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Selenium Species Bioaccessibility in Enriched Radish (*Raphanus sativus*): A Potential Dietary Source of Selenium

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An in vitro gastrointestinal method was employed to predict the potential bioavailability of selenium and its species from radish, belonging to the Brassicaceae family, grown in hydroponics media in the presence of inorganic selenium, such as Na_2SeO_3 and Na_2SeO_4. A low transformation of Se into organic forms was observed in radish plants grown in Se(VI)-enriched culture media. On the contrary, in those plants exposed to selenite, >95% of the total selenium was found as selenocystine ($SeCys_2$), selenomethionine (SeMet), and Se-methylselenocysteine (SeMetSeCys). The concentrations of these species in fresh samples remained almost unaltered after a simulated gastrointestinal digestion. Therefore, a high selenium content of Se-methylselenocysteine (65%), previously reported as a cancer chemopreventive species, remained in the potentially bioabsorbable fraction. As these plants usually undergo a short development cycle, these results suggest that radish enriched in selenite could be a good choice as an organoselenium supplement for the human diet and animal feed.

KEYWORDS: Selenium species; radish; in vitro digestion; LC-ICP-MS

INTRODUCTION

Selenium is an essential nutrient for humans;since it has been recognized as an integral component of different enzymes such as iodothrine 5′-deiodinase and glutathione peroxidase, which participate in the antioxidant protection of cells (*1*, *2*). Moreover, several studies have suggested that some organic forms of selenium could show anticarcinogenic properties against certain types of cancer (*1*−*4*). Even though the mechanism explaining the role of selenium in cancer inhibition is still unclear, it has been tentatively attributed to biological functions of selenoamino acids. Among all chemical forms of selenium, Se-methylselenocysteine seems to be one of the most effective (*1*−*4*). This is a nonproteinogenic amino acid found in the Allium and Brassicaceae families, which can be converted to methylselenol by cleavage of the Se-methyl group. It has been reported that methylselenol may provide better cancer protection (*1*−*4*).

The selenium content in plants varies depending on its concentration in soil and the accumulation capacity of the plant. Because the primary source to incorporate selenium into the body is through food (*5*, *6*), with the exception of fish, vegetables are the most relevant source of this element (*6*). In regions where the soil is low in selenium, diseases due to Se deficiency in humans have been detected, for example, hypothyroidism, cardiovascular disease, and weakening of the immune system. Se deficiency is the cause of endemic Keshan and Kashin−Beck diseases (*3*, *7*). Thus, it is important to find plants capable of tolerating and transforming selenium into bioactive compounds, which can be used as sources of Se-fortified food supplements for people inhabiting these regions.

Many *Allium* (*Allium cepa* L., *Allium sativum* L., *Allium schoenoprasum* L., etc.) and *Cruciferae* species (*Brassica juncea* and *Brassica oleracea*) have been the subject of several studies, as they are able to incorporate high quantities of selenium and to produce selenoamino acids, which are potentially bioactive for nutrition purposes and phytoremediation (*8*−*11*).

Radish (*Raphanus sativus*) belongs to the Cruciferae, also called Brassicaceae, family, and is an important vegetable in the human diet, which is widely used in many countries, usually consumed as salad (*12*). Several radish phytoextraction studies have shown its capacity to tolerate a moderate concentration of heavy metals without showing symptoms of toxicity (*13*−*16*).

Although the chemical form in which selenium is present in food affects its bioaccessibility, the determination of the total content of selenium and its species in food is not enough to evaluate its bioavailability. To achieve this goal it is necessary to carry out analytical speciation studies in the gastrointestinal absorpbable fraction. In vitro gastrointestinal digestion is a useful method to evaluate the potential elemental bioaccessible fraction from food and has been widely used for Se (*17*, *18*), Fe (*19*), Cd (*20*), Zn, and Cu (*18*) and for nonmetals such as carbon and nitrogen and minerals (*21*).

In vitro experiments provide a good estimation and are an alternative to human studies, because they are faster, cheaper, and simpler than in vivo experiments, especially when there is an increasing interest nowadays in reducing the use of laboratory animals for testing (*20*). However, the results of these simulations should be validated by in vivo experiments.

Therefore, the goals of this research were to study the uptake and transformation of selenium in radish and to determine the bioaccessible content of this element and its species in the

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gastrointestinal tract by using in vitro digestion methods. The ultimate objective was, in summary, to evaluate selenium-enriched radish as possible sources of selenium amino acids for the human diet.

EXPERIMENTAL PROCEDURES

Instrumentation. Dried roots and leaves of radish plant samples were digested for total multielemental determination in double-walled advanced composite vessels (ACV), using a 1000 W microwave sample preparation (MS) system microwave oven (CEM, Mattheus, NC).

A Sonoplus ultrasonic homogenizer (Bandelin) equipped with a titanium 3-mm-diameter microtip and fitted with an HF generator of 2200 W at a frequency of 20 kHz was used for the extraction of selenium species. The extracts obtained were centrifuged on an Eppendorf centrifuge 5804 F34-6-38.

An ICP-MS instrument (Thermo X Series-X7) fitted with a Meinhard nebulizer, an Impact bead quartz spray chamber, and a Peltier cooling system was used for elemental determination. This instrument was also coupled to a liquid chromatographic (LC) system for the determination of selenium species.

The samples were injected using a six-port Rheodyne 7725i sample injection valve fitted with a 100 μL loop. The chromatographic system also consisted of a CM4000 pump (Milton Roy). The separations of selenocompounds were carried out in an anion exchange column (Hamilton PRP-X100).

Reagents and Samples. All reagents used were of analytical grade. Bile salts and enzymes (i.e., pepsin, pancreatin, and α-amylase) were purchased from Sigma.

The enzymatic hydrolysis was achieved using a nonspecific protein, Protease XIV, purchased from Sigma. Selenomethionine (SeMet), selenomethylselenocystine (SeMetSeCys), and selenocystine ($SeCys_2$), also from Sigma, were dissolved with doubly deionized Milli-Q water (Millipore), and 3% hydrochloric acid was added for better dissolution of $SeCys_2$ and SeMetSeCys. Inorganic selenium solutions were prepared by dissolving sodium selenite (Na_2SeO_3) and selenate (Na_2SeO_4), purchased from Merck, in Milli-Q water. Stock solutions of 1000 mg L^{-1} were stored at 4 °C, whereas working standard solutions were prepared daily by dilution.

The mobile phase used for the anion exchange was 10 mM citric acid (Sigma) in 2% (v/v) methanol (Sharlab), adjusted at pH 5 with ammonium hydroxide.

Finally, the samples were digested in the microwave oven using 14 M nitric acid (Merck) and 30% hydrogen peroxide (Panreac).

Procedures. *Plant Growth and Samples.* The radishes (*R. sativus*) were germinated on inert coconut fiber substrate moistened with deionized water. After germination, radishes were grown in hydroponic culture using perlite as substrate, in vessels containing 0.1 strength Hoagland's solution (*22*) during 2 weeks. Afterward, Na_2SeO_3 and Na_2SeO_4 (1 mg L^{-1} each) were added to the vessels, and the solutions were renewed every 3 days for 40 days, until the cycle of the plants was completed. A control group of plants without selenium was grown in parallel. Then, plants were harvested, washed with deionized water, and divided into roots and stems. The samples were chopped and stored at −4 °C before analysis.

For total metal determination, the samples were dried at 50 °C in an oven for 48 h and later homogenized in an agate mortar.

Total Selenium and Essential Elements Determination. For the determination of Se, Cu, Mn, Zn, Fe, and Mo concentrations, ~25 mg of sample was digested with 2.5 mL of concentrated HNO_3 and 1 mL of H_2O_2 in an analytical microwave oven. The resulting solution was diluted to 25 mL with deionized water and the metal concentration determined by ICP-MS following conditions given in **Table 1**.

Selenium Species Determination. *Anion Exchange Chromatography−ICP-MS.* The extraction of selenium species from 250 mg of fresh sample and 25 mg of dried samples was performed by 2 min of sonication after the addition of 3 mL of deionized water and 10 mg of Protease XIV. The extracts were centrifuged at 7500*g* for 30 min using a 10 kDa cutoff filter. The supernatant was analyzed by anion exchange chromatography coupled to ICP-MS under the experimental conditions given in **Table 1**. The concentrations of selenium species were determined by monitoring ^{77}Se, ^{78}Se, and ^{82}Se isotopes using the standard addition method.

Table 1. Operating Conditions for Determinations by HPLC-ICP-MS

ICP-MS Conditions	
forward power	1250 W
plasma gas flow rate	15.0 L min^{-1}
auxiliary gas flow rate	0.73 L min^{-1}
carrier gas flow rate	0.7 L min^{-1}
nebulizer type	Meinhard
spray chamber type	impact bead quartz
data acquisition mode	time resolved analysis
isotopes monitored	^{77}Se, ^{78}Se, ^{82}Se, ^{57}Fe, ^{56}Fe, ^{95}Mo, ^{55}Mn, ^{64}Zn, ^{66}Zn, ^{63}Cu, and ^{65}Cu

Anion Chromatographic Parameters	
column	PRP X-100
mobile phase	10 mM ammonium citrate, pH 5
flow rate	1 mL min^{-1}
injection volume	100 μL

In Vitro Gastrointestinal Digestion Method. The in vitro gastric/intestinal digestion method employed was based on that described by Luten et al. (*23*). About 2.5 g of fresh radish sample was placed in a 25 mL glass vial containing 7.5 mL of gastric juice, which consisted of 6% w/v pepsin in 0.15 M NaCl at pH 1.8, adjusted with 6 M HCl. The vial was shaken for 1 min for initial degassing. The mixture were subsequently held in a thermostatic bath at 37 °C for 4 h.

After gastric digestion, saturated sodium bicarbonate was added until pH 6.8 was attained. Then, 5 mL of intestinal juice, which consisted of 1.5% w/v pancreatin, 0.5% w/v α-amylase, and 0.15 M NaCl, was added. The mixture was first energetically shaken for 1 min and later left to shake periodically for 4 h in a thermostatic bath at 37 °C. Once the digestion was completed, a 1 mL aliquot of the suspension was diluted to an appropriate volume and centrifuged at 7500*g* for 30 min. The supernatant was filtered through a 0.22 μm Millipore filter to reduce any microbial activity, and the solutions were stored at 4 °C until analysis.

The blanks of gastric and gastrointestinal digestions were obtained by adding 7.5 mL of gastric juice and 5 mL of intestinal juice, correspondingly, to 2.5 mL of Milli-Q water, and the above-described procedure was applied. The bioaccessible fraction is composed by the soluble metal fraction, and the bioaccessibility is calculated as the percentage of the Se present in the fraction with respect to the total content in the sample. All of the results have been expressed as the mean value ± standard deviation for $n = 3$.

RESULTS AND DISCUSSION

Selenium Accumulation and Effect on Distribution of Essential Metals. The total selenium concentrations in radish exposed to both inorganic chemical forms of selenium were 112 ± 7 and 120 ± 6 μg g^{-1} in the presence of selenite and selenate, respectively. The method was validated by analyzing white clover reference material (CRM-402), and no significant differences at a 95% confidence level were observed.

Several authors (*9*, *10*, *24*) have reported that selenium accumulation in plants exposed to Se(VI) is higher than that in plants grown in Se(IV)-enriched media. Differences between selenium uptake and growth have been attributed to the different mechanisms responsible of the selenate and selenite uptake and translocation into the plants.

Radishes grown in both selenium-enriched culture media did not apparently exhibit symptoms of toxicity; however, a reduction of ~25% in the growth of the roots of plants exposed to selenite was observed when compared with plants grown in the presence of Se(VI). Similar results have also been observed with other plants (*10*).

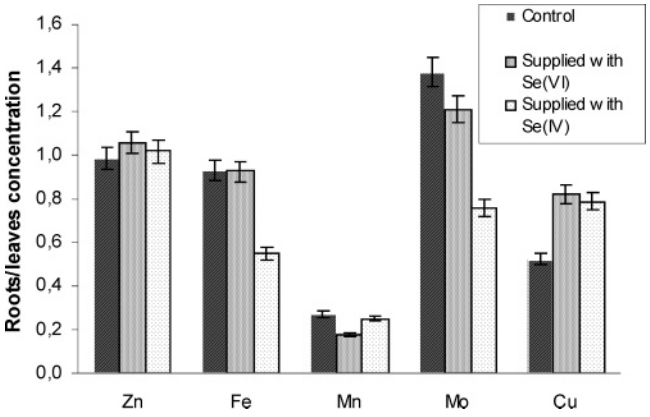

Figure 1. Root/stem concentration ratio for radish plants after growing for 40 days.

These results suggest that plants grown in enriched selenite media are quite tolerant to Se(IV).

To ensure the correct metabolism of Se-enriched plants, they have not only to show tolerance to this element, but it is also desirable that the accumulation of selenium does not alter the uptake and distribution of other essential elements and, thus, the nutritive value of the plants.

Hence, the translocation from roots to aerial parts of the essential elements in the nutritive Hoagland solution was determined to evaluate the effect of the presence of different chemical forms of Se in the culture media. These values were compared with the distribution in control plants grown without spiking selenium. Concentration ratios of roots to aerial parts of these essential elements are represented in **Figure 1**.

In general, plants exposed to selenite and selenate showed a slight decrease in the translocation of the tested elements, except for Cu and Zn, when compared to control plants. This observation shall be considered preliminary; to provide a definitive conclusion, a study with more population has to be performed. Depending on the element, the decrease is more or less noticeable, but a tendency to reduce the translocation can be observed when selenium is supplied as Se(IV). The smallest value for translocation in the presence of selenite was observed for molybdenum, being of ~40% with respect to control plants.

Therefore, it has been demonstrated that the chemical form of selenium can influence the uptake and translocation of essential metals in radish plants, which could be the cause of the growth reduction of those plants grown in selenite media, in which the translocations were slightly poor.

Speciation Studies. *Selenium Species Determination in Fresh and Dried Samples.* To release selenium presumably bound to proteins, enzymatic digestion of the samples was employed and analysis of Se species was carried out.

Radish plants grown in the presence of Se(VI) and Se(IV) accumulated similar quantities of total selenium, showing a certain independence of the chemical form present in the culture media. However, the selenium speciation results reported in **Table 2** show that the distribution of selenium is remarkably different depending on the selenium media.

In **Figure 2 a**,**b** are the chromatograms corresponding to the species analysis of fresh radish samples grown in the presence of both inorganic selenium forms.

In those plants grown in Se(IV)-enriched media, $SeCys_2$, SeMetSeCys, SeMet, and Se(VI) were identified, finding that ~95% of the selenium content was transformed in the organic species. In contrast, for those plants grown in selenate media, only 38% of the selenium was found to be present as organic forms, whereas the remaining selenium is Se(VI), which implies that in the latter case a lower transformation into Se-amino acids takes place. These differences in composition of organic selenium species for plants grown in selenite- and selenate-enriched media have been reported previously in the literature (*9*, *10*). The explanation could be found in the differences between the metabolism of these inorganic species. It has been proposed that whereas selenate is taken up and transported into the plants by using sulfate pathways without any further modifications, selenite is nonenzymatically reduced by an unidentified mechanism that produces selenoamino acids such as SeMet and $SeCys_2$, which presents a nonspecific incorporation into proteins, that can produce selenium toxicity (*1*, *9*, *10*, *24*, *25*). However, the total selenium accumulation in our study for both groups of plants was very similar and, as mentioned before, no perceptible symptoms of toxicity were observed in plants grown in selenite media. The most likely reason for the high selenium tolerance observed in these plants is the high production of SeMetSeCys (75%), linked with detoxification processes in plants. The synthesis of this nonproteinogenic selenoamino acid involves the reduction of the intracellular concentration of $SeCys_2$ and SeMet and was found to be responsible for the selenium tolerance increase in genetically modified *B. juncea* (*9*).

Table 2. Selenium Species Concentration in Fresh and Dried Samples of Radish Grown in the Presence of Selenite and Selenate[a]

	supplied with Na_2SeO_3		supplied with Na_2SeO_4	
	fresh Se ($\mu g\ g^{-1}$)	dried Se ($\mu g\ g^{-1}$)	fresh Se ($\mu g\ g^{-1}$)	dried Se ($\mu g\ g^{-1}$)
$SeCys_2$	6 ± 1	4.1 ± 0.5	19 ± 2	10 ± 1
SeMetSeCys	83 ± 7	4.0 ± 0.7	7 ± 1	2.2 ± 0.1
SeMet	18 ± 1	4.7 ± 0.4	20 ± 1	4.2 ± 0.1
Se(VI)	1.08 ± 0.24	35 ± 1	68 ± 5	94 ± 1

[a] Results, based on dry weight, are expressed as mean value ± standard deviation ($n = 3$).

Although radish is usually eaten fresh in salads and sandwiches, it is also customary in some eastern countries to cook it, preferably boiled (*12*). Therefore, with the purpose of establishing the stability of the selenium species when the radish is cooked, the analysis was carried out on samples dried at 50 °C.

The chromatograms of dried radish samples in **Figure 2c**,**d** show noticeable differences between dried and fresh samples. Concentrations of identified selenospecies for plants exposed to selenite and selenate are shown in **Table 2**, showing that $SeCys_2$, SeMet, and SeMetSeCys undergo a clear decrease when samples are dried at 50 °C. Whereas in fresh radish samples grown in the presence of selenite, SeMetSeCys is the principal species identified, when these samples are heated, a degradation of ~95% is observed. The presence of an unknown peak that eluted at 4.5 min and the increase of the Se(VI) concentration suggest that this new peak and selenate are results of the degradation of the organic species. This clearly shows the influence of the type and degree of meal processing in the incorporation of micronutrients species from food.

Bioaccessibility of Selenium and Its Species. The determination of the total selenium content and the distribution of its species in food is not enough to determine its bioaccessibility. For that it is necessary to determine the selenium amount and its speciation in the gastrointestinal extracts. The in vitro digestion method utilized in this work comprises a simulation of stomach and intestinal physiology. The analysis of total

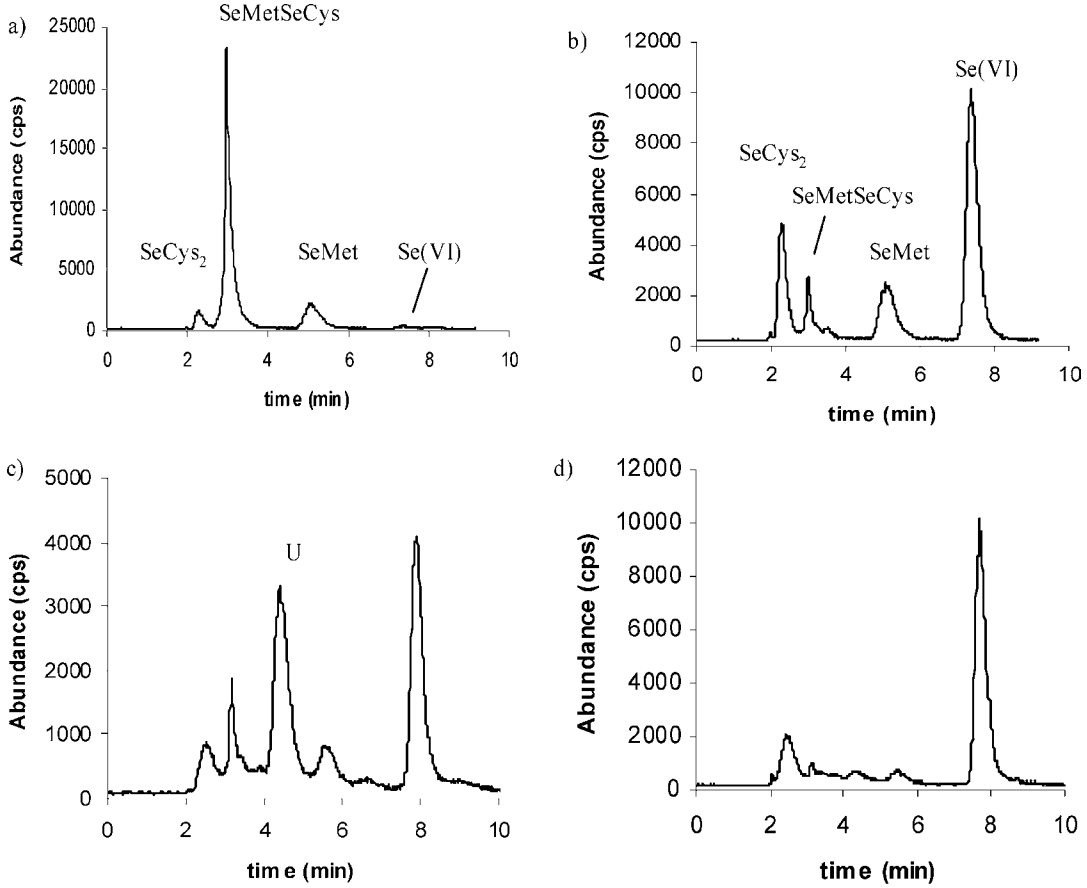

Figure 2. Typical chromatograms corresponding to fresh samples grown in the presence of (**a**) selenite and (**b**) selenate and to dried radish samples grown in the presence of (**c**) selenite and (**d**) selenate.

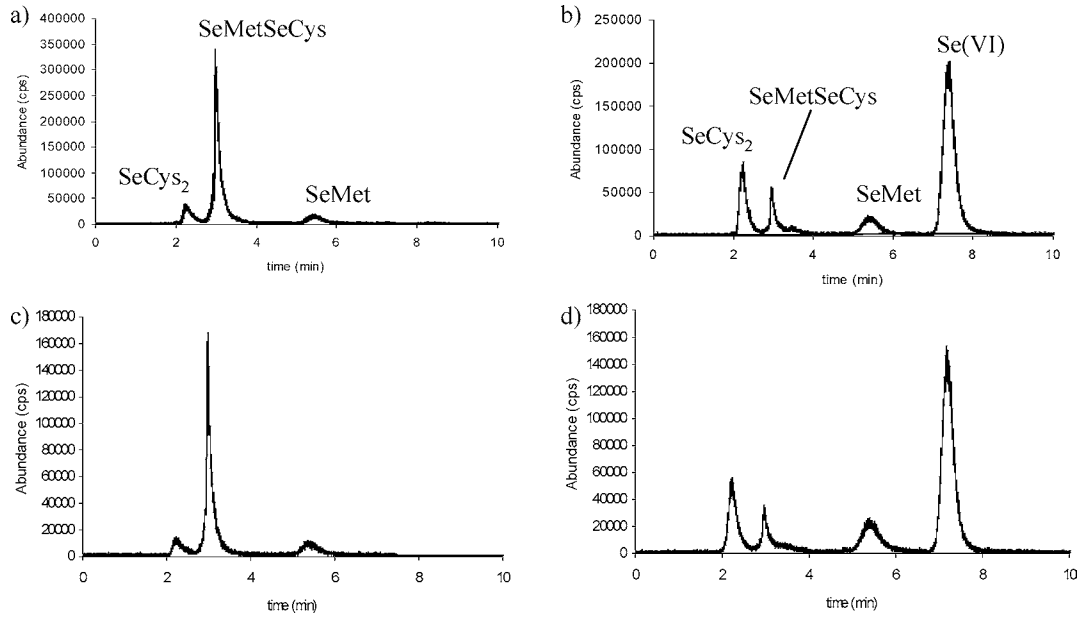

Figure 3. Chromatograms of selenospecies corresponding to gastric digestion for plants grown in the presence of (**a**) selenite and (**b**) selenate and gastrointestinal extracts of radish samples grown in the presence of (**c**) selenite and (**d**) selenate.

selenium and speciation studies in both steps were carried out to predict the bioaccessibility of organic and inorganic selenium forms.

The soluble selenium fractions after simulated gastric and gastrointestinal digestion of radish enriched with either Se(IV) or Se(VI) were ~70% in gastric extracts and increased to 90 and 100% for plants exposed to selenate and selenite, respectively.

The chromatograms of gastric and gastrointestinal extracts of radish exposed to Se(IV) and Se(VI), respectively, are shown in **Figure 3**. The content of bioaccessible selenospecies was determined by the method of standard addition, and the corresponding concentrations are reported in **Table 3**.

The species identified in radish plants enriched with selenite were $SeCys_2$, SeMet, and SeMetSeCys, the latter being the major

Table 3. Selenium Species Concentration in Gastric (G) and Gastrointestinal (GI) Simulated Extracts

	supplied with Na_2SeO_3		supplied with Na_2SeO_4	
	G digestion Se (μg g^{-1})	GI digestion Se (μg g^{-1})	G digestion Se (μg g^{-1})	GI digestion Se (μg g^{-1})
$SeCys_2$	6.7 ± 0.6	12 ± 1	17 ± 2	35 ± 4
SeMetSeCys	61 ± 3	73 ± 5	10 ± 2	8 ± 1
SeMet	5.6 ± 0.3	15 ± 3	8.3 ± 0.1	10 ± 1
Se(VI)			60 ± 2	73 ± 1

component of the sample. The distributions of selenium species in both simulated extracts were very alike. In contrast, in radish grown in selenate media, the species identified after digestion were $SeCys_2$, SeMetSeCys, SeMet, and Se(VI), and as in these fresh samples, ~60% of the selenium was present in inorganic form.

The distribution of the selenium species in the initial products was kept somewhat invariable in both samples during digestion, with a lower content of selenoamino acids in the bioabsorbable fraction in those radishes exposed to selenate, whereas all of the selenium in those exposed to selenite was in organic forms.

Because it is considered that selenium species found in the gastrointestinal juice constitute the bioaccessible fraction as a result of both gastric and intestinal digestion (*17*), the results described above suggest that almost all of the selenium content in radish is potentially bioaccessible after gastrointestinal digestion, although many other factors could affect its use (*6*, *26*, *27*). In the case of radish exposed to selenite, this bioaccessible fraction is fundamentally constituted by Se-methylselenocysteine, one of the best chemopreventive selenoamino acids (*1*, *4*).

Several dietary factors such as type and degree of food processing, diet composition, presence of other metals, concomitant ingestion of certain drugs, and physiology factors such as nutritional state, growth, and pregnancy, together with the presence or absence of dietary micronutrient inhibitors or enhancers substances, can reduce or promote the selenium bioaccessibility (*26*, *27*).

Conclusion. Total selenium accumulations in radishes grown in selenite- and selenate-enriched media were found to be very similar, and no symptoms of toxicity were observed. However, the chemical form of selenium present in the culture media affected the distribution of selenium species and the translocation of essential elements from roots to aerial parts, being more noticeable in plants exposed to selenite.

Selenium speciation studies also revealed noticeable differences in its distribution. A low transformation into organic forms was observed in radish grown in selenate, with Se(VI) being the major fraction identified. Meanwhile, in plants grown in selenite media, practically all of the selenium content was identified as selenoamino acids, from which 75% of it was found to be Se-methyl-selenocysteine.

The analysis of the gastrointestinal digestion showed that almost 100% of selenium present in fresh plants is found in the potentially bioaccessible fraction for both groups of plants. Nevertheless, the distribution of the species varies according to the characteristics of the enrichment media. In the case of radish plants grown in selenate media, this species remains as the principal component, whereas in those plants exposed to selenite, SeMetSeCys was the primary species observed. This result suggests that radish plants enriched in selenite media could be a source in human diets of the above-mentioned selenoamino acid, which can enhance cancer prevention.

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