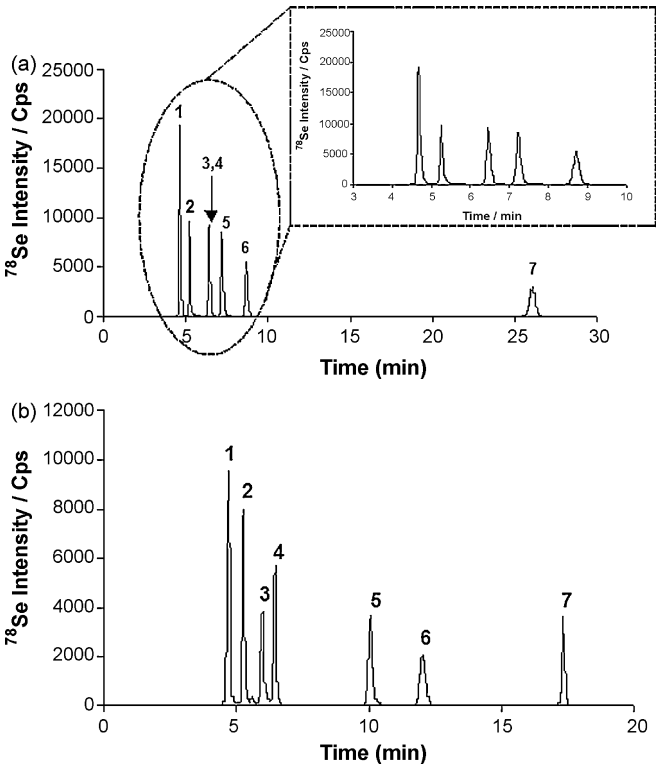

Fig. 2. Reversed-phase in-line with mixed-mode HPLC-ICP-MS profile of ^{78}Se obtained for a 10 ng g^{-1} Se standard mixture containing $SeCys_2$ (1), SeMC (2), MSA (3), SeMet (4), selenite (5), γ-glutamyl SeMC (6) and selenate (7) using (A) isocratic elution with 4 mM ammonium hydrogen citrate (pH 5.4) in 2% (v/v) MeOH at 0.9 mL min^{-1} and (B) the separation conditions given in Table 1. Inset in Fig. 2A shows chromatogram with an expanded scale.

Table 2
Limits of detection (LODs, 3σ criterion for three replicates in each case) obtained for selenium standards by reversed-phase with mixed-mode HPLC-ICP-MS (precisions for $n = 3$).

Se Species	LoD (ng kg^{-1})
$SeCys_2$	81 ± 3
SeMC	80 ± 3
MSA	140 ± 4
SeMet	120 ± 3
Selenite	123 ± 4
γ-glutamyl SeMC	180 ± 6
Selenate	121 ± 4

glutamyl-methyl-selenocysteine (γ-glutamyl-SeMC), and selenate by reversed-phase with mixed-mode HPLC-ICP-MS was achieved by switching between two citrate mobile phases of different pH and ionic strength [A: 4 mM ammonium hydrogen citrate, 2% MeOH (pH 4.2); B: 65 mM ammonium hydrogen citrate, 2% MeOH (pH 6.4)] within a single chromatographic run of 20 min. Buffer B was introduced after 7 min mainly to help significantly decrease the degree of retention of selenate on the stationary phase. Under such conditions, a fairly good separation of inorganic and organic As compounds (chromatogram not shown) such as arsenobetaine (AsB; t_R = 5.2 min) arsenite (t_R = 5.8 min), dimethylarsenic acid (DMA; t_R = 6.2 min) and arsenate (t_R = 10.4 min), that may coexist with Se species in biological samples, was also achieved.

The limits of detection (3σ criterion, $n = 3$) obtained for Se standard substances using the newly developed HPLC-ICP-MS method are summarised in Table 2. As shown in this table, Se LODs ≤180 ng kg^{-1} were achieved, demonstrating the potential of the methodology for the selective and sensitive detection of a number of inorganic and organic forms of Se relevant to nutrition, health and environment within a reasonable time frame.

3.2. Application to quantitative Se speciation analysis of Se-enriched watercress

Hydrolysis with enzymes and leaching with water by ASE using the procedures described above were found to extract 82.3 ± 8.0% and 66.0 ± 4%, respectively, of the total Se in the watercress sample.

Element speciation analysis of watercress extracts was undertaken using mixed-mode HPLC-ICP-MS. The HPLC-ICP-MS chromatograms of enzymatic and water extracts from watercress are shown in Fig. 3. Assignments based on retention time suggest that both extracts seem to contain Se species such as SeMC, SeMet and inorganic Se (with selenate being the major inorganic Se species). The presence of inorganic Se species in watercress extracts was confirmed using complementary anion-exchange HPLC on a Hamilton PRP-X100 column coupled to ICP-MS (data not shown) using a procedure described elsewhere [7]. Fig. 3 also suggests that most SeMet in watercress seems to be bound to proteins since it is primarily released by enzymatic hydrolysis. Moreover, three unknown Se peaks with retention times of 4.72, 13.7 and 15.3 min were detected in the two extracts. Since the main purpose of this study was to demonstrate the capabilities of the newly developed mixed-mode HPLC-ICP-MS method for the simultaneous detection/quantification of inorganic and organic Se species in complex biological samples, work on the identification of such unknown minor Se species in watercress by combining elemental and organic MS strategies has not been undertaken since it will only provide a deeper insight into the plant Se biochemistry. However, this will be addressed in following studies. Table 3 summarises the results obtained for total Se, SeMet, SeMC and selenate in the watercress extract obtained by enzymatic hydrolysis followed by mixed-mode HPLC-ICP-MS under optimal conditions. Hydrolysis with enzymes was selected for simultaneous quantitative analysis of the three

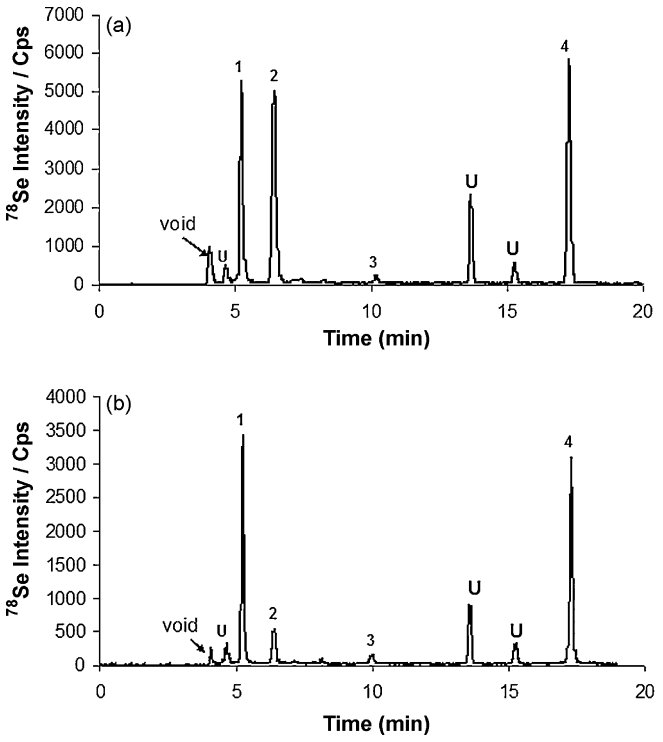

Fig. 3. Reversed-phase in-line with mixed-mode HPLC-ICP-MS profile of ^{78}Se obtained for (A) an enzymatic extract and (B) an ASE water extract from watercress under the conditions given in Table 1. Peak identity: SeMC (1), SeMet (2), selenite (3), selenate (4), U = unknown.

Table 3
Concentrations of total Se, SeMC, SeMet and selenate (on a dry weight basis) extractable from Se-enriched watercress by enzymatic hydrolysis. Precisions (as SD) are calculated from three independent determinations. In parentheses: percentage (w/w) of total Se content (on a dry weight basis).

Se fraction	Se concentration (μg g^{-1})
Total Se in solid[a]	7.44 + 0.90
Total Se (extracted from solid)	6.12 ± 0.60 (82.2 ± 8.1)
SeMC[b]	0.89 ± 0.02 (11.9 ± 0.3)
SeMet[b]	1.05 ± 0.07 (14.1 ± 0.9)
Selenate[b]	1.91 ± 0.10 (25.6 ± 1.3)

[a] Determined by microwave acid digestion. A recovery of total Se from the dogfish mussel CRM was 95 ± 4% ($n = 5$).

[b] Spike recovery of 102 ± 3% for SeMC, 94.9 ± 3% for SeMet and 98.3 ± 2% for selenate (see text for full details).

Se species because SeMet is known to occur in food-related samples incorporated into proteins and, therefore, hydrolysis with acids or enzymes is necessary to achieve best extraction efficiency for this Se compound [21], whilst selenate and SeMC are known to be easily extractable by leaching with aqueous solutions. The average recovery ($n = 3$) of Se species added before sample extraction was 102 ± 3% for SeMC, 94.9 ± 3% for SeMet and 98.3 ± 2% for selenate. The Se concentrations of species extractable in water such as SeMC and selenate were also obtained by ASE followed by mixed-mode HPLC-ICP-MS, used as a confirmatory method. The results obtained by ASE were 0.84 ± 0.04 μg g^{-1} Se (for SeMC) and 1.79 ± 0.13 μg g^{-1} Se (for selenate), which agree well with those obtained by enzymatic hydrolysis (see Table 3). As can also be seen in this table, 37% of the total Se extractable into the buffered enzymatic solution remains unidentified.

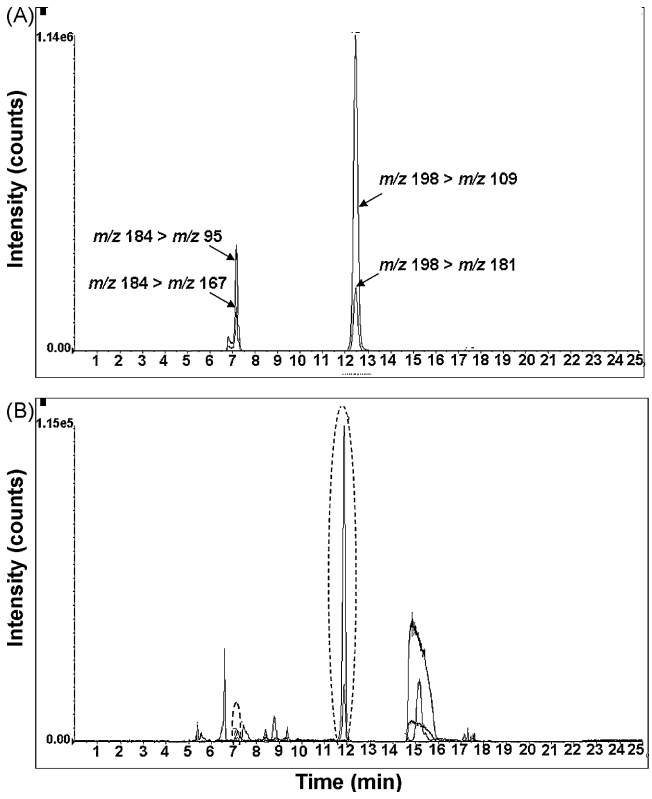

Fig. 4. HPLC-ESI-MS/MS chromatograms (SRMs of four transitions) of (A) a Se standard mixture containing SeMC and SeMet and (B) an enzymatic extract from watercress.

3.3. Verification of the presence of SeMC and SeMet using HPLC-ESI-MS/MS

The ammonium hydrogen citrate mobile phase used with mixed-mode HPLC is not compatible with ESI. Therefore, the on-line combination of reversed-phase HPLC (see Table 1 for conditions) with ESI-MS/MS in selected reaction monitoring mode (SRM) was used for verification of the identity of organic Se species including SeMC and SeMet (for which standards are available) detected by HPLC-ICP-MS in watercress enzymatic extracts. To do this, specific transitions from the precursor ions to product ions (obtained by CID) were selected to be monitored on the basis of the product ions obtained for SeMC and SeMet standards.

Fig. 4 shows the SRM chromatograms of the most abundant transitions m/z 184 > m/z 167 and m/z 184 > m/z 95, which occur at the retention of SeMC (7.0 min) and m/z 198 > m/z 181 and m/z 198 > m/z 109, which occur at the retention of SeMet (12.0 min) for a mixture of SeMC and SeMet standards (Fig. 4A) and an enzymatic extract from watercress (Fig. 4B). For both Se species, the relative abundance ratio of the two transitions obtained for the enzymatic extract was found to match the comparison standards within ±5%. These data are consistent with the presence of SeMet and SeMC in watercress.

4. Conclusions

The potential of combining mixed-mode (anion-exchange with reversed-phase) liquid chromatography with ICP-MS for the simultaneous determination of inorganic and organic forms of Se present at low parts-per-million levels in a complex plant matrix has been demonstrated for the first time. With the newly developed methodology, a relatively high chromatographic selectivity can be achieved for a range of Se species detected by ICP-MS within a reasonable time frame, offering a promising tool which should be further investigated for future application to study the interactions of Se with toxic metals such as As in biological tissues.

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References

[1] D.R. Ellis, D.E. Salt, Curr. Opin. Plant Biol. 6 (2003) 1.
[2] J. Meija, M. Montes Bayón, D.L. Le Duc, N. Terry, J.A. Caruso, Anal. Chem. 74 (2002) 5837.
[3] H. Goenaga Infante, R. Hearn, T. Catterick, Anal. Bioanal. Chem. 382 (2005) 957.
[4] M. Montes Bayón, T.D. Grant, J. Meija, J.A. Caruso, J. Anal. At. Spectrom. 17 (2002) 1523.
[5] M. Rayman, H. Goenaga Infante, M. Sargent, Br. J. Nutr. 100 (2008) 238.
[6] Z. Pedrero, Y. Madrid, Anal. Chim. Acta 634 (2009) 135.
[7] E. Warburton, H. Goenaga-Infante, J. Anal. At. Spectrom. 22 (2007) 370.
[8] O.E. Olson, E.J. Novacek, I.E. Whitehead, I.S. Palmer, Phytochem 9 (1970) 1181.
[9] M.A. Beilstein, P.D. Whanger, G.O. Yang, Biomed. Environ. Sci. 4 (1981) 392.
[10] S. Mounicou, J. Meija, J.A. Caruso, Analyst 129 (2004) 116.
[11] M. Montes Bayón, M.J. Díaz Molet, E. Blanco González, A. Sanz-Medel, Talanta 68 (2006) 1287.
[12] P.C. Uden, S.M. Bird, M. Kotrebai, P. Nolibos, J.F. Tyson, E. Block, E. Denoyer, Fresenius J. Anal. Chem. 362 (1998) 447.
[13] P.C. Uden, H.T. Boakye, C. Kahakachchi, R. Hafezi, P. Nolibos, E. Block, S. Johnson, J.F. Tyson, J. Anal. At. Spectrom. 19 (2004) 65.
[14] H. Goenaga-Infante, G. O'Connor, M. Rayman, R. Wahlen, J. Spallholz, R. Hearn, T. Catterick, J. Anal. At. Spectrom. 20 (2005) 864.
[15] E. Peachey, N. McCarthy, H. Goenaga-Infante, J. Anal. At. Spectrom. 4 (2008) 487.
[16] H. Goenaga-Infante, S.P. Joel, E. Warburton, C. Hopley, R. Hearn, S. Juliger, J. Anal. At. Spectrom. 22 (2007) 888.
[17] E.H. Larsen, M. Hensen, H. Paulin, S. Moesgaard, M. Reod, M. Rayman, J. AOAC Int. 87 (2004) 225.
[18] L. Ayouni, F. Barbier, J.L. Imbert, J.Y. Gauvrit, P. Lanteri, M.F. Grenier-Loustalot, Anal. Bioanal. Chem. 385 (2006) 1504.
[19] P. Vinas, I. Lopez-García, B. Merino-Merono, N. Campilo, M. Hernández-Cordoba, Anal. Chim. Acta 535 (2005) 49.
[20] H. Goenaga-Infante, G. O'Connor, M. Rayman, R. Hearn, K. Cook, J. Anal. At. Spectrom. 21 (2006) 1256.
[21] H. Goenaga Infante, R. Sturgeon, J. Turner, R. Hearn, M. Sargent, P. Maxwell, L. Yang, A. Barzev, Z. Pedrero, C. Cámara, V. Díaz Huerta, M.L. Fernández Sánchez, A. Sanz-Medel, K. Emese, P. Fodor, W. Wolf, R. Goldschmidt, V. Vacchina, J. Szpunar, L. Valiente, R. Huertas, G. Labarraque, C. Davis, R. Zeisler, G. Turk, E. Rizzio, L.G. Mackay, R.B. Myors, D.L. Saxby, S. Askew, W. Chao, W. Jun, Anal. Bioanal. Chem. 390 (2008) 629.