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# Selenolanthionine is the major water-soluble selenium compound in the selenium tolerant plant *Cardamine violifolia*☆

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ABSTRACT

*Background:* Selenium hyperaccumulation in plants often involves the synthesis of non-proteinaceous methylated selenoamino acids serving for the elimination of excess selenium from plant metabolism to protect plant homeostasis.
*Methods:* Our study aimed at the identification of the main selenium species of the selenium hyperaccumulator plant *Cardamine violifolia* (*Brassicaceae*) that grows in the wild in the seleniferous region of Enshi, China. A sample of this plant (3.7 g Se $kg^{-1}$ d.w.) was prepared with several extraction methods and the extracted selenium species were identified and quantified with liquid chromatography mass spectrometry set-ups.
*Results:* The *Cardamine violifolia* sample did not contain in considerable amount any of the organic selenium species that are often formed in hyperaccumulator plants; the inorganic selenium content (mostly as elemental selenium) accounted only for < 20% of total Se. The most abundant selenium compound, accounting for about 40% of total Se was proved to be selenolanthionine, a selenium species that has never been unambiguously identified before from any selenium containing sample. The identification process was completed with chemical synthesis too. The molar ratio of lanthionine:selenolanthionine in the water extract was ca. 1:8.
*Conclusions:* Finding selenolanthionine as the main organic selenium species in a plant possibly unearths a new way of selenium tolerance. This article is part of a Special Issue entitled Selenium research in biochemistry and biophysics - 200 year anniversary issue, edited by Dr. Elias Arnér and Dr. Regina Brigelius-Flohe.

## 1. Introduction

Metabolism of selenium in hyperaccumulator plants has been reviewed recently from several aspects [1–3]. Basically, selenium is uptaken in the form of selenate that is reduced into selenite and further to hydrogen selenide, which is metabolised into selenocysteine (Sec): this is the amino acid to be withdrawn from the amino acid pool to prevent the formation of dysfunctional plant proteins. The excess of this element is diverted in basically three directions: methylation of the selenium moiety in Sec yields Se-(methyl)selenocysteine, a non-proteinaceous amino acid derivative, that serves for further metabolism into the volatile dimethyl diselenide. Sec can also be converted into selenohomocysteine that opens the two basic other directions: one towards selenomethionine and ultimately until the volatile dimethyl selenide, while the second pathway ends up in the accumulation of selenohomolanthionine. Depending on the enzymatic activities, the plant species and plant tissues serving for the deposition of selenium, these three pathways may be also stopped or diverge to store intermediate or minor species such as gamma-Glu-methylselenocysteine [4], selenocystathionine [5], Se-(methyl)selenocysteine [6], gamma-Glu-methylselenomethionine [7] or even polyselenides [8]. Apart from the listed metabolic pathways, genuine compounds of specific sulphur metabolisms can also be synthesised in selenised form in considerable amounts due to the sulphate-selenate substrate-unspecific activity of the enzymes involved: e.g., several selenoglucosinolates could be identified in *Brassica nigra* after dedicated methanolic extraction [9].

On the other hand, non-accumulator and secondary accumulator plants can also store unaltered inorganic (selenate) or reduced

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(elemental) selenium species as an indication of exaggerated detoxification capacity. Therefore, when a novel plant species is considered for selenium speciation analysis, simple methods such as the quantification of water soluble selenium vs. total selenium content may not clearly distinguish between a highly active selenium metabolism that produces water soluble organic non-proteinaceous selenium species and a non-accumulator plant with selenate accumulation. Similarly, selenomethionine in hydrophobic or in denaturated plant proteins, elemental selenium and selenides are all insoluble in water based buffers and/or in water, thus no hint on metabolic pathways can be gained only from the ratio of water insoluble selenium vs. total selenium data.

In our study, *Cardamine violifolia*, a species of the *Cardamine* genus and of the *Brassicaceae* family was considered for selenium speciation. This plant is a selenium tolerant species that is native to the highly seleniferous region of Enshi, Hubei, China. *C. violifolia* has been characterized with selenium content in the leaves exceeding 0.6 g $kg^{-1}$ (dry weight) and has been shown to have around 14% of selenium in the form of water soluble proteins [10].

## 2. Materials and methods

### 2.1. Plant sample

*Cardamine violifolia* was identified and registered by the Wuhan Botanical Garden (Chinese Academy of Sciences; Wuhan, China). The sample was harvested in the natural seleniferous region Yutangba, Enshi (Hubei Province, China). The stems and the leaves of the plants were cleaned with deionised water, milled and lyophilised.

### 2.2. Reagents and standards

Heptafluorobutyric acid (HFBA; ≥99%), sodium borohydride (≥98%), Se-(methyl)selenocysteine hydrochloride (≥95%), D,L-lanthionine (≥98%), L-selenocystine (95%) and formic acid (~98% for MS) were supplied by the Merck-Sigma group (Schnelldorf, Germany). Deionised water (18.2 MΩ cm) was obtained from a Millipore purification system (Merck-Millipore; Darmstadt, Germany). Acetonitrile (Super Gradient Grade), and sodium sulphite (98%) were supplied by VWR (Radnor, Pennsylvania, USA), while carbon disulphide (extra pure), hydrochloric acid (37%), sodium selenite pentahydrate (≥99%), ammonia solution (25%), Pronase E (4,000,000 PU $g^{-1}$), hydrogen peroxide (a.r. 30 m/m%), the 1.000 g/l standards of S, Se and Rh were obtained from Merck. Nitric acid (a.r., 65 ≥ m/m%), formic acid (98–100%, used for liquid chromatographic purposes), pyridine (99.5%) and acetic acid (100%) were purchased from Scharlau (Barcelona, Spain). Ethanol (96%), Tris(hydroxymethyl)aminomethane (a.r.) and ammonium acetate (> 99%) were supplied by Reanal (Budapest, Hungary). Methanol (HPLC Gradient Grade) and D,L-dithiothreitol (≥98%) were obtained from Fisher Scientific (Loughborough, UK). β-Chloro-L-alanine (98%) was purchased from Santa Cruz Biotechnology (Dallas, USA). L-Selenomethionine (99 + %) was obtained from Acros Organics (Geel, Belgium).

### 2.3. Procedures

#### 2.3.1. Total sulphur and selenium determination

Microwave digestion of the samples, extraction residues and aliquots of extraction solutions was carried out in a CEM Mars-5 digestion system (CEM; Matthews, NC, USA). The samples (50 mg *C. violifolia*; entire extraction residues; 1.0–2.0 ml aliquots) were mixed with 5.0 ml $HNO_3$ in PTFE digestion tubes and after 24 h 3.0 ml $H_2O_2$ was added prior to the microwave digestion process. The pressure was raised to 250 psi over 20 min and held for 15 min. Total Se concentration was determined with an Agilent 7500ce ICP-MS (Agilent Technologies, Santa Clara, CA, USA) on the $^{77}Se$ and $^{82}Se$ isotopes by the method of standard addition using rhodium ($^{103}Rh$) as an internal standard.

The total S concentration was determined with a Perkin Elmer Optima 8000 ICP-OES instrument (Waltham, MA, USA) with external calibration at the wavelengths of 180.669, 181.975, 182.037 and 182.563 nm.

#### 2.3.2. Sequential extraction procedure

Sequential extraction protocol was adapted from Monicou et al. [11]:

1. Water extraction: 0.2 g of the *C. violifolia* sample was extracted with an ultrasonic probe (UP100H, Hielscher Ultrasound Technology, Teltow, Germany) at ambient temperature. The extraction was carried out in two steps: first with 6.0 ml deionised water for 2 × 1 min sonication then with 3.0 ml deionised water for 2 × 1 min. The supernatants were recovered by centrifugation (30 min at 4000*g*), then they were pooled and filled up to 10.0 ml. This procedure was carried out in six replicates.
2. Sulphite extraction: 5.0 ml of 1.0 M $Na_2SO_3$ was added to the water insoluble residue; the sample was vortexed and it was shaken in an orbital shaker at 180 1 $min^{-1}$ for 24 h at 37 °C. The supernatants and the residues were separated by centrifugation (30 min at 4000*g*). The residue was washed with 4.0 ml deionised water and the relevant supernatants ($Na_2SO_3$ and water) were pooled and made up to 10.0 ml with deionised water. This procedure was carried out in three replicates.
3. $CS_2$ extraction: this procedure was carried out with the three residues obtained after the sulphite extraction. First, 2.0 ml deionised water was added to the residues. The samples were vortexed, then 4.0 ml $CS_2$ was added, followed by vortexing and incubating for 4 h. The mixture was hand-shaken regularly in 20-min intervals. Afterwards the samples were centrifuged (10 min at 4000*g*) and three phases were separated: the upper water phase, the plant tissue debris and the $CS_2$-containing layer. These three phases were treated separately: the $CS_2$ phase was evaporated at room temperature and the dried-in residue was digested, while the plant debris and the water phase were digested as they were, without evaporation.

The selenium content of the extracts and phases was determined by ICP-MS following the $HNO_3$-$H_2O_2$ digestion procedure.

#### 2.3.3. Proteolytic extraction

250 mg of lyophilized plant material was enzymatically digested according to the method described by Shao et al. [12] with Pronase E in two subsequent steps in Tris-buffered medium. The supernatant arising from the treatments were decanted and made up to 10.0 ml with deionised water in a volumetric flask and filtered through 0.45 μm PTFE disposable syringe filters. The entire sample preparation was executed in three replicates.

#### 2.3.4. Strong anion exchange (SAX) chromatography

A PRP-X100 SAX column (250 mm × 4.1 mm × 10 μm; Hamilton, Reno, NV, USA) was applied with gradient elution made with ammonium acetate (buffer A, 10 mM; buffer B, 250 mM; pH 6.0) delivered at 1.5 ml $min^{-1}$. The program was as follows: 0–5 min, 100% A; 5–20 min, up to 40% B; 20–22 min, up to 100% B; 22–25 min, 100% B; 25–26 min, down to 0% B; 26–31 min, 0% B. Injection volume was 100 μl. Selenite was quantified using the method of three-point standard addition.

#### 2.3.5. Strong cation exchange (SCX) chromatography

A Zorbax 300-SCX column (150 mm × 4.6 mm × 5 μm, Agilent) equipped with a matching guard column was used. Gradient elution was done with pyridine formate (pH 2.2; buffer A: 1 mM; buffer B: 40 mM) delivered at 1.2 ml $min^{-1}$. The program was as follows: 0–2 min, 100% A; 2–15 min, up to 30% B; 15–16 min, up to 100% B; 16–20 min, 100% B; 20–21 min, 100% A. Lyophilized water extract of the *C. violifolia* sample was dissolved in buffer A and filtered through a

0.45 µm cellulose acetate filter. The injection volume was 5 µl (for mapping purposes) or 10 µl (for fraction collection). The peak with the highest abundance was repeatedly collected, frozen and lyophilized, then dissolved in the proper eluent for further IP-RP-ICP-MS and LC-ESI-QTOF-MS analysis.

*2.3.6. Ion-pairing reversed phase (IP-RP) chromatography*

An XTerra MS-$C_{18}$ (250 mm × 4.6 mm × 5 µm; Waters, Milford, MA, USA) column was used. The mobile phase consisted of deionised water (eluent A) and methanol (eluent B) both containing 0.05 v/v% HFBA. The flow rate was 0.6 ml $min^{-1}$ and the gradient elution program was: 0–2 min, 5% B; 2–10 min, up to 65% B; 10–15 min, 65% B; 15–16 min, down to 5% B; 16–22 min 5% B. Injection volume was 25 µl. Se-(methyl)selenocysteine was quantified using the method of three-point standard addition.

All (SAX, IP-RP and SCX) chromatographic set-ups were achieved by using an Agilent 1200 HPLC system connected to an Agilent 7500cs ICP-MS for element-specific detection of $^{77}Se$, $^{82}Se$ and $^{88}Sr$. In the case of IP-RP hyphenation, oxygen (40 ml $min^{-1}$) was used as optional gas.

*2.3.7. LC-ESI-QTOF-MS set-up*

For the HPLC–ESI-MS experiments, a 6530 Accurate Mass ESI-QTOF-MS system (Agilent) equipped with an Agilent 6220 derived dual ion spray source was applied. Chromatographic elution was provided by an Agilent 1200 HPLC system using a Zorbax XDB $C_{18}$ reversed phase (RP) HPLC column (50 mm × 2.1 mm × 3.5 µm; Agilent). Isocratic elution with deionised water containing 5 v/v% acetonitrile and 0.1 v/v% formic acid was carried out at the flow rate 0.35 ml $min^{-1}$. The ESI ion source was used in positive ionisation mode. The default fragmentor voltage was 170 V in MS and 145 V in MS/MS experiments. The other related instrumental parameters are described in the Supplementary material (SM Table S1).

*2.3.8. Chemical synthesis, purification and quantification of the selenolanthionine standard*

Selenolanthionine synthesis was based on a modified procedure originally described by Block et al. [13]. 200 mg L-selenocystine was suspended in 5 ml ethanol under argon atmosphere and 100 mg solid $NaBH_4$ was added. The solution was refluxed for 20 min, after an additional volume of ethanol (5 ml) was added and the solution was refluxed for further 20 min. Then 150 mg β-chloro-L-alanine was added to the still yellowish solution that was refluxed for 10 min and cooled to room temperature. The resulting suspension was filtered, the solid residue was dried and dissolved in 0.1 M HCl solution. Prior to SCX-ICP-MS analysis and purification the solution was 300-fold diluted with pyridine formate (1 mM, buffer A for SCX), filtered through a 0.45 µm cellulose acetate filter and injected onto the SCX-ICP-MS chromatographic set-up. The amount of residual L-selenocystine was quantified using the method of three-point standard addition to estimate the yield of selenolanthionine synthesis.

The peak corresponding to selenolanthionine was fractionated (50×), pooled and lyophilised. In order to determine its approximate concentration, an isocratic SCX method was applied with a three-point standard addition of Se-(methyl)selenocysteine as the closest eluting Se-species, according to a method introduced by Sloth et al. [14] for arsenic speciation. The chromatographic system was identical as described above for the SCX-ICP-MS analyses; the eluent was 1 mM pyridine formate (pH 2.2; 0–5 min).

*2.3.9. Quantification of lanthionine and selenolanthionine in the water extract of C. violifolia*

A QTRAP3200 triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems/Sciex; Foster City, CA, USA) was applied in MRM (multiple reaction monitoring) mode. The instrument was coupled to an Agilent 1100 series HPLC set-up using a Luna HILIC 200 Å (150 mm × 4.6 mm × 5 µm; Phenomenex, Torrance, CA, USA) HPLC column. For the gradient elution method, the mobile phase consisted of 98:1:1 v/v% deionised water/formic acid/acetonitrile (eluent A) and 98:1:1 v/v% acetonitrile/formic acid/deionised water (eluent B). The flow rate was 0.8 ml $min^{-1}$ and the gradient elution was performed as follows: 0–1 min 10% A, 1–10 min linear gradient up to 90% A, 10–11 min 90% A, 11–12 min linear gradient down to 10% A, 12–20 min 10% A. The injection volume was 5 µl and the column was kept at 25 °C.

The instrument was optimized in positive MRM mode for lanthionine and selenolanthionine by introducing a 2 mg $l^{-1}$ stock solution of each compound containing acetonitrile/water/formic acid (60:39:1; v/v%) with the help of a syringe pump at the flow rate of 10 µl $min^{-1}$. Lanthionine was monitored with the precursor ion set at *m/z* 209, while the transitions were set for 209/74, 209/120, and 209/146, with the quantifier product ion at *m/z* 120. The precursor ion for selenolanthionine was set to *m/z* 257 for the transitions at 257/168, 257/140, and 257/74, showing the most intensive (quantifier) product ion at *m/z* 168. The optimal parameters and other related instrumental data are described in the Supplementary material (SM Table S2).

## 3. Results

*3.1. Total selenium and sulphur contents and selenium distribution*

Total Se concentration was found to be 3.7 ± 0.2 g Se $kg^{-1}$ d.w. in the *C. violifolia* sample, which basically ranks this plant among the hyperaccumulator species. The concentration of sulphur was 15.2 g S $kg^{-1}$ d.w., which can be found in the range of sulphur accumulation of another selenium hyperaccumulator *Brassicaceae* species, e.g., *Stanleya pinnata var. pinnata* (14.0–19.3 g S $kg^{-1}$ d.w.; [15]), *Sinapis alba*, *Brassica arvense*, *B. oleracea*, and *B. juncea* (12.9–21.8 g S $kg^{-1}$ d.w.; [16]). However this high ratio of Se:S (0.24:1 m/m; 1:10 molar ratio) is unusual among the known *Brassicaceae* species and may relate to an exceptional selenium uptake, S and Se concentration data of the soil would be required to assess the relevant Se enrichment factor.

The distribution of selenium among different fractions is shown in Fig. 1. The aqueous extract was found to contain 60 ± 1.5% of Se originally present in the sample, while the cumulated amount of $Na_2SO_3$ extract (assigned as elemental selenium) and $CS_2$ extract (assigned as selenides) accounted for approximately 18% of the total Se content. There are different observations in the literature discussing the extraction efficiency and selectivity of $Na_2SO_3$ and $CS_2$ solvents in case of elemental selenium and selenides [17–20]; however, the considerable (16%) elemental selenium content is a feature of secondary and

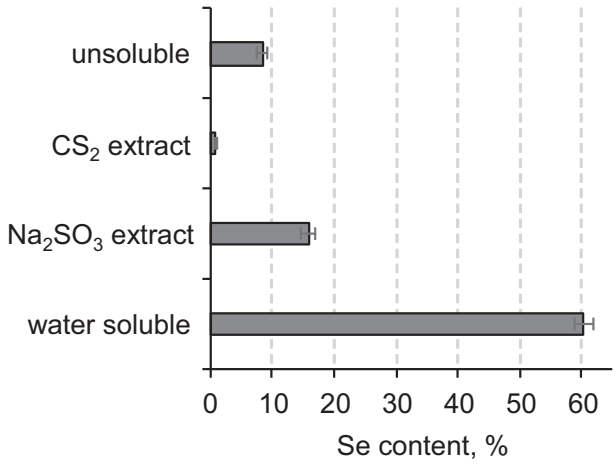


**Fig. 1.** Distribution of selenium in the *C. violifolia* sample on the basis of the sequential extraction procedure. Error bars indicate ± 1 standard deviation.

non-accumulator plants such as garlic [11] and Black-eyed Susan /*Thunbergia alata*/ [17]. This indicates *C. violifolia* might be listed among the secondary accumulators. It must be noted that the high fraction of elemental Se does not inherently exclude this *Cardamine* species out of the hyperaccumulators, as some *Astragalus* and *Stanleya* hyperaccumulator species have been shown to contain up to 35% of total Se content in this form [21]. Although, in this latter case, the high elemental Se fraction was found in the roots, not in the stem and leaves, and it was attributed to the microbial activity of endophytic bacteria or fungi.

The overall recovery was 88%. The missing amount from the mass balance can be attributed to the physical loss observed during the laborious collection of the $CS_2$ phase and therefore might be assigned as insoluble selenium after the triple stage extraction process.

### 3.2. Chromatographic characterization procedures

The high water-soluble selenium content, together with the considerable elemental selenium concentration can refer to the accumulation of inorganic (anionic) selenium species, selenate and selenite, therefore strong anion exchange chromatography (SAX) is a viable option for further characterization. As presented in Fig. 2a, the water-soluble fraction of *C. violifolia* contained only a negligible amount of selenite (48 μg $g^{-1}$ as Se) that accounts for only 1.3% of total selenium. Also, the SAX-ICP-MS chromatogram indicates that most of the soluble selenium compounds are either cationic or neutral at the pH of the separation (pH 6.0).

Efficient separation of selenoamino acids and their derivatives arising from proteolytic extracts on ion-pairing reversed phase chromatography (IP-RP) is a well-established method [22] and it has also been chosen in our study. Fig. 2b shows the elution of the proteolytic extract together with the indication of the elution time of Se-(methyl) selenocysteine and selenomethionine. Only the amount of Se-(methyl) selenocysteine could be quantified (13 μg $g^{-1}$ as Se; around 0.4% of total selenium) while selenomethionine was detected in traces. The two most abundant peaks could not be identified with any of the available standards with retention time matching; however, useful information could be gained as the two peaks showed retention with the anionic ion pairing agent used in the study (HFBA) at the acidic pH of the elution (pH < 3).

Therefore, the aqueous extract of the *C. violifolia* sample was then analysed with cation-exchange chromatography (Fig. 2c), showing one dominant peak at the retention time of 3.7 min and several minor ones. Finally, repeated fraction collection was carried out for the major peak to achieve a compound adequately pure for LC-ESI-QTOF-MS based identification. The collected and pooled fraction of the dominant peak was re-analysed and re-fractionated with the IP-RP chromatography (Fig. 2d), which indicated that the fraction indeed consists of mostly one compound.

### 3.3. Identification of selenolanthionine by HPLC-ESI-QTOF-MS and -MS/MS

The fraction of the dominant peak in the IP-RP chromatogram was introduced into the ESI-QTOF-MS instrument by the means of an RP-HPLC system. Two selenium-containing components with the mass to charge ratios ($m/z$) 257.0032 and 167.9555 were found in the chromatogram by visual seeking for the characteristic selenium isotopic pattern: Fig. 3 shows the recorded full scan spectrum, together with the inset of the selenium-containing isotopic pattern. As it was supposed that the ion at $m/z$ 167.9555 is the in-source fragment of the compound with $m/z$ 257.0032, extracted ion chromatograms (EIC) of both compounds were prepared (Fig. 4), showing the matching of the two ions. The correspondence was also proved by decreasing the fragmentor potential of the ion source from 170 V to 150 V, which practically eliminated the in-source fragment.

The elemental composition calculating tool of the instrument, together with the information that the molecule carries one selenium

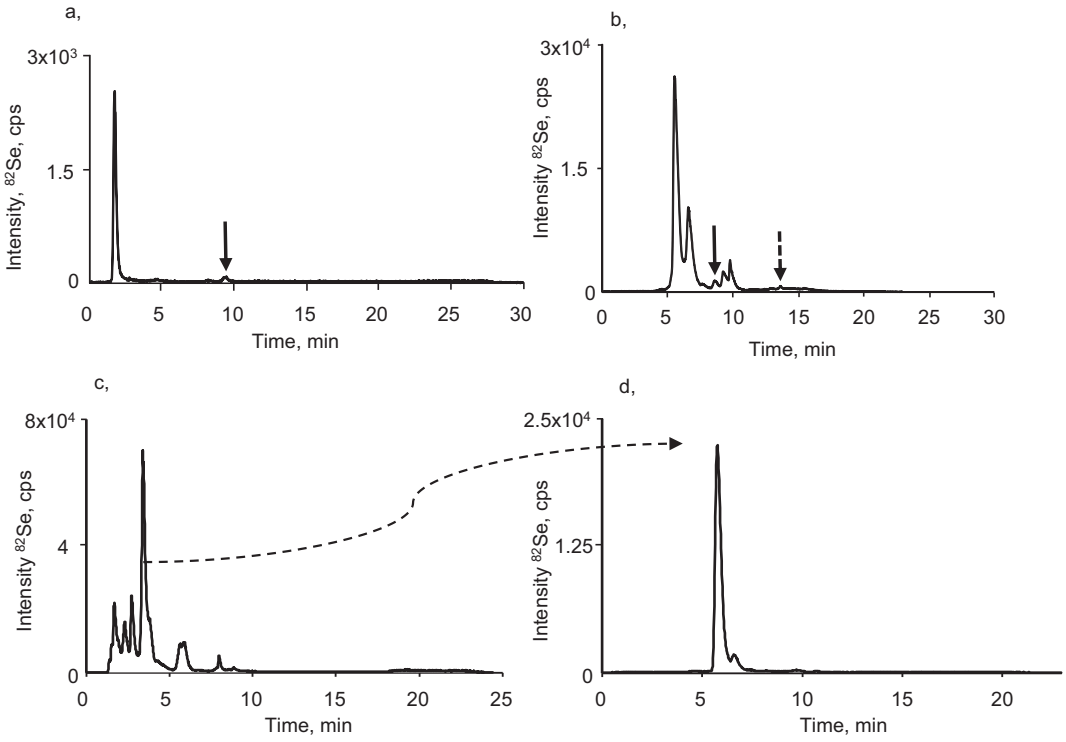


**Fig. 2.** (a) Strong anion exchange (SAX)–ICP-MS chromatogram of the *C. violifolia* water extract. The arrow indicates the retention time of selenite. (b) Ion-pairing reversed phase (IP-RP)–ICP-MS chromatogram of the *C. violifolia* water extract. The solid arrow indicates the retention time of Se-(methyl)selenocysteine, while the dashed arrow points at that of selenomethionine. (c) Strong cation exchange (SCX) – ICP-MS chromatogram of the *C. violifolia* water extract. The dashed line indicates the fraction collected for further analysis. (d) IP-RP–ICP-MS chromatogram of the fraction collected from the SCX chromatographic set-up.

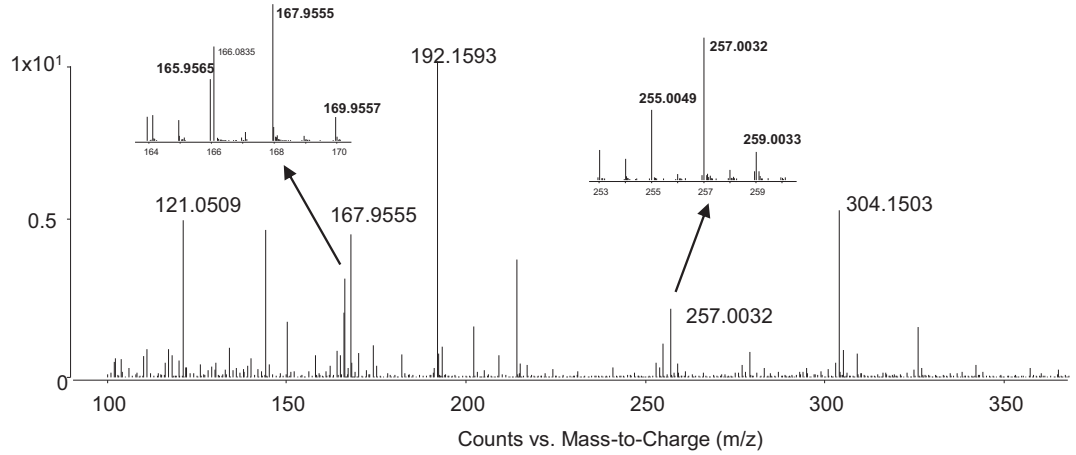


**Fig. 3.** HPLC-ESI-QTOF-MS full scan spectrum recorded at 0.502 min of the most abundant selenium containing fraction of *C. violifolia*. The spectrum presents the isotopologue patterns of selenolanthionine and its in-source fragment in the insets. Masses highlighted in bold refer to the $^{78}$Se, $^{80}$Se and $^{82}$Se isotopologues. The mass with *m/z* 166.0835 arrives from an interference on the $^{78}$Se isotopologue of the in-source fragment.

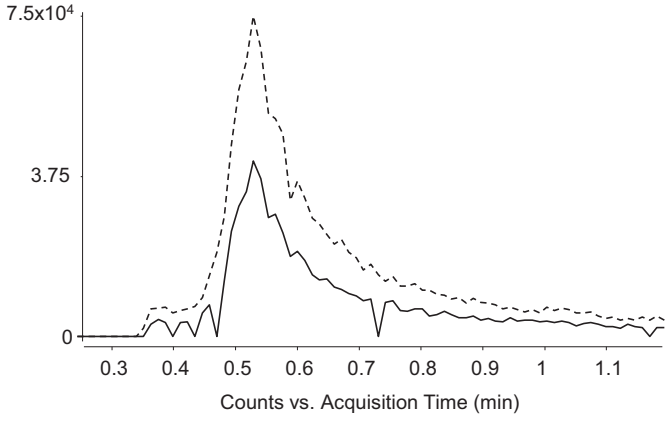


**Fig. 4.** Extracted ion chromatograms (EICs) of the ions *m/z* 257.003 (solid line) and *m/z* 167.955 (dashed line).

atom, found only one possible composition within 5 ppm ($C_6H_{13}N_2O_4Se^+$; theoretical *m/z* 257.0035, Δ = − 1.16 ppm; calculation settings are presented in SM Table S3). This composition, together with the observation of Block et al. [13] about the most significant fragment detected at *m/z* 168, indicates this molecule is selenolanthionine, a selenium species that has never been identified with unambiguous (at least electrospray mass spectrometry based) techniques before from any kind of selenium containing sample. Up to now, only one record on selenolanthionine has been published: Kotrebai et al. [23] assigned this species from selenized yeast in an HPLC-ICP-MS chromatogram on a shoulder of an eluting selenium containing peak with retention time matching.

For the further confirmation of the compound identity, an MS/MS experiment was carried out on the $^{80}$Se and $^{77}$Se isotope containing isotopologues. The results are shown in Fig. 5 and in Table 1.

However the isotopic pattern, the accurate mass data, the fragmentation pattern and the cationic chromatographic behaviour [24] all confirm the identity of this compound, the obvious lack of relevant scientific reports requires a higher level of verification, especially in the light of the fact that only the derivatives of selenolanthionine have been clearly identified from selenium containing plant [25] and yeast [26] samples. Therefore, it was found necessary to confirm the identification by the chemical synthesis of selenolanthionine as well.

### *3.4. Confirmation of the identification of selenolanthionine by chemical synthesis*

As the synthesis described by Block et al. [13] could not be completely reproduced, the synthetized material had to be analysed to achieve yield and purity data. The SCX-ICP-MS chromatogram (Fig. 6) shows that the synthetic product contained several selenium species in the mixture. The highest peak eluting at 5.3 min was identified as residual selenocystine based on retention time matching. The concentration of selenocystine was determined, which indicated that about 70% of this compound did not react, showing a yield of ≤ 30% for the selenolanthionine synthesis.

The synthetized compound eluting at 4.3 min (supposed to be selenolanthionine) was acquired with repeated fraction collection steps and it was first used for spiking the aqueous extract of the *C. violifolia* sample. As presented in Fig. 7, the SCX-ICP-MS chromatogram showed exact retention time matching with the peak of the previously identified selenolanthionine.

Afterwards, the synthetized compound and the *C. violifolia* extract spiked with the synthetized compound were analysed by the RP-LC-ESI-QTOF-MS in MS and MS/MS experiments. In the full-scan spectra the *m/z* 257.0038 and *m/z* 167.9557 ions could be found showing the selenium-containing isotopic pattern, and the MS/MS fragments showed matching with those detected from the *C. violifolia* extract (Fig. 8). The extracted ion chromatograms of selenolanthionine and its in-source fragment also showed matching in the spiked extract of *C. violifolia* (Fig. 9), which corresponds to another, orthogonal verification to the previous strong cation exchange chromatography.

It has been reported that *C. hupingshanesis* (an alternative name of *C. violifolia*) contains selenocystine and not selenolanthionine [27], which in that case would be in contrast to our results. These divergent results may possibly be explained by the fact that the prior report had analysed root samples as well while here we focused on stem and leaves. Alternatively, and perhaps more likely, it may also be that the HPLC method (that is, strong anion exchange at pH = 6.0) alone used in the prior report cannot easily separate selenocystine from selenolanthionine. That methodological challenge has indeed been discussed previously [28]. Because we have here used several complementary methods for analyses, including HPLC-ESI-QTOF-MS and chemical synthesis, we believe that our identification of selenolanthionine as the major metabolite should be reliable.

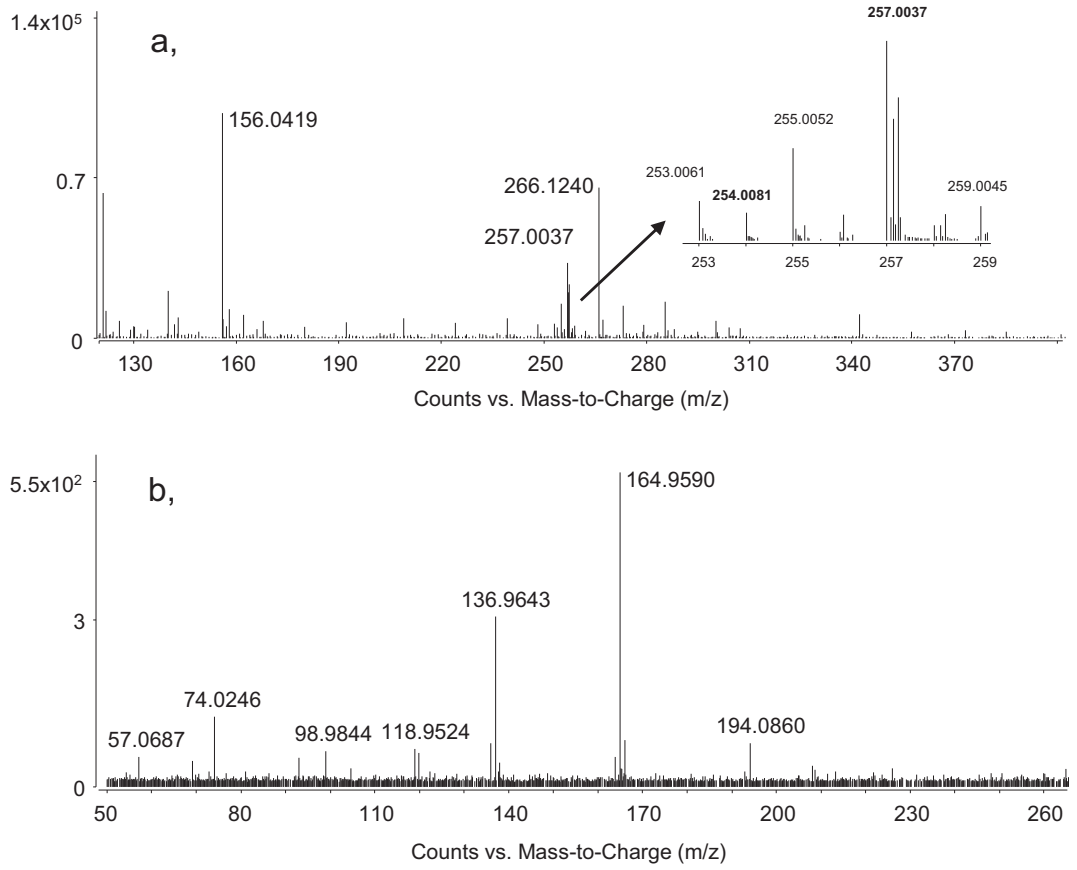


**Fig. 5.** (a) HPLC-ESI-QTOF-MS full scan spectrum of selenolanthionine prior to MS/MS analysis. The inset shows the isotopologue pattern, together with the fragmented ions highlighted in bold. (b) HPLC-ESI-QTOF-MS/MS collision induced dissociation (CID) spectrum of selenolanthionine fragmented on the $^{77}$Se isotopologue.

**Table 1**
MS/MS fragmentation data of selenolanthionine purified from *C. violifolia* water extract.

| Composition | Theoretical mass, *m/z* | Experimental mass, *m/z* | Δ, ppm |
|---|---|---|---|
| $C_6H_{13}N_2O_4(^{80}Se)^+$ | 257.0035 | 257.0037 | 0.78 |
| $C_6H_{13}N_2O_4(^{77}Se)^+$ | 254.0068 | 254.0081 | 5.12 |
| $C_3H_6NO_2(^{80}Se)^+$ | 167.9558 | 167.9551 | −4.17 |
| $C_3H_6NO_2(^{77}Se)^+$ | 164.9592 | 164.9590 | −1.21 |
| $C_2H_6NO(^{80}Se)^+$ | 139.9609 | 139.9606 | −2.14 |
| $C_2H_6NO(^{77}Se)^+$ | 136.9642 | 136.9643 | 0.73 |
| $C_2H_4NO_2^+$ | 74.0236 | 74.0246 | 13.5 |

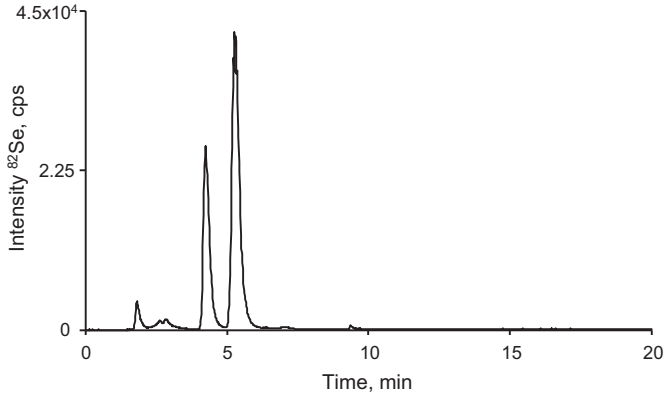


**Fig. 6.** SCX – ICP-MS chromatogram of the synthesised selenolanthionine standard after 100× dilution of the final reaction mixture. The peak eluting at 5.3 min refers to residual selenocystine, while selenolanthionine eluted at 4.3 min.

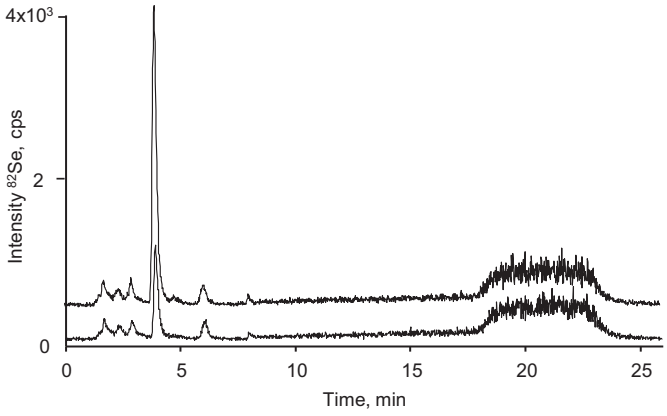


**Fig. 7.** SCX – ICP-MS chromatograms of the *C. violifolia* water extract (lower chromatogram) and that of the water extract spiked with the synthesised selenolanthionine standard (upper chromatogram).

### *3.5. Quantification of lanthionine and selenolanthionine in the water extract of C. violifolia*

Up to now, no information has been available on the lanthionine concentration in plants. On the other hand, the ratio of the sulphur and selenium analogues provides useful data on the bioaccumulation process, which – together with the total S and Se concentration – may give a hint about selenium metabolism [29].

As lanthionine standard is commercially available and selenolanthionine has been synthesised, the selective quantification of these two non-proteinaceous and cationic amino acids can be simultaneously carried out with hydrophilic interaction liquid chromatography (HILIC)

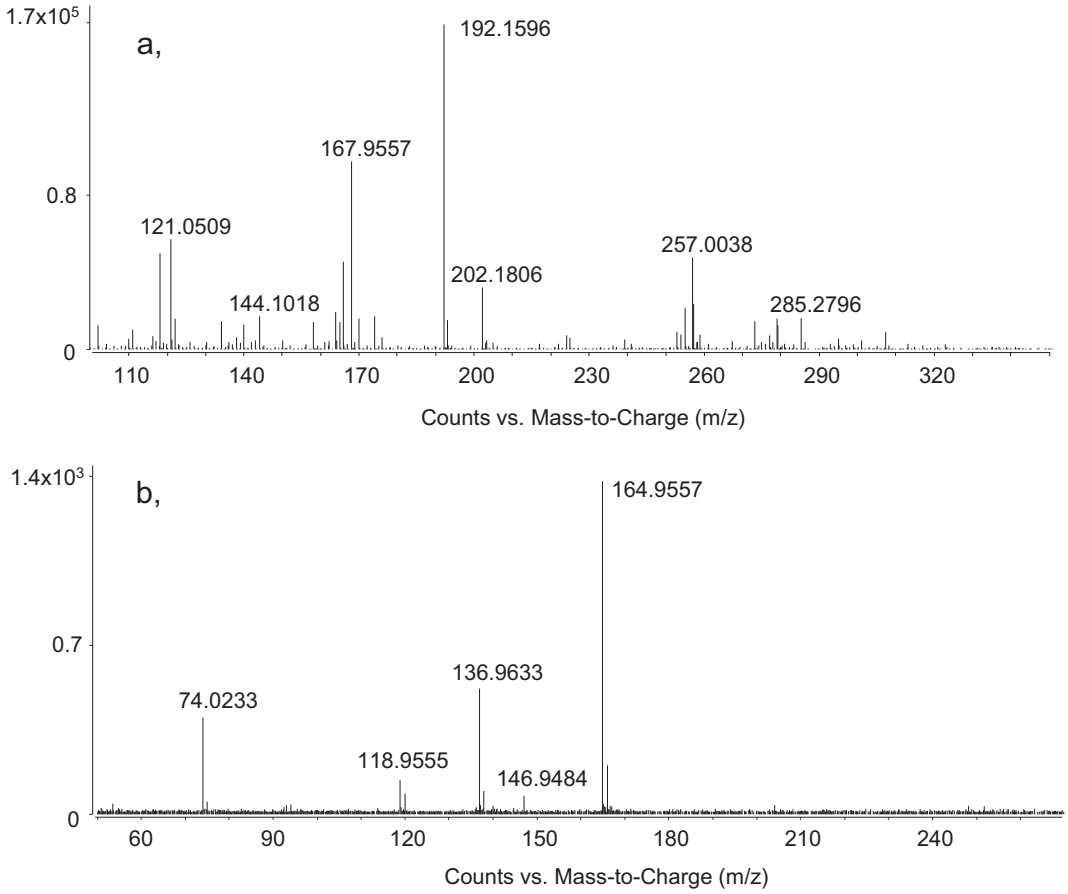


**Fig. 8.** (a) HPLC-ESI-QTOF-MS full scan spectrum of the synthesised selenolanthionine prior to MS/MS analysis. (b) HPLC-ESI-QTOF-MS/MS collision induced dissociation (CID) spectrum of the synthesised selenolanthionine fragmented on the $^{77}$Se isotopologue.

coupled to electrospray ionisation-triple quadrupole - mass spectrometry (ESI-$Q^3$-MS). Fig. 10a and b show the chromatograms of the two analytes; no considerable interferences could be observed at the monitored MRM transitions. The concentration of selenlanthionine was 4.8 mg $g^{-1}$ sample (that is equal to 1.5 mg Se $g^{-1}$) while lanthionine was present at 0.5 mg $g^{-1}$. These results refers to an about 1:8 molar ratio of lanthionine and selenolanthionine, which far exceeds the substitution rate for selenomethionine in the hyperaccumulator plant *Lecythis minor* seeds (1:2 for methionine:selenomethionine; [29]) and even more exceeds the quantified S:Se molar ratio of 10:1. However the quantification of selenolanthionine might be slightly biased due to the use of a non-commercial standard, it can be stated that selenolanthionine provides approximately 68% of selenium of the water-soluble fraction of *C. violifolia* and it accounts for about 40% of total selenium content. Such a high relative quantity for any non-proteinaceous selenium compound has been reported only for Se-(methyl)selenocysteine and γ-Glu-Se-(methyl)selenocysteine [1, 30].

## 4. Discussion

To the best of our knowledge, there have not been any known selenium related metabolic pathways that would include selenolanthionine. Basically, as this compound is a non-proteinaceous amino acid, it can fulfil the role of eliminating selenium, or, more directly, selenocysteine from being incorporated in plant proteins.

Concerning the chemical reactions potentially involved, lanthionine is known to be formed at high temperature at alkaline pH [31], or when samples with high protein content are exposed to high temperature in wet atmosphere for several hours (e.g., for 4 h at 130 °C with excess water) [32]. In the case of yeast and the 2,3-DHP-selenolanthionine

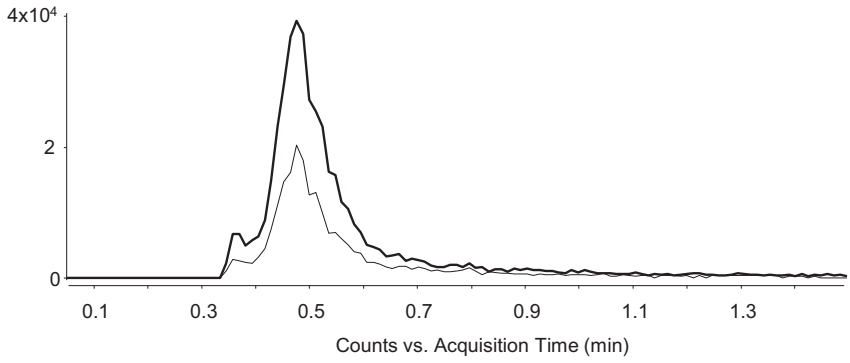


**Fig. 9.** EICs of the ions *m/z* 257.003 (lower line) and *m/z* 167.955 (upper line) from the *C. violifolia* water extract spiked with the synthesised selenolanthionine standard.

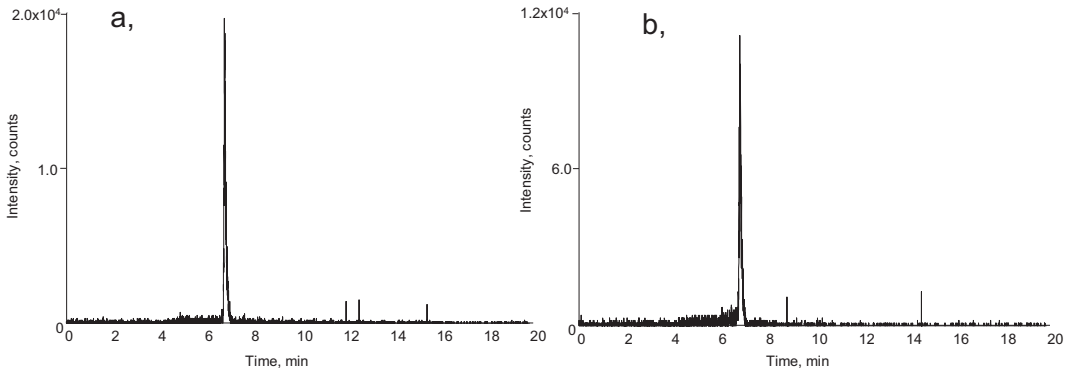


**Fig. 10.** (a) HILIC-ESI-$Q^3$-MS chromatogram of selenolanthionine from the *C. violifolia* water extract (MRM transition 257 → 168; retention time: 6.77 min). (b) HILIC-ESI-$Q^3$-MS chromatogram of lanthionine from the *C. violifolia* water extract (MRM transition 209 → 120; retention time: 6.83 min).

derivative, an oxidative cleavage process was proposed to form selenolanthionine, e.g., from a diselenide bridge containing metabolite, followed by a structural rearrangement [33] due to the sterilization related heat treatment. Evidently, none of these processes was concerned during sample treatment and preparation. Also, no selenocystine was detected in the *Cardamine* samples to support this theory.

Clearly, a comprehensive study dealing also with enzyme expression levels, biochemical aspects and the higher coverage of assigned selenium species are required to state if the outstanding level of selenolanthionine in *C. violifolia* belongs to a novel metabolomic process in selenium tolerance and accumulation.

## Transparency document

The http://dx.doi.org/10.1016/j.bbagen.2018.01.006 associated with this article can be found, in online version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2018.01.006.

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