Research Highlights

Speciation of selenium from sprouts grown in Se-enriched cultures are studied.

After growing, part of Se is transformed into SeMet and SeCys₂.

Seeds can grow without signs of damage until 2 mg Se L⁻¹.

The study contributes to a wider knowledge of the Se behaviour in plants.
Study of selenocompounds from Se-enriched culture of edible sprouts


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Abstract

Selenium is recognized as an essential micronutrient for humans and animals. One of the main sources of selenocompounds in the human diet is vegetables. Therefore, this study deals with the Se species present in different edible sprouts grown in Se-enriched media. We grew alfalfa, lentil and soy in a hydroponic system amended with soluble salts, containing the same proportion of Se, in the form of Se(VI) and Se(IV). Total Se in the sprouts was determined by acidic digestion in a microwave system and by ICP/MS. Se speciation was carried out by enzymatic extraction (Protease XIV) and measured by LC-ICP/MS. The study shows that the Se content of plants depends on the content in the growth culture, and that part of the inorganic Se was biotransformed mainly into SeMet. These results contribute to our understanding of the uptake of inorganic Se and its biotransformation by edible plants.

Keywords: Selenite, Selenate, Se-amino acids, edible plants, enzymatic extraction, LC-ICP/MS.
1. Introduction

Selenium (Se) is an essential element for humans and higher animals, since it is present in several selenoproteins and is a constituent of the anti-oxidant enzyme glutathione peroxidase (GPX), which contributes to preventing oxidative cellular degradation (Reilly, 2006). Since Se has potential health benefits, several studies have focused on Se speciation in human nutrition (Zeng & Combs, 2008; Hartikanen, 2005).

Regarding human nutrition, Se uptake via the diet is mainly from vegetables. Plants can absorb the main inorganic forms of Se, such as selenate or selenite, from soil or from other culture media (Sager, 2006). The variation in soil Se contents between different countries and locations causes significant differences in the Se in edible plants and consequently in the daily intake of Se (Scientific Committee on Food, 2000; Moreno et al., 2005; Spadoni et al., 2007). Interest in the dietary content of this nutrient has increased as the effects of Se deficiency on human health, with an intake of less than the recommended dietary allowance (RDA), have become known (Allen et al., 2006). For this reason, several countries, such as Finland, have introduced Se into fertilizers and its level in crops has increased considerably (Eurola, 2000).

In cultivated plants, the Se content can be increased via different fortification processes (Li et al., 2004; Lyons et al., 2003). Among them, a hydroponic system is an easy technique and has been widely used in several studies; the Se compound added to the solution is usually sodium selenate (Tsuneyoshi et al., 2006; Lintschinger et al., 2000). The present study also uses a hydroponic system applied to alfalfa, soy and lentil. These edible sprouts, used directly in various diets (in salads or soups), are sources of isoflavones, which exhibit a variety of biological activities that may influence the risk of different diseases (Lampe, 2003; Márton et al., 2010). Similar studies have been reported in the literature, in which selenite or selenate were added separately to the
hydroponic media (Lintschinger, 2000; Thavarajah, 2008; Sugihara, 2004). In the present study, mixtures of sodium selenite and sodium selenate are assayed in several ratios, in order to ascertain if the simultaneous presence of both compounds influences the absorption by plants and the biotransformation to organic Se compounds. The study compares the changes in Se speciation between control sprouts (with natural Se content) and those from Se-enriched cultures. It has to be considered that the nutritional bioavailability of Se from plants depends mainly on the Se compounds; so, Se speciation is assessed in the sprouts grown in all the experimental hydroponic assays studied.

2. Materials and methods

2.1. Materials

Alfalfa, soy and lentil seeds were purchased from a commercial nursery (medium diameter: 1-2 mm). The origin of the soy (INT-Salim) and alfalfa seeds (Certificated Seed R-1) was Spain, while the lentil seeds (Golden Line) were from Italy.

2.2. Plant experiment

Germination was carried out in a polypropylene container (1 L capacity and 4 cm in height) containing a mesh (pore diameter 0.3 cm). The growth solution consisted of tap water amended with selenium, in the form of Na$_2$SeO$_3$ and Na$_2$SeO$_4$, (1:1) at three concentrations: A (1 mg Se(IV) L$^{-1}$ and 1 mg Se(VI) L$^{-1}$), B (2.5 mg Se(IV) L$^{-1}$ and 2.5 mg Se(VI) L$^{-1}$) and C (4 mg Se(IV) L$^{-1}$ and 4 mg Se(VI) L$^{-1}$). 1L of the fortified solution was added to the containers, since
this volume was enough to allow the plants to grow. The containers with the growth solution were placed under a laboratory hood with constant air extraction. To control the tap water used in the hydroponic media, the main metals were determined by ICP-OES. In the experiments, all the equipment was cleaned with water and ethanol prior to use, to avoid microbiological activity and moreover residual chlorine was guaranteed in the tap water used.

2.3. Growing conditions

20 g of soy seeds and 15 g of alfalfa and lentil seeds were grown separately in the respective containers and in contact, through the mesh, with the Se-fortified aqueous solution (for three weeks). Seeds were submerged in the fortified solution for 24 h, according to the producer’s recommendations. We also used a control culture for each plant, which grew in tap water without selenium salts during the same period of time.

2.4. Sample collection

Three weeks after planting the seeds, all the sprouts were harvested and their shoots and roots were separated, cleaned and dried at 40ºC. Then, the samples were milled in a glass mortar and transferred to high-density polyethylene (HDPE) containers. They were stored at room temperature until analysis (in duplicate) of each part of the plant for each culture.

2.5. Analytical methods

Inorganic Se stock solutions were prepared to a concentration of 1000 mg L\(^{-1}\) from selenite 99% Na\(_2\)SeO\(_3\) (Aldrich, Milwaukee, WI, USA) and selenate 99% Na\(_2\)SeO\(_4\) (Aldrich). Organic Se stock solutions were also prepared to a concentration of 1000 mg L\(^{-1}\) from selenocysteine
(SeCys2), selenomethionine (SeMet) and selenomethyl-selenocysteine (SeMeSeCys) (Aldrich) with HCl 0.5%. All the standard solutions were kept at 4°C in closed opaque HDPE bottles, since the stability of Se species depends on storage time and conditions, notably on exposure to air and elevated temperature (Amoako et al., 2007; Liu & Bei, 2010).

_Aqua regia_ extractable Se in seeds was determined by following the appropriate ISO standard (ISO 11466, 1995), using 1 g of seeds. A digester bloc (P/Selecta model, RAT 4000051) with temperature control was used. Once at room temperature, the resulting suspension was filtered (Whatman 40) and the solid residue was washed several times with 0.5 mol HNO3 L⁻¹ (Hiperpur Panreac). The resulting filtrate, together with the washings, were diluted to 20 mL with 0.5 mol HNO3 L⁻¹, transferred to an HDPE bottle and stored at 4°C until analysis of total Se.

For the acid microwave digestion of the sprouts, a 0.2 g sample (weighed in a Teflon vessel) was mixed with 8 mL of HNO3 and 2 mL of H2O2 33% (Prolab). The resulting mixture was digested by a closed microwaver system (Milestone Ethos Touch Control, 1000 W), following the program: 10 min ramp from room temperature to 90°C; 5 min at 90°C; 10 min ramp from 90°C to 120°C; 10 min ramp from 120°C to 190°C; and 10 min at 190°C. After digestion, the samples were filtered (Whatman 40) and brought up to a total volume of 20 mL with double deionized water, transferred to an HDPE bottle and stored at 4°C until analysis. The Se contents from both _aqua regia_ extraction and acid MW digestion were measured using a 7500ce series inductively-coupled plasma mass spectrometer (ICP/MS) (Agilent Technologies) with an Ari Mist HP nebulizer (Burgener, Canada). Hydrogen was used as the reaction gas to prevent possible interferences, and Rh was used as the internal standard. The ion intensity at m/z 78 (⁷⁸Se) was monitored using time-resolved analysis software.
The total Se content of the *aqua regia* extracts from the Reference Materials was determined using an Atomic Fluorescence Spectrometer, (PSA Excalibur) with a hydride generator module (model 10.004, P.S. Analytical, Kent, UK). For HGAFS measurements, a pre-reduction step ensuring the quantitative reduction of Se (VI) to Se (IV) was required. Thus an aliquot of 5 mL of extract was placed in a sand bath at 170°C with 10 mL of 6 mol L\(^{-1}\) HCl for 30 min. Once at room temperature, the solution was diluted to 25 mL with 6 mol L\(^{-1}\) HCl. Hydride generation from Se (IV) was achieved with 6 mol L\(^{-1}\) HCl, at a flow rate of 8 mL min\(^{-1}\), and 1.5% NaBH\(_4\) in 0.4% NaOH, at a flow rate of 3 mL min\(^{-1}\).

For the enzymatic digestion of the sprouts, 0.3 g of vegetable samples and 30 mg of Protease XIV (Sigma Aldrich) was placed in a 40 mL HDPE tube with 10 mL of 25 mmol NH\(_4\)H\(_2\)PO\(_4\) L\(^{-1}\) at pH 7.5. The mixture was shaken for 16 h in a thermo-agitator water bath (Clifton NE5-28D) at 37°C. The resulting solution was centrifuged for 10 min at 3000 rpm. The extracts from the enzymatic digestion were first filtered consecutively through a 0.45 µm and a 0.20 µm nylon membrane. Se species were measured immediately after extraction. The extracts were analysed by LC-ICP/MS (Agilent Technologies, 1200 series, LC quaternary pump). The chromatographic anion exchange precolumn (20 x 2.0 mm, i.d. 10 µm) and column (250 x 4.1 mm, i.d. 10 µm) were the Hamilton PRP-X100 (Reno, NV, USA). The chromatographic mobile phase was prepared from a buffer of NH\(_4\)H\(_2\)PO\(_4\) 40 mmol L\(^{-1}\) (PA Panreac) adjusted to pH 7.0 with NH\(_3\) 25% (PA Panreac). The flow rate of the mobile phase was 1.5 mL min\(^{-1}\) and the injection volume was 50 µL. The detection of Se species by ICP/MS was carried out under the same conditions as those described above.
To assess the extraction efficiency and column recovery, the total Se present in the enzymatic extracts was determined by acidic microwave digestion of an aliquot of 4 mL of the extract and by ICP/MS, under the same conditions as for Se total determination described above.

3. Results and discussion

Some aspects on the plant responses from throughout the study can be highlighted. The amount of sprout biomass grown in the different cultures was assessed and compared. The average dry weights of sprouts, after the growth period, were: 4 g, 5 g and 17 g, in the control culture; 7 g, 2 g and 10 g, in culture A; and 2 g, 1 g and 8 g, in culture B, for alfalfa, lentil and soy respectively. According to these data, as the Se concentration increases in the culture medium, the biomass of the plant decreases. In a solution fortified with 4 mg kg\(^{-1}\) Se(IV) and 4 mg kg\(^{-1}\) Se(VI), culture C, inhibition of plant growth was observed and, consequently, they were not analysed further. We attributed this adverse effect only to the presence of high Se concentration, since possible salt toxicity from water could not be attributed to its composition (Ca (61.6 mg L\(^{-1}\)), Na (49.8 mg L\(^{-1}\)), Mg (13.7 mg L\(^{-1}\)), K (1.0 mg L\(^{-1}\))). The concentration of Na (in the form of Na\(_2\)SeO\(_3\) and Na\(_2\)SeO\(_4\)) added in the supplemented media was considered negligible. Alfalfa seemed to be the most vulnerable sprout; showing symptoms of growth inhibition and putrescence, even under the conditions of culture B. This is in agreement with other studies (Hajiboland & Amjad, 2008).

Moisture was determined in all the samples by drying at 105°C; the mean values were: 12% for sprouts and 16% for seeds. All the results in the study are expressed as mg Se kg\(^{-1}\) dry mass unless otherwise stated.
The results for total Se content in seeds were: 1002 ± 329, 1401 ± 122 and 479 ± 101 μg Se kg⁻¹ in alfalfa, lentil and soy seeds respectively. Several data of this kind are reported in the literature (Thavarajah et al., 2008; Lintschinger et al., 2000; Bañuelos et al., 2012). We considered that the values correspond to the natural contents of Se and this information was considered important in order to evaluate its contribution to total plant Se concentration. In the present study, three Reference Materials were used to study the accuracy of the results by aqua regia digestion. Due to a lack of Certified Reference Materials of seeds, two soil types and one fertiliser were analysed. CRM023-050 and CRM025-050 soil types (Natural Matrix Certified Reference Material) were from the Resource Technology Corporation (USA) with a reference value for total Se of 105 ± 9 and 518 ± 74 mg Se kg⁻¹, respectively. The Standard Reference Material for a fertiliser, SRM-695 (provided by the National Institute of Standards and Technology) had a reference value for total Se of 2.1 ± 0.1 mg Se kg⁻¹. The total Se content of the aqua regia extracts of the CRMs and SRM-695 were determined by applying the procedure based on the standard ISO (ISO 11466 1995). The extractable Se was measured by HG-AFS. The results for CRM023-050, CRM025-050 and SRM-695 were: 93 ± 10, 483 ± 8 and 2.2 ± 0.1 mg Se kg⁻¹, respectively. When comparing the aqua regia results for the Reference Materials with certified values, good agreement was found when considering the associated uncertainties of the certified values. The values obtained with aqua regia show that the determination of total Se in the seeds was quantitatively accurate.

In the present study, the shoots from the sprouts, and also the roots when available, were analysed to determine the total Se content and to study Se speciation. Table 1 shows the results, organized according to the parts of these edible vegetables and to the concentration ranges of Se sodium salts added. The results corresponding to control sprouts (non-amended) and to those
grown in the supplemented media: cultures A and B. The limits of detection (LOD) and quantification (LOQ) were calculated for the Se species and the values are shown at the bottom of Table 1, which also includes the extraction efficiencies, calculated as the ratio of the total Se in the extracts to the total Se in the sample.

From Table 1 it can be observed that when sprouts grown in the control experiments are compared with their respective seeds, the values in the sprouts are consistent with the Se content in the corresponding seeds. So, lentil seeds contained the highest natural Se concentration and their sprouts also had the highest value. The results for cultures A and B were also compared and the Se concentrations in shoots grown in culture B were in general almost twice those for A; but for lentil, the Se content did not increased from A to B.

Roots were only easily available from soy (growing in all the cultures) and from lentil (growing in control culture). We only compared the results for roots and shoots for soy, where the results show that the Se content in roots is higher than in shoots. A similar trend has been observed in studies of Se speciation in lentil grown in hydroponic media (Pedrero et al., 2007). In the soy roots, the total Se increased almost proportionally with the Se concentration in the culture solution.

Several studies of Se speciation in sprouts, grown in hydroponic Se-enriched media, have been reported and different extraction systems have been proposed (Cuderman et al., 2010; Sugihara et al., 2004; Lintschinger et al., 2000). Among them, enzymatic extraction with Protease XIV has been widely used and it provides high extraction efficiencies for Se species.

To study Se speciation, it should first be noted that during extraction, organic Se species may become oxidized (Ayouni et al., 2006; Pedrero et al., 2007).
Figure 1 (a) shows the chromatograms corresponding to a mixture of freshly prepared Se standard solutions (SeCys₂, SeMet, Se(IV) and Se(VI)) and that obtained four days after their preparation. The first peak (day 4) in Figure 1 was identified as SeOMet, the oxidized form of SeMet.

To check for the presence of the oxidized species (i.e., SeOMet) in the enzymatic extracts, the standard addition method was used. To prepare a stock standard solution of 500 mg Se L⁻¹ in the form of SeOMet, 50 µL of SeMet standard was oxidized with 50 µL of H₂O₂ (33%) and diluted with H₂O to 5 mL. Figure 1 (b) shows the increase of the SeOMet peak when an extract of lentil was spiked with different amounts of the SeOMet standard solution.

The standard addition was also used to identify all the Se species present in the enzymatic extracts from the sprouts. We also used an SeMeSeCys standard to check for its presence in the extracts, although this species was not detected in our experiments, in agreement Lintschinger et al., 2000. However, in some cases the occurrence of this species has been reported, although the experimental growth conditions were different from those used in the present study (Gergely et al., 2006; Yu et al., 2011).

Figure 2 (a) shows chromatograms of lentil, alfalfa and soy control extracts, where the main selenocompounds were quantified. Figure 2 (b) shows sample chromatograms of the enzymatic extracts from alfalfa, lentil and soy grown in culture A. In this figure, the chromatographic peaks were identified as: the inorganic forms Se(IV) and Se(VI); and the selenoamino acids SeCys₂ and SeMet, as well as the oxidized form of the latter: SeOMet. When comparing the chromatograms from the extracts corresponding to the control experiment and those grown in Se-enriched conditions, some differences can be observed. For example, in Figure 2(a), the chromatogram of the lentil extract shows the highest content of Se compounds. Figure 2(b), in contrast, shows that
lentil did not accumulate the highest concentration of Se (VI), as was observed for soy. Despite of this, the biotransformation of inorganic Se into SeMet was similar for both sprouts.

In Table 1, the results for the control sprouts show that Se species are naturally present in plants but their content is very low, and in some cases, some species were below the LOQ; this was the case for SeMet in alfalfa, despite it being the major species detected. For lentil, SeMet was also the major organic selenocompound quantified, though the contribution of inorganic species, mainly Se(VI), was not negligible. For soy, Se(VI) was the main species detected in the extracts, but the content of SeMet and SeCys$_2$ was also quantifiable. Table 1 shows that the concentration of Se species also increases with the Se content added to the hydroponic system. Despite this, the transformation of selenocompounds is different for the different plants. Although the concentrations of Se(IV) and Se(VI) in the fortified hydroponic media were in the same proportion, Se(VI) was more easily absorbed by the roots than Se(IV) was. So, the concentration of Se(VI) is from 8 to 35 times higher than the concentration of Se(IV), according to the results for each type of sprout. For all the sprouts, SeMet represents from 13% to 33% to the sum of the species quantified. For soy, the ratio of the SeMet present in shoots and roots was the same for cultures A and B. So we can conclude that the accumulation of Se in the tissue of soy plants in the form of SeMet depends on the amount of Se absorbed by the roots from the culture medium.

The extraction efficiency was at least 67%; typical for this kind of enzymatic hydrolysis using Protease XIV (Hawkesford & Zhao, 2007). Column recovery data, calculated as the ratio of the sum of Se species to the total Se in the extracts, were estimated to be in the range between 38% (for control lentil shoot) and 94% (for control lentil root).

**4. Conclusions**
The present study provides new data on the presence of selenocompounds in edible plants grown in hydroponic media. The Se contents of seeds of alfalfa, soy and lentil were determined to provide useful knowledge regarding the natural Se contents of these foodstuffs that are common sources of dietary Se.

These seeds can be grown in hydroponic media containing inorganic Se at up to 2 mg Se L\(^{-1}\) without there being signs of damage or growth inhibition in the plants. Sprouts grown without Se addition to the culture medium also contain selenoamino acids, but in very low concentrations that were considered natural contents. During the growth of the sprouts in the Se-enriched media, part of the inorganic Se was transformed into SeCys\(_2\) and a larger proportion was transformed into SeMet.

Acknowledgements

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References


### Table 1. Speciation of selenium present in alfalfa, lentil and soy, extracted by enzymatic digestion (Protease XIV) and determined by LC-ICP/MS. (na: not analysed).

<table>
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<tr>
<th>Plant species</th>
<th>Concentration ranges of fortifier</th>
<th>Vegetable part</th>
<th>Total Se (mg kg(^{-1}))</th>
<th>Se speciation (mg kg(^{-1}))</th>
<th>Se in the extract (mg kg(^{-1}))</th>
<th>Extraction efficiency (%)</th>
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<td></td>
<td></td>
<td>SeCys(_2)</td>
<td>Se(IV)</td>
<td>SeMet</td>
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<td>Alfalfa</td>
<td>Control</td>
<td>Shoot</td>
<td>1.5 ± 0.9</td>
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<td>&lt; 0.02</td>
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<td></td>
<td>Culture A</td>
<td>Shoot</td>
<td>132 ± 4</td>
<td>4.25 ± 0.04</td>
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<td>Culture B</td>
<td>Shoot</td>
<td>284 ± 1</td>
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<td>21.1 ± 0.5</td>
<td>20.1 ± 0.9</td>
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<td>Lentil</td>
<td>Control</td>
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<td>2.4 ± 0.1</td>
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<td>Control</td>
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<td></td>
<td>Root</td>
<td>1.0 ± 0.2</td>
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<td>0.03</td>
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<tr>
<td></td>
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<td>Shoot</td>
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FIGURE CAPTIONS

Figure 1. (a) Examples of chromatograms corresponding to a mixture of freshly prepared Se standard solutions and that obtained four days after preparation, obtained by LC-ICPMS. Standard solutions: 100 µg SeCys₂ L⁻¹, 100 µg Se(IV) L⁻¹, 100 µg SeMet L⁻¹ and 100 µg Se(VI) L⁻¹. (b) Example of a chromatogram corresponding to the identification of SeOMet in an enzymatic extract from lentil (culture A), using the standard addition method.

Figure 2. Examples of chromatograms obtained from enzymatic hydrolysis of sprouts analysed by LC-ICPMS. (a) Control sprouts; (b) Sprouts grown in culture A.