Anal Bioanal Chem (2015) 407:9029–9042
DOI 10.1007/s00216-015-9069-4

RESEARCH PAPER

Stable isotope tracing: a powerful tool for selenium speciation and metabolic studies in non-hyperaccumulator plants (ryegrass *Lolium perenne* L.)

Pamela Di Tullo[1,2] · Antoine Versini[3] · Maïté Bueno[1] · Isabelle Le Hécho[1] · Yves Thiry[2] · Philippe Biron[4] · Maryse Castrec-Rouelle[3] · Florence Pannier[1]

Received: 18 June 2015 / Revised: 16 September 2015 / Accepted: 21 September 2015 / Published online: 1 October 2015

Abstract Selenium is both essential and toxic for mammals; the range between the two roles is narrow and not only dose-dependent but also related to the chemical species present in foodstuff. Unraveling the metabolism of Se in plants as a function of Se source may thus lead to ways to increase efficiency of fertilization procedures in selenium deficient regions. In this study, stable-isotope tracing was applied for the first time in plants to simultaneously monitor the bio-incorporation of two inorganic Se species commonly used as foodstuff enrichment sources. Occurrence and speciation of Se coming from different Se sources were investigated in root and leaf extracts of ryegrass *(Lolium perenne* L.*)*, which had been co-exposed to two labeled Se species (^{77}SeIV and ^{82}SeVI). Although the plant absorbed similar amounts of Se when supplied in the form of selenite or selenate, the results evidenced marked differences in speciation and tissues allocation. Selenite was converted into organic forms incorporated mostly into high molecular weight compounds with limited translocation to leaves, whereas selenate was highly mobile being little assimilated into organic forms. Double-spike isotopic tracer methodology makes it possible to compare the metabolism of two species-specific Se sources simultaneously in a single experiment and to analyze Se behavior in not-hyperaccumulator plants, the ICP-MS sensitivity being improved by the use of enriched isotopes.

Keywords Selenium · HPLC-ICP-MS · Stable isotope tracer · Speciation · Plants · Metabolism

Electronic supplementary material The online version of this article (doi:10.1007/s00216-015-9069-4) contains supplementary material, which is available to authorized users.

✉ Florence Pannier
florence.pannier@univ-pau.fr

[1] Laboratoire de Chimie Analytique Bio-Inorganique et Environnement (LCABIE), Université de Pau et des Pays de l'Adour/ CNRS, UMR 5254, IPREM, Hélioparc, 2 Avenue du Président Angot, 64053 Pau Cedex 9, France

[2] Andra, Research and Development Division, Parc de la Croix Blanche, 1-7 rue Jean Monnet, 92298 Châtenay-Malabry Cedex, France

[3] Sorbonne Universités, UPMC Univ Paris 06, CNRS, EPHE, UMR7619 METIS, 4 place Jussieu, 75005 Paris, France

[4] UPMC Univ Paris 06, CNRS-iEES, Campus Inra-AgroParisTech, 78550 Thiverval-Grignon, France

Introduction

Selenium (Se) is an essential micronutrient required in humans and animals for the function of a number of selenium-dependent enzymes, such as glutathione peroxidase (GPx) and thioredoxin reductase [1]. It has been also demonstrated that certain Se-containing compounds (e.g., methylselenocysteine, γ-glutamyl-methyl-selenocysteine) are effective chemoprotective agents, reducing the incidence of certain types of cancer [2, 3]. However, at high dose, Se may be toxic to mammals [4, 5]. The concentration range between physiological requirement and lethality is very narrow, as consequences both Se deficiency and toxicity are common worldwide problems. Plants can play a pivotal role in this respect: For example, the abilities of plants to absorb, sequester, and volatilize selenium can be exploited to manage environmental Se contamination by phytoremediation strategies [6, 7]. On the other hand, Se-enriched plants may be useful as "Se delivery system" (in forage and crops) to supplement the diets of livestock and humans in Se-deficient regions [8, 9]. Moreover, the ability of some plants to transform selenium

into bioactive compounds (e.g., anticarcinogenic compounds) has also important implications for human health [10–12].

The correlation of bioavailability and toxicity of Se with its chemical form triggered interest in Se speciation in plants. Actually, in plants, Se supplied as inorganic form (low bioavailability, potentially toxic) could be converted to safer highly bioactive species, improving nutritional properties [4]. Nowadays, to increase Se concentration in crop plants on Se-deficient soils, it has become a widespread agricultural practice in many countries to use Se-enriched fertilizers. This form of agronomic biofortification is for example successfully practiced over 30 years in Finland, New Zealand, and Denmark [8, 12, 13]. Some chemical forms of selenium are more readily available to plant uptake than others, for example in soil, selenite, being more retained on solid phases, is less bioavailable than selenate [14]. Apart from the total Se uptake, different enrichment treatments normally undergo certain metabolic changes, leading to several final products showing diverse translocation and accumulation within plant compartments [15–17]. For this reason, it is important to evaluate which form of Se should be used for plant supplementation to obtain high content of this element in final plant and to know whether inorganic Se is efficiently assimilated into organic forms. Therefore, its distribution in different parts of the plant, as well as characterization and quantification of individual chemical species, including bioactive compounds, become an issue. Very few papers have been published so far on speciation analysis of Se in plants, and most of the reported works have been performed with Se hyper-accumulator plants *(*i.e., *Brassica*, *Allium)* which, being able to accumulate several thousand mg kg^{-1} of Se, were shown to be good candidate to investigate the formation of the different Se compounds after plant exposure [18, 19]. However, information available on accumulation and speciation of Se in non-accumulator plants (i.e., forages and grasses), which could serve as model for general environmental conditions, is still scarce, probably due to the lack of analytical approaches, allowing the detection of Se species at very low concentrations. Liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC/ICP-MS) is a widely accepted tool for screening and quantification of elemental species in plant tissue [20–22], but the detection and the accurate quantification of the separated Se species are not always possible, owing to the fact that species concentrations are below the detection limit. The low sensitivity for Se in ICP-MS is partly due to several interferences on major Se isotopes and the presence of many stable isotopes (^{74}Se (0.89 %), ^{76}Se (9.36 %), ^{77}Se (7.63 %), ^{78}Se (23.4 %), ^{80}Se (49.6 %), and ^{82}Se (8.73 %)). To overcome this issue, an attractive way to enhance the sensitivity of HPLC/ICP-MS measurement for Se and to get reliable species determination consists in the application of highly enriched stable isotopes as tracers in metabolic studies. Metabolites of a precursor labelled with an enriched stable isotope can be much more easily detected than Se with natural abundance. In addition, several nutritional forms of Se labelled with different isotopes can be simultaneously supplied into the growth media; consequentially, metabolites of different precursors can be traced in a single experiment. Various factors such as absorption rate, translocation and speciation as well as potential competition mechanisms between the added species can be thus compared using identical host plant under the same growing and detection conditions. However, if this isotopic tracer approach has been successfully applied in rats [23–25], and in environmental samples such as soils and sediments [26, 27], it has, to the best of our knowledge, never been performed in order to monitor Se biotransformations in plants.

In this study, a non-hyperaccumulator plant, ryegrass *(Lolium perenne* L.), was chosen as model system for being an easy and fast to grow plant. Moreover, ryegrass is one of the most important forage varieties in terms of contribution to pastures composition and an increase of Se accumulation has been previously evidenced in greenhouse experiments, as well as non negative effect on plant yield, when Se was supplied as selenite or selenate forms at low concentration [28]. Although there are few studies on the determination of total Se in ryegrass [29, 30], Se speciation in this plant has not been reported to our knowledge. We used two stable isotopes, ^{77}Se and ^{82}Se, to label respectively selenite and selenate sources added to hydroponic growth media (double spike) in order to monitor respective bio-incorporation of these two species in roots and leaves of ryegrass. HPLC/ICP-MS was used for Se species determination after water extraction and enzymatic hydrolysis of freeze-dried samples. The objectives were to determine both the capacity of ryegrass to take up and accumulate the Se inorganic forms and the corresponding metabolic products in different plant tissues.

Materials and methods

Instrumentation

Total Se concentration was determined with an Agilent 7500ce ICP-MS instrument (Agilent Technologies, Santa Clara, CA) equipped with an octopole collision/reaction cell (C/RC). The setting and acquisition parameters were previously optimized in our laboratory [27] (Table S1 in the Electronic Supplementary Material, ESM). An Agilent 1100 series HPLC pump was coupled to ICP-MS for speciation analyses. Chromatographic separation was carried out on a porous graphitic carbon stationary phase (PGC, Thermo Hypercarb column 10 cm×4.6 mm i.d) with a formic acid mobile phase (240 mmol L^{-1}, pH 2.4 adjusted with ammonia), delivered at 1 mL min^{-1} flow rate and a gradient program with increasing amount of methanol (from 1 to 35 %, as detailed in Table S1 in ESM). The column used for size exclusion

chromatography (SEC) was a Superdex Peptide 10/300 GL (Tricorn, Amercham Biosciences) with a fractionation range of 0.1–7 kDa and a total volume of 24 mL. The elution phase was a 30-mM Tris–HCl buffer (pH 7.5) at a flow rate of 0.6 mL min^{-1}. The calibration of the column was accomplished with a standard mixture of myoglobin (17 kDa), insulin (5.8 kDa), and vitamin B_{12} (1.35 kDa), with UV detection at 254 and 280 nm, giving a calibration curve log(MW)= 6.291—0.155(t_r), where t_r is the retention time in minutes and MW the molecular weight in Da.

SEC fractions were collected and analyzed off-line for Se total content and its speciation by PGC-HPLC/ICP-MS. For water extracts (roots and leaves), the eluate was collected every 3 min between 11 and 32 min of elution, leading to 7 fractions for each sample type. For driselase extract of roots, the eluate was collected every 3 min between 15 and 36 min (7 fractions), and for driselase extract of leaves between 21 and 32 min (4 fractions). The collection of fractions was repeated 6 times, and corresponding fractions were pooled (final volume of each fraction about 11 mL). The injection volumes were of 100 and 200 μL for RP and SEC chromatography, respectively.

Reagents and standards

Selenium stock standard solutions of 1000 mg L^{-1} were prepared in water and stored in the dark at 4 °C. $_{D,L}$-Selenomethionine (SeMet), $_{L}$-selenocystine ($SeCys_2$), methylselenic acid (MeSeOOH), sodium selenite (Se(IV)), sodium selenate (Se(VI)) and Se-methylselenocysteine (MeSeCys) were obtained from Sigma-Aldrich (SigmaUltra, ≥99 %). Working standard solutions were prepared freshly prior to use by diluting the respective stock solutions in ultra-pure water. All reagents used for mobile phases (formic acid, methanol, and ammonia) were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitric acid (Ultrex, J.T. Baker, Deventer, The Netherlands) and hydrogen peroxide 30 % (Fisher Scientific) were used for mineralization procedure. Tris(Hydroxymethyl)aminomethane, protease type XIV from *Streptomyces griseus* and driselase from *Basidiomycetes* used in extraction procedures were from Sigma-Aldrich. Highly enriched elemental $^{77}Se(0)$ and $^{82}Se(0)$ powders were purchased from Isoflex (Moscow, Russia).

Preparation of isotopically enriched Se tracers ($^{77}Se(IV)$ and $^{82}Se(VI)$)

Elemental Se was converted into Se(IV) and Se(VI), respectively, by nitric acid and a combined nitric acid/hydrogen peroxide oxidation reaction, according to Van Dael et al. [31]. Both standard solutions were characterized in terms of isotopic composition and of concentration by isotope dilution. Speciation analysis of the isotopically enriched Se standards was also performed to check complete conversion of elemental selenium into selenite and selenate. The results concerning the enriched solution are as follow: [^{77}Se]: 1015±3 mg kg^{-1}, [$^{77}Se(IV)$]: 1010±6 mg kg^{-1}, 98.3 % ^{77}Se and 1.7 % ^{78}Se and [^{82}Se] 947±4 mg kg^{-1}, [$^{82}Se(VI)$]: 950±5 mg kg^{-1}, 99.2 % ^{82}Se and 0.8 % ^{80}Se.

Plant growth and samples preparation

Ryegrass (*Lolium perenne* L.) plants (two replicates per treatment) were cultivated in a controlled atmosphere growth chamber (1 m^2 surface area and 1.5 m height) with a 10-h per day period (two 400 W metal halide discharge lamps, Growth spectra, MH400W, E40), a temperature of 25:23 °C (day/night), and relative humidity of 60–70 %. Dried seeds of English ryegrass (30 mg cm^{-2}) were planted on mesh screen (mesh diameter of 1 mm) stretched out at the top of white plastic container. The plants were grown in duplicate in two plastic tanks filled with 16 L of hydroponic solution (a modified Hoagland nutrient solution [32]).

After 2 weeks of plant growth, $^{77}Se(IV)$ and $^{82}Se(VI)$ tracers were simultaneously supplied in the medium at an initial concentration of approximately 100 μg L^{-1} of Se for each species. This concentration was chosen based on previous work to optimize growth conditions (unpublished data) showing that ryegrass growth is threatened by stunting, chlorosis, withering, and necrosis of leaves above 200 μg L^{-1}. Hydroponic solutions were replaced every 2 weeks with a fresh nutritive solution containing Se tracers at the same initial concentrations (about 100 μg L^{-1}). As the ryegrass plants grew up, the evapotranspiration gradually increased (up to 140 ml per hour), and the addition of deionized water was required to ensure stable nutrients concentrations. The water condensates drained out of the growth chamber were measured each day, and an equivalent volume of deionized water was therefore added in the plastic tanks.

As consequence of plant uptake, the tracer concentrations decreased during plant growth, aliquot of nutrient solutions were therefore analyzed before and after replacement (every 2 weeks) in terms of total Se content and speciation. After 6 weeks, the plants were harvested and the roots were separated from the leaves. Roots were then washed with ultra-pure water to remove all the traces of nutrient solution. Both tissues, from each culture repetition, were freeze-dried, ground with an automatic agate mortar obtaining four powdered homogenized samples (2 samples/plant compartment) and weighted (dry weight, DW).

Procedures

Total digestion

For the determination of total Se in *L. perenne* L., 0.250 g of powdered homogenized samples was digested with 2 mL of

HNO_3 and 0.5 mL of H_2O_2 in a closed vessel microwave digestion system (Ethos Touch Control, Milestone) assisted by a two-step temperature program (5 min up to 180 °C and held for 10 min, working with a maximum power of 1000 W). Finally, the digested samples were made up to 30 mL with ultra-pure water and filtered to 0.45 μm and analysed by ICP-MS. The four samples were digested and analysed in triplicate.

Validation of procedure was performed by digesting a certified reference material (Ryegrass ERM-CD281) measured total Se (0.022±0.001 mg kg^{-1} DW) being in complete agreement with theoretical value (0.023±0.004 mg kg^{-1} DW).

Sequential extraction protocol

Selenium species were extracted from the plant tissues using a sequential extraction protocol including the following steps: (1) ultra-pure water, (2) driselase (mixture of cell wall digesting enzymes), and (3) protease type XIV. For each step, different extraction conditions were tested and the optimized protocol was defined based on a compromise between extraction yield, species stability and time consuming.

According to the results of the optimization experiments (described below), the retained sequential extraction protocol for further analysis was as follow: about 0.1 g plant material was subjected to:

1. Extraction with 5 mL of ultra-pure water assisted by ultrasonic probe (SONICS VibraCell™) (25 % of energy, 20 s pulse,) for 4 min, repeated three times, for extraction of soluble selenium compounds. The supernatants collected at each extraction step were then pooled for analysis.
2. Enzymatic hydrolysis with driselase: the residue from water extraction was extracted twice with 5 mL of Tris–HCl buffer solution (8 mM, pH 7.5) containing 10 mg of driselase. The solution was kept at a constant temperature of 37 °C in a thermostat bath and constantly stirred for 24 h. The supernatants were then pooled for analysis.
3. Enzymatic hydrolysis with protease: 5 mL of a Tris–HCl buffer solution (8 mM; pH 7.5) containing 1 mM $CaCl_2$ and 20 mg of protease type XIV was added to the pellet from driselase extraction. The solution was incubated in a digitally controlled immersion thermostat bath for 20 h at 37 °C. After centrifugation, the supernatant was digested once again with 1 mL of Tris buffer solution containing 10 mg of protease XIV for 20 h at 37 °C.

For extracts obtained from enzymatic hydrolyses (driselase and protease), an aliquot of the solution was discarded for total analysis and the remaining was cut off filtered through a 100-kDa pore size membrane to eliminate high weight molecular compounds that might disturb chromatographic separation. Total Se content was determined before (noted as extracted ^{77}Se or ^{82}Se) and after cutoff filtration (noted as extracted $^{77}Se_{cut\text{-}off}$ and $^{82}Se_{cut\text{-}off}$).

Samples were sequentially extracted in triplicate, and a blank was subjected to the same procedure. Protease residues were mineralized for the determination of non-extracted Se allowing mass balance calculation. These precautions allowed to control that no contamination or loss occurred during extractions.

Extraction efficiencies are calculated as the percentage of total Se content in the freeze-dried plant tissues (DW).

Determination of Se in the digested samples and extracts

Endogenous and tracers (^{77}Se and ^{82}Se) Se concentrations, for total and speciation analyses, were determined by reverse isotope dilution (RID), with natural abundance Se standard used as a spike and, $^{78}Se/^{77}Se$ and $^{78}Se/^{82}Se$ ratios selected as measured isotopes pairs by using Eqs. 1 to 3 according to Hintelmann and Evans [33].

$$C = \frac{C' \times w' \times W_{at} \times \left(R \times {}^{77-82}A_{natural} - {}^{78}A_{natural}\right)}{w \times W'_{at} \times \left({}^{78}A_{sample} - R \times {}^{77-82}A_{sample}\right)} \quad (1)$$

With:

C: Se concentration in the sample

C': Se concentration of standard (natural abundance) spiked to the sample

w, w': masses of sample and spike

W_{at} ,W_{at}': atomic masses of sample and spike

R: $^{78}Se/^{77}Se$ or $^{78}Se/^{82}Se$ ratio measured after spike addition

$^{X}A_{sample}$: abundance of X isotope in the sample before spike addition

$^{X}A_{natural}$: abundance of X isotope in the natural standard used as spike

$$[Se_{ambient}] = \frac{C \times {}^{78}A_{sample}}{{}^{78}A_{natural}} \quad (2)$$

$$[Se_{tracer}] = \frac{C \times {}^{78}A_{sample} - \left[\left(\frac{{}^{78}A_{natural}}{{}^{77-82}A_{natural}}\right) \times C \times {}^{77}A_{sample}\right]}{\left(\frac{{}^{78}A_{tracer}}{{}^{77-82}A_{tracer}}\right) - \left(\frac{{}^{78}A_{natural}}{{}^{77-82}A_{natural}}\right)} \quad (3)$$

With:

$^{X}A_{tracer}$: abundance of isotopes 78 and 77 or 82 of tracers (i.e., $^{77}SeIV$ or $^{82}SeVI$) and natural abundance Se

Optimum spike-to-sample ratio was calculated for each sample to obtain minor error propagation during ID calculation [34].

All isotopes intensities were mathematically corrected from interferences due to the formation of SeH^+ and BrH^+ following the determination of Se and Br hydridation factors (f_{Se} and

f_{Br}) in Se and Br standards solutions measured each three samples (bracketing method; [27, 35]).

All isotope ratios obtained were further corrected for mass bias using an exponential model after determination of the mass bias factor with natural abundance Se standards [36]. C/RC conditions and correction method defined in Tolu et al. [27] were evaluated for reagents used in the present work showing a good accuracy and precision of measured isotopic ratios (error<4 %; RSD<5 %) [27].

Se concentrations were then expressed in micrograms of Se per kilogram based on precise weights of vegetal material (DW) and reagents used for extraction or mineralization.

Total uncertainties associated to concentrations of total Se and Se species were defined by combining analytical uncertainties to those related to the mean and standard deviation of values of extractions replicates (n=3). Analytical uncertainties were calculated according to random error propagation method along the calculation steps [27].

Values referring to the "whole plant" were calculated from Se tracer concentrations measured in each compartment (μg g^{-1} of Se) and corresponding compartment biomass (g).

Results and discussion

Nutritional solutions

Total concentrations of the two Se tracers were determined in aliquots of freshly prepared nutritional solution and solution after two weeks of growth. At the beginning, Se concentrations of both tracers amounted approximately to 100 μg L^{-1} (Table S2 in the ESM). After 2 weeks of plant growth, initial concentrations of both tracers decreased of about 60–66 % as result of plant uptake. Speciation analyses of nutritional solutions were performed as well in order to check eventual transformations of supplied Se species occurred during the considered period. Results resumed in Table S2 in the ESM showed that the tracers were stable in terms of speciation in the growth media remaining after 2 weeks of culture in the chemical form initially used for Se enrichment.

Comparative efficiencies of different extraction procedures

In order to monitor respective bio-incorporation of inorganic Se and elucidate possible further metabolism in root and leaf compartments, samples were sequentially extracted by reagent solutions designed to selectively leach different classes of selenium species. In this study, a sequential approach was designed consisting of extraction of (1) water soluble Se species, (2) Se bound to plant cell wall and (3) Se-amino acids incorporated into protein structure. Besides the maximum efficiency, the stability of Se species during the procedure should be taken into account when choosing extraction conditions. A preliminary study was thus performed to select appropriate extraction procedures in order to obtain maximum extraction efficiency as well as to ensure integrity of species. The first step of sequential extraction aimed to recover water-soluble Se species. Three different water extraction protocols were tested as follow: 0.1 g sample was extracted either once, twice or three times with 5 mL of ultra-pure water in an ice bath using an ultrasonic probe. In the case of repeated extractions, the supernatants were collected after centrifugation before further extraction(s). All supernatants were pooled together before analysis.

Figure 1a shows the results obtained considering total Se extractabilities (expressed as % $Se_{extracted}/Se_{total}$). For both samples and both tracers, Se recovery after three successive water extractions was almost twice higher compared to single extraction; it was thus selected as first step of sequential extraction protocol.

Comparison of total Se measured in extracts from once and twice repeated digestions with driselase evidenced a significant increase of extractability after two successive extractions with driselase (Fig. 1b) which were therefore retained for the sequential extraction protocol. A proteolytic digestion was performed on driselase residue as last step. Most widely reported conditions used amount of 30 mg protease type XIV for digestion of 0.1 g of plant material performed in 5 mL of Tris–HCl buffer solution (8 mM; pH 7.5) [37, 38]. We compared three different protocols to bring that amount; each protease addition was incubated 20 h at 37 °C: (1) single step digestion with 30 mg of enzyme, (2) first digestion step with 20 mg of enzyme followed by further digestion of supernatant adding 1 mL of buffer solution containing 10 mg of enzyme, and (3) first digestion step with 10 mg of enzyme followed by two successive digestions of supernatants obtained by adding 1 mL of buffer solution containing 10 mg of enzyme.

Differences in extracted selenium tracer recoveries were not statistically significant among the three procedures (RSD: 1–5 %). However, when the extraction procedure involved successive protease hydrolysis of supernatant, an increase in SeMet concentration was observed (20–60 % higher than one step digestion). A procedure including at least one further proteolytic digestion of supernatant seems thus to provide complete digestion of proteins and consequently release of seleno-amino acids.

For each step of extraction procedure, integrity of species during treatment (especially when applying successive ultrasonic rounds) was also verified. HPLC-ICPMS analyses of water and enzymatic extracts showed identical Se-containing peaks after successive extraction steps, indicating that no species degradation occurred during the treatments. Relative standard deviations obtained for total Se determinations were lower than 4 % for the selected procedures. Moreover, HPLC-ICPMS analyses of extracts showed superimposable

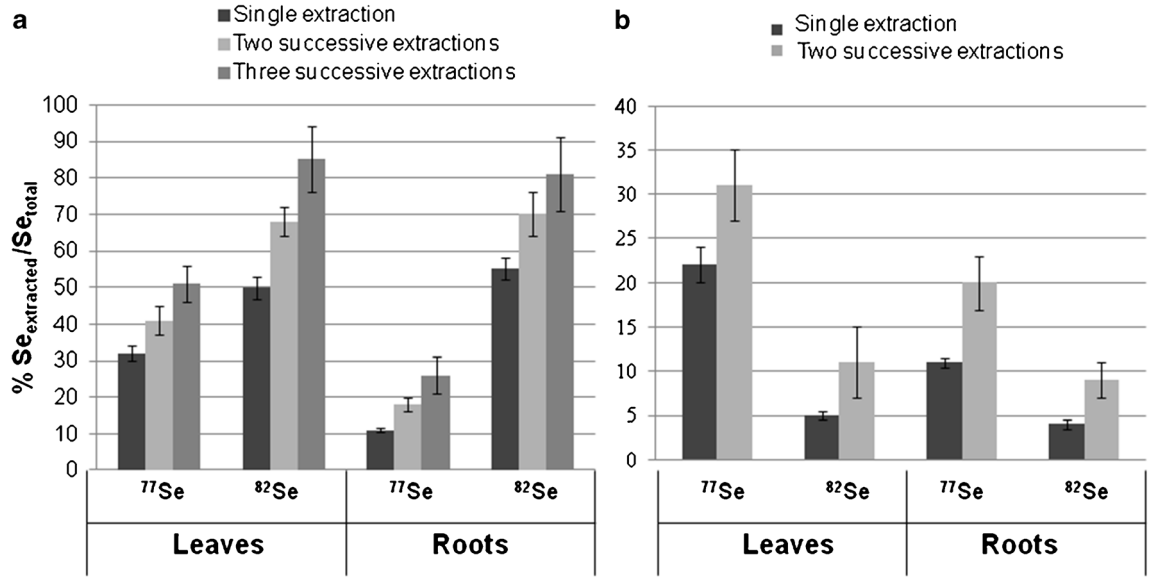

Fig. 1 Proportion of extracted Se (% $Se_{extracted}/Se_{tot}$) under tested conditions used for: **a** water extraction and **b** driselase extraction. *Error bars* represent SD for triplicate extractions of two samples/plant compartment

chromatograms for triplicate treated samples, confirming extractions procedures repeatability (RSD<5 %).

Allocation of ^{77}Se and ^{82}Se tracers in plant tissues

Total concentrations of both tracers and endogenous Se were measured in freeze-dried samples after digestion for each tissue type (roots and leaves). Total Se accumulation in the whole plant is the same for both tracers, accounting for 7.6± 0.8 and 7.3±1.2 mg kg^{-1} for ^{77}Se and ^{82}Se, respectively. These results indicate that, in used experimental conditions, the total amount of Se taken up by the plant is independent of the chemical species supplied in the grown media in agreement with previous studies performed in hydroponic solutions, both oxyanions being equally available for plant uptake [39]. However, different uptake could be observed in the case of plant cultivation on soil, where Se(IV) resulted to be less bioavailable being more strongly sorbed on soil solid phases compared to Se(VI) [14].

Although total Se uptake was similar, Se allocations and translocations in plant tissues (leaves or roots) strongly depend on supplied Se form used for the enrichment treatment (Tables 1 and 2). Namely, total ^{82}Se in leaves (8.1± 1.8 mg kg^{-1}) was found higher than that found in roots (5.1 ±0.2 mg kg^{-1}). On the contrary for ^{77}Se tracer, highest concentrations were observed in roots (23±1 and 3.0± 0.2 mg kg^{-1} for the roots and the leaves, respectively). The calculated translocation factor defined as the ratio between Se concentrations in leaves and roots is strongly higher for ^{82}Se (1.6±0.4) than that of ^{77}Se (0.13±0.01), suggesting an increased rate of Se translocation from roots to leaves in ryegrass after supplementation with Se(VI) versus Se(IV). These results are in agreement with previous studies performed with different plant species (soybean, wheat, broccoli, rice, Indian mustard, sugar beet, and maize) clearly demonstrating that Se translocation from roots to leaves is dependent on the form of Se supplied, selenate being much more easily transported than selenite or any organic species, like SeMet [6, 16, 17, 32, 40].

Regarding sequential extraction procedure, total Se concentrations measured in the three selective extracts and in digested residual pellet as well as the corresponding extraction efficiencies for leaves and roots samples are presented in Tables 1 and 2 for ^{77}Se and ^{82}Se tracers, respectively.

The first extraction step carried out on the plant tissue by mean of ultrapure water assisted by sonication extracted most of ^{82}Se present in roots and leaves samples, accounting for almost 80 % of total ^{82}Se present in the compartment. Driselase and protease digestions extracted almost the same amount of ^{82}Se, about 10 % in both samples. No significant decrease in ^{82}Se concentrations measured after 100 kDa cut-off filtration of enzymatic extracts was observed.

In roots, water-soluble ^{77}Se accounted for 25±1 % of total ^{77}Se. The digestion with driselase liberated an additional 20± 1 % (bound to cell walls) and the proteolytic lysis allowed recovery of an additional 12±1 %. Indeed, the remaining 46 ±2 % (residue) might correspond to ^{77}Se incorporated in more complex structures which could not be released with the above reagents. Different proportions of ^{77}Se were observed in leaves extracts compared to root extracts, accounting for 51 ±5, 31±4 and 16±2 % for water, driselase and protease extracts, respectively.

Comparison of total content before and after cut-off filter evidenced ^{77}Se losses of about 5 and 15 % related to total ^{77}Se in roots and leaves, respectively. It means that the tracer was

Table 1 Total and identified species concentrations of ^{77}Se tracer in leaves and roots. Corresponding proportions in extracts (expressed as % of total ^{77}Se in plant compartment) are given between brackets. Values are mean±SD from triplicate digestions or sequential extractions of two samples/plant compartment

μg kg^{-1} of ^{77}Se (% of total content in plant compartment)								
Extract	Total extracted Se	Total extracted Se_{cutoff}[a]	Se(IV)	Se(VI)	SeMet	MeSeCys	$SeCys_2$	MeSeOOH
LEAVES: $^{77}Se_{tot}$=2990±247								
Water	1499±153 (51±5)	–	12±2 (0.4±0.1)	<LOD[b]	14±1 (0.5±0.1)	3.1±0.4 (0.11±0.02)	<LOD[b]	<LOD[b]
Driselase	922±111 (31±4)	517±88 (18±4)	19±2 (0.6±0.1)	<LOD[b]	176±18 (6±0.2)	5±2 (0.2±0.1)	2.5±0.7 (0.09±0.03)	12±1 (0.4±0.1)
Protease	482±54 (16±2)	360±82 (12±3)	–	<LOD[b]	288±26 (10±1)	7.6±2.1 (0.3±0.1)	2.6±0.4 (0.19±0.04)	3.2±0.6 (0.11±0.03)
Residue	176±68 (6±2)							
ROOTS: $^{77}Se_{tot}$=22500±1150								
Water	5816±621 (25±1)	–	1630±40 (7±1)	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]
Driselase	4480±560 (20±1)	3475±223 (15±2)	41±3 (0.18±0.03)	<LOD[b]	288±29 (1.3±0.3)	115±17 (0.5±0.1)	34±6 (0.2±0.1)	<LOD[b]
Protease	2709±543 (12±1)	2467±548 (11±3)	20±5 (0.09±0.03)	<LOD[b]	1289±266 (6±2)	111±45 (0.5±0.3)	33±6 (0.14±0.04)	42±5 (0.15±0.04)
Residue	10507±828 (46±2)							

[a] Measured in supernatant after 100 kDa cutoff filtration of driselase and protease extracts

[b] Range of LOD for ^{77}Se species=0.2–3 μg kg^{-1}

Table 2 Total and identified species concentrations of ^{82}Se tracer in leaves and roots. Corresponding proportions in extracts (expressed as % of total ^{82}Se in plant compartment) are given between brackets. Values are mean±SD from triplicate digestions or sequential extractions of two samples/plant compartment

μg kg^{-1} of ^{82}Se (% of total content in plant compartment)								
Extract	Total extracted Se	Total extracted Se_{cutoff}[a]	Se(IV)	Se(VI)	SeMet	MeSeCys	$SeCys_2$	MeSeOOH
LEAVES: $^{82}Se_{tot}$=8050±1775								
Water	6217±1390 (77±8)		<LOD[b]	6021±1589 (75±13)	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]
Driselase	832±144 (11±3)	552±138 (7±3)	31±4 (0.4±0.1)	< LOD[b]	279±34 (4±1)	54±9 (0.7±0.2)	7.4±0.3 (0.09±0.02)	29±3 (0.4±0.1)
Protease	567±82 (7±1)	543±93 (7±2)	<LOD[b]	< LOD[b]	488±48 (6±1)	14±3 (0.2±0.1)	5.7±1.2 (0.07±0.01)	5.6±1.3 (0.07±0.01)
Residue	91±22 (1.1±0.1)							
ROOTS: $^{82}Se_{tot}$=4950±490								
Water	4013±435 (81±13)		<LOD[b]	4003±334 (81±8)	<LOD[b]	<LOD[b]	<LOD[b]	<LOD[b]
Driselase	465±70 (10±1)	379±78 (8±2)	44±9 (0.9±0.3)	<LOD[b]	56±13 (1.1±0.4)	34±6 (0.7±0.2)	<LOD[b]	<LOD[b]
Protease	480±130 (10±1)	400±67 (8±2)	7±1 (0.3±0.1)	<LOD[b]	341±71 (7±2)	17±4 (0.15±0.04)	<LOD[b]	<LOD[b]
Residue	129±79 (1.6±0.4)							

[a] Measured in supernatant after 100 kDa cutoff filtration of driselase and protease extracts

[b] Range of LOD for ^{82}Se species=0.7–5 μg kg^{-1}

incorporated into HMW macromolecules (>100 kDa) released after cell wall digestion. On the contrary, no significant decrease of ^{77}Se concentration after cut-off was observed in protease extracts.

Although these results display a near-complete extraction (up to 90 %) for both tracers in leaves and for ^{82}Se in roots, a large amount of ^{77}Se remained in the unextracted fraction of roots.

Quantitative speciation of Se

Species determination by PGC-HPLC/ICP-MS

PGC separation was firstly performed to obtain information on the occurrence of Se metabolites coming from the two precursors (^{77}Se(IV) and ^{82}Se(VI)) and their distribution in the plants. Identification of Se containing chromatographic peaks in extracts was performed by spiking commercially available Se standards. Complete separation and elution of available standards can be achieved within the first 10 min of the separation run. Quantification of identified Se species was then performed by standards addition method based on peak areas.

Quantitative results (concentrations and proportions) for identified Se species determined in the different extracts and plant compartments are presented in Tables 1 and 2 for ^{77}Se and ^{82}Se tracers, respectively. Chromatograms of roots water extracts revealed the presence of tracers as inorganic Se forms (^{77}Se(IV) and ^{82}Se(VI)) used for Se enrichment in the growing media. ^{82}Se(VI) amounts to 100 % of total water extracted ^{82}Se and consequently 81 % of total ^{82}Se in roots. On the contrary, ^{77}Se(IV) only accounts for 29 % of water extracted ^{77}Se and 8 % of total ^{77}Se measured in roots, although no additional chromatographic peaks containing ^{77}Se were detected. Regarding leaves samples, for ^{82}Se, similar to that observed in roots water extract, only ^{82}Se(VI) could be detected corresponding to almost 100 % of total ^{82}Se extracted with water. For ^{77}Se, two major peaks corresponding to ^{77}Se(IV) and ^{77}SeMet were observed. A smaller peak was also detected with a retention time corresponding to that of ^{77}MeSeCys. Calculated extraction efficiencies for ^{82}Se(VI) are in agreement with results obtained for others plants (chicory, dandelion, lamb's lettuce, and parsley) that showed water extraction recovery of selenate in leaves of different plants ranging from 45 to 85 % [41].

Example chromatograms corresponding to driselase extracts of roots and leaves are shown in Fig. 2. For both samples, two major Se peaks matching the retention times of MeSeCys and SeMet and a minor selenocystine peak were detected. Some Se-containing peaks whose retention times do not match the ones of any Se available standard were also detected. In roots extracts, these peaks were more intense for ^{77}Se isotope than for ^{82}Se one. The opposite was observed for leaves. An increase of baseline signal was observed between 15 and 30 min (methanol gradient) mostly for ^{77}Se tracer, suggesting some elution of Se compounds or Se peptides.

Considering quantitative data obtained in the Se fraction released by driselase hydrolysis, it appears that both inorganic Se tracers were metabolized by the plant into species associated with cell wall, actually no more ^{82}Se(VI) could be detected and ^{77}Se(IV) only accounted for between 1.2 and 3.6 % of total extracted ^{77}Se measured after cutoff ($^{77}Se_{cut\text{-}off}$). SeMet and MeSeCys are the most abundant detected known Se species; their concentrations varying as a function of plant compartment and Se tracer. SeMet was present at a higher concentration in leaves compared to roots. As already observed in water extracts, most of ^{77}Se were present as unknown compounds, quantified species accounting for 14 and 42 % of total extracted $^{77}Se_{cut\text{-}off}$ in roots and leaves, respectively. In roots extract, a large amount of ^{82}Se was also undetected, accounting for almost 64 % of total extracted $^{82}Se_{cut\text{-}off}$.

The chromatographic profiles of protease XIV extracts of roots and leaves are shown in Fig. 3. Two main peaks were detected, corresponding to SeMet and MeSeCys by standards matching retention times. MeSeOOH, Se(IV) and $SeCys_2$ could be detected at μg kg^{-1} level for ^{77}Se in roots and for ^{82}Se in leaves. Additional peaks corresponding to unknown selenium-containing species were also observed, some of them eluting similarly than those observed in the driselase extract. For the two tracers, similar profiles in terms of detected species were observed, however with different intensities.

According to the quantitative speciation data for both tracers in roots and leaves after proteolytic digestion, SeMet represents the main extracted species accounting for 80 and 90 % of total extracted $Se_{cut\text{-}off}$ in leaves and for 52 and 90 % in roots for ^{77}Se and ^{82}Se, respectively.

Little amount of $SeCys_2$ could be observed in protease extract which could reflect either the unspecific ($SeCys_2$ detection) or the specific (SeCys detection) selenium incorporation into the plant proteins. In fact, in these extraction conditions, the detection of SeCys is not possible as it is rapidly oxidized to $SeCys_2$.

Selenium distribution as a function of molecular weight (SEC /ICP-MS)

Considering the large proportion of unknown species in aqueous and driselase extracts, an insight into speciation of selenium in these extracts was attempted by analytical scale size-exclusion chromatography that allows obtaining information about the molecular weight of extracted seleno-molecules.

For ^{77}Se tracer, water extracts of leaves and roots show similar elution profiles with a peak eluting in the exclusion volume and two peaks in the <1.3 kDa range, but with important discrepancies in peaks intensity, higher in roots than in leaves, as shown in Fig. 4a, b. The first peak (RT 12 min)

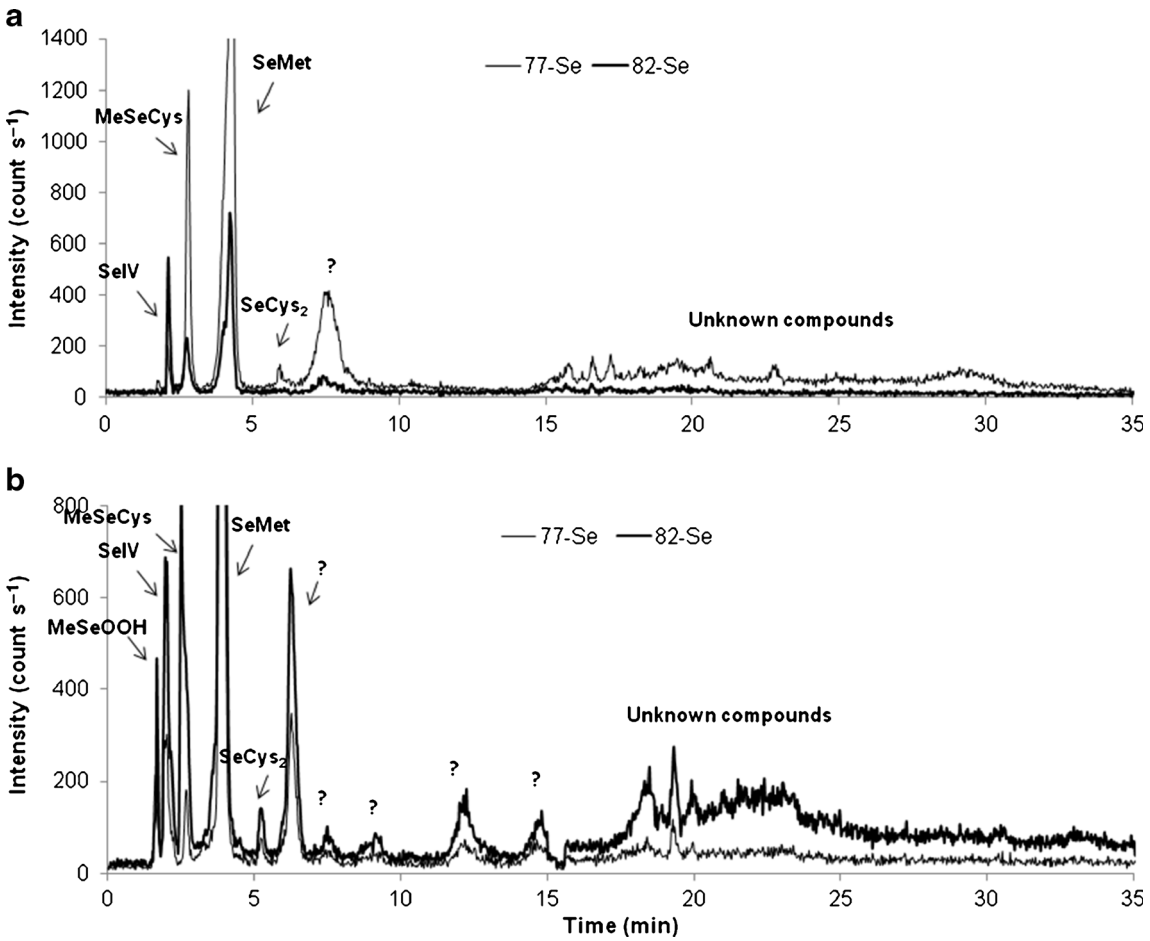

Fig. 2 PGC–HPLC ICP-MS example chromatograms of driselase extracts (diluted 2.5 times) of **a** roots and **b** leaves. The *y*-scale of both figures is expanded as the high amount of SeMet would not allow showing the smaller peaks

corresponding to the exclusion volume probably corresponds to selenium containing proteins. A baseline increase was observed between 14 and 23 min, probably corresponding to elution of Se-containing peptides. In the low molecular weight (LMW) region, two major peaks were also observed which may correspond to the free seleno-amino acids, inorganic selenium and small selenium metabolites. In the case of ^{82}Se, the SEC/ICP-MS profiles for leaves and roots water extracts showed only one peak eluting in LMW region corresponding thus to selenate, as previous PGC chromatographic analysis showed that selenate was the only species detected in water extract (100 % of extracted ^{82}Se). Chromatograms of driselase extracts of leaves and roots samples indicate, for both tracers, the presence of selenium species eluting after the 1.3 kDa marker region, corresponding to LMW species. SEC profiles, showing two major peaks at higher elution times, were quite similar for leaves and roots extracts, but differed among the two tracers. In root extracts, peak intensity at *m*/*z* 77 was higher than the one at *m*/*z* 82, whereas on the contrary was observed in leaves extracts. Moreover, some minor Se species eluting between 14 and 23 min could be observed only in SEC chromatograms of ^{77}Se tracer in roots extracts.

Subsequently, in order to analyze Se distribution as a function of the molecular weight, SEC fractions were collected, and their selenium content was determined off-line. The sum of Se content measured in all collected fractions was equal to the total selenium amount in extract, evidencing no loss during the chromatographic separation. The proportions of ^{77}Se in each collected fraction of water extracts (% of total extracted ^{77}Se) as a function of calibrated molecular weight fractions are summarized in histogram depicted in Fig. 4c.

In water extracts of roots and leaves, respectively, 64 and 50 % of total extracted ^{77}Se are associated to the fraction lower than 1.3 kDa, corresponding mainly to inorganic selenium, free amino acids and small peptides. For leaves and root driselase extracts, about 90 % of total extracted ^{77}Se appears in this low molecular weight fraction. These results indicate that ^{77}Se associated with cell wall is mostly in the form of low molecular weight metabolites (less than 1.3 kDa).

Multidimensional chromatography was further applied by PGC–HPLC/ICP-MS analysis of SEC collected fractions. Namely, fractions eluting in the LMW region (<1.3 kDa) were directly analyzed, while first fractions collected in the HMW area were subjected to enzymatic hydrolysis with protease

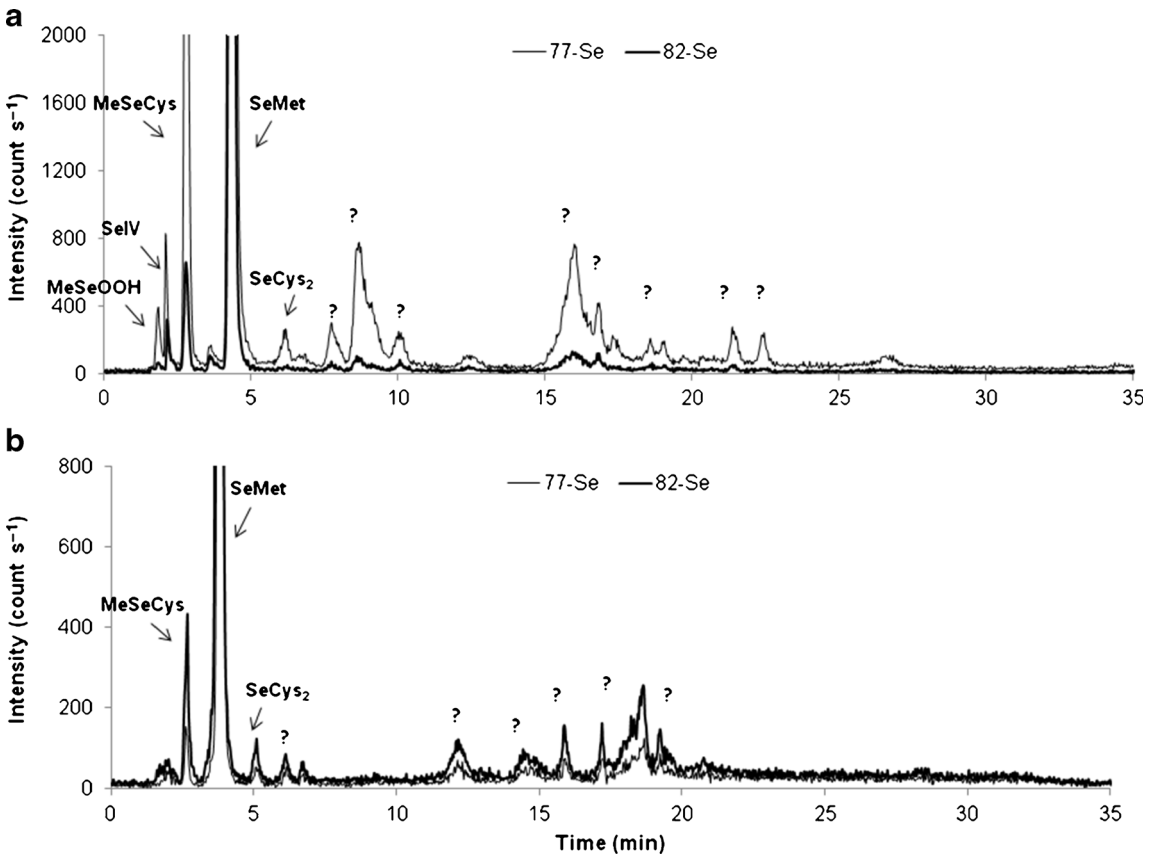

Fig. 3 PGC–HPLC-ICP MS example chromatograms of protease extracts **a** roots and **b** leaves. The *y*-scale of both figures is expanded as the high amount of SeMet would not allow showing the smaller peaks

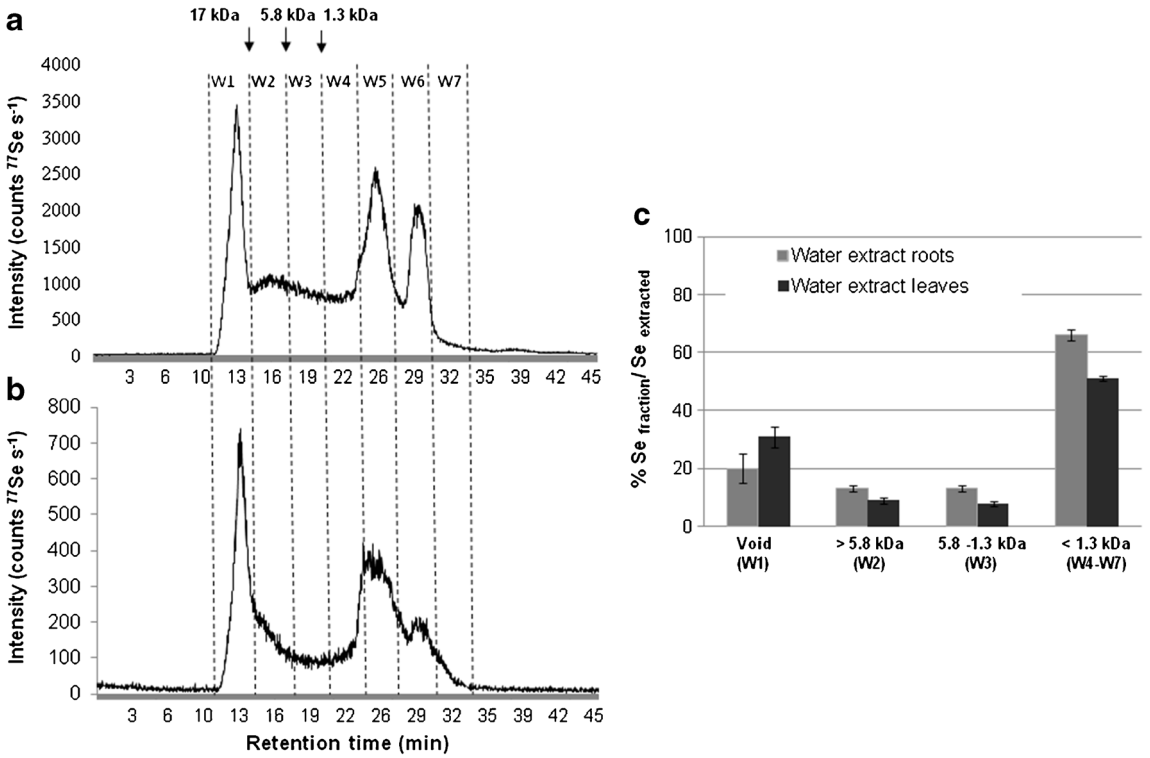

Fig. 4 SEC-ICP MS profile of ^{77}Se tracer in water extracts of: **a** roots and (**b**) leaves (*arrows* indicate the retention time of calibration molecules (myoglobine 17 kDa, insulin 5.8 kDa and vitamin B_{12} 1.35 kDa). **c** Proportion of ^{77}Se in defined molecular weight fractions

type XIV in order to release selenium species bound to proteins. For 77-Se tracer, chromatographic profiles of water extract HMW fractions (W1, W2, and W3) of roots and leaves after proteolytic digestion reveal the presence of two major peaks with retention times matching those corresponding to SeMet and $SeCys_2$ standards. A little amount of 77-selenite was detected in fraction W1 of root sample. No additional peak corresponding to unknown species could be observed. In each collected fraction above 1.3 kDa, quantification of seleno-amino acids do not account for the total ^{77}Se measured in the fraction (26–83 % of total ^{77}Se in each fraction). This result indicates that all the soluble macromolecules could not be hydrolyzed by the proteolytic digestion, suggesting the non-protein nature of these compounds and/or an incomplete digestion.

With regard to root driselase extracts, no Se-containing peaks could be detected after proteolytic digestion of fractions, suggesting a non-protein nature of Se species eluting in HMW region and indicating that dissolution of the cell walls using a mixture of pectinolytic enzymes does not release any protein. However, a significant amount of low molecular weight compounds could be released only after the destruction of the wall structure.

In the case of LMW fractions obtained from water and driselase extracts of both samples, PGC–HPLC/ICP-MS analysis confirmed the presence and amount of Se compounds previously determined by direct analysis of extracts, but no others species could be detected, probably due to their relatively low concentration in the analyzed samples.

Metabolism of $^{77}Se(IV)$ and $^{82}Se(VI)$ in ryegrass

The comparison of the proportions of Se species versus total Se uptaken by the whole plant indicates strong differences between both tracers, suggesting different metabolisms (Table 3). These results demonstrate that applied Se(VI) is less efficiently converted into organo-selenium compounds than Se(IV). Precisely, supplementation with $^{82}Se(VI)$ leads to an accumulation of Se essentially as selenate, accounting for almost 75 % of total Se in whole plant, the rest being converted into organic compounds such as SeMet (9 %) and unknown metabolites (14 %). This low conversion yield might be due to the energy-demanding reduction step of Se(VI) into Se(IV) which is required for selenate transformation into seleno-amino acids in the metabolic pathway of selenium in plants [6, 42].

Opposite to $^{82}Se(VI)$, added $^{77}Se(IV)$ is readily metabolized by the plant; actually, the remaining amount of inorganic Se(IV) corresponds to only 6 % of total Se content in plant. SeMet coming from the ^{77}Se tracer is the most abundant identified organic species, accounting for 19 % of total amount and, mostly incorporated into protein structures and to less extent as free soluble amino acid. However, the major pool of ^{77}Se (about 37 %) is represented by unknown compounds, and a large amount of the tracer remains un-extracted (32 % of total ^{77}Se in the whole plant).

Comparing the occurrence of Se metabolites and their distribution in the plant obtained by different selective extraction procedures, a possible metabolic pathway as function of the chemical species could be proposed as depicted in Fig. 5a, b for ^{82}Se and ^{77}Se, respectively. After $^{82}Se(VI)$ absorption by root via sulfate transporter [15, 17], ^{82}Se appears to be mainly translocated to the leaves with little amount remaining in the roots. ^{82}Se tracer is weakly metabolized in roots remaining essentially in form of highly mobile inorganic Se(VI), leading to significant rate of translocation. The little amount of $^{82}Se(IV)$ observed in root extracts (1 % of total ^{82}Se in roots) suggests small transformation of $^{82}Se(VI)$ into organic compounds through a reduction pathway via selenite. Actually, Se(VI) can be reduced to Se(IV) that can be converted downstream to selenide [43], a part of which is metabolized into organo compounds such as SeMet, SeCys MeSeCys and others LMW metabolites (Fig. 5a).

On the basis of chromatographic profiles and decrease in ^{77}Se abundance from root to leaf, it is suggested that Se(IV), after absorption through plant roots, is reduced into selenide that in turn is quickly transformed into organic Se before transport to leaves. SEC/ICP-MS profiles of water extracts of roots and leaves suggested incorporation of ^{77}Se tracer supplied in form of selenite to both HMW and LMW compounds. HPLC/ICP-MS analysis of fractions eluting in HMW region after proteolytic digestion indicated the incorporation of ^{77}Se tracer into soluble proteins and peptides mostly as SeMet, and the occurrence of non-protein macromolecules containing Se. The high metabolism rate into high molecular weight compounds probably restricts further translocation to the leaves of the plant which supports the total analysis results. The highest recovery of ^{77}Se tracer obtained after enzymatic hydrolysis of roots compared to water extraction indicates that exposure of the ryegrass to Se(IV) promotes its transformation into organic compounds and incorporation into proteins or molecules associated with cell wall.

Our findings are consistent with results from previous speciation studies carried out on different accumulator and not accumulators plants species supplied with Se(VI) or Se(IV). These works report that in selenate-treated plants, Se is translocated to leaves predominantly as selenate, whereas in selenite-treated plants Se is quickly converted into organic Se before transport, only a small amount of selenite being directly transported from root to leaf [12, 15, 17, 20, 44–47]. However, some differences are observed if we compare the proportion of selenite-derived compounds obtained in this study with those

Table 3 Proportion of Se species related to total Se tracer content in whole plant and plant compartments

Species	^{77}Se			^{82}Se		
	Whole plant	Roots	Leaves	Whole plant	Roots	Leaves
Se(IV)	6 %	8 %	1 %	0.5 %	1 %	0.4 %
Se(VI)	–	–	–	75 %	81 %	75 %
SeMet	19 %	12 %	36 %	9 %	8 %	10 %
$SeCys_2$	4 %	1 %	9 %	0.1 %	–	–
MeSeCys	2 %	1 %	3 %	1 %	1 %	0.1 %
MeSeOOH	0.3 %	0.1 %	0.5 %	0.1 %	–	1 %
Unknown compounds[a]	37 %	31 %	50 %	14 %	–	0.1 %
Unextracted compounds	32 %	47 %	–	–	–	–

[a] Difference between total Se and sum of identified species

obtained with accumulator plants. Namely, Se-hyperaccumulator species (*Brassica*, *Astragalus*) and secondary accumulators *(Allium)* metabolize primarily Se into various non-proteinogenic Se amino-acids such as γ-glutamyl-selenocysteine, selenocystathionine or methylselenocysteine, this latter being the most abundant organo compound (up to 80 % of total Se) [18–21]. Selenium tolerance of these plants seems to be related to the synthesis of these compounds, allowing plants to accumulate high amounts of selenium without toxicity symptoms [48]. Kotrebai et al. reported that proportions of selenium compounds in selenium-enriched samples are dependent on the total selenium concentrations, being present mainly as SeMet and γ-glutamyl-selenocysteine in samples with Se contents below 333 mg kg^{-1} and as MeSeCys in those with higher concentration [49]. Similarly, Cubadda et al. found an increasing amount of MeSeCys (until 21–68 %) with increasing Se supply in wheat [50]. In the present study, the low presence of MeSeCys (<2 %) could, thus, indicate that applied concentrations did not require a response from the ryegrass to minimize Se-induced toxicity.

Conclusion

This work presents for the first time the application of double Se-stable isotopes tracing in plant (exposed to ^{77}Se(IV) and ^{82}Se(VI)), including determination of tracers distribution and speciation analysis, in order to simultaneously monitor the bio-incorporation of two inorganic species commonly used as plant enrichment sources.

A sequential extraction protocol, targeting different Se fractions in cell plant, and based on combination of various state-of-the-art extraction procedures in plant was optimized in order to reach maximum Se recoveries and to ensure stability of species. Through the use of isotopically enriched tracers, it was shown that inorganic selenium can be taken up and biotransformed, i.e., converted into organic compounds and/or incorporated into Se-containing proteins. In general, total selenium measurement showed that supplementation with Se(VI) resulted in similar total Se level in whole plant compared to that enriched with Se(IV). However, allocation in plant compartments and metabolism of Se depends on the

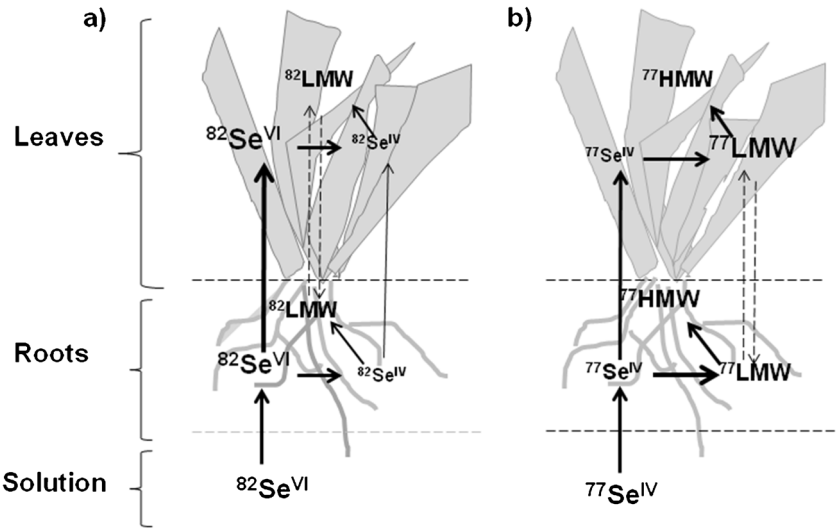

Fig. 5 Schematic overview of proposed metabolism pathway following uptake of **a** ^{82}Se(VI) and **b** ^{77}Se(IV). *Font size* is proportional to the relative concentration of the species and thickness of arrows is proportional to transport rate

nature of initial inorganic species. Selenite was found to be readily metabolized in roots into both HMW and LMW compounds and showed low mobility to leaves, while supplementation with selenate resulted in its accumulation in leaves essentially as original inorganic form. Therefore, at equal bioavailability, the degree of metabolic conversion of Se(IV) into organic species is largely higher than the one of selenate, leading to lower accumulation in leaves. To conclude, the present labeling methodology demonstrates several merits, such as an increase of detection sensitivity allowing speciation study in not accumulator plants, and the possibility of simultaneous monitoring of different species, allowing comparing biological responses to various compounds of the same element. This opens new approaches for its application to other types of plant for a better understanding of their metabolic pathways. Further investigations regarding identification by molecular mass spectrometry of unknown Se-containing peaks detected in the different extracts are required to better substantiate possible Se metabolic pathway.

Acknowledgments This work was financially supported by the National Radioactive Waste Management Agency (Andra) in the frame of its PhD program 2011 and the program "Needs Environment 2012" (CNRS-ANDRA-IRSN-EDF).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Rayman MP (2012) Selenium and human health. Lancet 379:1256–1268. doi:10.1016/S0140-6736(11)61452-9
2. Rayman MP (2005) Selenium in cancer prevention: a review of the evidence and mechanism of action. Proc Nutr Soc 64:527–542
3. Chiang EC, Shen S, Kengeri SS, Xu H, Combs GF, Morris JS, Bostwick DG, Waters DJ (2009) Defining the Optimal Selenium Dose for Prostate Cancer Risk Reduction: Insights from the U-Shaped Relationship between Selenium Status, DNA Damage, and Apoptosis. Dose-Response 8:285–300. doi:10.2203/dose–response.09-036
4. Ellis DR, Salt DE (2003) Plants, selenium and human health. Curr Opin Plant Biol 6:273–279
5. Sager M (2006) Selenium in agriculture, food, and nutrition. Pure Appl Chem. doi:10.1351/pac200678010111
6. Terry N, Zayed AM, De Souza MP, Tarun AS (2000) Selenium in higher plants. Annu Rev Plant Biol 51:401–432
7. Zhao F-J, McGrath SP (2009) Biofortification and phytoremediation. Curr Opin Plant Biol 12:373–380. doi:10.1016/j.pbi.2009.04.005
8. Lyons G, Stangoulis J, Graham R (2003) High-selenium wheat: biofortification for better health. Nutr Res Rev 16:45–60. doi:10.1079/NRR200255
9. Broadley MR, White PJ, Bryson RJ, Meacham MC, Bowen HC, Johnson SE, Hawkesford MJ, McGrath SP, Zhao F-J, Breward N, Harriman M, Tucker M (2006) Biofortification of UK food crops with selenium. Proc Nutr Soc 65:169–181
10. Ip C, Birringer M, Block E, Kotrebai M, Tyson JF, Uden PC, Lisk DJ (2000) Chemical speciation influences comparative activity of selenium-enriched garlic and yeast in mammary cancer prevention. J Agric Food Chem 48:2062–2070
11. Auger J, Yang W, Arnault I, Pannier F, Potin-Gautier M (2004) High-performance liquid chromatographic–inductively coupled plasma mass spectrometric evidence for Se-"alliins" in garlic and onion grown in Se-rich soil. J Chromatogr A 1032:103–107. doi:10.1016/j.chroma.2003.11.077
12. Nielsen GG, Gupta UC, Lamand M, Westermarck T, Nielsen GG, Gupta UC, Lamand M, Westermarck T (1984) Selenium in soils and plants and its importance in livestock and human nutrition. Adv Agron 37:397–460
13. Eurola M, Hietaniemi V (2000) Report of the Selenium Monitoring Programme 1997–1999. Agricultural Research Centre of Finland
14. Fernández-Martínez A, Charlet L (2009) Selenium environmental cycling and bioavailability: a structural chemist point of view. Rev Environ Sci Biotechnol 8:81–110. doi:10.1007/s11157-009-9145-3
15. Arvy MP (1993) Selenate and selenite uptake and translocation in bean plants (Phaseolus vulgaris). J Exp Bot 44:1083–1087. doi:10.1093/jxb/44.6.1083
16. Zayed A, Lytle CM, Terry N (1998) Accumulation and volatilization of different chemical species of selenium by plants. Planta 206:284–292
17. Li H-F, McGrath SP, Zhao F-J (2008) Selenium uptake, translocation and speciation in wheat supplied with selenate or selenite. New Phytol 178:92–102. doi:10.1111/j.1469-8137.2007.02343.x
18. Montes-Bayón M, Yanes EG, Ponce de León C, Jayasimhulu K, Stalcup A, Shann J, Caruso JA (2002) Initial studies of selenium speciation in *Brassica juncea* by LC with ICPMS and ES-MS detection: an approach for phytoremediation studies. Anal Chem 74:107–113. doi:10.1021/ac0106804
19. Mounicou S, Vonderheide AP, Shann JR, Caruso JA (2006) Comparing a selenium accumulator plant (Brassica juncea) to a nonaccumulator plant (Helianthus annuus) to investigate selenium-containing proteins. Anal Bioanal Chem 386:1367–1378. doi:10.1007/s00216-006-0707-8
20. Pedrero Z, Madrid Y (2009) Novel approaches for selenium speciation in foodstuffs and biological specimens: a review. Anal Chim Acta 634:135–152. doi:10.1016/j.aca.2008.12.026
21. Pyrzynska K (2009) Selenium speciation in enriched vegetables. Food Chem 114:1183–1191. doi:10.1016/j.foodchem.2008.11.026
22. Ogra Y, Katayama A, Ogihara Y, Yawata A, Anan Y (2013) Analysis of animal and plant selenometabolites in roots of a selenium accumulator, Brassica rapa var. peruviridis, by speciation. Metallomics 5:429–436. doi:10.1039/C2MT20187A
23. Suzuki KT, Doi C, Suzuki N (2008) Simultaneous tracing of multiple precursors each labeled with a different homo-elemental isotope by speciation analysis: distribution and metabolism of four parenteral selenium sources. Pure Appl Chem. doi:10.1351/pac200880122699
24. González Iglesias H, Fernández Sánchez ML, García Alonso JI, Sanz-Medel A (2007) Use of enriched 74Se and 77Se in combination with isotope pattern deconvolution to differentiate and determine endogenous and supplemented selenium in lactating rats. Anal Bioanal Chem 389:707–713. doi:10.1007/s00216-007-1499-1
25. Sánchez-Martínez M, Pérez-Corona T, Martínez-Villaluenga C, Frías J, Peñas E, Porres JM, Urbano G, Cámara C, Madrid Y (2014) Synthesis of [77Se]-methylselenocysteine when preparing sauerkraut in the presence of [77Se]-selenite. Metabolic transformation of [77Se]-methylselenocysteine in Wistar rats determined by LC–IDA–ICP–MS. Anal Bioanal Chem 406:7949–7958. doi:10.1007/s00216-014-8224-7
26. Collins RN, Tran ND, Bakkaus E, Avoscan L, Gouget B (2006) Assessment of isotope exchange methodology to determine the sorption coefficient and isotopically exchangeable concentration

of selenium in soils and sediments. Environ Sci Technol 40:7778–7783. doi:10.1021/es061528s
27. Tolu J, Di Tullo P, Le Hécho I, Thiry Y, Pannier F, Potin-Gautier M, Bueno M (2014) A new methodology involving stable isotope tracer to compare simultaneously short- and long-term selenium mobility in soils. Anal Bioanal Chem 406:1221–1231. doi:10.1007/s00216-013-7323-1
28. Cartes P, Gianfreda L, Mora ML (2005) Uptake of selenium and its antioxidant activity in ryegrass when applied as selenate and selenite forms. Plant Soil 276:359–367. doi:10.1007/s11104-005-5691-9
29. Hopper JL, Parker DR (1999) Plant availability of selenite and selenate as influenced by the competing ions phosphate and sulfate. Plant Soil 210:199–207
30. Munier-Lamy C, Deneux-Mustin S, Mustin C, Merlet D, Berthelin J, Leyval C (2007) Selenium bioavailability and uptake as affected by four different plants in a loamy clay soil with particular attention to mycorrhizae inoculated ryegrass. J Environ Radioact 97:148–158. doi:10.1016/j.jenvrad.2007.04.001
31. Van Dael P, Lewis J, Barclay D (2004) Stable isotope-enriched selenite and selenate tracers for human metabolic studies: a fast and accurate method for their preparation from elemental selenium and their identification and quantification using hydride generation atomic absorption spectrometry. J Trace Elem Med Biol 18:75–80. doi:10.1016/j.jtemb.2004.04.005
32. Longchamp M, Angeli N, Castrec-Rouelle M (2013) Selenium uptake in Zea mays supplied with selenate or selenite under hydroponic conditions. Plant Soil 362:107–117. doi:10.1007/s11104-012-1259-7
33. Hintelmann H, Evans RD (1997) Application of stable isotopes in environmental tracer studies—measurement of monomethylmercury (CH3Hg+) by isotope dilution ICP-MS and detection of species transformation. Fresenius J Anal Chem 358:378–385. doi:10.1007/s002160050433
34. Webster RK (1960) Methods of Geochemistry. A.A. Smales and L.R. Wagner
35. Hinojosa Reyes L, Marchante Gayn JM, Garca Alonso JI, Sanz-Medel A (2003) Determination of selenium in biological materials by isotope dilution analysis with an octapole reaction system ICP-MS. J Anal At Spectrom 18:11–16. doi:10.1039/b209213a
36. Encinar JR, Alonso JIG, Sanz-Medel A, Main S, Turner PJ (2001) A comparison between quadrupole, double focusing and multicollector ICP-MS instruments. J Anal At Spectrom 16:315–321. doi:10.1039/B006145J
37. Shah M, Kannamkumarath SS, Wuilloud JCA, Wuilloud RG, Caruso JA (2004) Identification and characterization of selenium species in enriched green onion (Allium fistulosum) by HPLC-ICP-MS and ESI-ITMS. J Anal At Spectrom 19:381. doi:10.1039/b312320k
38. Montesbayon M, Molet M, Gonzalez E, Sanzmedel 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. doi:10.1016/j.talanta.2005.07.040
39. Bitterli C, Bañuelos GS, Schulin R (2010) Use of transfer factors to characterize uptake of selenium by plants. J Geochem Explor 107:206–216. doi:10.1016/j.gexplo.2010.09.009
40. Zhang Y, Pan G, Chen J, Hu Q (2003) Uptake and transport of selenite and selenate by soybean seedlings of two genotypes. Plant Soil 253:437–443
41. Mazej D, Osvald J, Stibilj V (2008) Selenium species in leaves of chicory, dandelion, lamb's lettuce and parsley. Food Chem 107:75–83. doi:10.1016/j.foodchem.2007.07.036
42. de Souza MP, Lytle CM, Mulholland MM, Otte ML, Terry N (2000) Selenium Assimilation and Volatilization from Dimethylselenoniopropionate by Indian Mustard. Plant Physiol 122:1281–1288
43. Pilon-Smits EAH, Quinn CF (2010) Selenium Metabolism in Plants. In: Hell R, Mendel R-R (eds) Cell Biol. Met. Nutr. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 225–241
44. de Souza MP, Pilon-Smits EAH, Lytle CM, Hwang S, Tai J, Honma TSU, Yeh L, Terry N (1998) Rate-limiting steps in selenium assimilation and volatilization by Indian mustard. Plant Physiol 117:1487–1494
45. Wróbel K, Wróbel K, Kannamkumarath SS, Caruso JA, Wysocka IA, Bulska E, Świa tek J, Wierzbicka M (2004) HPLC–ICP-MS speciation of selenium in enriched onion leaves—a potential dietary source of Se-methylselenocysteine. Food Chem 86:617–623. doi:10.1016/j.foodchem.2003.11.005
46. Ximénez-Embún P, Alonso I, Madrid-Albarrán Y, Cámara C (2004) Establishment of selenium uptake and species distribution in lupine, Indian mustard, and sunflower plants. J Agric Food Chem 52:832–838. doi:10.1021/jf034835f
47. Kápolna E, Shah M, Caruso JA, Fodor P (2007) Selenium speciation studies in Se-enriched chives (Allium schoenoprasum) by HPLC-ICP–MS. Food Chem 101:1398–1406. doi:10.1016/j.foodchem.2006.03.048
48. Neuhierl B, Thanbichler M, Lottspeich F, Böck A (1999) A family of S-methylmethionine-dependent thiol/selenol methyltransferases. Role in selenium tolerance and evolutionary relation. J Biol Chem 274:5407–5414
49. Kotrebai M, Tyson JF, Uden PC, Birringer M, Block E (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. doi:10.1039/a906320j
50. Cubadda F, Aureli F, Ciardullo S, D'Amato M, Raggi A, Acharya R, Reddy RAV, Prakash NT (2010) Changes in selenium speciation associated with increasing tissue concentrations of selenium in wheat grain. J Agric Food Chem 58:2295–2301. doi:10.1021/jf903004a