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A simplified method for inorganic selenium and selenoaminoacids speciation based on HPLC–TR–HG–AFS

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ABSTRACT

A simplified speciation method for the determination of selenite, selenate and three selenoaminoacids (selenocystine, selenomethylselenocysteine and selenomethionine) has being developed, based on the coupling of high performance liquid chromatography (HPLC), thermoreduction (TR), hydride generation (HG) and atomic fluorescence spectrometry (AFS). Most of the existing methods based on AFS detection employ a two step procedure to reduce selenate to selenite before HG: (i) Ultraviolet radiation followed by (ii) heating, to produce volatile hydrides of the selenium compounds. The proposed simplified method HPLC–TR–HG–AFS does not require ultraviolet radiation. Instead, KBr dissolved in a HCl solution is added during the heating step (thermoreduction), resulting in an effective hydride generation of the selenium species. Different variables (temperature, HCl and $NaBH_4$ concentrations) have been optimized, using both univariant and multivariant experimental designs. The proposed method is therefore less complex and allows limits of detection, reproducibility and repeatability values similar or better than the existing AFS detection methods described in the literature. A Certified Reference Material (SELM-1 with certified selenomethionine content) and a Se-enriched algae sample have been successfully analyzed with the proposed method. The results were also compared to an alternative technique (GC–MS) that provided similar results.

1. Introduction

Selenium is an essential element for higher organisms, although the range between deficiency and toxicity is narrow [1,2]. Selenium is found in biological samples in form of non-volatile inorganic selenium (selenite and selenate) and several organic selenium species (e.g., selenoaminoacids). In order to better understand the metabolism of selenium in organisms, analytical methods for the determination of selenium compounds in biological tissues must be readily available [3–5]. Therefore, speciation of selenium is of great importance in this respect, as this type of analysis gives information regarding the individual species. Speciation analysis allows to distinguish between non-covalently bound inorganic selenium, organic selenium species that are the result of the replacement of S by Se during supplementation (as it happens in selenomethionine), and other selenoaminoacids that participate in the genetically encoded incorporation of selenium into true selenoproteins [6–9].

Most methods for the speciation analysis of non-volatile selenium compounds are based on a separation technique, such as gas chromatography (GC) or high performance liquid chromatography (HPLC), which is coupled on-line to a suitable elemental or mass-selective detector. Certain approaches require the conversion of the analyte species into a form that is suitable for detection. If GC is the separation technique of choice, all selenoaminoacids must be derivatized (e.g., with chloroformates or cyanogen bromide) to volatile compounds prior to their detection either by mass spectrometry (MS) or inductively coupled plasma–mass spectrometry (ICP–MS) [10–14].

Liquid chromatography has the advantage that it can be employed both for the separation of inorganic selenium (selenite and selenate) and selenoaminoacids. The separation of the selenium species can be accomplished by using reversed-phase columns in combination with an ion-pairing agent [15–18], size-exclusion [19] or ion exchange columns [20–21]. HPLC has been coupled to MS [22] and ICP–MS [8,9,21,23]. Owing to its inherent selectivity and sensitivity, atomic fluorescence spectrometry (AFS), combined with hydride generation (HG), has gained in recent years a wide use as an element-specific detector [24].

A critical step in AFS is the necessity to convert the selenium compounds into Se(IV), in order to obtain afterwards the

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corresponding volatile hydride (SeH_2), a feature that is considered in detail in the existing literature. The early published approaches for this involve the use of either microwave radiation with a redox reagent (concentrated HBr/$KBrO_4$) [24] after the chromatographic separation to reduce Se(VI) and the selenoaminoacids, followed by HG and AFS detection. UV radiation [26] has also been employed as intermediate step to convert some selenoaminoacids into Se(IV). However, using only UV radiation as derivatization step does not allow the reduction of Se(VI) to Se(IV) [23]. Some authors have point in this sense that UV radiation has to be employed in combination with a reducing agent (e.g., potassium iodide) [27].

Nowadays, some authors have employed a commercially available device (Speciation Heated Coil) consisting of two Teflon reactions coils (a long one wrapped around a UV lamp, and a second short one wrapped around a heating block), in combination with concentrated HCl [18,28–31] for derivatization before hydride generation. Under these conditions, Se(VI) is effectively reduced to Se(IV), although the long reaction coils results in broaden peaks with some overlapping. Also, the need of a long resident time of the selenium species in the reaction coils requires low flow rates of the HPLC pump, ca. 0.5 ml min^{-1}, resulting in long retention times of ca. 35 min.

The present study considers the development of a simplified selenium speciation method (HPLC–TR–HG–AFS) that does not require the use of UV radiation for the decomposition of the selenoaminoacids and the reduction of Se(VI) to Se(IV). Instead, we propose a thermoreduction (TR) step before the hydride generation step, based on use of the short heated reaction coil of the Speciation Heated Coil device, and in the presence of KBr dissolved in concentrated HCl. This simplified method eliminates the use of dangerous UV radiation, reduces the length of the reaction coils, and allows faster flow rates of the chromatographic mobile phase. This results in shorter retention times in comparison with those methods described in the literature. The optimization of the experimental variables (temperature, HCl and $NaBH_4$ concentrations) was performed both by a univariant approach and multivariant experimental design. The proposed method was validated using a Certified Reference Material (SELM-1) and comparing the results of the certified value of the selenomethionine content with other analytical technique (GC–MS). This method has also been applied to an algae sample enriched with selenium.

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Solutions were prepared with ultra pure Milli-Q water obtained from an Elix Advantage System (Millipore). The selenium compounds that were used in this study were: sodium selenite, sodium selenate, selenomethionine (SeMet), selenocystine (SeCyst) (Aldrich) and selenomethylselenocysteine (SeMetSeCys) (Flucka). Stock solutions of 1000 mg Se l^{-1} were prepared for selenite and selenate, and 500 mg Se l^{-1} (in 0.1 M HCl) for the three selenoaminoacids, and stored at 4 °C. Working solutions were prepared daily. The mobile phase for liquid chromatography was prepared with K_2HPO_4 and KH_2PO_4 (Merck). HCl 37% (v/v) (Albus) and $NaBH_4$ (Ridel de Hean) were employed for hydride generation. Protease and lipase for enzymatic hydrolysis of the samples, and KBr for thermoreduction were purchased from Sigma-Aldrich. Certified Reference Material SELM-1 (National Research Council of Canada), corresponding to yeast enriched with selenium, was employed for quality control.

2.2. Instrumentation

A schematic diagram of the instrumental coupling HPLC–TR–HG–AFS employed for selenium speciation is depicted in Fig. 1. The operating conditions are summarized in Table 1. The HPLC consisted of a Jasco PU-2080 Plus quaternary pump equipped with a Rheodyne 7125 injector and a 200 µl loop for sample introduction. The isocratic separation of the selenium compounds was accomplished by means of a strong anion exchange column (PRP-X100, Hamilton). The mobile phase employed for chromatographic separation was 80 mM potassium phosphate solution at pH 6.

The Speciation Heated Coil (PS Analytical) employed for thermoreduction allows performing two types of sample treatment, just changing the position of a two-way switching valve. In the first position of the valve, labelled as "UV+heating", the samples passes first through a 9 m long Teflon coil placed around a 9 W ultraviolet radiation source, and then through a 3 m Teflon tube wrapped around a heating block (maximum heating temperature of 200 °C). If the valve is switched to the second position, labelled as "Heating only", the sample passed only through the 3 m Teflon tube wrapped around the heating block. The internal diameter of the tubing is 0.5 mm. In this work, thermoreduction of the selenium compounds was achieved after chromatographic separation, with the valve of the Speciation Heated Coil placed in the "Heating only" position, at a temperature of 150 °C. The outlet of the chromatographic column was mixed with a reducing solution (5% KBr in 6 M HCl) and pumped into the Speciation Heated Coil with a peristaltic pump of the AFS instrument.

HG-AFS was performed employing an atomic fluorescence spectrometer (Millenium Excalibur, PS Analytical), equipped with two peristaltic pump, a glass gas-liquid separator, and a selenium boosted discharge hollow cathode lamp (Photron). Argon was added at the gas-liquid separator to carry the hydrides to the AFS detector. The flame of the detector was sustained by the hydrogen

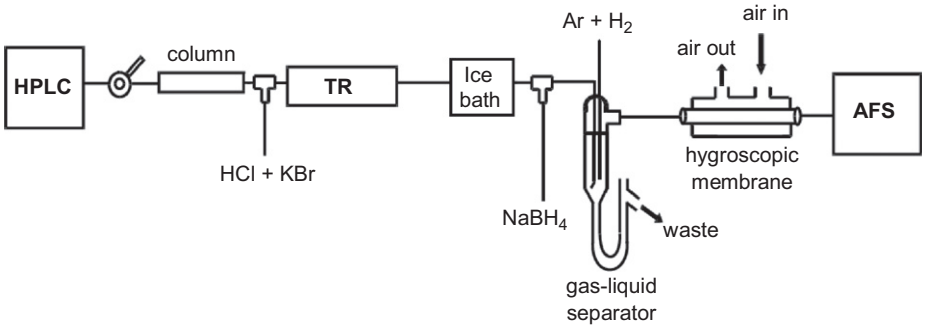

Fig. 1. Scheme of the instrumental coupling for selenium speciation based on high performance liquid chromatography–thermoreduction–hydride generation–atomic fluorescence spectrometry (HPLC–TR–HG–AFS).