Synthesis of [77]Se-methylselenocysteine when preparing sauerkraut in presence of [77]Selenite. Metabolism of [77]Se-methylselenocysteine in Wistar rats determined by LC-ID-ICP-MS

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Abstract

The use of enriched Se isotopes as tracers has provided important information on Se metabolism. However, selenium isotopes are expensive and difficult to obtain. A simple and cheap strategy based on the production of [77]Se-methylselenocysteine (Me[77]SeCys) when preparing sauerkraut in presence of [77]Selenite was developed. The resulting Me[77]SeCys was used for evaluating the metabolic pathway of MeSeCys in Wistar rats by feeding them with an AIN-93M diet containing 20 % of sauerkraut enriched in Me[77]SeCys. Organs (liver, kidney, brain, testicles and heart) were obtained after 7 days of treatment and subjected to total selenium and selenium speciation analysis by HPLC-ID-ICP-MS. Analysis of [77]Se labeled organs shown a prominent increase (more than a 100% of Se level enhancement) of selenium in kidney and heart, whereas in liver, selenium concentration only increased up to 20 %, remaining constant in brain and testicles. [77]Se-enriched sauerkraut supplementation does not alter the concentration of other essential elements in comparison to the controls, except in the case of heart and kidney, in which selenium was positively correlated with Mg, Zn, Cu and Mo. HPLC-ICP-MS analysis of hydrolyzed extracts after carbamidomethylation of the [77]Se labeled organs shows the presence of [77]SeCys and an unknown Se-containing peak, the identity of which could not be verified by ESI/MS/MS. Low amounts of Me[77]SeCys were found in [77]Se-labeled liver and kidney extracts, suggesting the incorporation of this selenium species in its intact form.

Introduction

Selenium (Se) is an essential element to humans and plays an important role in a number of biological processes involving: immune and reproductive systems, thyroid function [1] and redox balance [2-5]. Therefore, improving Se status may benefit long-term health while strengthening the immune response. The dietary considerations of Se have led to a widespread scientific interest regarding the development of Se-enriched food products [6-10]. However, selenium metabolism, once ingested, has not been clearly stated. Selenium is commonly transformed into selenide which is assumed to be the intermediate for either the excretion or for the synthesis of proteins, where selenium is present as selenocysteine (SeCys), in selenoproteins, or as selenomethionine (SeMet) in selenium-containing proteins [11]. On the other hand, methylselenol is another intermediate compound involved in selenium metabolic pathway. This species is supposed to be the responsible for the anticarcinogenic effect of selenium [12], and it is obtained from methylselenocysteine (MeSeCys) through β-lyase reaction. A comprehensive understanding of selenium species metabolism is necessary in order to elucidate its physiological role. In this line, the use of isotopic labeled selenium is an easy way to monitor selenium metabolisms once ingested. Enriched stable isotopes are used to safely estimate the absorption and retention, or to study the uptake and metabolism of minerals in human physiological studies. As selenium is mainly covalently bound in selenoamino acids, the need for intrinsic labeling utilizing the natural biosynthetic pathways in living organisms is evident. However, the production of selenium isotopes is expensive and difficult to obtain. In a previous work [8], MeSeCys has been reported as the main metabolic product when preparing fermented cabbage (sauerkraut) in presence of Se(IV). Based on this work, a simple and cheap method was used for producing Me^{77}SeCys when preparing sauerkraut in presence of

[77]Selenite. Moreover, metabolism of MeSeCys was evaluated by feeding rats with a diet containing Me[77]SeCys-enriched sauerkraut. For this purpose, the resulting [77]Se-labeled metabolites were analyzed in different organs (kidney, liver, heart, brain, and testicles) by HPLC-ICP-MS and quantified by HPLC-ID-ICP-MS. The obtained results could help to provide further insights on the mechanism of action of MeSeCys. Further, the work illustrates how natural processes (such as lactic fermentation), which imply inorganic selenium biotransformation, can be useful for preparing selenium stable isotopes as tracers to investigate selenium metabolism and also to achieve rapid speciation analyses and quantification of Se by HPLC-ICP-MS.

Materials and methods

Instrumentation

An analytical microwave oven (CEM, Matthews, USA) was used for the acid digestion process.

Enzymatic hydrolysis of samples was carried out by using an Optic Ivymen System constant temperature incubator shaker (Comecta S.A., Spain).The extracts from the enzymatic hydrolysis were centrifuged in an Eppendorf centrifuge 5804 R F34-6-38 (Germany).

An Agilent HP-7700 Series ICP-MS (USA) equipped with an octapole reaction cell was used for monitoring [77]Se, [78]Se and [80]Se isotopes. Argon-based polyatomic interferences were reduced by using H_2 as the cell gas. For chromatographic experiments, a high-pressure pump Jasco PU-2089 (Italy) was used as the sample delivery system under isocratic conditions. The pump was fitted with a six-port Rheodyne 7725i sample injection (Rohner Park, USA). The separation was performed on an anionic exchange

PRP-X100 (250 x 4.1 mm, 10 µm) (Hamilton, Switzerland), and a reversed-phase ion pairing C8 Zorbax R_x-C_8 (250 x 3.0 mm, 5 µm) (Agilent, USA). The operating conditions for ICP-MS measurements as well as the chromatographic parameters are summarized in Table 1.

With the purpose of verifying the tentatively identified selenium species, ESI-MS/MS analysis were conducted by using a 6520 Q-TOF mass spectrometer (Agilent, USA) tandem quadrupole mass spectrometer in the selected positive-ion mode and by employing chromatographic conditions that were compatible with ESI-MS/MS

Reagents and standards

All chemicals and reagents used were of analytical grade and solutions were prepared with de-ionized water (18 M Ω cm) obtained from a Milli-Q water purification system unit (Millipore, USA). Stock standard solutions of SeMet, SeMeSeCys and SeCys$_2$ (Sigma-Aldrich, Germany) were prepared by dissolving them in 3% hydrochloric acid (37%, Merck, Germany). Inorganic selenium solutions were obtained by dissolving sodium selenite (Na_2SeO_3) and selenate (Na_2SeO_4), purchased from Merck, in 2% HNO_3 (60%, Sharlau, Spain).

[77]Se-enriched standard (purity 99.66%) (Cambridge Isotope Laboratories, USA) was dissolved in a minimum volume of sub-boiled HCl and diluted with Milli-Q water. The concentration of [77]Se-enriched standard was determined by reverse IDA-ICP-MS.

For the enzymatic extraction, Tris-HCl buffer (pH 7.5) (Fluka; Germany), the nonspecific protease *Streptomyces griseus* (protease XIV) and *Candida rugose* (Lipase) (Sigma-Aldrich, Germany) were used

Carbamidomethylation (CAM) of samples was performed by using urea (99.5 %, Sigma-Aldrich, Germany), dithiothreitol (DTT) (99 %, Sigma-Aldrich, Germany) and iodoacetamide (IAM) (Sigma-Aldrich, Germany).

Selenium species separation by anionic-exchange chromatography was achieved by using 10 mM citric acid (Sigma) in 2% MeOH (99.9%, Scharlau, Spain) adjusted to pH 5 with ammonium hydroxide (Fluka, Germany) as mobile phase. The mobile phase for Zorbax C8 reversed-phase chromatography was 0.1% trifluoroacetic acid, (TFA) (Sigma-Aldrich, Germany) in 2% MeOH.

Fresh cabbages were provided by Bejo Iberica S. L. (Madrid, Spain) and fermented immediately upon reception.

Preparation of ^{77}Se-enriched sauerkraut. Synthesis of Me^{77}SeCys

White cabbages (*Brassica oleracea* L. var. *capitata* cv. Megaton) grown in the North region of Spain (La Rioja) during winter season of 2008 were selected among five Spanish cultivars, based on their glucobrassicin content [13].

The cabbage heads were prepared by removing the outer leaves and their central core, as described in a previous work [8]. The edible part of cabbages was then shredded into about 2 mm thick strips using a shredder (Moka Express, Spain). Subsequently, 0.5% NaCl and 0.5 mg of sodium ^{77}selenite per kg of fresh cabbage (6.38 mg Se/kg of dry matter) were added. Shredded cabbage and brine were mixed thoroughly, transferred to sterile polyethylene vessels (8L) and tightly pressed to exclude air. Fermentations were performed spontaneously by the indigenous microbiota present on raw cabbage. Sauerkrauts, without addition of Se, were also prepared and considered as controls. Fermentations were carried out in 3 batches (4 kg per batch) at room temperature (22–25º C) for 7 days. On the third day, cabbage was pricked to remove releasing gases.

After fermentation, samples were freeze-dried, milled and stored at -20 ºC under vacuum until their characterization and subsequent administration to animals.

Animal experiments

Thirty male Wistar rats with an average initial body weight of 156 ± 2 g were distributed in three experimental groups (n = 10). The experiments lasted for 10 days, 3 days of adaptation and 7 days of the experimental period during which animals were housed in individual stainless-steel metabolic cages designed for food intake control. The cages were located in a well-ventilated thermostatically controlled room (21 ± 2 °C), with relative humidity ranging from 40 to 60 % and 12h light/dark cycle. Food intake was controlled daily in all experimental groups and the animals consumed the different experimental diets *ad libitum*. At the end of the experimental period, rats were fasted for 12 h prior to be sacrificed by CO_2 inhalation. Liver, kidney, heart, brain, and testicles were extracted, weighed, and immediately frozen in liquid nitrogen and stored at -80ºC. All experiments were undertaken according to Directional Guides Related to Animal Housing and Care [14] and all procedures were approved by the Animal Experimentation Ethics Committee of the University of Granada.

Rats were fed with an AIN-93M diet containing 20 % of freeze-dried [77]Se-enriched sauerkraut. AIN-93M diets were formulated following the recommendations of the American Institute of Nutrition [15], to meet the nutrient recommendations of adult rats [16], with casein supplemented with 0.5 % methionine as the sole source of protein (12 %). Addition of a 20 % as freeze-dried cabbage was selected as the expense of cellulose and wheat starch with the aim of establishing a reasonable content of cabbage in the diet that could resemble that of a general population. A control group fed with AIN-93M

diet containing a 20 % of freeze-dried non Se-enriched sauerkraut was also used in this study.

Total selenium and essential elements determination in rat organs after feeding with a diet containing Me^{77}SeCys- enriched sauerkraut

Selenium and essential elements concentration was determined in rat organs (kidney, liver, heart, brain, testicles). Approximately 0.10 g of freeze-dried sample was digested with 1.00 mL of concentrated nitric acid and 0.30 mL of 30% hydrogen peroxide in an analytical microwave oven at 130 °C for 15 min with a ramp temperature of 10 min following conditions described previously [17]. The resulting solutions were diluted to 10.00 mL with deionized water. The concentration of essential elements was measured by ICP-MS by using the external calibration method and following the experimental conditions given in Table 1. Selenium concentration was determined by conventional and reverse mode of IDA-ICP-MS in control and intrinsically ^{77}Se labeled rat organs, respectively. For that purpose, an appropriate amount of ^{77}Se spike solution was added to samples just before applying the acid digestion step (a selenium solution of natural abundance was added when analyzing intrinsically ^{77}Se labeled samples by reverse IDA-ICP-MS analysis).

For IDA-ICP-MS analysis, isotope intensities were corrected with a detector dead time of 30 ns. Interferences caused by SeH$^+$ were corrected with a selenium factor (f_{Se}) ranged from 2.2-2.4 % of the Se signal, similar values to those reported before [18, 19]. Mass bias discrimination was computed every 5 analyses and a mass bias factor (K) of *ca.* - 4% per mass unit, in agreement with other authors [19, 20], was used to correct

[78]Se/[77]Se and [80]Se/[77]Se isotope ratio. Concentration of selenium was calculated by using the IDA equation described by García-Alonso *et al*. [21].

Selenium speciation in the intrinsically [77]Se labeled organs

A conventional enzymatic hydrolysis [22] consisting in incubation of 0.1 g of sample in 3 mL of 30 mM Tris-HCl (pH 7.5) with 30 mg of Protease XIV and 20 mg of Lipase at 37 °C for 20 h was performed. To improve the efficiency of SeCys extraction and to preserve the integrity of such species, samples were carbamidomethylated before applying enzymatic hydrolysis, based on the procedure reported by other authors [22, 23]. Briefly, 0.1 g of freeze-dried sample was incubated with 2 mL of 7 M urea in 0.1 M Tris-HCl (pH 7.5) and sonicated for 10 min in an ultrasound bath for protein denaturation. The solution was shaken and incubated at 25 °C in the dark with 30 µL of 0.2 M DTT in 0.1 M Tris-HCl and 40 µL of 0.5 M IAM for 1 h each step in order to reduce Se-Se, S-Se and S-S bonds and to protect them by methylation. To remove the excess of IAM, 375 µL of DTT was added and shaken for 1 h. The resulting solution was diluted with 0.1 M Tris-HCl until a final concentration of 1 M urea was reached. Finally, enzymatic hydrolysis was performed by 20 h of incubation at 37 °C using a protease/lipase solution mixture. The extracts were centrifuged at 10,233 *g* for 20 min and the supernatants were filtered through 0.22 µm Nylon filter and analyzed by anionic exchange and reversed-phase ion pairing C8 columns coupled to the ICPMS Identification of selenium compounds was carried out by matching retention times and by spiking experiments. Recovery values were calculated as the sum of selenium in separated selenium compounds relative to the total selenium concentration obtained after performing microwave acid digestion. Selenium species quantification in organs

from control rats was performed by HPLC species-unspecific IDA-ICP-MS which allow us the quantification of both identified and unidentified species. For that purpose, a ^{77}Se-enriched standard solution of the appropriate known concentration was continuously mixed (at 0.1 mL min^{-1}) with the effluent of the column (at 0.9 mL min^{-1}) through a T piece and directly nebulized into the plasma. Conventional intensity chromatograms (counts s^{-1}) were converted into mass flow chromatograms (ng min^{-1}) after applying an adequate mathematical treatments based on the on-line isotope dilution equation [21]. The same corrections as in conventional IDA-ICP-MS used for total Se determination were applied to ^{78}Se/^{77}Se and ^{80}Se/^{77}Se isotope ratios before using the on-line isotope dilution equation to obtain the mass flow chromatograms. The amount of selenium corresponding to each chromatographic peak was achieved by area integration. ^{77}Se-labeled metabolites in the intrinsically ^{77}Se labeled organs were quantified by unspecific IDA-ICP-MS was carried out following the same procedure as above but replacing ^{77}Se spike solution by a selenium solution of natural abundance.

With the aim of identifying unknown selenium species, purification and isolation of the corresponding chromatographic peak was carried out by anion exchange and reversed-phase chromatography, respectively. Fractions collected were pooled, lyophilized and re-dissolved in water/methanol (1:1) prior to ESI MS/MS analysis. The ion-spray voltage was set at 4.5 KV and the fragmentor voltage at 150 V. Nitrogen was used as nebulizer, drying and collision gas. Samples were analyzed by using reversed-phase C18 chromatography coupled to ESI/MS/MS at a flow of 0.2 mL min^{-1}.

Results and discussion

Characterization of AIN-93M animal diet and ^{77}Se enriched sauerkraut

Prior to administering the diet to the experimental animals, both sauerkraut and AIN-93M diet were characterized in terms of total selenium content and occurrence of selenium species. The concentration of selenium in non enriched and [77]Se-enriched sauerkraut was 0.07 ± 0.01 and 5.21 ± 0.03 µg g^{-1}, respectively. The diet used to feed animals (AIN-93M with 20 % sauerkraut) provides selenium concentration values of 0.10 ± 0.04 and 1.21 ± 0.04 µg g^{-1} when non-enriched and [77]Se enriched sauerkraut is employed, respectively. Regarding Se-species, selenium mainly appeared as Se(IV) and SeMet (Fig 1a) in the AIN-93M diet, whereas Me[77]SeCys was the major Se-species found in [77]Se-enriched sauerkraut (Fig. 1b). Selenium compounds were not detected in control sauerkraut. The results indicate that [77]Se enriched sauerkraut preparation is an easy and cheap alternative to obtain Me[77]SeCys. Synthesis of Me[77]SeCys implies harsh chemical conditions and long-time procedures [24] which are not affordable for most laboratories working on speciation. Once Me[77]SeCys-enriched sauerkraut was prepared, it was employed to evaluate MeSeCys metabolism in Wistar rats fed with a diet containing 20% of [77]Se-enriched sauerkraut.

Selenium accumulation in rat organs after feeding them with a diet containing 20% of [77]Se-enriched sauerkraut. Effect on essential elements distribution.

Total selenium content in different organs (liver, kidney, heart, testicles and brain) from rats fed with a diet containing 20% of [77]Se-enriched or non-enriched sauerkraut (control group) was determined by IDA-ICP-MS after performing acid digestion. The results represented in Table 2 show that liver, testicles and kidney were the organs of the control group of rats with the highest selenium content, with selenium values of 3.7 ± 0.2, 3.4 ± 0.3 and 1.9 ± 0.2 µg g^{-1}, respectively. This could be due to the fact that liver

and kidney are considered to be selenium -accumulating organs [25] and, by the other hand, selenium is required for sperm synthesis in testicles [26]. Analysis of [77]Se labeled organs shown a prominent increase (more than a 100% of Se level enhancement) of selenium in kidney and heart, whereas in liver, selenium concentration only increased up to 20 %, remaining constant in brain and testicles. Interestingly, although liver is the organ with the highest Se level in the control group, it did not experiment an important increase when rats were fed with a 20% of [77]Se-enriched sauerkraut diet. The data obtained evidence the selenium tendency to be accumulated in certain organs. Selenium accumulation in kidney could be explained as a part of a detoxification process prior to be excreted. The reason by which selenium level in heart is increased remains unclear but since this organ is quite sensitive to selenium status changes in the body [27], it could be more affected by the supplementation of selenium. In contrast, the selenium level remains constant in organs of important biological activity, such as brain or reproductive system in order to protect the body against selenium status modifications [28].

To ensure appropriate growth of experimental animals, they not only have to tolerate selenium, but supplementation with selenium should not alter the uptake and distribution of other essential elements (Cu, Mo, Mg, and Zn) or their biological functions. For this purpose, the concentration of several essential elements in the intrinsically [77]Se labeled rat organs were measured (Table 2). In general, selenium supplementation with a diet containing 20% of [77]Se-enriched sauerkraut does not alter the concentration of these elements in comparison to the controls, except in the case of heart and kidney. It is interesting to highlight that these two organs were those with the highest selenium increase after feeding rats with Me[77]SeCys. To date, the number of studies on the influence of selenium supplementation and others metals on accumulation

and distribution of micronutrients in organs and tissues are scarce and the existing ones are sometimes controversial. Pedrero *et al.* [23] determined selenium and essential elements in organs of catfish fed with a selenium-enriched garlic-based diet (mainly as γ-glutamilmethylselenocysteine). Selenium supplementation did not have any effect on Zn and Cu accumulation and distribution in catfish organs. Cabañero *et al*. [29] evaluated Se-Hg interaction in chicken after supplementing them with selenium as selenite. Results showed that the concentration of Cu, Zn and Fe in kidney remains unaltered after long-term selenium exposure. In contrast, in other study [30] performed with broiler chickens supplemented with selenized yeast, selenium was positively correlated with Fe, Zn and Cu and negatively correlated with Cd. Based on the mentioned results, it is clear that trace elements may interact at the levels of absorption, distribution and retention. Deficiency or supplementation of one of them may alter their balance and trigger the antagonistic or synergistic effects. The alteration of trace elements balance may also affect the antioxidant system, since several trace elements such as Se, Cu, Zn and Fe are integrant of various antioxidant enzymes. The influence of selenium on trace metal distribution depends on the level and the chemical form of supplemented selenium, the animal and the type of the examined tissue.

Se speciation in the intrinsically [77]Se labeled and control rat organs

In the present work, the distribution of selenium species in the different organs was evaluated by HPLC-IDA-ICP-MS after species extraction by using carbamidomethylation process followed by enzymatic hydrolysis. Because of selenocysteine degradation, it is necessary to use a sample treatment procedure based on reduction with dithiotreitol (DTT) followed by acetylation with iodoacetamide(IAM) to

prevent oxidation of selenocysteine [22]. Therefore, commercially available Se-standards and samples were carbamidomethylated following the procedure described at Material and Methods Section. Two different chromatographic columns (anionic exchange and reversed-phase chromatography) coupled to ICPMS were used for analyzing the extracts of the different organs.

The anion exchange HPLC-ICP-MS chromatogram in Fig. 2a shows that ^{77}SeCys and a ^{77}Se-containing peak observed at 6 minutes were detected in the hydrolyzed extract of ^{77}Se labeled rat liver by using carbamidomethylation and enzymatic hydrolysis. In addition, the Se-containing peak found at 6 minutes matched the retention time with CAM-Se(IV) standard (Fig. 2a and 2d). However, when sample treatment was performed without applying a previous carbamidomethylation step, only SeCys was detected in liver extracts (Fig 2b), suggesting that the Se-containing peak observed at 6 minutes might be a Se-unknown peak originated when carbamidomethylation is applied prior enzymatic hydrolysis.

The anionic exchange HPLC-ICP-MS chromatographic profiles in Figure 3 show the distribution of the two selenium isotopes ^{78}Se and ^{77}Se in control and ^{77}Se-labeled organs. As it was previously mentioned, ^{78}SeMet and ^{78}Se(IV) were the main Se-species found in control diet, whereas Me^{77}SeCys was the main Se-specie found in ^{77}Se-enriched sauerkraut diet. Selenocysteine was detected in all ^{77}Se-labeled organs and control organs. Low amounts of Me^{77}SeCys in ^{77}Se-labeled liver and kidney extracts were found, suggesting the incorporation of this selenium compound in its intact form. The detection of low amounts of MeSeCys in liver and kidney in its intact form after feeding animals with such species has been reported by other authors [31, 32]. Suzuki *et al.* [31] evaluated the metabolisms of ^{77}SeMet and Me^{76}SeCys orally administrated in

Wistar rats. The results indicated that MeSeCys was incorporated into selenoprotein P (SelP) slightly more than or at a comparable level to that of SeMet. SeMet and MeSeCys are suggested to be transformed into selenide through the β-lyase reaction but differently: SeMet is transformed into SeCys through the trans-selenation reaction, and then into selenide through the β-lyase reaction for SeCys. MeSeCys is transformed into methylselenol through the β-lyase reaction for MeSeCys, and then through the demethylation reaction into selenide. The results obtained in the present work are in agreement with those reported by Suzuki *et al.*[31], since the presence of SeCys, released from selenoproteins, in these metabolically active glandular visceral tissues (heart, kidney, liver, brain and) can be explained as a result of the conversion of the species initially present in the supplemented diet..

Since species identification using a unique chromatography column should not be used as conclusive, a reversed-phase separation mechanism was also employed. The chromatograms obtained corresponding to the hydrolyzed extracts of [77]Se-labeled organs are shown in Figure 4 and clearly demonstrate the presence of SeCys and an unknown Se-containing peak.

The origin of the unknown Se-containing peak is not clear but it could be formed by any of the three species administrated to the animals since selenium was present as [77]Se (from Me[77]SeCys) and as [78]Se (from [78]SeMet or [78]Selenite). The metabolism pathway of SeMet and MeSeCys involves the production of Se intermediates such as methylselenol, selenide or methylseleninicacid [31, 33] before the production of selenoproteins, or the formation of selenosugars and trimethylselenonium (TMSe) as urinary metabolites [33]. In the present work, TMSe was not observed in liver or kidney.. The unknown Se-

containing peak was also detected in heart and brain suggesting that it is not a Se-urinary metabolite. As the Se-unknown peak was only detected when carbamidomethylation was applied, its presence could be also attributed to artifacts formation during carbamidomethylation . With the aim of identifying the unknown peak found in carbamidomethylated tissues, ESI MS/MS was unsuccessfully applied. Further, the coupling of C18 reversed-phase LC to ESI MS/MS in order to suppress possible interferences from the matrix which can affect the ionization also failed. The low amount of selenium in the tested organs made difficult to identify the unknown Se-containing peak.

Since the identification of one of the Se-species was not possible, quantification of species was carried out by species-unspecific IDA-ICP-MS by continuously mixing an enriched [77]Se solution (selenium solution of natural abundance in the case of reverse IDA) with the effluent of the column. By using this mode of IDA both identified and unidentified species can be quantified. Figure 5 shows the mass flow chromatograms of control and Se-enriched liver extracts obtained by anionic exchange IDA-ICP-MS. Table 3 shows the total content of selenium, recovery values (calculated as the amount of selenium extracted relative to the total selenium concentration obtained after acid digestion), the concentration of Se-species and the fraction of total selenium (calculated as the sum of selenium in separated Se species relative to the total Se extracted) from both control and [77]Se labeled rat organs. Recovery values ranged from 52 to 100%, being the liver the organ which provides a minimum selenium extraction yield.

In general, control samples presented a higher content of CAM-SeCys and a lower content of the unknown species than [77]Se-labeled organs. Formation of the unknown Se-containing peak seems to be favored by presence of MeSeCys. The organs with higher content of either SeCys or the unidentified species were liver, kidney and testicles and,

after supplementation, testicles diminished the content of both species while heart increased concentration of the unknown Se-containing peak.

Conclusions

Preparation of sauerkraut in the presence of selenite represents a cheap and easy way for obtaining Me^{77}SeCys as tracer in either quantitative speciation or metabolism studies. The method could be considered as an alternative to commonly practices of preparing labeled MeSeCys which imply the application of harsh chemical conditions. Supplementation with an AIN-93M diet containing 20 % of Me^{77}SeCys-enriched sauerkraut to Wistar rats showed the presence of ^{77}SeCys and unknown Se-containing peak in all ^{77}Se labeled rat organs. Moreover, IDA-ICP-MS analysis of ^{77}Se labeled organs showed a prominent increase (more than a 100% of Se level enhancement) of selenium in kidney and heart. ^{77}Se-enriched sauerkraut supplementation does not alter the concentration of other essential elements in comparison to the controls, except in the case of heart and kidney, in which selenium was positively correlated with Mg, Zn, Cu and Mo. We have also demonstrated how natural processes (such as lactic acid fermentation) that imply inorganic selenium biotransformation can be useful to prepare selenium stable isotopes as tracers to investigate selenium metabolism and also to achieve rapid speciation analyses and quantification of Se by HPLC-ICP-MS.

Acknowledgments

References

1. Rayman MP (2002) The argument for increasing selenium intake. P Nutr Soc 61: 203-215.

2. Clark LC, Graham GF, Gross EG, Krongrad A, Lesher JL Jr, Park HK, Sanders BB Jr, Smith CL, Taylor JR (1996) The nutritional prevention of cancer with selenium 1983-1993: a randomized clinical trial. J Am Med Assoc 276: 1957-1963.

3. Ip C (1998) Lessons for basic research in selenium and cancer prevention. J Nutr 128: 1845-1854.

4. Abdulah R, Miyazaki K, Nakazawa M, Koyama H (2005) Chemical forms of selenium for cancer prevention. J Trace Elem Med Bio 19: 141-150.

5. Jackson MI, Combs GF Jr (2008) Selenium and anticarcinogenesis: underlying mechanisms. Curr Opin Clin Nutr Metab Care 11: 718-726.

6. Pedrero Z, Madrid Y, Cámara C (2006) Selenium species bioaccessibility in enriched radish (*Raphanus sativus*): A potential dietary source of selenium. J Agr Food Chem 54: 2412-2417.

7. Alzate A, Cañas B, Pérez-Mungía S, Hernández H, Pérez-Conde C, Gutiérrez AM, Cámara C (2008) Evaluation of the inorganic selenium biotransformation in Se-enriched yogurt by HPLC-ICP-MS. J Anal Food Chem 56: 8728-8736.

8. Peñas E, Martínez-Villaluenga C, Frías J, Sánchez-Martínez MJ, Pérez-Corona MT, Madrid Y, Cámara C, Vidal Valverde C (2012) Se improves índole glucosinolate hydrolysis products content, Se-methylselenocysteine content, antioxidant capacity and potential anti-inflammatory properties of sauerkraut. Food Chem 132: 907-914.

9. Alzate A, Pérez-Conde C, Gutiérrez AM, Cámara C (2010) Selenium-enriched fermented milk: A suitable dairy product to improve the selenium intake for humans. Int Dairy J 20: 761-769.

10. Sánchez-Martínez M, Galvao, P. da Silva E, Pérez-Corona T, Cámara, Ferreira SLC, Madrid Y (2012) Selenite biotransformation during brewing. Evaluation by HPLC-ICP-MS. Talanta 88: 272-276.

11. Szpunnar J (2005) Advances in analytical methodology for bioinorganic speciation analysis: metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomicc. Analyst 130: 442-465.

12. Ip C, Dong Y, Ganther HE (2002) New concepts in selenium chemoprevention. Cancer metast Rev 21: 281-289.

13. Peñas E, Frías J, Martínez-Villaluenga C, Vidal Valverde C (2011) Bioactive compounds, myrosinase activity and antioxidant capacity of White cabbages grown in different locations of Spain. J Agr Food Chem 59: 3772-3779.

14. European Union Council (2010) Directive 2010/63/UE on the Protection of Animals used for Scientific Purposes. Official Journal of the European Union, Strasbourg.

15. Reeves PG, Nielsen FH, Fahey GC (1993) AIN-93G purified diets for laboratory rodents: final report of the American Institute of Nutrition *Ad Hoc* Writing Committee on the reformulation of the AIN-76A rodent diet. J Nutr 123: 1939-1951.

16. National Research Council (1995) In: (ed) Nutrient Requirements of Laboratory Animals Fourth Revised Edition. National Academy Press, Washington D.C.

17. Cabañero AI, Madrid Y, Cámara C (2004) Selenium and mercury bioaccessibility in fish samples: an in vitro digestion method. Anal Chim Acta 526: 51-61.

18. Pedrero A, Ruiz–Encinar J, Madrid Y, Cámara C (2007) Application of species-specific isotope dilution analysis to the correction for selenomethionine oxidation in Se-enriched yeast simple extracts during storage. J Anal Atom Spectrom 22: 1061-1066.

19. Hinojosa-Reyes L, Marchante-Gayón JM, García-Alonso JI, Sanz-Medel A (2003) Determination of selenium in biological materials by isotope dilution analysis with an octapole reaction system ICP-MS. J Anal Atom Spectrom 18: 11-16.

20. Kirby JK, Lyons GH, Karkkainen MP (2008) Selenium speciation and bioavailability in biofortified products using species-unspecific isotope dilution and revered phase ion pairing-inductively coupled plasma-mass spectrometry. J Agr Food Chem 56: 1772-1779.

21. García-Alonso JI, Rodríguez P (2011) In: C. Cámara C, Pérez-Conde C (eds) Análisis químico de trazas, Editorial Síntesis S.A., Spain.

22. Bierla K, Dernovics M, Vacchina V, Szpunar J, Bertin G, Lobinski R (2008) Determination of selenocysteine and selenomethionine in edible animal tissues by 2D size-exclusion reversed-phase HPLC-ICP MS following carbamidomethylation and proteolytic extraction. Anal Bioanal Chem 390: 1789-1798.

23. Pedrero Z, Murillo S, Cámara C, Schram E, Luten JB, Feldmann I, Jakubowski N, Madrid Y (2011) Selenium speciation in different organs of African catfish (*Clarias gariepinus*) enriched through a selenium-enriched garlic based diet. J Anal Atom Spectrom 26: 116-125.

24. Otha Y, Suzuki N, Kobayashi Y, Hirano S (2011) Rapid speciation and quantification of selenium compounds by HPLC-ICP MS using multiple standards labelled with different isotopes. Isot Environ Healt 47: 330–340.

25. Ciardullo S, Aureli F, Coni E, Guandalini E, Iosi F, Raggi A, Rufo A, Cubadda F (2008) Bioaccumulation potential of dietary arsenic, cadmiun, lead, mercury, and selenium in organs and tissues of rainbow trout (*Oncorhyncus mykiss*) as a function of fish growth. J Agr Food Chem 56: 2442-2451.

26. D. Behne, H. Weiler, A. Kyriakopoulos, Effects of selenium deficiency on testicular morphology and function in rats, J. Reprod. Fertil. 106 (1996) 291-297.

27. Patching SG, Gardiner RHE (1999) Recent developments in selenium metabolism and chemical speciation: A review. J Trace Elem Med Bio 13: 193-214.

28. Behne D, Weiss-Nowak C, Kalcklösch M, Westphal C, Gessner H, Kyriakopoulos (1995) A Studies on the distribution and characteristics of new mammalian selenium-containing proteins Analyst 120: 823-825.

29. Cabañero AI, Madrid Y, Cámara C (2005) Study of mercury-selenium interaction in chicken liver by size exclusion chromatography inductively coupled plasma mass spectrometry. J Anal Atom Spectrom 20: 847-855.

30. Pappas, AC Zoidis E, Georgiou CA, Demiris N, Surai PF, Fegeros K (2011) Influence of organic selenium supplementation on the accumulation of toxic and essential elements involved in the antioxidant system of chicken. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 28: 446-454.

31. Suzuki KT, Doi C, Suzuki N (2006) Metabolism of [76]Se-methylselenocystine compared with that of [77]Se-selenomethionine and [82]Se-selenite. Toxicol Appl Pharm 217: 185-195.

32. Suzuki Y, Hashiura Y, Sakai T, Yamamoto T, Matsukawa T, Shinohara A, Furuta N (2013) Selenium metabolism and excretion in mice after injection of [82]Se-enriched selenomethionine. Metallomics 5: 445-452.

33. Suzuki KT, Somekawa L, Suzuki N, (2006) Distribution and reuse of [76]Se-selenosugar in selenium-deficient rats. Toxicol Appl Pharm 216: 303-308.

Figure Captions

Fig. 1. Anion-exchange HPLC-ICP-MS of a hydrolyzed extract of a) control (m/z = 78) and b) [77]Se-enriched sauerkraut (m/z = 77) diets.

Fig. 2. Anion exchange HPLC-ICP-MS chromatograms of hydrolyzed extracts of [77]Se-labeled liver (monitoring on m/z = 78 and 77) obtained by a) enzymatic hydrolysis after carbamidomethylation, b) enzymatic hydrolysis without carbamidomethylation, and chromatograms of c) a mixture of Se-standards and d) a mixture of carbamidomethylated Se-standards.

Fig. 3. Anion exchange HPLC-ICP-MS chromatograms of hydrolyzed extracts corresponding to control (left panel) and [77]Se-labeled organs (right panel) a) kidney, b) heart, c) testicles and d) brain.

Fig. 4. Reversed-phase C8 column HPLC-ICP-MS chromatograms of hydrolyzed extracts corresponding to [77]Se-labeled organs a) kidney, b) heart, c) testicles and d) brain.

Fig. 5. Mass flow chromatograms by anion exchange-ID-ICP-MS of hydrolyzed extracts corresponding to: a) control liver (at 78/77 isotope ratio measurement) and b) [77]Se labeled-liver (at 77/78 isotope ratio measurement).
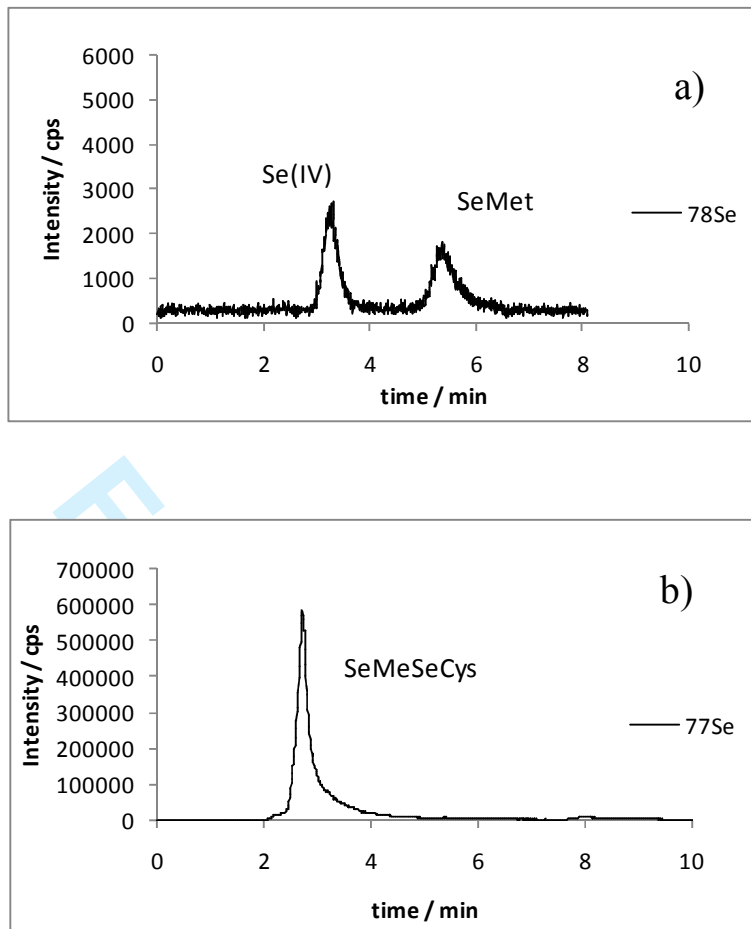
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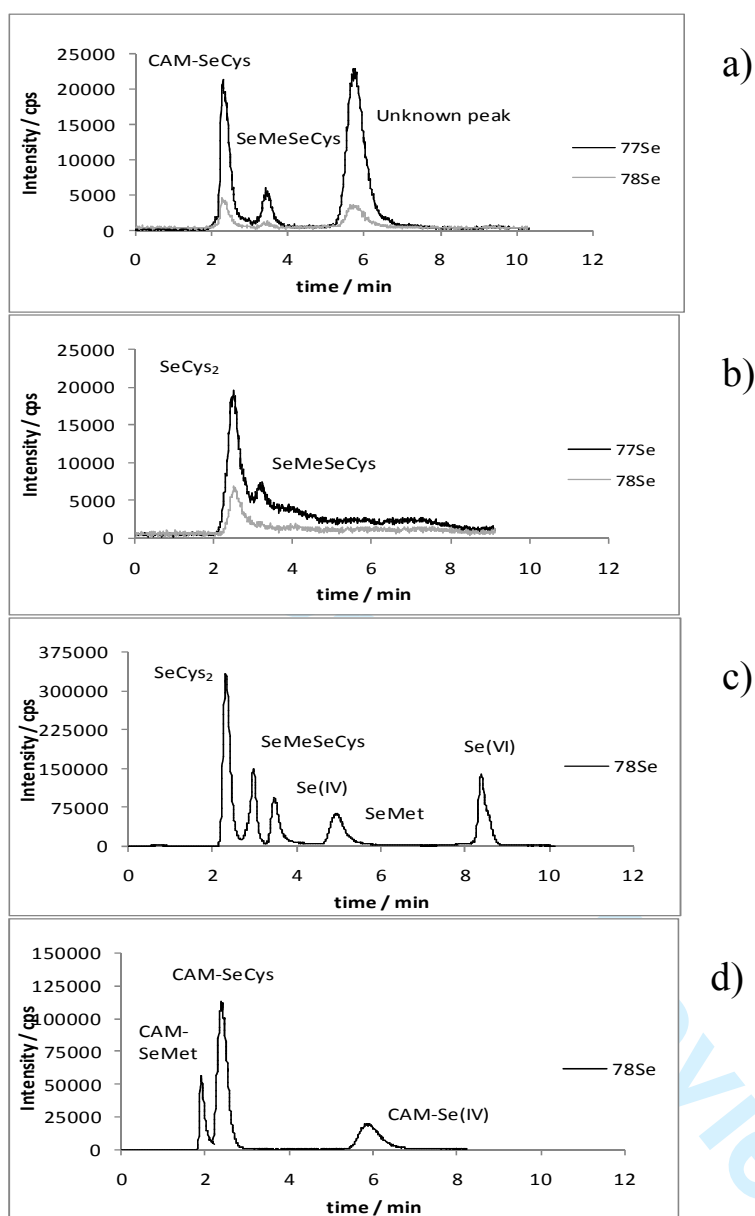46
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Fig 1

Fig. 2

CONTROL ORGANS   **77Se-LABELLED ORGANS**
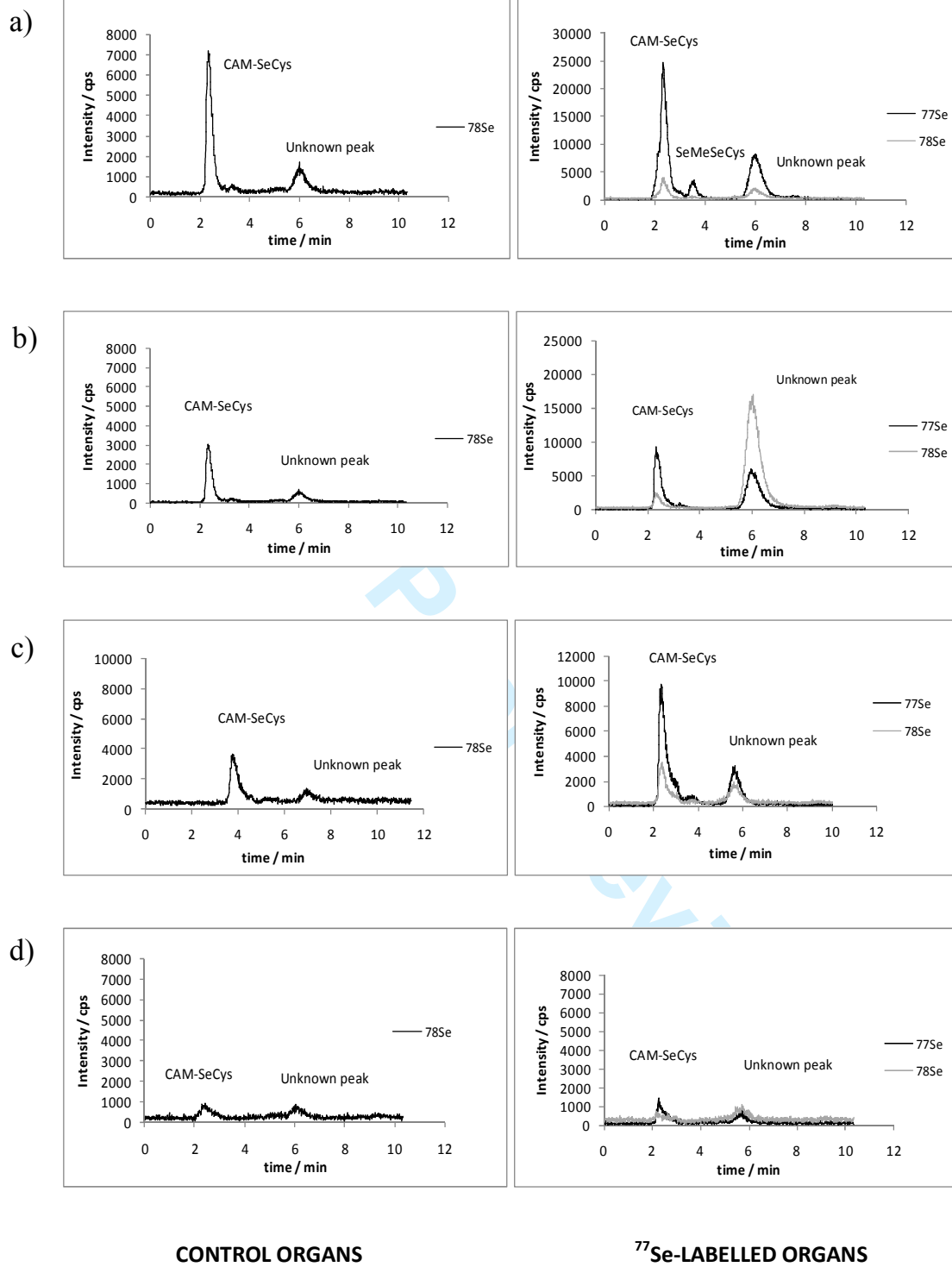
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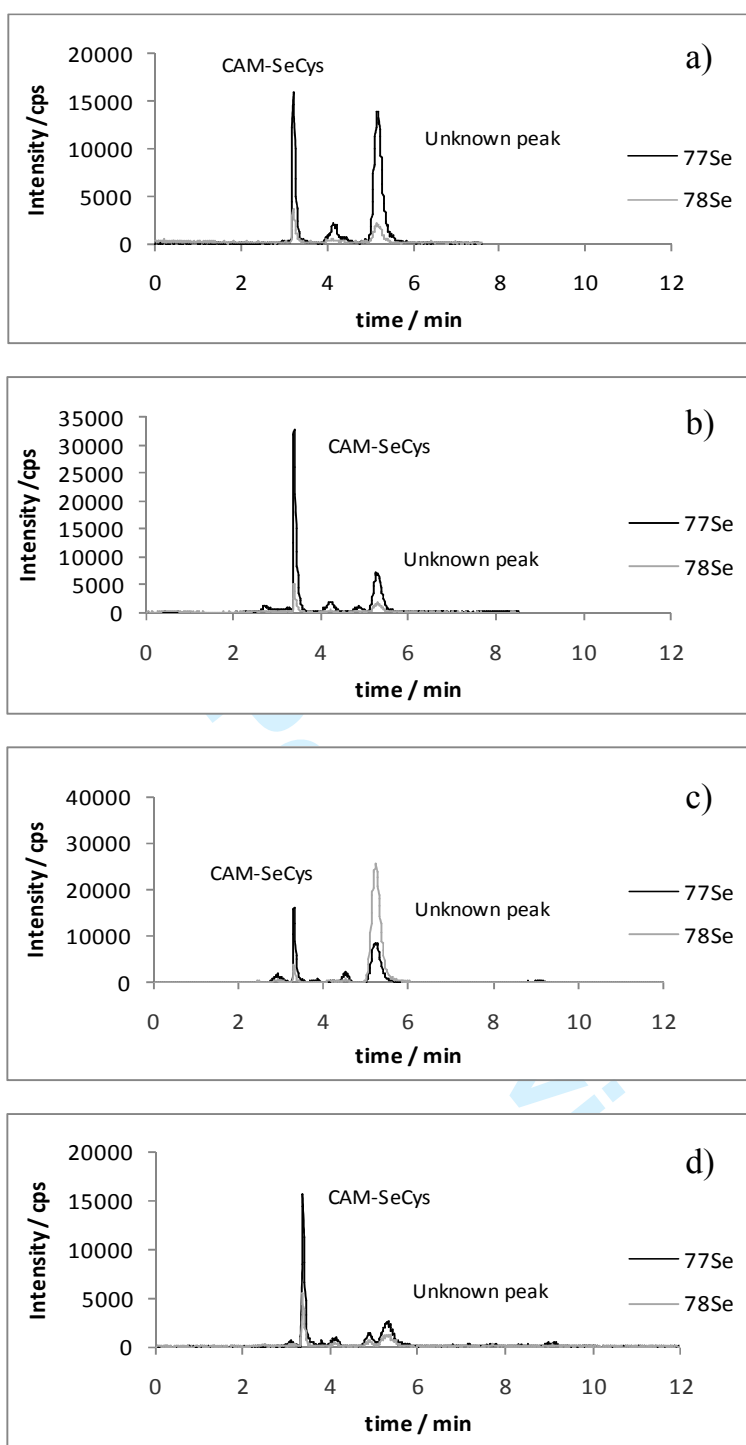
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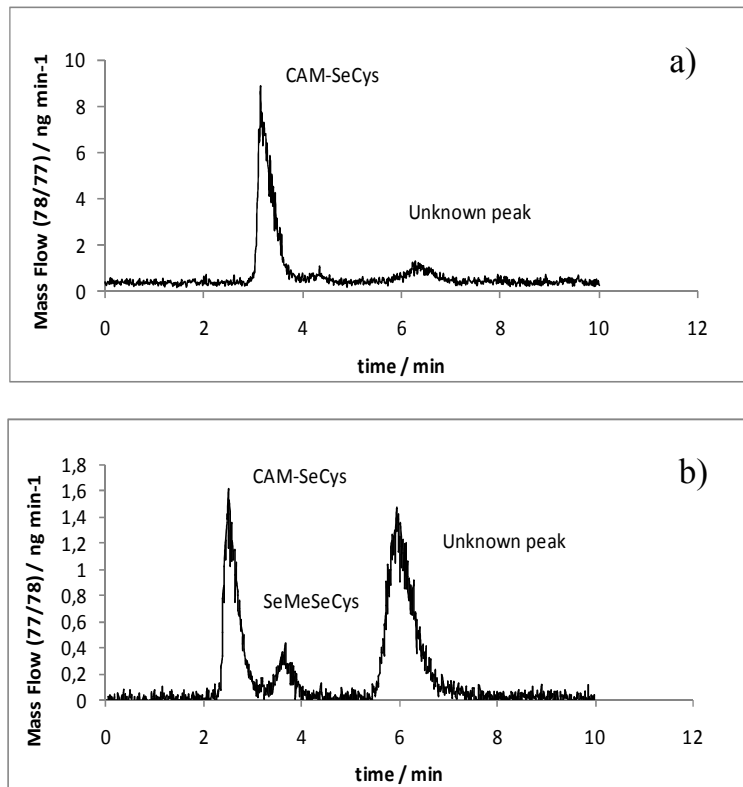
Fig. 5

Table 1. Instrumental operating conditions for determinations by HPLC-ICP-MS.

ICP-MS conditions	
Forward power	1550 W
Plasma gas flow rate	15.0 L min^{-1}
Auxiliary gas flow rate	1.26 L min^{-1}
Carrier gas flow rate	1.1 L min^{-1}
H$_2$ reaction gas flow rate	4.5 mL min^{-1}
Nebulizer type	Meinhard
Spray chamber type	Scott-double pass
Isotope monitored	^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{63}Cu, ^{65}Cu, ^{24}Mg, ^{95}Mo, ^{98}Mo, ^{64}Zn, ^{66}Zn, ^{79}Br, ^{81}Br

LC parameters			
Analytical Column	Mobile phase	Flow Rate	Injection Volume
PRP X -100	10 mM Ammonium citrate, 2% MeOH, pH 5	1.0 mL min^{-1}	100μL
Zorbax RP-C8	0.1% TFA, 2% MeOH, pH 1.98	1.0 mL min^{-1}	100μL

Table 2. Concentration of selenium and essential elements (μg g^{-1} dry mass) in control and intrinsically ^{77}Se-labeled organs

	Selenium		Cupper		Magnesium		Molibdenum		Zinc	
	Control	^{77}Se-labeled organs	Control	^{77}Se-labeled organs	Control	^{77}Se-labeled organs	Control	^{77}Se-labeled organs	Control	^{77}Se-labeled organs
Liver	3.7 ± 0.2	4.4 ± 0.3	12.4 ± 0.4	11.0 ± 0.5	834 ± 23	744 ± 29	1.68 ± 0.02	1.62 ± 0.06	109 ± 4	97 ± 2
Kidney	**1.9 ± 0.2**	**4.1 ± 0.3**	**5.7 ± 0.1**	**16.2 ± 0.2**	**308 ± 4**	**544 ± 13**	**0.39 ± 0.02**	**1.1 ± 0.3**	**39 ± 1**	**98 ± 11**
Heart	**0.8 ± 0.1**	**1.70 ± 0.05**	**6.7 ± 0.1**	**18.2 ± 0.6**	**449 ± 6**	**792 ± 42**	**0.29 ± 0.02**	**0.84 ± 0.07**	**42 ± 1**	**108 ± 4**
Testicles	3.4 ± 0.3	2.7 ± 0.1	7.4 ± 0.4	8.7 ± 0.5	1098 ± 59	1138 ± 4	0.26 ± 0.02	0.28 ± 0.03	140 ± 4	140 ± 4
Brain	0.46 ± 0.06	0.6 ± 0.1	7.2 ± 0.2	7.4 ± 0.1	577 ± 20	596 ± 10	0.16 ± 0.03	0.13 ± 0.02	54 ± 1	61 ± 4

All the concentrations are given as the mean ± standard deviation. N= 3 replicates.

Total selenium concentration was determined by conventional and reverse mode of ID-ICP-MS in control and intrinsically ^{77}Se labeled rat organs, respectively.

Essential element concentration was determined by ICP-MS using external calibration method.

Table 3. Concentration (μg Se g^{-1} dry mass) of selenium-species contained in control and intrinsically [77]Se-labeled organs.

	Total Se	Se extracted	Recovery (%)	CAM-SeCys	MeSeCys	Unknown species	Fraction of total selenium (%)
Control Liver	3.7 ± 0.2	2.01 ± 0.01	54 ± 3	1.09 ± 0.09	-	$0.15 \pm 0{,}01$	62 ± 4
[77]Se-labeled Liver	4.4 ± 0.3	2.29 ± 0.07	52 ± 1	0.42 ± 0.06	0.13 ± 0.02	0.63 ± 0.01	52 ± 3
Control Kidney	1.9 ± 0.2	1.89 ± 0.02	99 ± 4	1.02 ± 0.03	-	0.39 ± 0.01	74 ± 4
[77]Se-labeled Kidney	4.1 ± 0.3	3.71 ± 0.01	91 ± 3	0.87 ± 0.02	0.39 ± 0.01	0.56 ± 0.02	51 ± 3
Control Heart	0.8 ± 0.1	0.78 ± 0.01	98 ± 4	0.43 ± 0.01	-	0.16 ± 0.01	76 ± 3
[77]Se-labeled Heart	1.70 ± 0.05	1.63 ± 0.03	96 ± 3	0.26 ± 0.02	-	0.80 ± 0.03	65 ± 4
Control Testicles	3.4 ± 0.3	3.48 ± 0.04	102 ± 5	0.52 ± 0.01	-	0.33 ± 0.01	25 ± 1
[77]Se-labeled Testicles	2.7 ± 0.1	2.73 ± 0.02	101 ± 2	0.33 ± 0.01	-	0.10 ± 0.01	16 ± 2
Control Brain	0.46 ± 0.06	0.45 ± 0.01	98 ± 1	0.10 ± 0.01	-	-	22 ± 1
[77]Se-labeled Brain	0.6 ± 0.1	0.54 ± 0.02	90 ± 4	0.13 ± 0.01	-	0.08 ± 0.01	39 ± 1

Concentration of selenium species are given as mean ± standard deviation (n = 3).

The fraction of total Se (%) denotes the ratio of the sum of Se species concentrations to total selenium extracted.

Se-species quantification was achieved by HPLC post column ID-ICP-MS by monitoring [78]Se/[77]Se and [77]Se/[78]Se isotope ratios in control and [77]Se-labeled organs, respectively.