Journal of Chromatography A, 1216 (2009) 7001–7006

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

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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ARTICLE INFO

Article history:
Received 24 June 2009
Received in revised form 14 August 2009
Accepted 18 August 2009
Available online 24 August 2009

Keywords:
Selenium
ICP-MS
ESI MS/MS
Mixed-mode liquid chromatography
Anion-exchange liquid chromatography
Reversed-phase liquid chromatography
Se-enriched plants
Watercress
Accelerated solvent extraction
Enzymatic hydrolysis

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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doi:10.1016/j.chroma.2009.08.047

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