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Title: Bio-fortification and isotopic labelling of Se metabolites in onions and carrots following foliar application of Se and ^{77}Se

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Keywords: Foliar application; Onion; Carrot; Methylselenocysteine; Intrinsic ^{77}Se labeling; HPLC-ESI-MS/MS.

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Abstract: The aims were to bio-fortify onions by foliar application of selenium (Se) and to intrinsically label bioactive Se-metabolites in onion and carrot by enriched, stable ^{77}Se for use in human physiological studies. Onion bulbs and leaves were enriched in Se by repeated foliar spraying of 10 or 100 $\mu\text{g Se mL}^{-1}$ solutions of sodium selenite (Se(IV)) or sodium selenate (Se(VI)). ICP-MS analysis of onion leaves and bulbs showed that the Se concentration was enhanced by up to a factor of approximately 50 and 200 in bulbs and leaves, respectively. HPLC-ICP-MS analysis of proteolytic plant extracts showed that foliar application of Se(IV) gave rise to bio-synthesis of a higher fraction of the desired organic Se species and was better tolerated by the plants than Se(VI). Based on these findings onions and carrots were bio-fortified by foliar application of a solution of ^{77}Se (IV) that was enriched to 99,7 % as ^{77}Se . The ^{77}Se - labeled metabolites in onions were predominantly γ -glutamyl- ^{77}Se -selenomethyl-selenocysteine (γ -glu-Me $^{77}\text{SeCys}$), ^{77}Se -methylselenocysteine (Me $^{77}\text{SeCys}$) and ^{77}Se -selenomethionine ($^{77}\text{SeMet}$). Furthermore, we report here for the first time the finding in carrots of the bioactive Me $^{77}\text{SeCys}$, the identity of which was verified by HPLC-ESI-MS/MS.

National Food Institute



Food Chemistry

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Submission of a manuscript for publication

On behalf of the author team I hereby submit a revised manuscript for publication in Food Chemistry, Bio-fortification and isotopic labelling of Se metabolites in onions and carrots following foliar application of Se and ^{77}Se .

Best regards,

Erik Huusfeldt Larsen
Professor
Food Chemistry

Reviewer #1:

The manuscript describes a good effort in the production and characterisation of food enriched with ^{77}Se species of importance to human health at typical concentration levels of relevance to clinical intervention trials. Increased levels of specific forms of Se in the diet have been shown to reduce the risk of certain diseases including cancer but the mechanisms by which Se acts and the mediating effects observed have still to be further elucidated. In this vein, the present work is timely and important. Therefore, I welcome this contribution. However, I have some reservations and comments, which should be addressed by the authors before the paper can be considered suitable for publication in Food Chemistry. They are the following:

- Page 5, greenhouse experiments : Were control plants grown and analysed for their total Se and Se speciation content ?. This is important but missing throughout.

Results for the control plants were already given in Table S2. To more clearly explain that control plants (no Se amendment) were included, this has been explained on page 5 under the section 2.2 Greenhouse experiments

- Page 6 and throughout, 'enzymatic hydrolysis with Protease XIV' : Blank chromatograms of enzymatic hydrolysates are missing throughout and should be added. The reason for this is that the levels of $^{80}\text{SeMet}$ in Protease XIV blanks have been found significant and batch dependent.

To address this remark the text in bold has been added on page 11 to explain that no SeMet peaks were detected from the proteolytic enzyme

The chromatograms corresponding to the extracts of the control samples did not show any peaks exceeding the baseline noise (3σ) of the chromatograms and therefore remained undetectable by HPLC-ICP-MS. The risk of blank chromatographic peaks originating from the proteolytic enzyme or occurring because of memory from previous injections could therefore be disregarded.

- Page 7 and throughout , 'multiple reaction monitoring (MRM)' : It should be replaced by 'selected reaction monitoring (SRM)'.

MRM was changed to SRM throughout

- Page 7 : It is not clear to the reader which LC method has been used for which purpose. It seems that different LC methods have been used in connection with ICP-MS and ESI MS. By doing this, the potentiality of using elemental and combined organic MS for species identification has not been fully exploited. Please provide an explanation.

Yes, two different chromatographic methods were used for LC-ICP-MS and for LC-ESI-MS/MS. We did not make an attempt to combine both MS detectors with the same LC method. The mobile phases used for a number of years for speciation using ICP-MS detection were however, not compatible with ESI. Therefore when using ESI-MS detection a different mobile phase was required.

- Page 10, Fig S1A : Identification of species eluting in the chromatographic void volume (e.g. inorganic Se) is unacceptable and should be removed. Again, is the SeMet peak observed also present in the enzymatic blank ?

Yes, I agree. Annotations for peaks eluting with the void volume has been removed. Regarding the possible SeMet blank, please see comment above

- -Page 11, line 239 : Again, is SeMet present in the blank at a similar level ?

Regarding the possible SeMet blank, please see comment above

- Page 15, line 331, 'Fig.3' : It should read 'Fig. 4'.

Yes, has been corrected

- Page 15, last para before conclusions :, '.was spiked with an authentic standard' : Was this a ⁷⁷Se-enriched MeSeCys standard ?. If not, why looking at the transitions m/z 181 > m/z 164, m/z 181 > m/z 119 ?. Some discussion would be welcome. How did the ratio of the two transitions agreed with that of the standard ?

No, a MeSeCys standard with natural isotopic composition was used, and the transitions recorded in A and B corresponded to Me⁸⁰SeCys and in C and D to Me⁷⁷SeCys. I agree that a better explanation will help the reader to better understand what we did and why we did it. Therefore the text on page 15-16 under the heading 3.5. Mass spectrometric confirmation of Me⁷⁷SeCys in carrot has been revised.

- Conclusions, lines 354-355 : This is purely speculative since Se(VI) could be a product of sample preparation. Otherwise, please provide enough evidence for the presence of Se(VI) in onions.

As I don't have experimental evidence to further discuss the finding of Se(VI) in Se(IV) treated plants I have moderated the discussion on page 11-12 and deleted this aspect in the conclusion section

Reviewer #2:

The objective of the study was to investigate effects of bio-fortification of selected vegetables by foliar application selenium (Se). This paper is a prolongation and complement of previous work on plant-derived selenium compounds published by corresponding author (Larsen) and his team. Some of their work has already been published in Food Chemistry, (Kapolna, 2007; 2009).

This kind of study presents an original research and certainly contributed to an understanding the metabolism of Se in the plants and its conversion to organic Se species which are potentially beneficial to human health.

The submitted manuscript is in general clearly written and organized, but I proposed some minor suggestions relating to the references cited in the text.

According to the "Guide for Authors", one should follow the instruction stated as a rule: "For 2-6 authors all authors are to be listed at first citation. At subsequent citations use first author et al.. When there are more than 6 authors, first author et al. should be used throughout the text."

The following points listed below should be corrected:

Page 3, line 40: (Lee et al., 1996) - 5 authors Page 3, line 47: (Larsen et al., 2002) - 6 authors Page 3, line 50: (Kápolna et al., 2009) - 5 authors Page 3, line 53: (Finley et al., 1999) - 5 authors Page 5, line 87-88; 92-93: Latin names should be in italic Page 9, line 197: (Meija et al., 2002) - 5 authors Page 9, line 201: (Fang et al., 2009) - 6 authors Page 12, line 263: (Kotrebai et al., 2000) - 5 authors Page 19; ref. "Finely" to correct into "Finley" Page 20; ref. "Łobinski" to correct into "Lobinski"

All citations are now correctly written in the text

Re: Bio-fortification and isotopic labelling of Se metabolites in onions and carrots following foliar application of Se and ⁷⁷Se (manuscript for publication)

Highlights:

- Biofortification and intrinsic labeling of onions and carrots by an enriched stable selenium isotope was successfully achieved using foliar spraying (application by foliar uptake).
- Selenite was superior to selenate in spray solutions as it was non-toxic to the plants up to 100 µg Se/mL.
- Carrots and onions were enriched in selenium up to two hundred of times relative to control and primarily bioactive selenium species were detected.
- The enrichment by ⁷⁷Se was close to 100% of all Se in the plants
- We identified for the first time the existence of methylselenocysteine in carrot

Bio-fortification and isotopic labelling of Se metabolites in onions and carrots following foliar application of Se and ⁷⁷Se

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ABSTRACT

The aims were to bio-fortify onions by foliar application of selenium (Se) and to intrinsically label bioactive Se-metabolites in onion and carrot by enriched, stable ^{77}Se for use in human physiological studies. Onion bulbs and leaves were enriched in Se by repeated foliar spraying of 10 or 100 $\mu\text{g Se mL}^{-1}$ solutions of sodium selenite (Se(IV)) or sodium selenate (Se(VI)). ICP-MS analysis of onion leaves and bulbs showed that the Se concentration was enhanced by up to a factor of approximately 50 and 200 in bulbs and leaves, respectively. HPLC-ICP-MS analysis of proteolytic plant extracts showed that foliar application of Se(IV) gave rise to bio-synthesis of a higher fraction of the desired organic Se species and was better tolerated by the plants than Se(VI). Based on these findings onions and carrots were bio-fortified by foliar application of a solution of $^{77}\text{Se(IV)}$ that was enriched to 99,7 % as ^{77}Se . The ^{77}Se - labeled metabolites in onions were predominantly γ -glutamyl- ^{77}Se -selenomethyl-selenocysteine (γ -glu-Me $^{77}\text{SeCys}$), ^{77}Se -methylselenocysteine (Me $^{77}\text{SeCys}$) and ^{77}Se -selenomethionine ($^{77}\text{SeMet}$). Furthermore, we report here for the first time the finding in carrots of the bioactive Me $^{77}\text{SeCys}$, the identity of which was verified by HPLC-ESI-MS/MS.

Keywords: Foliar application; Onion; Carrot; Methylselenocysteine; Intrinsic ^{77}Se labeling; HPLC-ESI-MS/MS.

1. Introduction

Selenium (Se) is an essential trace element to humans and plays an important role in a number of biological processes (Lee, Park, Park, Chittum & Hatfield, 1996). The mean dietary intake of Se has been estimated to $43 \mu\text{g day}^{-1}$ in the adult Danish population (Larsen, Rokkjaer & Chistensen, 2007), and is in good agreement with the recommended intake at 40 and $50 \mu\text{g Se day}^{-1}$ for women and men, respectively. This recommendation however, does not take into consideration the possible protective effect of Se against development of certain cancer forms when supplemented at supra-nutritional doses (Clark et al., 1996). The food groups that contribute the most to the Se intake are meat (30%) followed by bread and cereals (18%) and fish (14%). Individuals that mainly consume a vegetable-based diet may be at risk of a too low intake of this element (Larsen, Andersen, Møller, Petersen, Mortensen & Petersen, 2002) and supplementation or consumption of certain novel foods or plants fortified with Se represent possible ways of increasing the total dietary intake of Se. Bio-fortification of plants can be carried out through Se fertilization via root uptake (Makela et al., 1995) or via foliar application (Kápolna, Hillestrøm, Laursen, Husted & Larsen, 2009).

In human physiological studies, enriched, stable isotopes are used to safely estimate the absorption and retention or to study the uptake and metabolism of minerals (Egan, Smith, Houk, & Serass 2009); (Finley, Duffield, Ha, Vanderpool & Thomson, 1999); (Sloth, Larsen, Bügel & Moesgaard 2003). Successful use of extrinsic labeling by enriched isotopes relies on the full equilibration between the enriched isotope added to food and its naturally occurring counterparts in the food matrix. In contrast, for a mineral like Se that is covalently bound to organic ligands, intrinsic labeling of the living crop plant by the stable isotope has proven necessary (Sandström 1996). As Se is mainly covalently bound in selenoamino acids, the need for intrinsic labeling

utilizing the natural biosynthetic pathways in plants is evident (McKenzie, Arthur & Beckett, 2002). The advantage of foliar application compared with soil fertilization with Se for bio-fortification is that losses caused by soil adsorption, chemical or microbiologically mediated conversions or losses are not likely to occur. Furthermore, the direct foliar uptake route ensures a high degree of assimilation by the plant, which is beneficial when only a small amount of a costly enriched isotope is available for bio-fortification (Kápolna et al., 2009).

The objectives of this work were to study the degree of fortification by Se in onions using foliar application of Se(IV) or Se(VI) sodium salts and to study the resulting bio-synthesized Se species at two concentration levels of these inorganic Se species. Furthermore, the enrichment and identification of occurring Se metabolites following foliar application of ^{77}Se was of interest for future use of the enriched plants in human physiological studies.

2. Experimental

2.1. Chemicals

All reagents were of analytical grade and were obtained from sources specified elsewhere (Kápolna et al., 2009). For the isotopic labeling of plants, enriched ^{77}Se isotope (purity 99.66%) was purchased from Isoflex USA (Isoflex, San Francisco, USA). The synthesis of $\text{Na}_2^{77}\text{SeO}_3$ from elemental ^{77}Se was carried out according to a published method (Toda & Hioki, 1995). Two reference materials, NIST-RM 8436 Durum Wheat Flour (National Institute of Science and Technology, Gaithersburg, USA) and CRM-BCR-402 White Clover (Institute of Reference Materials and Methods, Geel, Belgium) were analyzed in parallel with the samples for control of

accuracy of quantitative Se analyses. Purified water (resistivity $>18 \text{ M}\Omega \text{ cm}^{-1}$) was obtained from an Element Milli-Q apparatus (Millipore, MA, USA).

2.2. Greenhouse experiments

For enrichment of the plants by ^{77}Se , onions (*Allium cepa* CV Sturon) and carrots (*Daucus carota ssp. sativus* CV Bolero) were cultivated at 18°C and 15°C day and night temperatures, respectively, with light providing a cycle with 16 h at $250\text{-}300 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ followed by an 8 h night period. Onions were planted and carrots sown in a 2:1 mixture of soil (Pindstrup type 2, Denmark) and washed quartz sand (1.2-2.0 mm grain size). Aphids were controlled biologically with a solution of BD5 bio soap in water (1.5 mL L^{-1}) and by *Aphidius colemani* and *Chrysoperla carnea* (Borregaard BioPlant ApS, Denmark). During the 21 weeks cultivation period of carrots and onions, 350 mL or 175 mL of a nutrient solution containing 2.9 % nitrate, 1.9 % ammonium, 0.8 % phosphorus, 3.7 % potassium, 0.4 % magnesium, 0.8 % sulfur and essential plant micronutrients were applied in weeks 10-15. Carrots and onions were sprayed 6 times during weeks 16-19 with an aqueous solution of $^{77}\text{Se(IV)}$ sodium salt at $50 \mu\text{g Se mL}^{-1}$ containing surfactant at $5 \mu\text{L L}^{-1}$ for all sprayings (TWEEN 20, Sigma-Aldrich, Copenhagen, Denmark). A total of 75 mg Se was used for each crop corresponding to 102 mg Se applied per m^2 soil surface area. Control plants (no Se enrichment) were cultivated according to the same procedure.

For optimization of the conditions for the Se enrichment (natural isotope abundance) onions were grown from seeds and were cultivated for 21 weeks. Foliar application of Se was performed 4 times (week 14-17) by sprayings of aqueous solutions of Se(IV) or Se(VI) sodium salts at 10 or $100 \mu\text{g Se mL}^{-1}$ as. An unsprayed control group was also included. The treatments with low

or high concentrations of Se(IV) or Se(VI) corresponded to application of 11.6 or 116 mg Se m⁻² soil surface area, respectively.

After harvesting, soil and adhered dust were removed from the plant surfaces by first soaking in deionised water, then in Milli-Q water for 30 minutes and a final rinse in Milli-Q water. The plant parts were minced and freeze-dried separately (Christ Freeze Dryers, Beta 1-8, Montreal Biotech, Inc., Dorval, Canada). The moisture content was determined at 92 % and 84 % in the onion leaves and bulbs, and at 87% and 90% in the carrot leaves and roots, respectively. Finally, the dried samples were homogenised using a commercial coffee-grinder.

2.3. Sample preparation for Se and Se speciation analyses

Prior to Se analysis by inductively coupled plasma mass spectrometry (ICP-MS), the samples were digested by concentrated nitric acid using a microwave system equipped with quartz vessels operated at a maximum pressure and temperature of 70 bar and 250°C, respectively (Multiwave, Anton Paar, Graz, Austria). Prior to Se speciation analysis the Se content in the samples was liberated by enzymatic hydrolysis using Protease XIV (Sigma-Aldrich, Copenhagen, Denmark) for 24 hours at 37°C (Kápolna et al., 2009); (Larsen, Sloth, Hansen, & Moesgaard 2003). Before identification of Se species by mass spectrometric analysis, the proteolytic plant extracts were evaporated under a flow of nitrogen and were re-dissolved in the HPLC mobile phase. To further confirm the identification, a separate portion of the sample digest was spiked with the authentic Se species at 100 ng Se mL⁻¹.

2.4. Instrumentation

An Agilent 7500ce ICP-MS (Agilent Technologies, Tokyo, Japan) equipped with an octopole reaction cell was used for detection of ^{77}Se , ^{78}Se and ^{80}Se . Argon-based polyatomic interferences were reduced using H_2 as cell gas at optimised flow rate. A commercial nebulizer (Micromist, Glass Expansion, West Melbourne, Australia) was used for the sample introduction under standard plasma conditions. A solution of yttrium at 5 ng Y mL^{-1} was continuously introduced as an internal standard for total Se analyses. The method of standard additions was used for the quantification of Se in the samples. For the ion exchange chromatographic separations and detection of the Se species liberated from the plant samples, an Agilent 1100 HPLC (Agilent Technologies, Palo Alto, CA, USA) was coupled with ICP-MS or with ESI-MS/MS for Se or Se species selective detection, respectively. The anion exchange HPLC system consisted of a strong anion exchange column (ION-120, Transgenomic, UK) (120 mm x 4.6 mm x 5 μm) used in combination with a mobile phase containing salicylic acid. The cation exchange HPLC column (Ionospher 5C, Varian BV, Middelburg, The Netherlands) (100 mm x 3.0 mm x 5 μm) was used in combination with a mobile phase containing pyridinium formate (Kápolna et al., 2009). External five-point standard curves were used for the speciation quantifications. Qualitative analysis was conducted by observing that authentic standards spiked to the sample extracts co-eluted with the analytes. All analyses were performed in triplicate based on the detailed instrumental conditions in Supplementary information Table S1. Data were collected and evaluated using the Agilent Chemstation ICP-MS chromatographic software. Se species, which were tentatively identified with HPLC-ICP-MS on the basis of co-elution with authentic standards, were separated by cation exchange HPLC (Supplementary information Table S1) using a Security Guard (4.0 x 2.0 mm) pre-column (Phenomenex, Torrance, CA, USA). For the speciation work blanks were taken through the

entire analytical procedure (including proteolytic digestion), and the absence of any of the separated Se species was controlled by monitoring Se as ^{80}Se . The base-line noise of these chromatograms was used for estimation of the limit of detection.

For the purpose of verification of the tentatively identified Se species, experiments using ESI-MS/MS were conducted using a Quattro Micro (Waters, Milford, MA, USA) tandem quadrupole mass spectrometer in the selected reaction monitoring (SRM) mode using chromatographic conditions that were compatible with ESI-MS/MS (Supplementary information Table S1). In the positive ion mode the SRM transitions m/z 197.7 \rightarrow (180.8 and 151.7) and m/z 183.7 \rightarrow (122.7 and 166.8) were used for identification of $^{80}\text{SeMet}$ and $\text{Me}^{80}\text{SeCys}$, respectively. Similarly, SRM transitions m/z 194.7 \rightarrow (177.7 and 148.7) and m/z 180.7 \rightarrow (163.8 and 119.7) were used for identification of $^{77}\text{SeMet}$ and $\text{Me}^{77}\text{SeCys}$, respectively. Data were collected and evaluated using the Mass Lynx 4.0 software.

2.5 Data analysis

Chromatograms were processed with Origin 7.0 (Microcal Software Inc., Northampton, MA, USA) and Microsoft® Excel 2000 (Microsoft Corporation, Redmond, Washington, USA) was applied for statistical evaluations. Repeated analyses of the CRMs, which were run in parallel with the plant samples, were used to assure satisfactory accuracy of the Se analyses. The uncertainty of the mean values of Se determined in the CRMs was used to decide if the analyses deviated from the certified values by applying the Student's t-tests or a one-sided analysis of variance. All concentration values presented in this paper are expressed on the basis of dry mass of the sample, except where stated otherwise.

3. Results and discussion

3.1. Bio-fortification by Se using foliar application

The objective of the experiment with foliar application of Se to onion plants was to identify the optimum concentration level of Se(IV) or Se(VI) in the foliar spraying solutions for effective bio-fortification by organic Se metabolites in the plants. The second objective was to avoid any visible toxic effects to the plants, such as stunted growth, chlorosis, withering and necrosis of leaves (Trelease & Beath, 1949). Following the foliar application by Se(VI) serious damage to the onion leaves occurred for the $100 \mu\text{g Se mL}^{-1}$ concentration in the spray solution, suggesting that the upper tolerable limit had been exceeded for this Se species. In contrast, no signs of toxicity were observed for the same Se concentration when applying Se(IV). The analytical results (Supplementary information Table S2) showed that the fortification amounted to 22-213 times relative to controls, depending on plant part and treatment. For both treatments, the Se content of the onion leaves was significantly higher than that in the corresponding bulbs. Application of Se as Se(VI) caused a higher Se concentration in the leaves than that resulting from application of Se(IV) ($P < 0.05$). The fortification by Se in the onion bulbs was roughly a factor of two higher when spraying with Se(VI) in comparison with Se(IV). The assimilation rates of Se, which was estimated as the mass of Se recovered in the harvested onion biomass divided by the mass of Se used for foliar spraying, were 10 % and 11 % for Se(IV) and Se(VI), respectively, for the $10 \mu\text{g Se mL}^{-1}$ spraying solutions. For the $100 \mu\text{g Se mL}^{-1}$ concentration in solution the same rates were 3% and 5%, respectively. Thus, the estimated Se assimilation rates for onion were significantly lower than those reported (Kápolna et al., 2009) for carrots (range 29-48%). The rather modest assimilation rates for onion were ascribed to a limited absorption capacity of the onion leaves because of a

relatively low surface area compared to that of carrot leaves, or explained by the possible phyto-volatilization of the absorbed Se as gaseous selenide species through the leaves (Meija et al., 2002).

The degree of Se bio-fortification obtained for onions in the present study (Supplementary information Table S2) compare well with that reported in similar studies on foliar Se enrichment of crop plants. For rice plants a 6.5– 56 times increase in the Se content was observed when applying Se(IV) (from 0 g ha⁻¹ to 100 g ha⁻¹) by foliar spraying (Fang, Zhang, Catron, Chan, Hu & Caruso, 2009). Cabbage, radish, onion and garlic were enriched by applying Se(VI) at 20 mg Se m⁻² twice during the growth period (Slejkovec & Goessler, 2005). The highest Se concentrations were detected in onion and radish leaves (37.4 and 37.1 mg kg⁻¹) followed by the leaves of garlic (19.6 mg kg⁻¹) and cabbage (11.9 mg kg⁻¹). When only considering results for the edible parts of these vegetables, the highest Se accumulation was obtained in cabbage (11.9 mg kg⁻¹), followed by radish root (8.2 mg kg⁻¹), garlic cloves (6.6 mg kg⁻¹) and onion bulb (5.6 mg kg⁻¹). For use in human nutrition and physiology studies however, not only the degree of Se fortification in crop plants, but also its incorporation into bio-active metabolites biosynthesized by the plant is essential in human studies related to promotion of good health and prevention of disease.

3.2. Se speciation in onion samples

For plants that were fortified with Se(IV) at 100 µg Se mL⁻¹ the efficiency of the enzyme-assisted method of extraction was 79 ± 2 % and 98 ± 2 % (n=3) for onion leaves and bulbs, respectively, calculated as the sum of Se in separated Se species relative to the total Se concentration. For plants fortified with Se(VI) solution at 100 µg Se mL⁻¹ however, the corresponding values were 78 ± 2 % and 46 ± 3 % (n=3). These results showed that the Se species contained in the Se(IV) fortified plants were almost fully accounted for, whereas a significant

fraction of Se remained non-extractable from the bulbs, and thereby of unknown identity, when fortification was carried out by foliar application of Se(VI).

The HPLC separation methods with ICP-MS detection were applied to the enzymatic extracts of onion leaves and bulbs for characterization of the Se metabolites in the fortified plants. The chromatogram corresponding to the fortified onion showed (Supplementary information Fig. S1A) that the major compound in onion extracts was assigned to γ -glu-MeSeCys whereas the two minor peaks were assigned to MeSeCys and SeMet on the basis of co-elution with spiked authentic standards. The corresponding anion exchange chromatogram (Supplementary information Fig. S1B) showed a peak eluting at T_R 11 minutes, which was similarly assigned to Se(VI), whereas separation of SeMet and MeSeCys was not achieved when using this HPLC system. The Se species detected in bulbs and leaves of the onions sprayed with $10 \mu\text{g Se mL}^{-1}$ as Se(IV) or Se(VI) showed the same proportion of the same Se species, only at lower concentrations (data not shown). This finding suggests that the plant's metabolic pathways respond equally to foliar spraying with both Se concentrations used. The chromatograms corresponding to the extracts of the control samples did not show any peaks exceeding the baseline noise (3σ) of the chromatograms and therefore remained undetectable by HPLC-ICP-MS. The risk of blank chromatographic peaks originating from the proteolytic enzyme or occurring because of memory from previous sample injections could therefore be disregarded.

The quantitative results for Se species detected in the onion bulbs following application of Se at $100 \mu\text{g Se mL}^{-1}$ are shown in Fig. 1A. The predominant Se species was γ -glu-MeSeCys regardless of which of the two inorganic forms of Se that were used for foliar application. Two other organic Se compounds, SeMet and MeSeCys, were detected in the samples at 5-10 % of the concentration of the former predominant Se species. The quantitative data for onion leaves in

Fig. 1B show, that the concentration of γ -glu-MeSeCys was similar to, and that SeMet was found at significantly higher concentration than in the bulb.

In contrast to Se(VI), Se(IV) remained undetectable in onion bulbs and leaves for both inorganic forms of applied Se. Apparently Se(IV) was effectively metabolized to organic Se species, whereas the rate limiting reduction of Se(VI) to Se(IV) caused build-up of this oxidized species both in the leaves and, after translocation, also in the plant's storage tissue, *i.e.* the bulb. Whether the moderate amount of Se(VI) found in the onion's leaves and bulb (Figure 1 A and B) following foliar application of Se(IV) was a result of the plant's natural metabolism or caused by an analytical artifact remains to be investigated in future studies. MeSeCys, which was detected in the onions' parts, is a precursor of the predominant organic Se species in the onion plant, namely the dipeptide γ -glu-MeSeCys. Overall, the results demonstrate that Se was present as organic Se species in onion, particularly following foliar application of Se(IV). Therefore, this inorganic Se species was preferred for bio-fortification of onions to achieve the desired organic Se compounds.

In a study on foliar application of Se to onions and garlic used Se(VI) at $10 \mu\text{g mL}^{-1}$ in the spraying solution (Slejkovec & Goessler 2005), the Se content of the two enriched plant samples were $1.92 \text{ mg Se kg}^{-1}$ and $3.16 \text{ mg Se kg}^{-1}$, respectively, and Se(VI) was the predominant Se species found in both plants. Also SeMet was reported in garlic, but the identity of a chromatographic peak ascribed to MeSeCys was not confirmed. In comparison, the results of the present study demonstrated that a higher degree of enrichment by Se in onion was achieved, and that a wider range of especially organic Se species was accounted for in the fortified onion bulbs and leaves. Other studies of Se enrichment of Allium plants grown in soil or in hydroponic cultures have demonstrated that γ -glu-MeSeCys and MeSeCys were biosynthesized from inorganic Se amended to the growth medium (Kápolna & Fodor 2006); (Kápolna, Shah, Caruso, & Fodor 2007); (Kotrebai, Birringer, Tyson, Block & Uden, 2000); (Larsen et al., 2006); (McSheehy et al., 2000);

(Montes-Bayón, Díaz Molet, Blanco González & Sanz-Medel, 2006) and suggested that the metabolic products are similar for root or foliar uptake routes of Se.

3.3. Enrichment and intrinsic labeling of onions and carrots by enriched ^{77}Se

The results and observations, including fortification rate, occurrence of organic Se species and observed signs of toxicity, from the foliar application experiments with onions were used to plan and conduct similar experiments aiming at fortification and intrinsic labeling of organic Se species by ^{77}Se . Because Se(IV) was preferred, this species was synthesized from elemental ^{77}Se and used in the foliar spraying solution. In order to minimize wasting the precious ^{77}Se isotope because of unforeseen stress or adverse effects to the plants, a safety factor of two was applied and consequently a final Se concentration as $^{77}\text{Se(IV)}$ of $50 \mu\text{g } ^{77}\text{Se mL}^{-1}$ was used for the foliar spraying solution for onions and carrots. Following foliar application, the absorbed enriched isotope was metabolized along with Se with a natural isotopic abundance naturally present at low concentration in the plant. In order to estimate the concentration of Se originating from the foliar application of ^{77}Se , the natural background of ^{77}Se was calculated from the measured signal intensity corresponding to the non-enriched ^{78}Se and subtracted from the ICP-MS signal intensity corresponding to all ^{77}Se . The total Se content in the plant (all isotopes) was calculated as the sum of the concentration of the enriched ^{77}Se and the concentration of natural abundance Se determined from ICP-MS measurement of ^{78}Se (Sloth, Larsen, Bügel, & Moesgaard 2003).

The results in Table 1 show that the achieved isotopic purity of ^{77}Se in the carrots and onion plants was 99% or higher, or about 14 times the natural abundance of this isotope. Consequently, virtually all Se in carrots and onions originated from the foliar application of ^{77}Se . In comparison with the results reported for Se in onions that were bio-fortified with Se(IV) at $100 \mu\text{g}$

Se mL⁻¹ (Supplementary information Table S2), the corresponding Se concentrations in the ⁷⁷Se fortified onion samples were at the expected level bearing in mind that the Se concentration in the ⁷⁷Se(IV) spraying solution was 50 µg Se mL⁻¹.

3.4. Se speciation in the intrinsically ⁷⁷Se labelled plants

The cation exchange HPLC-ICP-MS chromatograms for onion bulb, onion leaves and carrot root in Fig. 2 and 3 show that γ -glu-Me⁷⁷SeCys, Me⁷⁷SeCys and ⁷⁷SeMet were detected in the proteolytic plant extracts. The absence of chromatographic peaks corresponding to ⁸⁰Se-containing Se species (chromatographic tracing not shown) was in accordance with the low content of Se from the natural background. The chromatograms in Fig. 2A-B show that γ -glu-Me⁷⁷SeCys, Me⁷⁷SeCys and a small amount of ⁷⁷SeMet was detected in onion bulb, and that the predominant Se species in onion leaves included ⁷⁷SeMet and Me⁷⁷SeCys. These findings confirmed the findings in onions from the foliar application of natural abundance Se(IV). The reason for the absence in the onion leaves of the storage form of the latter selenoamino acid, γ -glu-MeSeCys, remains unknown. Results from analysis of the onion leaves by anion exchange HPLC analysis showed, compared to the onion bulbs, that the former plant part contained a higher amount of both inorganic ⁷⁷Se species (chromatograms not shown). The chromatogram corresponding to the isotopically labelled carrot roots (Fig. 3) indicated that Me⁷⁷SeCys and ⁷⁷SeMet were present in addition to a small peak of unknown identity at T_R 2.5 minutes.

The quantitative results for the Se species detected in the edible parts of the ⁷⁷Se enriched plants are presented in Table 2. The major fraction of ⁷⁷Se in onions and carrots existed as organic ⁷⁷Se species. In an earlier study a low concentration of γ -glu-MeSeCys was tentatively identified in carrot at 0.1 µg Se g⁻¹ by HPLC-ICP-MS (Kápolna et al., 2009), but in the present study

only its precursor Me⁷⁷SeCys was found. The observed differences in Se species contained in the plants following foliar application of Se indicate, that variations in the assimilation and metabolites of Se indeed occurred between cultivation trials. Possibly these differences may have been induced by differences in growth conditions including climate, number of fertilizer applications or stress factors of unknown nature. Consequently, when information about the content and identity of bio-synthesized Se species is warranted, chemical characterization of each crop batch is mandatory.

3.5. Mass spectrometric confirmation of Me⁷⁷SeCys in carrot

The qualitative and quantitative results for Se species reported in this work rely on correct assignment of identity to the HPLC peaks. Although co-elution with authentic standard spiked to the sample extracts and use of two HPLC-systems combined with ICP-MS detection provide a high degree of certainty, analysis by HPLC-ESI-MS/MS provides unequivocal information on the identity of the Se-containing molecular species. The tentative identification of MeSeCys in carrots based on results from HPLC-ICP-MS analysis therefore required further proof of identity.

The specific MS/MS transitions for Me⁸⁰SeCys and for Me⁷⁷SeCys were monitored in the pre-concentrated extract of a ⁷⁷Se-enriched carrot sample that contained $2.8 \pm 0.1 \mu\text{g Se g}^{-1}$. The chromatograms in Fig. 4 (panels A and B) show that Me⁸⁰SeCys was absent in the sample as no signal was recorded for the two transitions corresponding to ⁸⁰Se. This was in accordance with the high degree of isotopic enrichment by ⁷⁷Se in the sample reported in Table 1. When spiking the sample with the authentic MeSeCys (natural isotope composition) however, Me⁸⁰SeCys was eluted and detected at T_R 6.00 minutes. This shows that the chromatographic separation and the SRM detection of Me⁸⁰SeCys were possible for this matrix. In the unspiked ⁷⁷Se enriched carrot sample

however, Me⁷⁷SeCys was detected (panel C) by monitoring the corresponding ⁷⁷Se-specific transitions. When the sample was spiked with the authentic standard that contained Me⁷⁷SeCys as part of its natural Se isotope composition, this Se species was detected and co-eluted with the plant's natural content of Me⁷⁷SeCys (panel D). The combined results, including chromatographic retention times and the isotope and species-specific mass transitions demonstrated that Me⁷⁷SeCys was present in the ⁷⁷Se-fortified carrot root. To the best of our knowledge this is the first identification of MeSeCys in carrot. In combination with the previous tentative finding of γ -glu-MeSeCys in carrot (Kápolna et al., 2009), the unequivocal finding of MeSeCys in carrot in the present work provided new information on the existence of this bioactive metabolite in a crop of widespread use in the human diet.

4. Conclusions

Foliar application of Se as Se(IV) was an effective and cost-effective method of bio-fortification of onion and carrot by bioactive Se species. Application of Se(IV) in solutions at 50-100 $\mu\text{g Se mL}^{-1}$ did not cause any visible symptoms of toxicity to the plants. Following its uptake, the metabolism of Se(IV) in the plants studied led to a high degree of conversion to organic Se species, which were potentially beneficial to human health. In contrast, foliar application of Se(VI) at 100 $\mu\text{g Se mL}^{-1}$ led to signs of toxicity and to less efficient incorporation into organic Se species. The method of foliar application was well-suited for bio-fortification and intrinsic ⁷⁷Se labeling of organic Se species in crop plants. Given these advantages, this bio-fortification method was useful for production of crop plants for human intervention studies where intrinsically labeling and a high enrichment factor by enriched stable ⁷⁷Se is desired. In the ⁷⁷Se-fortified carrots, Me⁷⁷SeCys was for the first time detected by HPLC-ICP-MS and its identity verified by ESI-MS/MS.

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Figure captions

Fig. 1. Quantitative results for Se species identified in onion bulbs (A) and onion leaves (B) following foliar application of Se(IV) or Se(VI) at 100 $\mu\text{g Se mL}^{-1}$. The columns and error bars represent mean values \pm 95% confidence intervals (n=3).

Fig. 2. Cation exchange HPLC-ICP-MS chromatograms of ^{77}Se -labelled onion samples overlaid with samples spiked with a mixture of authentic Se standards. The proteolytic extracts correspond to onion bulb (A) and onion leaf (B) enriched with Se following foliar application of $^{77}\text{Se(IV)}$ at 50 $\mu\text{g Se mL}^{-1}$ (see Table 1).

Fig. 3. Cation exchange HPLC-ICP-MS chromatograms of ^{77}Se -labelled carrot overlaid with a sample spiked with a mixture of authentic Se standards. The proteolytic extracts correspond to carrot root enriched with Se following foliar application of $^{77}\text{Se(IV)}$ at 50 $\mu\text{g Se mL}^{-1}$ (see Table 1).

Fig. 4. Cation exchange HPLC-ESI-MS/MS chromatograms of a proteolytic extract of a ^{77}Se enriched carrot root sample. Panels A and C correspond to unspiked samples and panels B and D to samples spiked with an authentic standard of MeSeCys. For MS/MS transitions, see Experimental section.

References

- Clark, L. C., Combs, G. F. Jr., Turnbull, B. W., Slate, E. H., Chalker, D. K., Chow, J., Davis, L. S., Glover, R. A., Graham, G. F., Gross, E. G., Krongrad, A., Leshner, J. L. Jr., Park, H. K., Sanders, B. B. Jr., Smith, C. L. & Taylor, J. R. (1996), Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group, *Journal of the American Medical Association* 276, 1957-1963.
- Egan, C. B., Smith, F. G., Houk, R. S. & Serass, R. E. (2009), Zinc absorption in women: comparison of intrinsic and extrinsic stable-isotope labels, *American Journal of Clinical Nutrition* 91, 547-553.
- Fang, Y., Zhang, Y., Catron, B., Chan, Q., Hu, Q. & Caruso, J. A. (2009), Identification of selenium compounds using HPLC-ICPMS and nano-ESI-MS in selenium-enriched rice *via* foliar application, *Journal of Analytical Atomic Spectrometry* 24, 1657-1664.
- Finley, J. W., Duffield, A., Ha, P., Vanderpool, R. A. & Thomson, C. D. (1999), Selenium supplementation affects the retention of stable isotopes of selenium in human subjects consuming diets low in selenium, *British Journal of Nutrition* 82, 357-360.
- Kápolna, E. & Fodor, P. (2006), Speciation analysis of selenium enriched green onions (*Allium fistulosum*) by HPLC-ICP-MS, *Microchemical Journal* 84, 56-62.
- Kápolna, E., Hillestrøm, P. R., Laursen, K. H., Husted, S. & Larsen, E. H. (2009), Effect of foliar application of selenium on its uptake and speciation in carrot, *Food Chemistry* 115, 1357-1363.
- Kápolna, E., Shah, M., Caruso, J. A. & Fodor, P. (2007), Selenium speciation studies in Se-enriched chives (*Allium schoenoprasum*) by HPLC-ICP-MS, *Food Chemistry* 101, 1398-1406.
- Kotrebai, M., Birringer, M., Tyson, J. F., Block, E. & Uden, P. C. (2000), Selenium speciation in enriched and natural samples by HPLC-ICP-MS and HPLC-ESI-MS with perfluorinated carboxylic acid ion-pairing agents, *Analyst* 125, 71-78.
- Larsen, E. H., Andersen, N. L., Møller, A., Petersen, A., Mortensen, G. K. & Petersen, J. (2002), Monitoring the content and intake of trace elements from food in Denmark, *Food Additives and Contaminants* 19, 33-46.
- Larsen, E. H., Lobinski, R., Burger-Meyer, K., Hansen, M., Ruzik, R., Mazurowska, L., Rasmussen, P. H., Sloth, J. J., Scholten, O. & Kik, C. (2006), Uptake and speciation of selenium in garlic cultivated in soil amended with symbiotic fungi (mycorrhiza) and selenate, *Analytical and Bioanalytical Chemistry* 385, 1098-1108.
- Larsen, E. H., Rokkjaer, I., & Chistensen, T. Danish monitoring system for foods 1998-2003. Content of As, Cd, Hg, Ni, Pb and Se and dietary intake by children and adults. In: Caroli S ed. *The determination of chemical elements in food: applications for atomic and mass spectrometry* 2007. 297-332.

- Larsen, E. H., Sloth, J. J., Hansen, M. & Moesgaard, S. (2003), Selenium speciation and isotope composition in ^{77}Se -enriched yeast using gradient elution HPLC separation and ICP-dynamic reaction cell-MS, *Journal of Analytical Atomic Spectrometry* 18, 310-316.
- Lee, B., Park, S., Park, J., Chittum, H. & Hatfield, D. (1996), Molecular biology of selenium and its role in human health, *Molecules and Cells* 6, 509-520.
- Makela, A. L., Wang, W. C., Hamalainen, M., Nanto, V., Laihonon, P., Kotilainen, H., Meng, L. X. & Makela, P. (1995), Environmental effects of nationwide selenium fertilization in Finland, *Biological Trace Element Research* 47, 1-3.
- McKenzie, R. C., Arthur, J. R. & Beckett, G. J. (2002), Selenium and the regulation of cell signaling, growth, and survival: Molecular and mechanistic aspects, *Antioxidants & Redox Signaling* 4, 339-351.
- McSheehy, S., Yang, W., Pannier, F., Szpunar, J., Lobinski, R., Auger, J. & Potin-Gautier, M. (2000), Speciation analysis of selenium in garlic by two-dimensional high-performance liquid chromatography with parallel inductively coupled plasma mass spectrometric and electrospray tandem mass spectrometric detection, *Analytica Chimica Acta* 421, 147-153.
- Meija, J., Montes-Bayón, M., Le Duc, D. L., Terry, N. & Caruso, J. A. (2002), Simultaneous monitoring of volatile selenium and sulfur species from Se accumulating plants (wild type and genetically modified) by GC/MS and GC/ICPMS using solid-phase microextraction for sample introduction, *Analytical Chemistry* 74, 5837-5844.
- Montes-Bayón, M., Díaz Molet, M. J., Blanco González, E. & Sanz-Medel, A. (2006), Evaluation of different sample extraction strategies for selenium determination in selenium-enriched plants (*Allium sativum* and *Brassica juncea*) and Se speciation by HPLC-ICP-MS, *Talanta* 68, 1287-1293.
- Sandström, B. Overview of isotope methods and inorganic nutrient metabolism. In: Mellon F ed. *Stable isotopes in human nutrition* 1996. 3-9.
- Slejkovec, M. & Goessler, W. (2005), Accumulation of selenium in natural plants and selenium supplemented vegetable and selenium speciation by HPLC-ICPMS, *Chemical Speciation and Bioavailability* 17, 63-73.
- Sloth, J. J., Larsen, E. H., Bügel, S. & Moesgaard, S. (2003), Determination of total selenium and ^{77}Se in isotopically enriched human samples by ICP-dynamic reaction cell-MS, *Journal of Analytical Atomic Spectrometry* 18, 317-322.
- Toda, E. & Hioki, A. (1995), Determination of impurities in high-purity selenium by inductively coupled plasma mass spectrometry after matrix separation with thiourea, *Analytical Sciences* 11, 115-118.
- Trelease, S. F., & Beath, O. A. *Selenium: its geological occurrence and its biological effects in relation to botany, chemistry, agriculture, nutrition, and medicine*, New York: Trelease & Beath, 1949. 292.

Table 1

Concentration ($\mu\text{g Se g}^{-1}$ dry mass) of selenium and yield (g m^{-2}) of carrots and onions enriched by foliar application of ^{77}Se (IV) at $50 \mu\text{g Se mL}^{-1}$ ^a

Plant and sub-sample		Yield	Total Se concentration	^{77}Se fraction (%)
Carrot	Leaf	3871	$25.0 \pm 0.3^{\text{A}}$	99.4
	root	18299	$2.8 \pm 0.1^{\text{C}}$	99.1
Onion	Leaf	6231	$6.3 \pm 0.6^{\text{B}}$	98.2
	Bulb	4762	$6.8 \pm 0.4^{\text{B}}$	99.4

^a All selenium concentrations are given as mean \pm one standard deviation (n=3). Total Se concentration denotes sum of naturally occurring and isotopically enriched concentration. Values with different superscripts in capital letters are significantly different ($P < 0.05$). ^{77}Se fraction denotes abundance of this isotope and is given relative to the total selenium concentration.

Table 2Concentration ($\mu\text{g } ^{77}\text{Se g}^{-1}$ dry mass) of selenium-species contained in intrinsically ^{77}Se -labelled carrot and onion ^a

Plant and sub-sample		γ -glu-MeSeCys	MeSeCys	SeMet	Se(IV)	Se(VI)	Fraction of total Se
Carrot	Leaf	n.a.	n.a.	n.a.	n.a.	n.a.	
	Root	< 0.09	1.23 \pm 0.05 ^E	1.47 \pm 0.07 ^C	0.20 \pm 0.03 ^H	0.071 \pm 0.018 ^I	65
Onion	Leaf	< 0.09	0.87 \pm 0.04 ^F	1.31 \pm 0.03 ^D	0.70 \pm 0.04 ^G	0.94 \pm 0.04 ^F	61
	Bulb	1.83 \pm 0.02 ^B	2.41 \pm 0.03 ^A	0.23 \pm 0.03 ^H	< 0.06	< 0.06	106

^a Concentrations of selenium species are given as mean \pm one standard deviation (n=3). Values below the limit of detection are indicated < . Values with different superscripts are significantly different (P<0.05). The fraction of total Se (%) denotes the ratio of the sum of Se species concentrations to total selenium concentration (Table 3). n.a. denotes that samples were not available.

Figure(s)

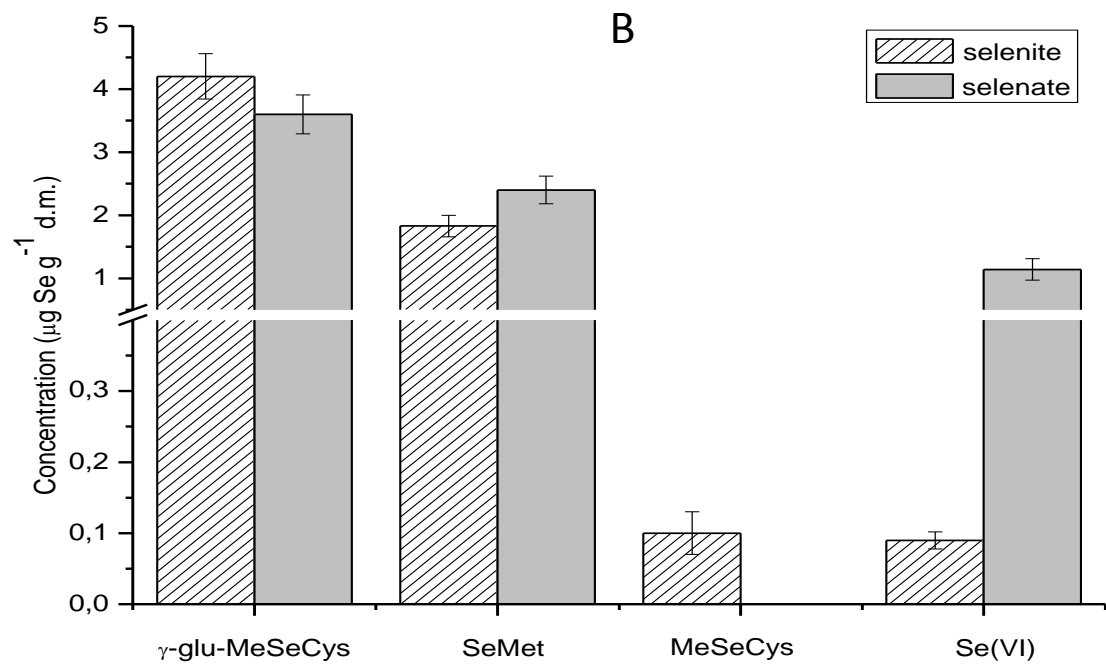
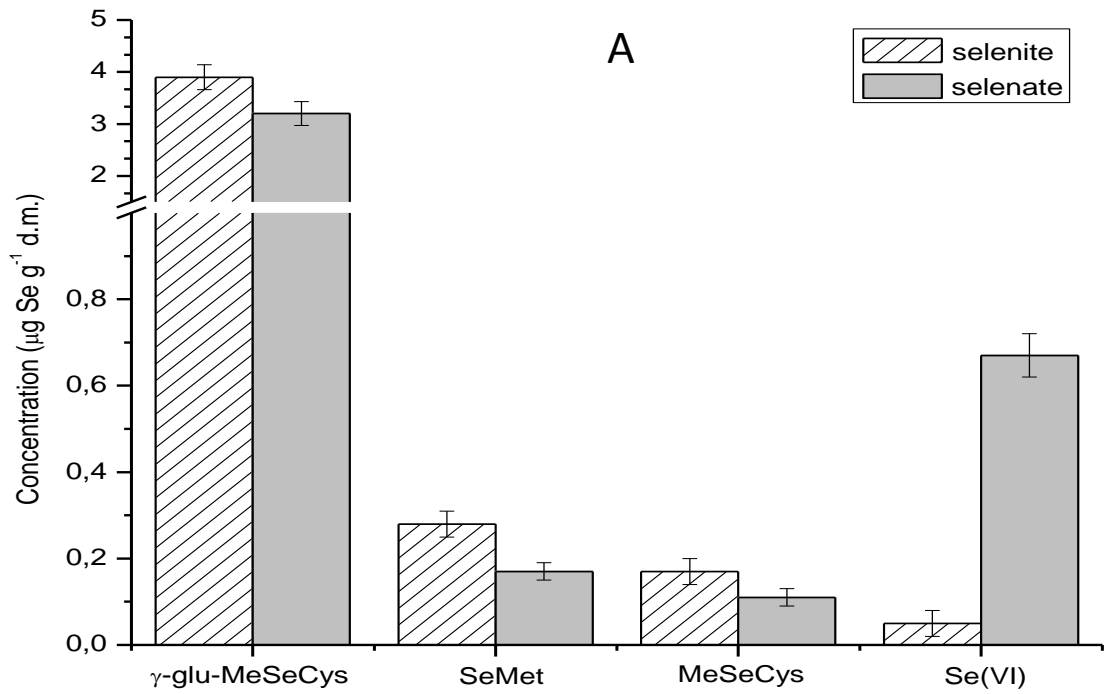


Fig. 1

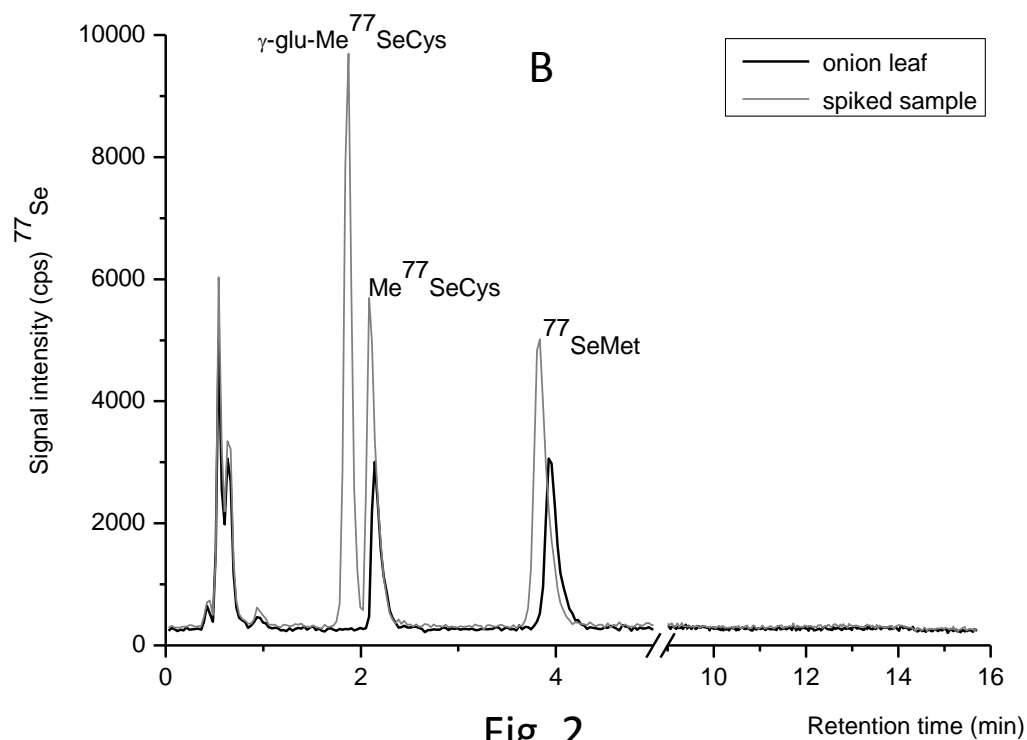
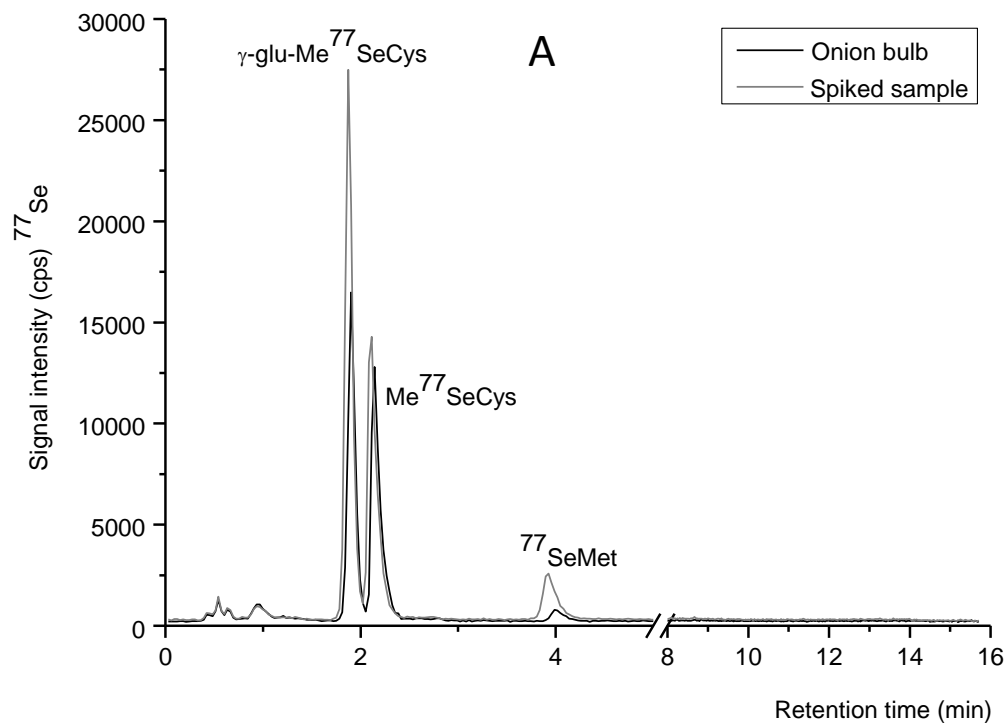


Fig. 2

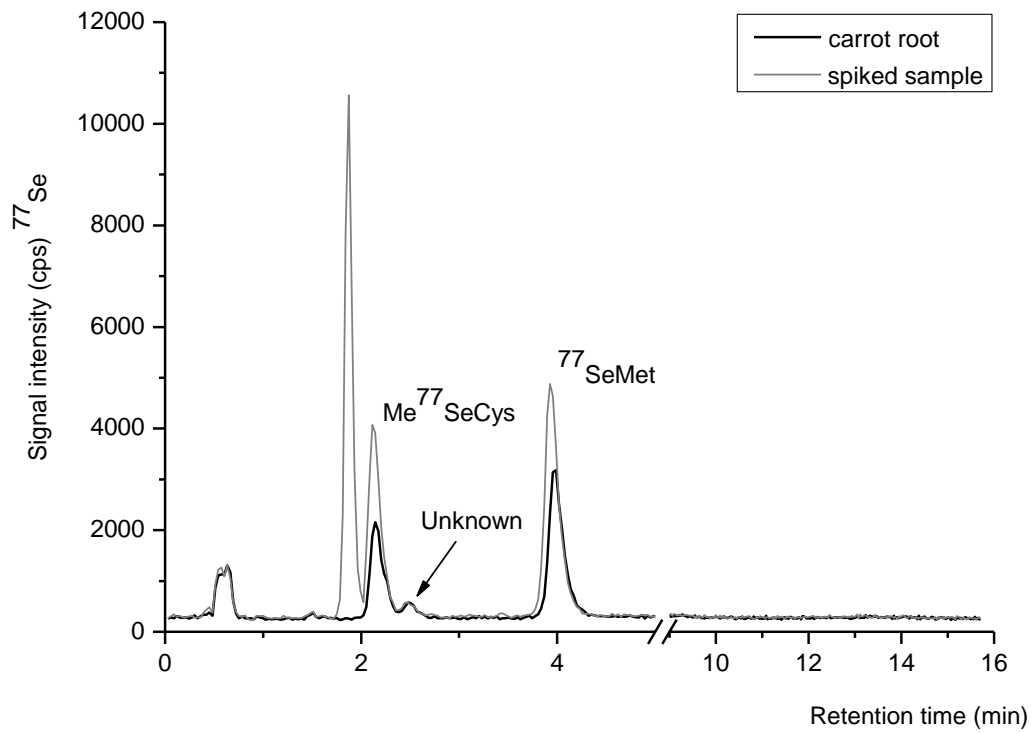


Fig. 3

Figure(s)

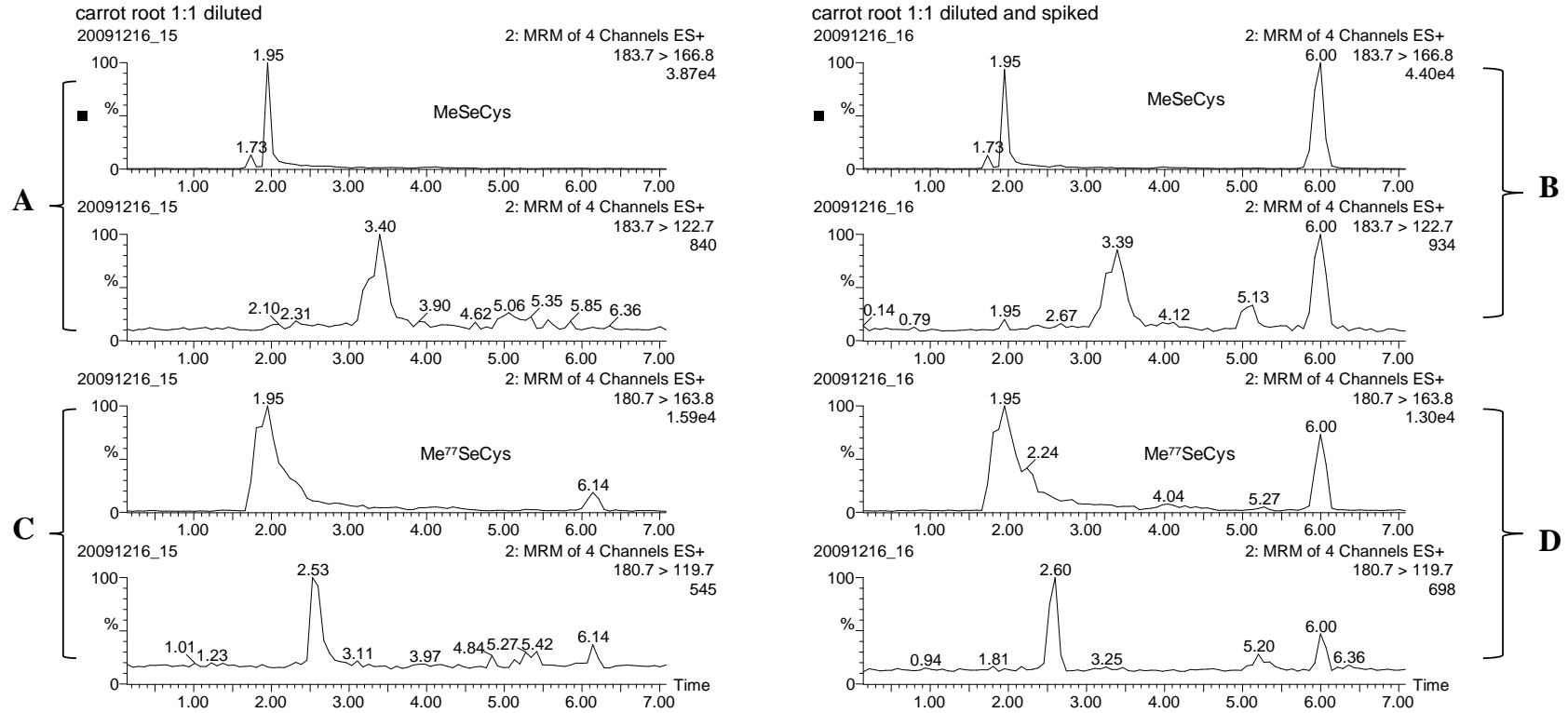


Fig. 4

Table S1Instrumental settings for HPLC-ESI-MS/MS

ESI-MS/MS settings

Auxiliary gas, N ₂ (L hr ⁻¹)	490
Nebulizer gas, N ₂ (L hr ⁻¹)	60
Collision gas, Ar (psi)	8
Needle voltage (V)	3150
Cone temperature (°C)	120
Nebulizer temperature (°C)	400
Cone voltage (V)	12
Collision voltage (V)	15

SCX HPLC settings

HPLC column	Chrompack IonoSpher 5C (150 x 2.0mm; 5 µm)
Mobile phases	(A): 1.5 mM ammonium formate 5% (v/v) MeOH, pH 3.1 (B): 7 mM ammonium formate 5% (v/v) MeOH, pH 3.5
Gradient elution programme	0.0 – 2.5 min: 80% A – 20% B 2.6 – 11.0 min: 100% B 11.1 – 18.0 min: 80% A – 20%B
Flow rate (mL min ⁻¹)	0.25
Column temperature (°C)	20
Injection volume (µL)	25

Table S 2

Concentration ($\mu\text{g Se g}^{-1}$ dry mass) and degree of fortification (times) by selenium in onions enriched by foliar application of solutions of Se(IV) or Se(VI) at $100 \mu\text{g Se mL}^{-1}$ ^a.

Treatment	Se-species applied	Se concentration		Fortification relative to control
		Leaf	Bulb	
10 $\mu\text{g Se mL}^{-1}$	None (Control)	< 0.045	< 0.045	
	Se (IV)	$4.0 \pm 0.1^{\text{F}}$	$1.1 \pm 0.1^{\text{H}}$	89
	Se (VI)	$5.2 \pm 0.2^{\text{D}}$	$2.1 \pm 0.1^{\text{G}}$	24
				116
100 $\mu\text{g Se mL}^{-1}$	None (Control)	< 0.045	$0.2 \pm 0.1^{\text{I}}$	47
	Se (IV)	$7.8 \pm 0.1^{\text{C}}$	$4.3 \pm 0.1^{\text{E}}$	173
	Se (VI)	$9.6 \pm 0.1^{\text{A}}$	$9.1 \pm 0.1^{\text{B}}$	22
				213
			45	

^a All concentrations are given as mean \pm one standard deviation (n=3) for ⁸⁰Se. Concentration values with different superscripts in capital letters are significantly different (P<0.05). Values below the limit of detection are indicated as <.

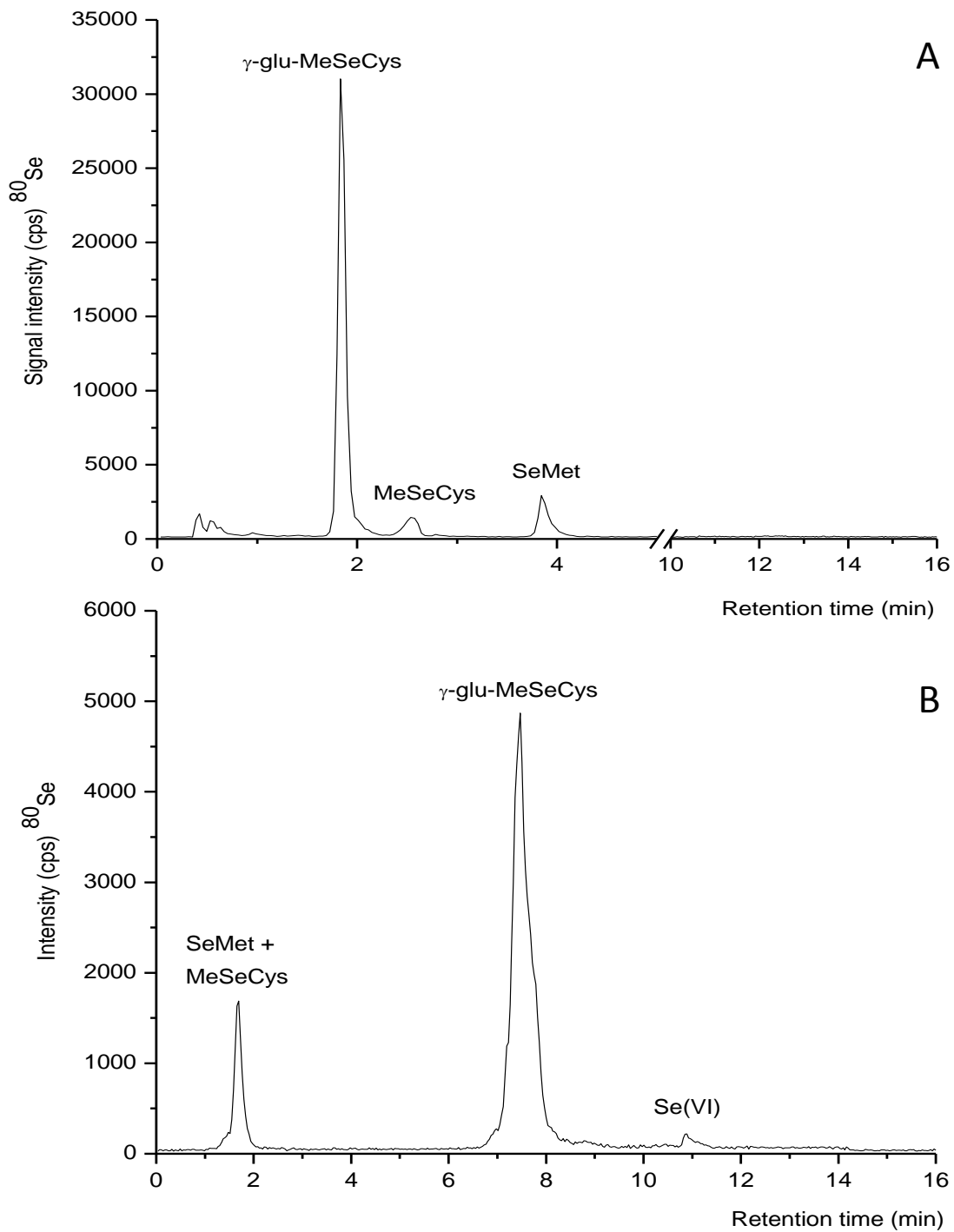


Fig. S1