Research Highlights

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

After growing, part of Se is transformed into SeMet and $SeCys_2$.

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_2SeO_3 and Na_2SeO_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_2SeO_3 (Aldrich, Milwaukee, WI, USA) and selenate 99% Na_2SeO_4 (Aldrich). Organic Se stock solutions were also prepared to a concentration of 1000 mg L^{-1} from selenocysteine

99 ($SeCys_2$), selenomethionine (SeMet) and selenomethyl-selenocysteine (SeMeSeCys) (Aldrich)
100 with HCl 0.5%. All the standard solutions were kept at 4ºC in closed opaque HDPE bottles, since
101 the stability of Se species depends on storage time and conditions, notably on exposure to air and
102 elevated temperature (Amoako et al., 2007; Liu & Bei, 2010).
103 *Aqua regia* extractable Se in seeds was determined by following the appropriate ISO standard
104 (ISO 11466, 1995), using 1 g of seeds. A digester bloc (P/Selecta model, RAT 4000051) with
105 temperature control was used. Once at room temperature, the resulting suspension was filtered
106 (Whatman 40) and the solid residue was washed several times with 0.5 mol HNO_3 L^{-1} (Hiperpur
107 Panreac). The resulting filtrate, together with the washings, were diluted to 20 mL with 0.5 mol
108 HNO_3 L^{-1}, transferred to an HDPE bottle and stored at 4ºC until analysis of total Se.
109 For the acid microwave digestion of the sprouts, a 0.2 g sample (weighed in a Teflon vessel) was
110 mixed with 8 mL of HNO_3 and 2 mL of H_2O_2 33% (Prolab). The resulting mixture was digested
111 by a closed microwaver system (Milestone Ethos Touch Control, 1000 W), following the
112 program: 10 min ramp from room temperature to 90ºC; 5 min at 90ºC; 10 min ramp from 90ºC to
113 120ºC; 10 min ramp from 120ºC to 190ºC; and 10 min at 190ºC. After digestion, the samples
114 were filtered (Whatman 40) and brought up to a total volume of 20 mL with double deionized
115 water, transferred to an HDPE bottle and stored at 4ºC until analysis. The Se contents from both
116 *aqua regia* extraction and acid MW digestion were measured using a 7500ce series inductively-
117 coupled plasma mass spectrometer (ICP/MS) (Agilent Technologies) with an Ari Mist HP
118 nebulizer (Burgener, Canada). Hydrogen was used as the reaction gas to prevent possible
119 interferences, and Rh was used as the internal standard. The ion intensity at m/z 78 (^{78}Se) was
120 monitored using time-resolved analysis software.

121 The total Se content of the *aqua regia* extracts from the Reference Materials was determined
122 using an Atomic Fluorescence Spectrometer, (PSA Excalibur) with a hydride generator module
123 (model 10.004, P.S. Analytical, Kent, UK). For HGAFS measurements, a pre-reduction step
124 ensuring the quantitative reduction of Se (VI) to Se (IV) was required. Thus an aliquot of 5 mL
125 of extract was placed in a sand bath at 170ºC with 10 ml of 6 mol L^{-1} HCl for 30 min. Once at
126 room temperature, the solution was diluted to 25 mL with 6 mol L^{-1} HCl. Hydride generation
127 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
128 0.4% NaOH, at a flow rate of 3 mL min^{-1}.
129 For the enzymatic digestion of the sprouts, 0.3 g of vegetable samples and 30 mg of Protease
130 XIV (Sigma Aldrich) was placed in a 40 mL HDPE tube with 10 mL of 25 mmol $NH_4H_2PO_4$ L^{-1}
131 at pH 7.5. The mixture was shaken for 16 h in a thermo-agitator water bath (Clifton NE5-28D) at
132 37ºC. The resulting solution was centrifuged for 10 min at 3000 rpm. The extracts from the
133 enzymatic digestion were first filtered consecutively through a 0.45 µm and a 0.20 µm nylon
134 membrane. Se species were measured immediately after extraction. The extracts were analysed
135 by LC-ICP/MS (Agilent Technologies, 1200 series, LC quaternary pump). The chromatographic
136 anion exchange precolumn (20 x 2.0 mm, i.d. 10 µm) and column (250 x 4.1 mm, i.d. 10 µm)
137 were the Hamilton PRP-X100 (Reno, NV, USA). The chromatographic mobile phase was
138 prepared from a buffer of $NH_4H_2PO_4$ 40 mmol L^{-1} (PA Panreac) adjusted to pH 7.0 with NH_3
139 25% (PA Panreac). The flow rate of the mobile phase was 1.5 mL min^{-1} and the injection volume
140 was 50 µL. The detection of Se species by ICP/MS was carried out under the same conditions as
141 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_2SeO_3 and Na_2SeO_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 105ºC; 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.

164 The results for total Se content in seeds were: 1002 ± 329, 1401 ± 122 and 479 ± 101 μg Se kg^{-1}
165 in alfalfa, lentil and soy seeds respectively. Several data of this kind are reported in the literature
166 (Thavarajah et al., 2008; Lintschinger et al., 2000; Bañuelos et al., 2012). We considered that the
167 values correspond to the natural contents of Se and this information was considered important in
168 order to evaluate its contribution to total plant Se concentration. In the present study, three
169 Reference Materials were used to study the accuracy of the results by *aqua regia* digestion. Due
170 to a lack of Certified Reference Materials of seeds, two soil types and one fertiliser were
171 analysed. CRM023-050 and CRM025-050 soil types (Natural Matrix Certified Reference
172 Material) were from the Resource Technology Corporation (USA) with a reference value for
173 total Se of 105 ± 9 and 518 ± 74 mg Se kg^{-1}, respectively. The Standard Reference Material for a
174 fertiliser, SRM-695 (provided by the National Institute of Standards and Technology) had a
175 reference value for total Se of 2.1 ± 0.1 mg Se kg^{-1}. The total Se content of the *aqua regia*
176 extracts of the CRMs and SRM-695 were determined by applying the procedure based on the
177 standard ISO (ISO 11466 1995). The extractable Se was measured by HG-AFS. The results for
178 CRM023-050, CRM025-050 and SRM-695 were: 93 ± 10, 483 ± 8 and 2.2 ± 0.1 mg Se kg^{-1},
179 respectively. When comparing the *aqua regia* results for the Reference Materials with certified
180 values, good agreement was found when considering the associated uncertainties of the certified
181 values. The values obtained with *aqua regia* show that the determination of total Se in the seeds
182 was quantitatively accurate.
183 In the present study, the shoots from the sprouts, and also the roots when available, were
184 analysed to determine the total Se content and to study Se speciation. Table 1 shows the results,
185 organized according to the parts of these edible vegetables and to the concentration ranges of Se
186 sodium salts added. The results corresponding to control sprouts (non-amended) and to those

187 grown in the supplemented media: cultures A and B. The limits of detection (LOD) and
188 quantification (LOQ) were calculated for the Se species and the values are shown at the bottom
189 of Table 1, which also includes the extraction efficiencies, calculated as the ratio of the total Se
190 in the extracts to the total Se in the sample.

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

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

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

207 To study Se speciation, it should first be noted that during extraction, organic Se species may
208 become oxidized (Ayouni et al., 2006; Pedrero et al., 2007).

209 Figure 1 (a) shows the chromatograms corresponding to a mixture of freshly prepared Se
210 standard solutions ($SeCys_2$, SeMet, Se(IV) and Se(VI)) and that obtained four days after their
211 preparation. The first peak (day 4) in Figure 1 was identified as SeOMet, the oxidized form of
212 SeMet.
213 To check for the presence of the oxidized species (i.e., SeOMet) in the enzymatic extracts, the
214 standard addition method was used. To prepare a stock standard solution of 500 mg Se L^{-1} in the
215 form of SeOMet, 50 µL of SeMet standard was oxidized with 50 µL of H_2O_2 (33%) and diluted
216 with H_2O to 5 mL. Figure 1 (b) shows the increase of the SeOMet peak when an extract of lentil
217 was spiked with different amounts of the SeOMet standard solution.
218 The standard addition was also used to identify all the Se species present in the enzymatic
219 extracts from the sprouts. We also used an SeMeSeCys standard to check for its presence in the
220 extracts, although this species was not detected in our experiments, in agreement Lintschinger et
221 al., 2000. However, in some cases the occurrence of this species has been reported, although the
222 experimental growth conditions were different from those used in the present study (Gergely et
223 al., 2006; Yu et al., 2011).
224 Figure 2 (a) shows chromatograms of lentil, alfalfa and soy control extracts, where the main
225 selenocompounds were quantified. Figure 2 (b) shows sample chromatograms of the enzymatic
226 extracts from alfalfa, lentil and soy grown in culture A. In this figure, the chromatographic peaks
227 were identified as: the inorganic forms Se(IV) and Se(VI); and the selenoamino acids $SeCys_2$ and
228 SeMet, as well as the oxidized form of the latter: SeOMet. When comparing the chromatograms
229 from the extracts corresponding to the control experiment and those grown in Se-enriched
230 conditions, some differences can be observed. For example, in Figure 2(a), the chromatogram of
231 the lentil extract shows the highest content of Se compounds. Figure 2(b), in contrast, shows that

232 lentil did not accumulate the highest concentration of Se (VI), as was observed for soy. Despite
233 of this, the biotransformation of inorganic Se into SeMet was similar for both sprouts.
234 In Table 1, the results for the control sprouts show that Se species are naturally present in plants
235 but their content is very low, and in some cases, some species were below the LOQ; this was the
236 case for SeMet in alfalfa, despite it being the major species detected. For lentil, SeMet was also
237 the major organic selenocompound quantified, though the contribution of inorganic species,
238 mainly Se(VI), was not negligible. For soy, Se(VI) was the main species detected in the extracts,
239 but the content of SeMet and $SeCys_2$ was also quantifiable. Table 1 shows that the concentration
240 of Se species also increases with the Se content added to the hydroponic system. Despite this, the
241 transformation of selenocompounds is different for the different plants. Although the
242 concentrations of Se(IV) and Se(VI) in the fortified hydroponic media were in the same
243 proportion, Se(VI) was more easily absorbed by the roots than Se(IV) was. So, the concentration
244 of Se(VI) is from 8 to 35 times higher than the concentration of Se(IV), according to the results
245 for each type of sprout. For all the sprouts, SeMet represents from 13% to 33% to the sum of the
246 species quantified. For soy, the ratio of the SeMet present in shoots and roots was the same for
247 cultures A and B. So we can conclude that the accumulation of Se in the tissue of soy plants in
248 the form of SeMet depends on the amount of Se absorbed by the roots from the culture medium.
249 The extraction efficiency was at least 67%; typical for this kind of enzymatic hydrolysis using
250 Protease XIV (Hawkesford & Zhao, 2007). Column recovery data, calculated as the ratio of the
251 sum of Se species to the total Se in the extracts, were estimated to be in the range between 38%
252 (for control lentil shoot) and 94% (for control lentil root).
253
254 **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.

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TABLES

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).

Plant species	Concentration ranges of fortifier	Vegetable part	Total Se (mg kg^{-1})	Se speciation (mg kg^{-1})				Se in the extract (mg kg^{-1})	Extraction efficiency (%)
				$SeCys_2$	Se(IV)	SeMet	Se(VI)		
Alfalfa	Control	Shoot	1.5 ± 0.9	< 0.03	< 0.02	< 0.2	< 0.06	1.8 ± 0.9	120
	Culture A	Shoot	132 ± 4	4.25 ± 0.04	19 ± 5	13.6 ± 0.1	21.41 ± 0.04	89.5 ± 21.5	68
	Culture B	Shoot	284 ± 1	4.0 ± 0.3	21.1 ± 0.5	20.1 ± 0.9	30 ± 2	189 ± 5	67
Lentil	Control	Shoot	2.4 ± 0.1	0.10 ± 0.04	0.15 ± 0.05	1.15 ± 0.05	0.3 ± 0.1	1.8 ± 0.1	75
		Root	4.7 ± 0.2	0.22 ± 0.01	0.22 ± 0.03	0.9 ± 0.1	0.4 ± 0.1	4.6 ± 1.0	98
	Culture A	Shoot	98 ± 1	4.3± 0.3	5.61 ± 0.03	16.1 ± 0.4	53.3 ± 0.2	88.4 ± 0.1	90
	Culture B	Shoot	111 ± 3	10.1 ± 0.2	8.5 ± 0.2	24 ± 8	31 ± 2	111 ± 1	100
Soy	Control	Shoot	0.8 ± 0.4	< 0.01	0.07 ± 0.01	0.107 ± 0.001	0.52 ± 0.04	na	
		Root	1.0 ± 0.2	< 0.01	0.03	< 0.07	0.51	1.0 ± 0.3	100
	Culture A	Shoot	158 ± 7	2.60 ± 0.02	16.1 ± 0.2	14.9 ± 0.1	85 ± 2	132 ± 10	84
		Root	257 ± 8	5.0 ± 0.6	14.5 ± 0.2	13.0 ± 0.1	118 ± 4	184 ± 28	72
	Culture B	Shoot	188 ± 19	1.9 ± 0.1	17.5 ± 0.1	29.1 ± 1	70 ± 3	na	
		Root	750	3.7	71.9	40.6	499	na	

	$SeCys_2$	Se(IV)	SeMet	Se(VI)
LOD / (mg kg^{-1})	0.01	0.01	0.07	0.02
LOQ / (mg kg^{-1})	0.03	0.02	0.2	0.06

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 $SeCys_2$ 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.

figure 1
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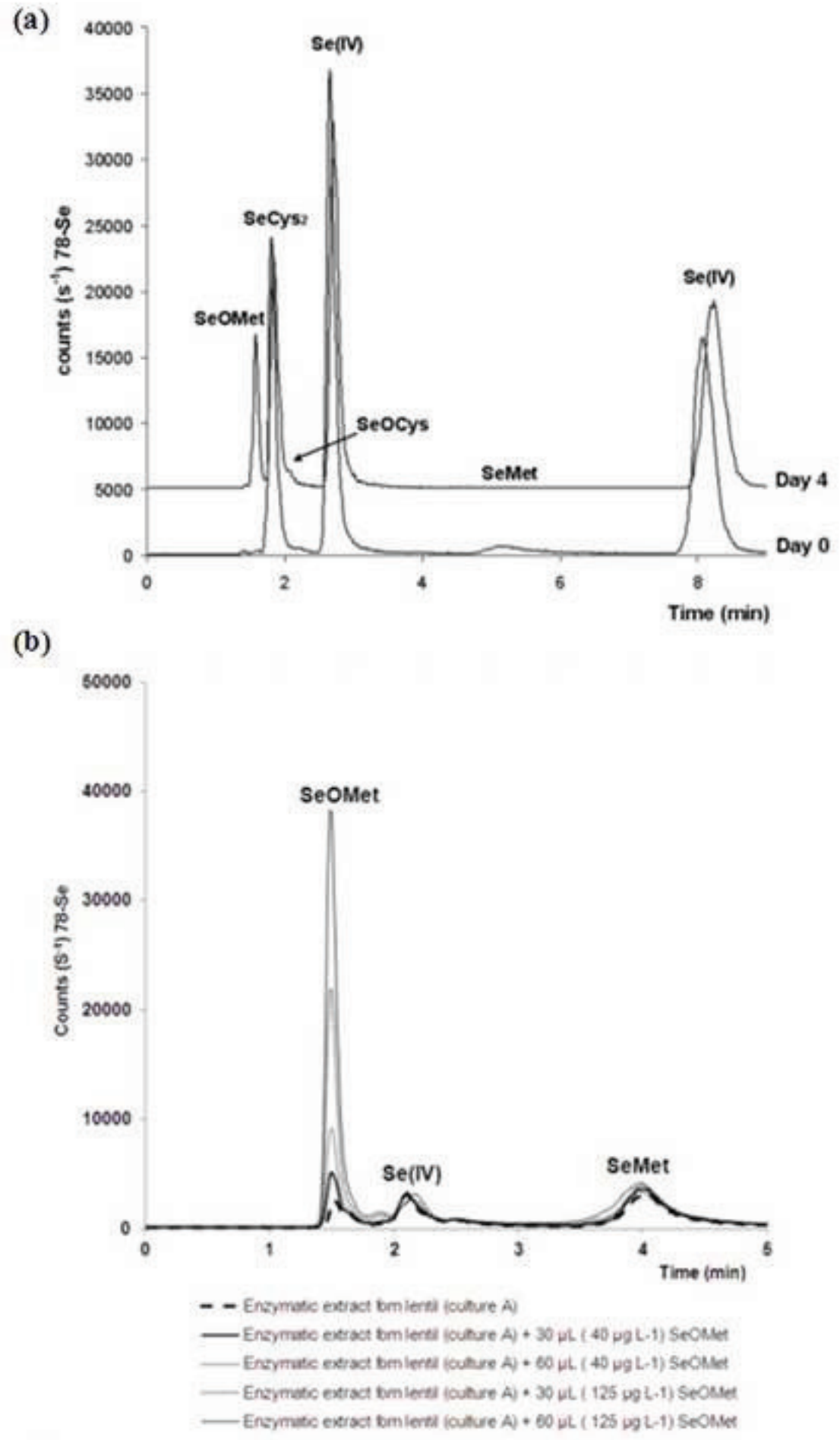

figure 2

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