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# Quantification of *Se*-Methylselenocysteine and Its γ-Glutamyl Derivative from Naturally Se-Enriched Green Bean (*Phaseolus vulgaris vulgaris*) After HPLC-ESI-TOF-MS and Orbitrap $MS^n$-Based Identification

**Shuxun Shao · Xiubo Mi · Laurent Ouerdane · Ryszard Lobinski · Juan Francisco García-Reyes · Antonio Molina-Díaz · Andrea Vass · Mihály Dernovics**

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**Abstract** Orthogonal liquid chromatographic (ion exchange, reversed phase, and ion pairing) and mass spectrometric [electrospray ionization (ESI)-TOF-MS, ESI-Orbitrap MS, and inductively coupled plasma mass spectrometry (ICP-MS)] methods were addressed to identify and quantify selenium species from a naturally Se-enriched green bean (*Phaseolus vulgaris vulgaris*) sample after proteolytic digestion. While selenomethionine (10.1 mg/kg as Se) and selenate (9.5 mg/kg as Se) could be quantified in a straightforward way by anion exchange LC-ICP-MS technique, a multistep purification protocol was required to identify *Se*-methylselenocysteine and γ-glutamyl-*Se*-methylselenocysteine in an unambiguous way prior to quantification by using either in-source fragmentation (LC-ESI-TOF-MS) or collision-induced dissociation (LC-ESI-Orbitrap MS). Finally, *Se*-methylselenocysteine (2.6 mg/kg as Se) and γ-glutamyl-*Se*-methylselenocysteine (1.2 mg/kg as Se) could contribute to the overall selenium recovery of 72 %. This sample is the first of the Faboideae subfamily and *Phaseolus* ssp. to be speciated to such an extent for selenium including γ-glutamyl-*Se*-methylselenocysteine, a highly potential selenium species, which makes this bean material an ideal candidate for functional food purposes.

**Keywords** Selenium · Speciation · Orbitrap · *Phaseolus* · γ-Glutamyl-*Se*-methylselenocysteine

S. Shao · X. Mi
State Key Laboratory of Ore Deposit Geochemistry, Institute of Geochemistry, Chinese Academy of Sciences, 46 Guanshui Road, Guiyang 550002, China

X. Mi
College of Earth Science, University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China

L. Ouerdane · R. Lobinski
Laboratoire de Chimie Analytique Bio-Inorganique et Environnement, UMR 5254, CNRS/UPPA, Hélioparc, 2 av. Pierre Angot, 64053 Pau, France

J. F. García-Reyes · A. Molina-Díaz
Analytical Chemistry Research Group, Department of Physical and Analytical Chemistry, University of Jaén, Campus Las Lagunillas, Edif B3, 23071 Jaén, Spain

A. Vass · M. Dernovics (✉)
Department of Applied Chemistry, Corvinus University of Hungary, Villányi út 29-33, 1118 Budapest, Hungary
e-mail: mihaly.dernovics@uni-corvinus.hu

R. Lobinski
Department of Chemistry, Warsaw University of Technology, Noakowskiego 3, 00-664 Warsaw, Poland

## Introduction

One of the most promising challenges of selenium speciation is to pinpoint and quantify Se species with supposed anticancer effects (Brozmanová et al. 2010; Rayman et al. 2008). The SELECT trial which stopped in 2008 (Lippman et al. 2009) concluded that selenomethionine, the most studied organic selenium compound, did not possess direct anticancer properties in the case of selenium-replete individuals. Therefore, attention should be focused, on one hand, on identifying Se compounds that could be responsible for successful clinical trials (Lippman et al. 2005) and, on the other hand, on showing which food and food-related matrices contain other potentially more active Se species, such as *Se*-methylselenocysteine (chosen for an actual clinical trial of the National Cancer Institute;

ClinicalTrials.gov identifier: NCT01497431) and γ-Glu-*Se*-methylselenocysteine (Ip et al. 2000; Stan et al. 2008).

Concerning the second option, i.e., to analyze food matrices for targeted Se species, the synergic use of elemental and molecular mass spectrometric instrumentation is almost inevitable. Indeed, identification on the basis of co-elution of standards and assigned chromatographic peaks in a high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC-ICP-MS) analysis does not provide an unambiguous prove on the identity of Se species (Ogra 2008; Ogra and Anan 2009). Misidentification of selenium compounds (e.g., selenocysteine) and the detection of artifacts are considered an important topic in this field (Dernovics and Lobinski 2008; Dernovics et al. 2006). Clearly, when aiming at the identification of unknown or less abundant Se species, a consecutive process of three or even four chromatographic HPLC-ICP-MS cleanup steps prior to HPLC-high-resolution-electrospray ionization (ESI)-MS analysis might be also required (Dernovics et al. 2007). The application of this multistep approach is therefore a function of species concentration and the complexity of the matrix (Szpunar and Lobinski 2002). Besides, samples with artificially increased selenium content, e.g., high-selenium yeast and high-selenium garlic (more than hundreds of milligram Se per kilogram), are usually addressed to provide a final species concentration that is high enough to monitor throughout the complete cleanup procedure.

There has also been a continuous effort to characterize less exceptional samples from selenium speciation point of view in order to facilitate the access to adequate selenium intake through staple food, which often involves a labor-intensive sample preparation approach. In the case of low-selenium meat and offal samples, multistep sample preparation including the unusual derivatization and cleanup of selenomethionine was required to achieve reproducible quantitative results (Bierla et al. 2008). Studies on plant matrices with naturally moderate selenium content such as lentils, wheat, and rice usually end up in limited speciation information because of the low individual concentration of species and in quantifying selenomethionine as the most frequently identified species (Hart et al. 2011; Pedrero et al. 2007; Premarathna et al. 2012). At the same time, the assignment of selenium species from samples with moderate selenium content might be advanced if the species have previously been identified from the same types of matrices with higher selenium concentration.

In our study, a naturally high-selenium green bean (*Phaseolus vulgaris*) sample was addressed. This plant is not a selenium hyperaccumulator; its usual selenium concentration hardly reaches 0.08 mg $kg^{-1}$ dry weight (d.w.), and artificial Se enrichment (e.g., with foliar spraying) can only slightly increase this level to around 2 mg $kg^{-1}$ d.w., rendering selenium speciation difficult without applying mass spectrometry-based instrumentation (Smrkolj et al. 2007). On the other hand, when naturally grown in high-selenium soils, *P. vulgaris* can be the highest selenium-containing edible vegetable (Huang et al. 2013). Therefore, our goal was to identify and quantify the selenium species from this plant matrix with the comprehensive use of HPLC-ICP-MS and ESI-MS methods.

## Experimental

### Reagents and Standards

Standard solutions (1.000 g $L^{-1}$) of Se and Rh, Pronase E enzyme (4,000 PU $mg^{-1}$), $H_2O_2$ (analytical reagent grade (a.r.), 30 m/m%), and $HNO_3$ (a.r., >65 m/m%) were ordered from Merck (Darmstadt, Germany). Gradient grade methanol and acetonitrile (ACN) were bought from VWR Fisher Scientific (VWR International, Budapest, Hungary). Ammonium acetate (a.r.), Tris-hydroxymethyl-aminomethane (Tris; a.r.), and HCl (37 m/m%) were purchased from Reanal (Budapest, Hungary). Sodium selenate, D,L-selenomethionine, *Se*-methylselenocysteine, HCOOH (~98 %, puriss), dithiothreitol (DTT), and heptafluorobutyric acid (HFBA) were purchased from the Sigma-Aldrich Group (Schnelldorf, Germany). γ-Glu-*Se*-methylselenocysteine was purchased from PharmaSe (Lubbock, TX, USA). Milli-Q water (18.2 MΩ/cm, Millipore, Molsheim, France) was used throughout.

### Sample

The green bean (*P. vulgaris vulgaris*) sample was harvested in the natural seleniferous region of Jianshi County, Enshi, China. The sample was cleaned, dried, milled, and homogenized.

### Instrumentation

For sample preparation purposes, the Hielscher UP100H ultrasonic probe (Teltow, Germany) was used with full cycle time and amplitude of 100 %. The samples were digested using the CEM Mars-5 microwave unit equipped with HP-500 vessels (CEM, Matthews, NC, USA). ICP-MS Agilent 7500cs (Agilent, Santa Clara, CA, USA) was used to monitor the isotopes of $^{77}Se$ and $^{82}Se$ for identification/quantification purposes and $^{103}Rh$ to correct for instrumental drifts. The instrument was coupled to the Agilent 1200 HPLC system. The HPLC-ICP-MS analysis was executed with the use of 5 % $O_2$ as optional gas (50 ml $min^{-1}$) in the case of organic solvent-based eluents.

For the screening of selenium species, the Agilent 6220 Accurate-Mass ESI-TOF-MS was used with a dual ion spray source. The related instrumental parameters are summarized in Table 1.

For the identification of selenium compounds including fragmentation studies, the Accela High Speed Liquid Chromatograph (Thermo Fisher Scientific, Waltham, MA, USA)

**Table 1** Instrumental parameters of the ESI-TOF-MS setup

| 6220 Accurate Mass ESI-TOF-MS (Agilent) | |
|---|---|
| ESI source | Dual ESI (Agilent) |
| Operational mode | Positive |
| Mass accuracy | <2 ppm |
| Mass resolution | >10,000 |
| Detection frequency | 4 GHz |
| Fragmentor voltage | 150 V |
| Curtain voltage | 65 V |
| Drying gas | 13 L/min |
| Capillary voltage | 800 V |
| Nebulizer pressure | 40 psig |
| Gas temperature | 325 °C |
| Data analysis software | Mass Hunter Acquisition B.02.01 with SP3 |
| | Mass Hunter Qualitative Analysis B.03.01 with SP3 |

system was connected to a hybrid linear ion trap/Orbitrap mass analyzer (LTQ-Orbitrap Velos; Thermo Fisher Scientific) used in either full scan mode or in product ion ($MS^n$) mode. The instrument was equipped with an Ion Max ESI electrospray ion source (Thermo Fisher Scientific). The ion source was operated in the positive ion mode at 4 kV. Capillary temperature was set to 300 °C. Nitrogen sheath gas was set to 15 units, while the auxiliary and sweep gases were set to 0 unit. The resolving power of the Orbitrap (full-width half-height, FWHM) was set to nominal 60,000 (at $m/z=400$; 1-s scan cycle time) in full scan mode. For the $MS^n$ experiments, the product ions from the $[M+H]^+$-charged target ions were generated in the LTQ trap at a collision energy setting of 35 % and using an isolation width of 2 Da. The data recorded from either 100.00 (full scan mode) or 60.00 ($MS^n$ mode) to 1000.00 were processed with Xcalibur 2.0 software (Thermo Fisher Scientific).

Both instruments were tuned according to the manufacturers' guidelines.

## Enzymatic Sample Preparation

Two hundred fifty milligrams of dried and milled bean sample was mixed with 5.0 ml Tris buffer (pH 6.8, 0.1 M) and 50 mg of Pronase E. The sample was shaken overnight at 37 °C, and then, another 50 mg of enzyme was dissolved in 3.0 ml buffer, added to the sample, and shaken for 24 h. The supernatant was decanted and made up to 10.0 ml with deionized water in a volumetric flask and filtered through 0.45-μm PTFE disposable syringe filters.

## Determination of Total Selenium Content

To determine the total selenium content in the bean sample and in the remaining drags after the enzymatic digestion, the samples were digested with 5.0 ml nitric acid and 3.0 ml of $H_2O_2$ using the following program: at 0–15 min, the pressure raised to 250 psi; at 15–35 min, it was kept at 250 psi; and at 35–50 min, it was cooled down to 70 psi. The total Se concentration was determined with the Agilent 7500cs ICP-MS on the $^{77}Se$ and $^{82}Se$ isotopes by the method of standard addition using Rh as the internal standard.

## Strong Anion Exchange Chromatography for the Characterization and Quantification of Selenium Species

The PRP-X100 SAX column (250 mm×4.1 mm×10 μm; Hamilton, Reno, NV, USA) fitted with a matching guard column filled with the identical phase was used. Gradient elution was made with ammonium acetate (buffer A, 25 mM; buffer B, 250 mM; pH 5.5) delivered at 1.5 ml $min^{-1}$. The program was as follows: 0–4 min, 100 % A; 4–20 min, up to 100 % B; 20–

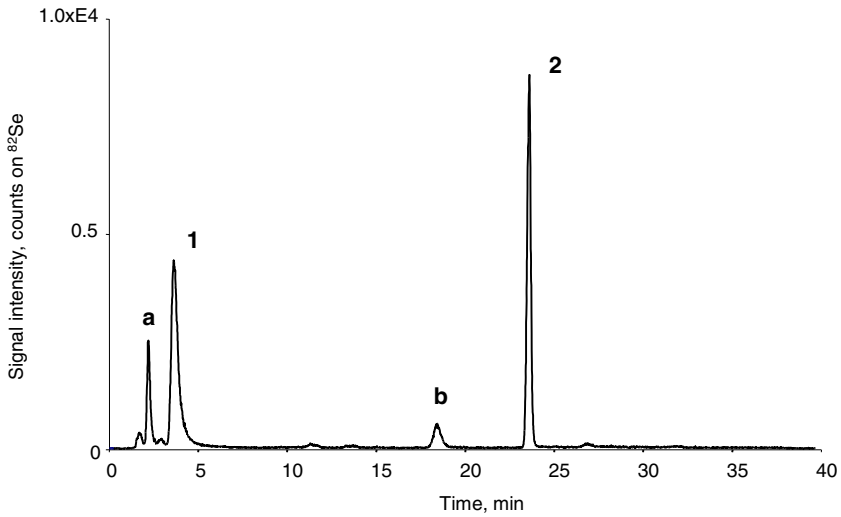



**Fig. 1** SAX-ICP-MS chromatogram of the enzymatically digested bean sample. *1* Selenomethionine. *2* Selenate. Peaks assigned with *letters* (*a*, *b*) were collected for further purification and identification purposes

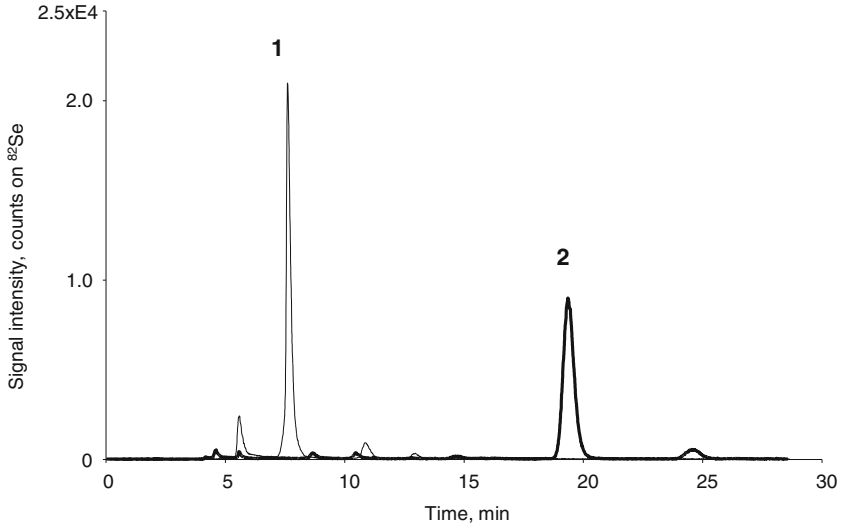



**Fig. 2** RP-HPLC-ICP-MS cleanup chromatograms of the two fractions collected from the SAX separation. The *thin line* and the abundant compound (*1*) arise from the peak *a* of the SAX separation, while the *thick line* and the compound (*2*) arise from the peak *b*

30 min, 100 % B; and 30–32 min, down to 0 % B. The sample was dissolved in 0.5 ml of buffer A. Injection volume was either 50 μl (for mapping purposes, quantification, and column recovery determination) or 100 μl (for fraction collection).

Selenomethionine and selenate were individually quantified using the method of three-point standard addition. Four aliquots of 100 μl of the enzymatic digested samples were mixed with 50 μl 0.1 m/m% DTT and standard solutions of 0, 2, 5, and 10 ng selenomethionine (as Se), respectively. All four solutions were made up to 1 ml using eluent A. In the case of selenate, the quantification was carried out with the same standard addition procedure except for using DTT.

Concerning the two unidentified Se-containing peaks, the related fractions were collected separately, frozen, and lyophilized.

## Reversed Phase-HPLC-ICP-MS Cleanup of Selenium Compounds Prior to ESI-MS Analysis

The two peaks with the unidentified selenium compounds were cleaned up with a HPLC-ICP-MS setup. The column was an XTerra MS18 (4.6 mm×250 mm×5 μm; Waters, Milford, MA, USA) used in isocratic mode. The eluent was 5 V/V% ACN with 0.1 V/V% HCOOH in Milli-Q water. Detection was carried out with the 7500 cs ICP-MS on $^{77}$Se and $^{82}$Se. The fractions collected from the strong anion exchange chromatography (SAX) separation of the enzymatic digested sample were dissolved in 500 μl of the HPLC eluent, and then, 50 μl aliquots were injected for characterization and fraction collection.

## HPLC-ESI-TOF-MS Analysis

The dried samples were dissolved in 200 μl of 0.1 V/V% HCOOH in Milli-Q water, and 10 μl was injected on the Zorbax SB-$C_{18}$ column (Agilent; 4.6 mm×150 mm×5 μm). Flow rate was 450 μl min$^{-1}$. Eluent A was 0.1 V/V% HCOOH in Milli-Q water, and B was 0.1 V/V% HCOOH in ACN. The gradient was as follows: 0–5 min, 5 % B; 5–15 min, up to 50 % B; 15–20 min, up to 100 % B; 20–25 min, 100 % B; 25–26, down to 5 % B; and 26–32 min, 5 % B.

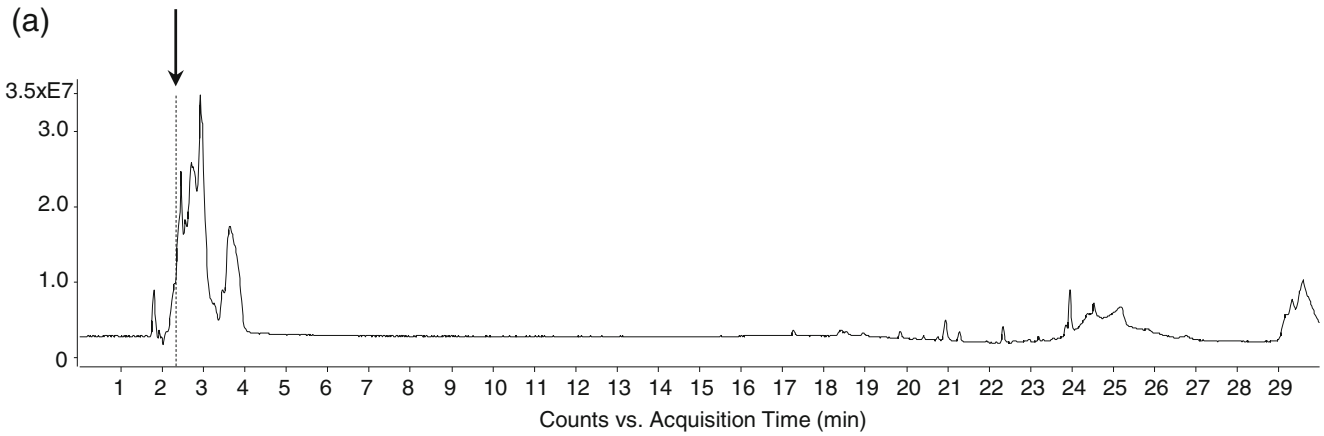
## HPLC-ESI-Orbitrap MS Analysis

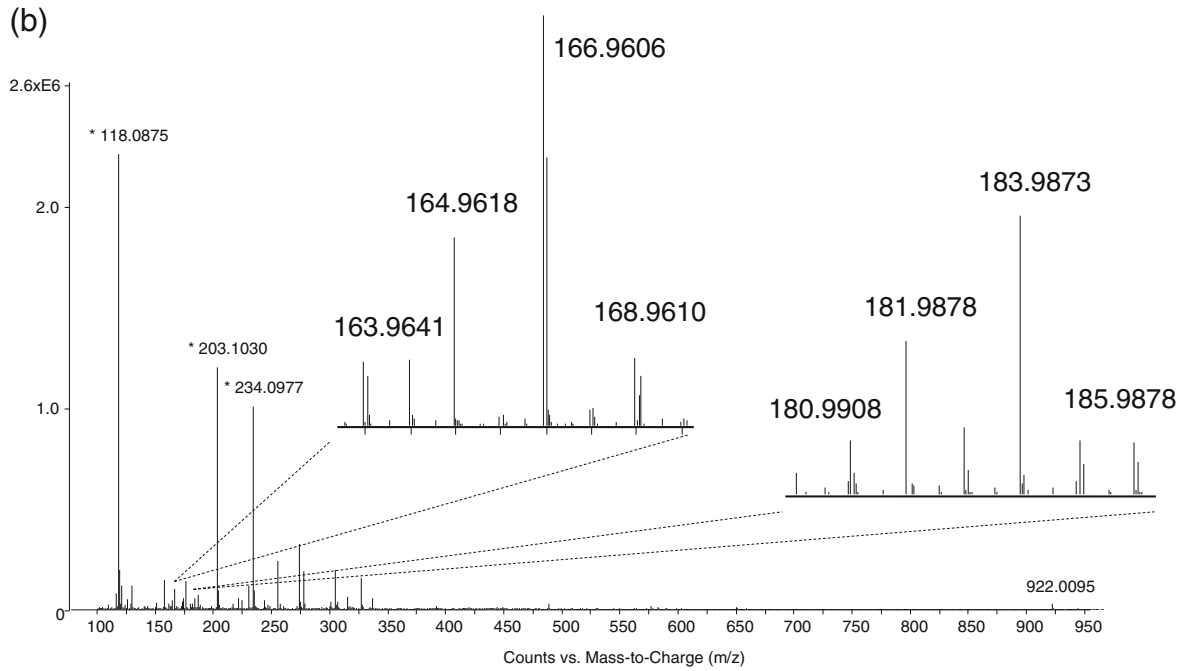
The dried samples were dissolved in 200 μl of 0.1 V/V% HCOOH in Milli-Q water, and 10 μl was injected on the Zorbax SB-$C_{18}$ column. Flow rate was 400 μl min$^{-1}$. Eluent A was 0.1 V/V% HCOOH in Milli-Q water, and B was 0.1 V/V% HCOOH in ACN. The gradient was as follows: 0–5 min, 5 % B; 5–15 min, up to 50 % B; 15–20 min, up to 100 % B; 20–25 min, 100 % B; 25–26, down to 5 % B; and 26–32 min, 5 % B.

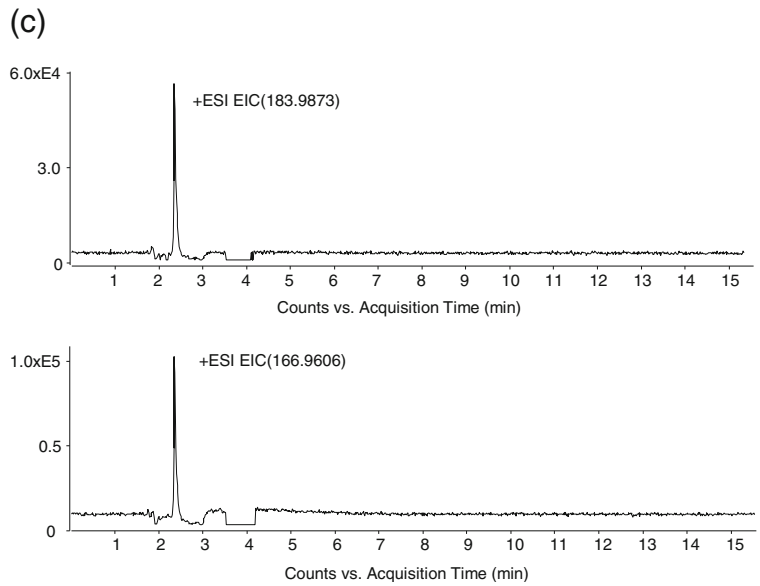
## Quantification of *Se*-Methylselenocysteine

*Se*-methylselenocysteine was quantified using the method of three-point standard addition with an ion-pairing reversed phase HPLC-ICP-MS setup. Eluent A was 0.1 V/V% HFBA in Milli-Q water, while eluent B was 0.1 V/V% HFBA in methanol. The corresponding fraction collected from the SAX separation of the enzymatic digested sample was dissolved in

**Fig. 3** **a** HPLC-ESI-TOF-MS total ion chromatogram of the fraction collected at the theoretical retention time of *Se*-methylselenocysteine from the SAX-HPLC separation (see the peak *a* on Fig. 1 and the *thin line* on Fig. 2). The *dashed line* and the *arrow* indicate the $t_R$=2.35 min. **b** The full scan spectrum at $t_R$=2.35 min indicates the presence of selenium-containing molecules at *m/z* 183.9873 ($C_4H_{10}NO_2Se^+$, $[M+H]^+$, $\delta$=0.93 ppm) and at *m/z* 166.9606 ($C_4H_7O_2Se^+$, $[M+H]^+$, $\delta$=0.12 ppm). **c** Extracted ion chromatograms (*EIC*) of the selenium-containing molecules. The matching shapes indicates that the compound could be identified as *Se*-methylselenocysteine


(a)
3.5xE7
3.0
2.0
1.0
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Counts vs. Acquisition Time (min)


(b)
2.6xE6
2.0
1.0
0
* 118.0875
166.9606
164.9618
163.9641
168.9610
* 203.1030
* 234.0977
183.9873
181.9878
180.9908
185.9878
922.0095
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950
Counts vs. Mass-to-Charge (m/z)


(c)
6.0xE4
3.0
0
+ESI EIC(183.9873)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Counts vs. Acquisition Time (min)
1.0xE5
0.5
0
+ESI EIC(166.9606)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Counts vs. Acquisition Time (min)

500 μl water, and then, 100 μl aliquots were mixed with 50 μl of 0.1 m/m% DTT and standard solutions of 0, 0.2, 0.5, and 1.0 ng *Se*-methylselenocysteine (as Se), respectively. All four solutions were made up to 1 ml using eluent A.

One hundred microliters of mixture was then injected onto the Thermo Hypersil column (Keystone $C_{18}$; 250 mm×4.6 mm× 5 μm; Thermo Fisher Scientific, Pittsburgh, PA, USA). The gradient was as follows: 0–5 min, 5 % B; 5–15 min, 50 % B; 15–20 min, 50 % B; 20–21 min, 5 % B; and 21–28 min, 5 % B. Flow rate was 0.9 ml $min^{-1}$. The column temperature was set to 30 °C.

## Results and Discussion

### Total Selenium Content

The bean sample contained 32.4 mg $kg^{-1}$ Se d.w. This value is higher than the concentration level achieved by Smrkolj et al. (2007) for *P. vulgaris* by foliar spraying and is higher than the concentration reported for an unspecified kidney bean sample from the same geographic region (Huang et al. 2013). This level of Se provides the possibility for the reliable detection and quantification of selenium species.

### SAX-Based Characterization and Quantification of Selenium Species

The quantification of selenomethionine after protease XIV-based hydrolysis followed by SAX HPLC-ICP-MS coupling is a well-established procedure and referenced by the EFSA and several publications (EFSA 2012; Polatajko et al. 2005). Also, selenate can be unambiguously quantified with this technique due to the adequate retention on anion exchange systems (Kozak et al. 2012; Mounicou et al. 2009; Pedrero and Madrid 2009).

As shown in Fig. 1, the SAX-ICP-MS chromatogram of the enzymatically digested bean sample is dominated by selenomethionine (10.1 mg/kg as Se) and selenate (9.5 mg/kg as Se). The presence of selenomethionine in selenium-enriched *P. vulgaris* beans and other bean plants in the Faboideae subfamily such as soybean (*Glycine max*) and lima bean (*Phaseolus lunatus*) has been reported by Smrkolj et al. (2007), Chan et al. (2010), and Nigam and McConnell (1973) and indirectly assigned by Cao et al. (2012). The accumulation of inorganic selenium in the edible part of the plant is regarded as a consequence of the insufficient rate of selenium metabolism, and it is reported in several mushroom and plant species (Dernovics et al. 2002; Ximénez-Embún et al. 2004).

The enzymatic extraction recovery was 77 %, while the two most abundant species accounted for 60 % of total selenium content of the bean sample. The quantification of the unassigned peaks on the SAX chromatogram could have theoretically completed the selenium mass balance. According to the reports on selenized bean samples, *Se*-methylselenocysteine is frequently synthesized by the Faboideae subfamily (Nigam and McConnell 1973; Smrkolj et al. 2006; Thavarajah et al. 2007); therefore, its presence should be considered. Indeed, the retention time ($t_R$=2.51 min) of the first unassigned peak matched that of *Se*-methylselenocysteine, but quantifying this species close to the void volume of the column could have resulted in biased results. The other unassigned peaks of the SAX chromatogram eluting at 18.22 min did not match the retention time of any other species previously detected in bean samples. The two unidentified peaks were thus recovered by fractionation for further purification by reversed phase chromatography as it possesses high orthogonality after SAX to provide sufficient separation from the matrix.

### Identification of *Se*-Methylselenocysteine and γ-Glu-*Se*-Methylselenocysteine by HPLC-ESI-TOF-MS and HPLC-ESI-Orbitrap MS

Figure 2 presents the reversed phase (RP)-HPLC-ICP-MS cleanup of the two fractions. Both fractions contained several low abundant selenium species that were co-eluting during the SAX separation, leaving one highly abundant species in each fraction that could now be adequately purified for electrospray mass spectrometry-based identification.

Figure 3a shows the HPLC-ESI-TOF-MS total ion chromatogram (TIC) of the fraction collected at the theoretical retention time of *Se*-methylselenocysteine from the SAX-HPLC separation. The full scan spectra were both manually searched for the characteristic isotope pattern of selenium, and database-directed search for known selenium species was also run. The only hit was found at *m*/*z* 183.9873 ($C_4H_{10}NO_2Se^+$, $[M+H]^+$, $\delta$=0.93 ppm) at the retention time of 2.35 min. As the typical in-source fragment at *m*/*z* 166.9606 ($C_4H_7O_2Se^+$, $\delta$=0.12 ppm) (Infante et al. 2004) was also present (Fig. 3b) with the identically shaped extracted ion chromatogram (EIC) compared to the ion *m*/*z* 183.9873 (Fig. 3c), the compound could be identified as *Se*-methylselenocysteine.

Figure 4a presents the TIC of the HPLC-TOF-MS of the fraction collected at 18.22 min from the SAX-HPLC separation. The screening of the full scan spectra resulted in only one selenium-containing compound, eluting at 4.42 min, with the mass-to-charge ratio of 313.0300 (see Fig. 4b for the full scan spectrum and Fig. 4c for the EIC). This *m*/*z* value and the isotopic pattern can relate to two previously described compounds, γ-Glu-*Se*-methylselenocysteine and *N*-acetylselenocystathionine with the same elemental composition ($C_9H_{17}N_2O_5Se^+$, $[M+H]^+$, $\delta$=0.89 ppm). None of these compounds have been found in any Faboideae plant; γ-Glu-*Se*-methylselenocysteine has been reported from selenized yeast, *Allium* ssp., and hyperaccumulator plants (Kotrebai et al. 2000; McSheehy et al. 2000; Nigam and McConnell 1969), while *N*-

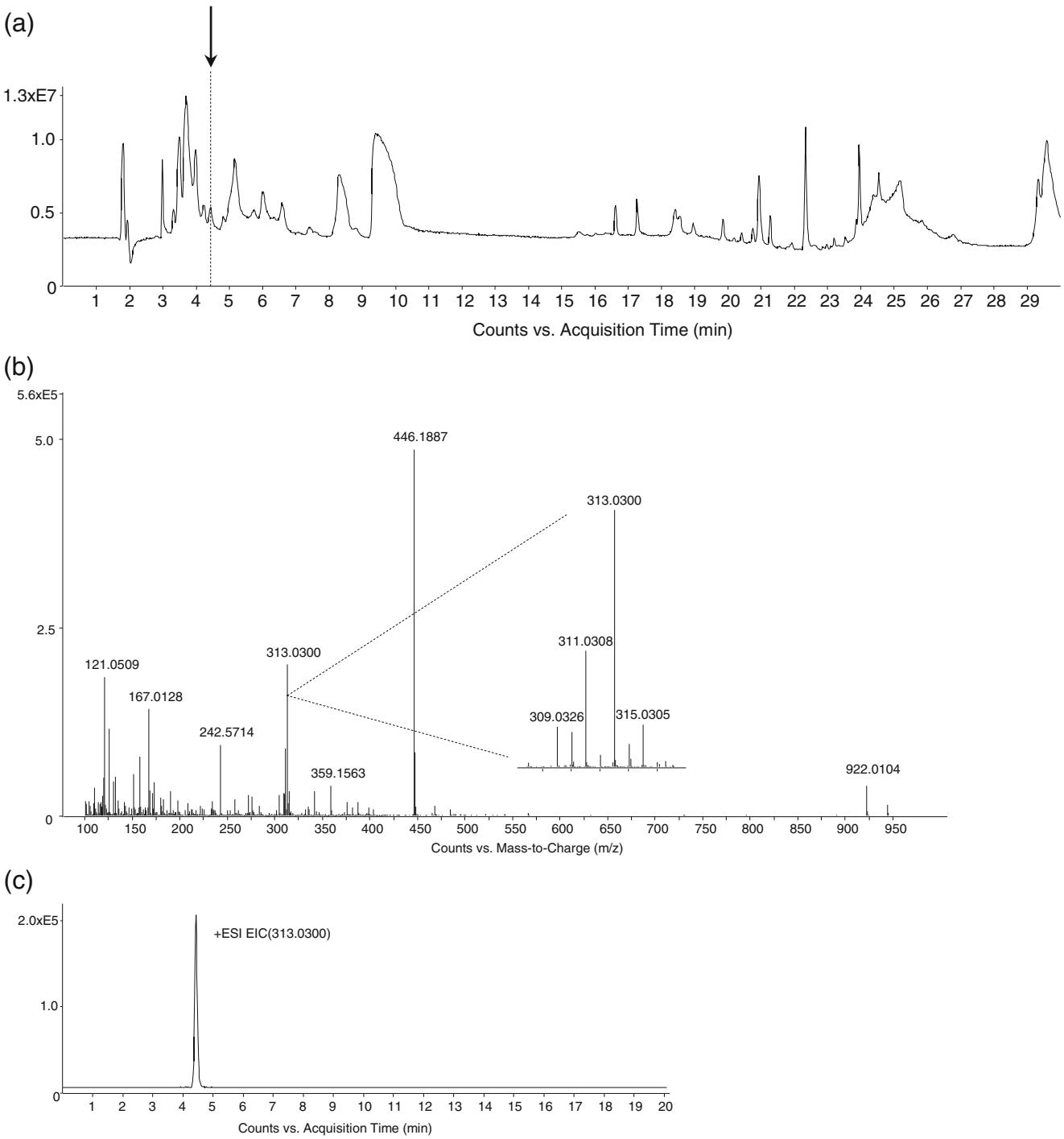
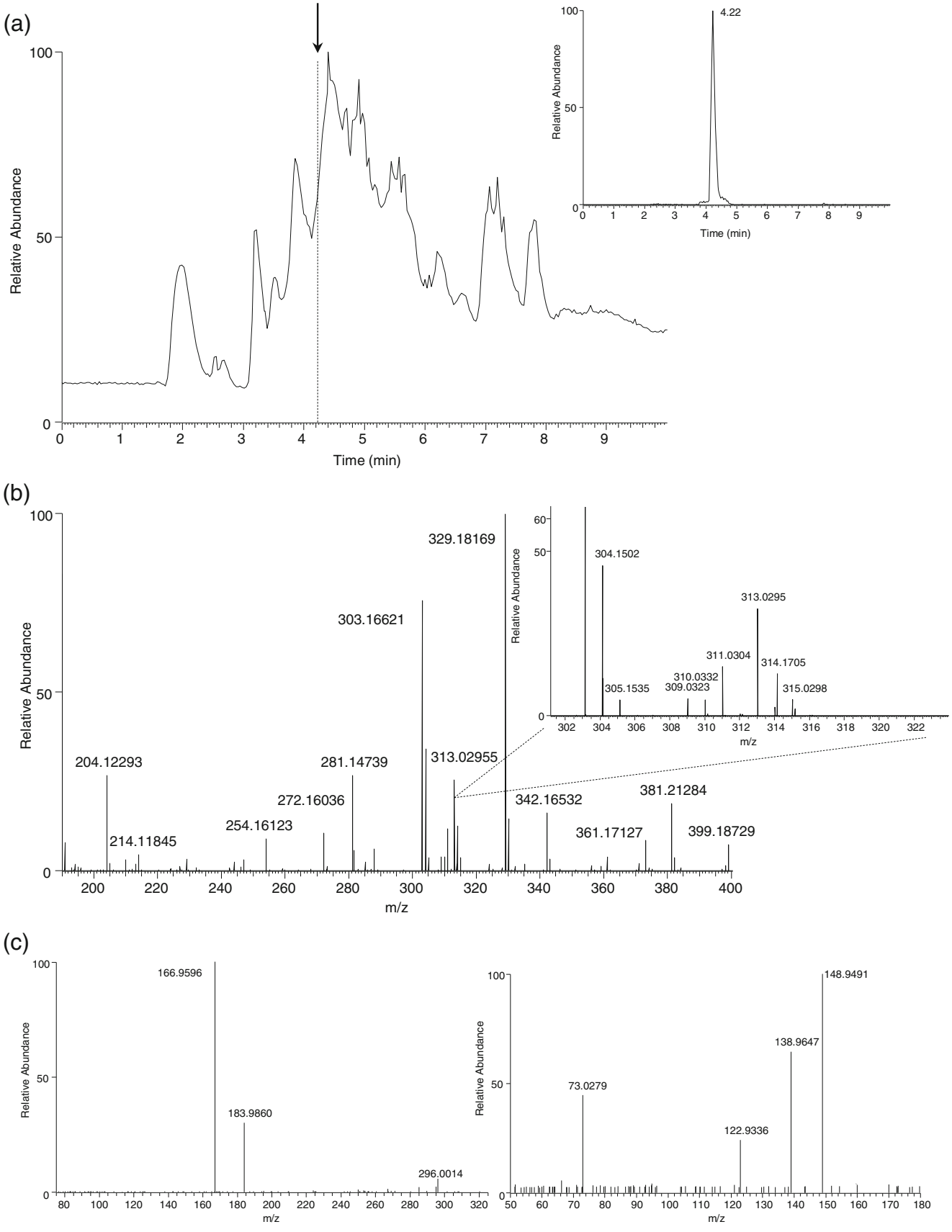



**Fig. 4** **a** HPLC-ESI-TOF-MS total ion chromatogram of the fraction collected from the SAX-HPLC separation (see the peak *b* on Fig. 1 and the *thick line* on Fig. 2). The *dashed line* and the *arrow* indicate the $t_R$=4.42 min. **b** The full scan spectrum at $t_R$=4.42 min indicates the presence of a selenium-containing compound at *m/z* 313.0300 ($C_9H_{17}N_2O_5Se^+$, $[M+H]^+$, $\delta$= 0.89 ppm). **c** EIC of the selenium-containing compound at *m/z* 313.0300

acetyl-selenocystathionine has only been identified in selenized yeast samples up to now (Dernovics et al. 2009). As no characteristic in-source fragments for any of the two compounds could be assigned at the retention time of the selenium species with *m/z* 313.0300, collision-induced dissociation (CID) was required to identify the compound unambiguously.

Figure 5a shows the TIC and the EIC at *m/z* 313.0300, while the related full scan spectrum of the HPLC-ESI-Orbitrap MS analysis is presented in Fig. 5b ($\delta$=−0.54 ppm). Figure 5c presents the $MS^2$ and $MS^3$ fragmentation data. All of the obtained fragments are in agreement with the fragmentation characteristic of γ-Glu-*Se*-methylselenocysteine, including *m/z* 296.0014


(a)
Relative Abundance
Time (min)
4.22
(b)
329.18169
303.16621
313.02955
204.12293
281.14739
272.16036
254.16123
214.11845
342.16532
381.21284
361.17127
399.18729
304.1502
313.0295
311.0304
314.1705
310.0332
309.0323
305.1535
315.0298
m/z
(c)
166.9596
183.9860
296.0014
148.9491
138.9647
73.0279
122.9336

◀ **Fig. 5** **a** HPLC-ESI-Orbitrap MS total ion chromatogram of the fraction collected from the SAX-HPLC separation (see the peak *b* on Fig. 1 and the *thick line* on Fig. 2), together with the EIC recorded for *m/z* 313.0300. The *dashed line* and the *arrow* indicate the $t_R$=4.22 min. **b** The full scan spectrum at $t_R$=4.22 min indicates the presence of a selenium-containing compound at *m/z* 313.0295 ($C_9H_{17}N_2O_5Se^+$, $[M+H]^+$, $\delta$=−0.54 ppm). **c** $MS^2$ (for *m/z* 313.0295 on the left) and $MS^3$ (for *m/z* 166.9596 on the right) fragment ion spectra

($C_9H_{14}NO_5Se^+$, $\delta$=−5.89 ppm), *m/z* 183.9860 ($\delta$=−6.14 ppm), *m/z* 166.9596 ($\delta$=−5.87 ppm), *m/z* 148.9491 ($C_4H_5OSe^+$, $\delta$=−6.11 ppm), *m/z* 138.9647 ($C_3H_7OSe^+$, $\delta$=−6.91 ppm), *m/z* 122.9336 ($C_2H_3OSe^+$, $\delta$=−6.18 ppm), and *m/z* 73.0279 ($C_3H_5O_2^+$, $\delta$=−6.98 ppm), arising from the *Se*-methylselenocysteine residue.

The sulfur analogue of γ-Glu-*Se*-methylselenocysteine is a highly abundant characteristic non-proteinaceous amino acid derivative of bean plants, and it is regarded as suboptimal for nutrition (Giada et al. 1998; Padovese et al. 2001; Taylor et al. 2008). However it is formed through the activity of γ-Glu-transferase, an enzyme involved in stress adaptation, the concentration of γ-Glu-*S*-methylcysteine is not influenced by stress, but by plant species and protein composition (Taylor et al. 2008). The reason why γ-Glu-*Se*-methylselenocysteine has not been reported from Faboideae can be the actual high concentration of selenium compared to the experiments of Smrkolj et al. (2007). From higher taxonomy levels, Fabaceae, γ-Glu-*Se*-methylselenocysteine has only been found in sweet clover (*Melilotus indicus*; Wu et al. 1997), a Se-tolerant grassland legume species, and in the selenium hyperaccumulator two-grooved milk vetch (*Astragalus bisulcatus*; Freeman et al. 2006; Nigam and McConnell 1969).

## Quantification of *Se*-Methylselenocysteine and γ-Glu-*Se*-Methylselenocysteine

In order to achieve adequate retention, *Se*-methylselenocysteine was quantified by standard addition on an ion-pairing (IP)-RP-HPLC-ICP-MS setup (Fig. 6) that resulted in 2.6 mg/kg *Se*-methylselenocysteine as Se (Kotrebai et al. 2000; Mounicou et al. 2009). The amount of γ-Glu-*Se*-methylselenocysteine was determined by SAX-HPLC-ICP-MS and corrected for purity (83 %), according to the RP-HPLC cleanup chromatogram. The final concentration was 1.2 mg/kg γ-Glu-*Se*-methylselenocysteine as Se. This is the highest amount of γ-Glu-*Se*-methylselenocysteine found in naturally selenium-enriched food-related samples, and it is superseded only by selenium accumulator plants (Freeman et al. 2006; Wu et al. 1997) and artificially enriched yeast and *Allium* ssp. (Infante et al. 2005; Kápolna et al. 2012; Kotrebai et al. 1999; Larsen et al. 2006; Ohta et al. 2011; Yoshida et al. 2005). On the other hand, this concentration of γ-Glu-*Se*-methylselenocysteine is equal to about one third of total γ-Glu peptides in *P. vulgaris* bean samples (Giada et al. 1998), which is comparable to the nonspecific replacement ratio of Se in methionine in selenium-enriched yeast samples (Bierla et al. 2013; McSheehy et al. 2005).

The cumulated amount of selenium from the four quantified species resulted in 93 % of the enzymatically extracted selenium, which indicates 72 % total recovery of selenium of the bean sample. However this value is in agreement with the reported results on bean samples (Smrkolj et al. 2007), there is still a relatively high amount of Se that cannot be accessed by proteolytic activity. The formation of selenosugars of plant cell wall origin (Aureli et al. 2012), a recently described process, can be cited as a possible pathway to deposit excess selenium without resulting in amino acid-derived species. On the other hand, this naturally enriched bean material can be an optimal raw material for functional food production due to the combination of favorable bean characteristics such as high protein content, modest cropping requirements, and moderate selenium concentration together with bioavailable selenium species (Hurst et al. 2010).

After unambiguous identification with ESI-TOF-MS and ESI-Orbitrap MS, the final quantification of all the four identified species could be carried out with conventional HPLC-ICP-MS instrumentation due to the commercial availability of

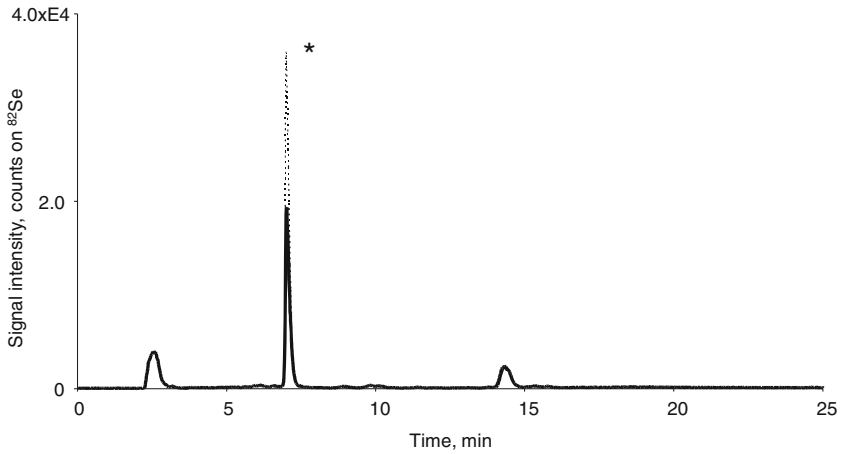



**Fig. 6** IP-RP-HPLC-ICP-MS chromatogram of the quantification of *Se*-methylselenocysteine. The compound assigned with *asterisk* at the $t_R$=7.0 was identified and quantified by standard addition (*thin dashed line*)

standards. Taking into consideration optional ways to decrease quantification limits, e.g., by addressing $^{80}Se$ in collision cell mode, and applying narrow or microbore HPLC setup, the presented methods may be adapted for bean samples with lower selenium concentration.

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**Conflict of Interest** Shuxun Shao declares that he has no conflict of interest. Xiubo Mi declares that he has no conflict of interest. Laurent Ouerdane declares that he has no conflict of interest. Ryszard Lobinski declares that he has no conflict of interest. Juan Francisco García-Reyes declares that he has no conflict of interest. Antonio Molina-Díaz declares that he has no conflict of interest. Andrea Vass declares that she has no conflict of interest. Mihály Dernovics declares that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

## References

Aureli F, Ouerdane L, Bierla K, Szpunar J, Prakash NT, Cubadda F (2012) Identification of selenosugars and other low-molecular weight selenium metabolites in high-selenium cereal crops. Metallomics 4:968–978

Bierla K, Dernovics M, Vacchina V, Szpunar J, Bertin G, Lobinski R (2008) Determination of selenocysteine and selenomethionine in edible animal tissues by 2D size-exclusion reversed-phase HPLC-ICP MS following carbamidomethylation and proteolytic extraction. Anal Bioanal Chem 390:1789–1798

Bierla K, Bianga J, Ouerdane L, Szpunar J, Yiannikouris A, Lobinski R (2013) A comparative study of the Se/S substitution in methionine and cysteine in Se-enriched yeast using an inductively coupled plasma mass spectrometry (ICP MS)-assisted proteomics approach. J Proteome 87:26–39

Brozmanová J, Mániková D, Vlcková V, Chovanec M (2010) Selenium: a double-edged sword for defense and offence in cancer. Arch Toxicol 84:919–938

Cao JJ, Gregoire BR, Zeng H (2012) Selenium deficiency decreases antioxidative capacity and is detrimental to bone microarchitecture in mice. J Nutr 142:1526–1531

Chan Q, Afton SE, Caruso JA (2010) Selenium speciation profiles in selenite-enriched soybean (*Glycine max*) by HPLC-ICPMS and ESI-ITMS. Metallomics 2:147–153

Dernovics M, Lobinski R (2008) Characterization of the selenocysteine-containing metabolome in selenium-rich yeast. J Anal At Spectrom 23:744–751

Dernovics M, Stefánka Z, Fodor P (2002) Improving selenium extraction by sequential enzymatic processes for Se-speciation of selenium-enriched *Agaricus bisporus*. Anal Bioanal Chem 372:473–480

Dernovics M, Ouerdane L, Tastet L, Giusti P, Preud'homme H, Lobinski R (2006) Detection and characterization of artefact compounds during selenium speciation analysis in yeast by ICP-MS-assisted MALDI MS, oMALDI MS/MS and LC-ES-MS/MS. J Anal At Spectrom 21:703–707

Dernovics M, Garcia-Barrera T, Bierla K, Preud'homme H, Lobinski R (2007) Standardless identification of selenocystathionine and its gamma-glutamyl derivatives in monkeypot nuts by 3D liquid chromatography with ICP-MS detection followed by nanoHPLC-Q-TOF-MS/MS. Analyst 132:439–449

Dernovics M, Far J, Lobinski R (2009) Identification of anionic selenium species in Se-rich yeast by electrospray QTOF MS/MS and hybrid linear ion trap/orbitrap $MS^n$. Metallomics 1:317–329

EFSA (2012) Scientific opinion on safety and efficacy of selenium in the form of organic compounds produced by the selenium-enriched yeast *Saccharomyces cerevisiae* NCYC R646 (Selemax 1000/2000) as feed additive for all species. EFSA J 10:2778–2794

Freeman JL, Zhang LH, Marcus MA, Fakra S, McGrath SP, Pilon-Smits EAH (2006) Spatial imaging, speciation, and quantification of selenium in the hyperaccumulator plants *Astragalus bisulcatus* and *Stanleya pinnata*. Plant Physiol 142:124–134

Giada MDLR, Miranda MTM, Marquez UML (1998) Sulphur gamma-glutamyl peptides in mature seeds of common beans (*Phaseolus vulgaris* L.). Food Chem 61:177–184

Hart DJ, Fairweather-Tait SJ, Broadley MR, Dickinson SJ, Foot I, Knott P, McGrath SP, Mowat H, Norman K, Scott PR, Stroud JL, Tucker M, White PJ, Zhao FJ, Hurst R (2011) Selenium concentration and speciation in biofortified flour and bread: retention of selenium during grain biofortification, processing and production of Se-enriched food. Food Chem 126:1771–1778

Huang Y, Wang Q, Gao J, Lin Z, Banuelos GS, Yuan L, Yin X (2013) Daily dietary selenium intake in a high selenium area of Enshi, China. Nutrients 5:700–710

Hurst R, Armah CN, Dainty JR, Hart DJ, Teucher B, Goldson AJ, Broadley MR, Motley AK, Fairweather-Tait SJ (2010) Establishing optimal selenium status: results of a randomized, double-blind, placebo-controlled trial. Am J Clin Nutr 91:923–931

Infante HG, O'Connor G, Rayman M, Wahlen R, Entwisle J, Norris P, Hearn R, Catterick T (2004) Selenium speciation analysis of selenium-enriched supplements by HPLC with ultrasonic nebulisation ICP-MS and electrospray MS/MS detection. J Anal At Spectrom 19:1529–1538

Infante HG, O'Connor G, Rayman M, Wahlen R, Spallholz JE, Hearn R, Catterick T (2005) Identification of water-soluble gamma-glutamyl-Se-methylselenocysteine in yeast-based selenium supplements by reversed-phase HPLC with ICP-MS and electrospray tandem MS detection. J Anal At Spectrom 20:864–870

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

Kápolna E, Laursen KH, Husted S, Larsen EH (2012) Bio-fortification and isotopic labelling of Se metabolites in onions and carrots following foliar application of Se and 77Se. Food Chem 133:650–657

Kotrebai M, Birringer M, Tyson JF, Block E, Uden PC (1999) Identification of the principal selenium compounds in selenium-enriched natural sample extracts by ion-pair liquid chromatography with inductively coupled plasma- and electrospray ionization-mass spectrometric detection. Anal Commun 36:249–252

Kotrebai M, Birringer M, Tyson JF, Block E, Uden PC (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

Kozak L, Rudnicka M, Niedzielski P (2012) Determination of inorganic selenium species in dietary supplements by hyphenated analytical system HPLC-HG-AAS. Food Anal Methods 5:1237–1243

Larsen EH, Lobinski R, Burger-Meyer K, Hansen M, Ruzik R, Mazurowska L, Rasmussen PH, Sloth JJ, Scholten O, Kik C (2006) Uptake and speciation of selenium in garlic cultivated in soil

amended with symbiotic fungi (mycorrhiza) and selenate. Anal Bioanal Chem 385:1098–1108

Lippman SM, Goodman PJ, Klein EA, Parnes HL, Thompson IM, Kristal AR, Santella RM, Probstfield JL, Moinpour CM, Albanes D, Taylor PR, Minasian LM, Hoque A, Thomas SM, Crowley JJ, Gaziano JM, Stanford JL, Cook ED, Fleshner NE, Lieber MM, Walther PJ, Khuri FR, Karp DD, Schwartz GG, Ford LG, Coltman CA (2005) Designing the Selenium and Vitamin E Cancer Prevention Trial (SELECT). J Natl Cancer Inst 97:94–102

Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, Parnes HL, Minasian LM, Gaziano JM, Hartline JA, Parsons JK, Bearden JD III, Crawford ED, Goodman GE, Claudio J, Winquist E, Cook ED, Karp DD, Walther P, Lieber MM, Kristal AR, Darke AK, Arnold KB, Ganz PA, Santella RM, Albanes D, Taylor PR, Probstfield JL, Jagpal TJ, Crowley JJ, Meyskens FL Jr, Baker LH, Coltman CA Jr (2009) Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). JAMA 301:39–51

McSheehy S, Yang W, Pannier F, Szpunar J, Lobinski R, Auger J, Potin-Gautier M (2000) Speciation analysis of selenium in garlic by two-dimensional high-performance liquid chromatography with parallel inductively coupled plasma mass spectrometric and electrospray tandem mass spectrometric detection. Anal Chim Acta 421:147–153

McSheehy S, Kelly J, Tessier L, Mester Z (2005) Identification of selenomethionine in selenized yeast using two-dimensional liquid chromatography-mass spectrometry based proteomic analysis. Analyst 130:35–37

Mounicou S, Dernovics M, Bierla K, Szpunar J (2009) A sequential extraction procedure for an insight into selenium speciation in garlic. Talanta 77:1877–1882

Nigam SN, McConnell WB (1969) Seleno amino compounds from *Astragalus bisulcatus* isolation and identification of gamma-L-glutamyl-Se-methyl-seleno-L-cysteine and Se-methylseleno-L-cysteine. BBA - Gen Subj 192:185–190

Nigam SN, McConnell WB (1973) Biosynthesis of Se-methylselenocysteine in lima beans. Phytochemistry 12:359–362

Ogra Y (2008) Integrated strategies for identification of selenometabolites in animal and plant samples. Anal Bioanal Chem 390:1685–1689

Ogra Y, Anan Y (2009) Selenometabolomics: Identification of selenometabolites and specification of their biological significance by complementary use of elemental and molecular mass spectrometry. J Anal At Spectrom 24:1477–1488

Ohta Y, Suzuki N, Kobayashi Y, Hirano S (2011) Rapid speciation and quantification of selenium compounds by HPLC-ICP MS using multiple standards labelled with different isotopes. Isot Environ Health Stud 47:330–340

Padovese R, Kina SM, Barros RMC, Borelli P, Lanfer Marquez UM (2001) Biological importance of gamma-glutamyl-*S*-methylcysteine of kidney bean (*Phaseolus vulgaris* L.). Food Chem 73:291–297

Pedrero Z, Madrid Y (2009) Novel approaches for selenium speciation in foodstuffs and biological specimens: a review. Anal Chim Acta 634: 135–152

Pedrero Z, Encinar JR, Madrid Y, Camara C (2007) Identification of selenium species in selenium-enriched *Lens esculenta* plants by using two-dimensional liquid chromatography-inductively coupled plasma mass spectrometry and [Se-77]selenomethionine selenium oxide spikes. J Chromatogr A 1139:247–253

Polatajko A, Banas B, Encinar JR, Szpunar J (2005) Investigation of the recovery of selenomethionine from selenized yeast by two-dimensional LC-ICP MS. Anal Bioanal Chem 381:844–849

Premarathna L, McLaughlin MJ, Kirby JK, Hettiarachchi GM, Stacey S, Chittleborough DJ (2012) Selenate-enriched urea granules are a highly effective fertilizer for selenium biofortification of paddy rice grain. J Agric Food Chem 60:6037–6044

Rayman MP, Infante HG, Sargent M (2008) Food-chain selenium and human health: spotlight on speciation. Br J Nutr 100:238–253

Smrkolj P, Germ M, Kreft I, Stibilj V (2006) Respiratory potential and Se compounds in pea (*Pisum sativum* L.) plants grown from Se-enriched seeds. J Exp Bot 57:3595–3600

Smrkolj P, Osvald M, Osvald J, Stibilj V (2007) Selenium uptake and species distribution in selenium-enriched bean (*Phaseolus vulgaris* L.) seeds obtained by two different cultivations. Eur Food Res Technol 225:233–237

Stan SD, Kar S, Stoner GD, Singh SV (2008) Bioactive food components and cancer risk reduction. J Cell Biochem 104:339–356

Szpunar J, Lobinski R (2002) Multidimensional approaches in biochemical speciation analysis. Anal Bioanal Chem 373:404–411

Taylor M, Chapman R, Beyaert R, Hernández-Sebastia C, Marsolais F (2008) Seed storage protein deficiency improves sulfur amino acid content in common bean (*Phaseolus vulgaris* L.): redirection of sulfur from gamma-glutamyl-*S*-methyl-cysteine. J Agric Food Chem 56:5647–5654

Thavarajah D, Vandenberg A, George GN, Pickering IJ (2007) Chemical form of selenium in naturally selenium-rich lentils (*Lens culinaris* L.) from Saskatchewan. J Agric Food Chem 55:7337–7341

Wu L, Guo X, Banuelos GS (1997) Accumulation of seleno-amino acids in legume and grass plant species grown in selenium-laden soils. Environ Toxicol Chem 16:491–497

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

Yoshida M, Sugihara S, Inoue Y, Chihara Y, Kondo M, Miyamoto S, Sukcharoen B (2005) Composition of chemical species of selenium contained in selenium-enriched shiitake mushroom and vegetables determined by high performance liquid chromatography with inductively coupled plasma mass spectrometry. J Nutr Sci Vitaminol 51: 194–199