

Water-soluble organic selenometabolites of alfalfa (*Medicago sativa* L.) green biomass-derived fractions

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ABSTRACT

Background: Tolerance of plants towards selenium, a non-essential microelement for higher plants, is a key issue when designing either the indirect (selenium-depletion from highly seleniferous soils) or directed (selenized feed production) enrichment of selenium in forages. Alfalfa (*Medicago sativa* L.), the well-known forage crop of the Fabaceae family, has been gaining considerable interest due to its application as a green manure, as a cover crop, or in soil remediation by nitrogen fixation.

Objective: The goal of our study was to assess into which selenocompounds alfalfa plants biotransform the excess selenium uptake from the soil. Selenocompounds (other than selenomethionine and inorganic forms) accumulated in the fiber and the so-called brown juice by-product fractions of processed alfalfa biomass were targeted. **Methodology:** Inductively coupled plasma – mass spectrometry assisted multidimensional (strong anion exchange, strong cation exchange, reversed phase) orthogonal chromatographic purification was applied to supply Se-containing fractions in adequately high purity for electrospray high-resolution mass spectrometry (used for the first time for this matrix) analyses.

Results: As a total, 30 selenocompounds (with isomers) were described, showing the abundance of the derivatives of selenohexose, selenohomolanthionine, and 2,3-dihydroxypropionic acid. Out of the 30 selenocompounds, 15 could be assigned the elemental composition, and the tentative structure of five compounds including among others deamino-2-oxo-selenohomolanthionine, deamino-hydroxy-selenohomolanthionine, and the dimer of 2,3-dihydroxypropionyl-selenohomocysteine could be presented.

Conclusions: The studied fractions arising from the standard alfalfa processing technology contained a wide variety of selenocompounds whose origin can be either the plant's inherent Se metabolism or the processing technology itself. The importance of negative mode data acquisition has been highlighted, as out of the 30 compounds, 16 could be detected exclusively in this electrospray ionization mode.

1. Introduction

Among forage crops, perennial legume alfalfa (*M. sativa* L.) is of particular importance for its nutritional and agroecological impacts. Alfalfa is widely grown in many regions around the world, with a global cultivated area of 32 Mha yielding annually about 211 Mt fresh biomass [1]. In Europe, the alfalfa cultivated area represents about 7.12 Mha [2].

Alfalfa is conventionally used for making hay and silage in livestock production. However, biorefining fresh green biomass is an emerging solution to produce value-added products and platform chemicals,

maximizing the potential of green crops. Alfalfa is the most promising feedstock for green biorefineries, alongside grasses, clover, and different green residues, which can also be used [3,4]. The basic protocol of a green biorefinery starts with the mechanical fractionation of green biomass, resulting in fiber-rich press cake and green liquid juice. The colour of green juice is due to the chlorophylls it contains, which are susceptible to weak acids and heat. The further processing mainly concentrates the soluble proteins in the green juice by thermal or acid precipitation and then separates them from the aqueous fraction [5]. The main product, leaf protein concentrate (LPC), could substantially

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substitute soybean, which is currently the primary source of protein in animal feed. The aqueous phase, the so-called brown juice, is the most abundant (up to 50 %) waste stream of the green biorefinery process [6]. During microwave coagulation, the transformation of the chlorophyll content in the green juice into pheophytin and pheophorbide contributes to the yellowish-brown or greenish-brown colour of brown juice.

The biochemical composition of brown juice makes it a promising intermediate for further research and valorization. Up to date, it has been used as a soil conditioner and fertilizer [7]. The simplest method of application is directly to the soil of field crops by irrigation. In addition to organic components, brown juice is also rich in macro- and micro-nutrients. Among the microelements, the selenium (Se) content of untreated alfalfa stems and leaves varies between 0.07 and 0.33 and 0.52–0.86 mg kg⁻¹, of which 0.1–0.2 mg L⁻¹ selenium remains in the brown juice during the wet fractionation process [8]. Since selenium is essential for the development of farm animals, Se-enriched forage crops are an opportunity to meet feed quality needs within a biofortification strategy for crops [9]. The agronomic enrichment of alfalfa offers the possibility that the LPC from the harvested green biomass can, in addition to being a source of protein, also fulfill the selenium requirements of animals, as underlined by Kovács et al. [10]. In this latter study, a strong anion exchange (SAX)-HPLC-ICP-MS (high performance liquid chromatography-inductively coupled plasma mass spectrometry) set-up was addressed for the speciation of selenium that was found mainly in the forms of selenomethionine and selenate in selenium-enriched alfalfa. Along with the fractions from green biomass, selenomethionine was present in only a small proportion in free form, as it was mainly incorporated into proteins, accumulating in the LPC. In the brown juice, the anionic selenate form predominated. At the same time, the analysis revealed the presence of a set of selenocompounds in the water extract of the brown juice and in the proteolytic extract of the fiber fractions that cannot be matched with the LC-ICP-MS setup through spiking with any of the available standards.

Selenium-enriched brown juice can be of added value if it is used for industrial purposes such as the cultivation of microalgae, for which, contrary to higher plants, selenium is essential [11]. However, when determining the final use of any selenium-enriched biomass, the highest possible characterization (speciation) of selenium is a clear demand that cannot be actually fulfilled with the sole use of HPLC-ICP-MS instrumentation. Indeed, identification of selenometabolites on the basis of available authentic standards is limited to less than a dozen compounds, while selenium-enriched plants contain a plethora of selenometabolites [12–14]. Assigning and tentatively identifying these selenometabolites call for the application of high-resolution electrospray mass spectrometry (HR-ESI-MS), often assisted by preliminary orthogonal chromatographic purification to achieve adequate purity even for the detection (“spotting”) of selenium-containing compounds [15,16].

The goal of our study was to characterize the unknown selenium species of Se-enriched alfalfa fractions, fiber, and brown juice extracted during the wet processing of alfalfa green biomass that had to be left unidentified during the former SAX-HPLC-ICP-MS analysis focusing only on the most often quantified selenium species, the inorganic ones (selenite, selenate, elemental Se) and selenomethionine. To achieve this, two-dimensional orthogonal purification was involved, followed by the HR-ESI-MS-based characterization (accurate mass, elemental composition, and MS/MS analyses) of the assigned selenocompounds. High emphasis was put on the screening of the negative mode electrospray data, which represent a generally dismissed part of selenium speciation [17]. When applicable, providing tentative structures was also challenged.

2. Materials and methods

2.1. Plant and soil materials

Seeds of alfalfa (*M. sativa* L. ‘Tápiószelei 1’ cultivar) were provided

by Tedej Ltd, Hungary. The soil used for the pot experiment was topsoil (0–25 cm) collected from the demonstration garden of the University of Debrecen (Debrecen, Hungary; 47° 32′ 0″; 21° 38′ 0″ E). The soil was dried, crushed, and sieved before use.

2.2. Plant seed sowing, selenium treatment and the processing of fresh green biomass

The pot experiment was conducted in a greenhouse environment and was described in detail by Kovács et al. [8]. In brief, seeds of alfalfa (*M. sativa* L. ‘Tápiószelei 1’ cultivar) were sown in “190 + 2 × 80 × 350 mm” polyethylene plant containers (volume: 9 L) filled with 8 kg of sieved and dried soil. Seed sowing was conducted at a rate of 0.29 g seed pot⁻¹.

For the actual study, selenium treatment was achieved with selenite (Na₂SeO₃; 99 %, Merck-Sigma Group; Darmstadt, Germany) at the application rate of 50 mg Se kg⁻¹ soil. The treatment was carried out only once after seed germination by soil irrigation. Afterwards, the soil moisture content of pots was kept at 75 % water saturation using tap water (checked by weighing the pot each time it was watered) during the whole experimental period. Altogether, four harvests were done, out of which the biomass arising from the first harvest was taken for the study. The total selenium contents of the stem and leaf parts of alfalfa were determined to be 0.10 mg Se kg⁻¹ d.w. (dry weight) and 0.86 mg Se kg⁻¹ d.w. for the control plants and 55.42 mg Se kg⁻¹ d.w. and 145.6 mg Se kg⁻¹ d.w. for the selenium enriched plants, respectively, which indicates a more than 100-fold enrichment.

The green biomass of the plants was harvested at the green bud stage 39 days after seed sowing, taking into account the harvesting practices of alfalfa. The fresh alfalfa biomass was fractionated by a twin-screw juicer (Angel Juicer 5500, Angel Ltd, Busan, South Korea) resulting in green juice and fiber (‘F’) fractions. Then the green juice fraction was microwave coagulated at 80±5 °C concentrating the soluble proteins. The protein aggregates contained in the leaf protein concentrate (LPC) fraction were separated from the brown juice (‘BJ’) by cloth filtration. The ‘BJ’ fraction was frozen and kept for further measurements at –20 °C to prevent chemical changes or spoilage. The fiber fraction (‘F’) was freeze-dried (Alpha 1–4 LSC Basic; Martin Christ Ltd., Osterode am Harz, Germany) and ground for further analysis. The total protein content of the control and selenium enriched ‘F’ and ‘BJ’ fractions were as follows: 11.69 m/m% and 7.52 m/m% (d.w.) for the ‘F’ fractions, and 1.41 m/m % and 1.18 m/m% (fresh weight) for the ‘BJ’ fractions, respectively; that is, the protein content considerably decreased in the selenium enriched plants at the selenium application rate of 50 mg Se kg⁻¹ soil, compared to the control plants.

In our previous work, the total selenium content of leaf shoots and their processed fractions (fiber (‘F’; 171.0±1.86 mg Se kg⁻¹ d.w.) and brown juice (‘BJ’; 78.5±1.3 mg Se kg⁻¹ fresh weight)) were measured [10]. Taking into account the results of growth parameters and total selenium content, selenium speciation analyses were performed by strong anion exchange (SAX) HPLC-ICP-MS. The main forms of Se in the plant samples were selenomethionine (SeMet) and Se(VI), together with unknown selenium forms in the ‘F’ and ‘BJ’ fractions of fractionated alfalfa. For this reason, in the present work, we focused only on the identification of unknown components in these two fractions obtained from the plants exposed to the 50 mg Se kg⁻¹ soil treatment.

2.3. Sample preparation and chromatographic fraction collection for selenium speciation

Brown juice (‘BJ’) was diluted two-fold with deionized water (Millipore, Molsheim, France) in a centrifuge tube and sonicated for 10 min. After that, the sample was centrifuged at 8000 × g for 10 min, then the supernatant was filtered through a 0.22 µm pore-sized hydrophilic polytetrafluoroethylene (PTFE) syringe filter and subjected to the SAX-HPLC-ICP-MS analysis.

Concerning the fiber ('F'), 200 mg-sized subsamples were weighted into 15-ml centrifuge tubes in five replicates with 10 ml deionized water and sonicated for 10 min. Afterwards, the supernatants were discarded, and the solid residues were enzymatically digested with Pronase E (from *Streptomyces griseus*, 4000,000 PU g⁻¹, Merck-Sigma Group) in a water bath at 37°C overnight. About 40 mg of enzyme per sample was dissolved in 9.0 ml of 100 mM tris(hydroxymethyl)aminomethane (TRIS) buffer and mixed with the plant residues. The pH of the buffer was adjusted to pH=9.0 with 1.0 M HCl solution (Merck-Sigma Group) to compensate for the increasing acidity during the proteolysis. An additional 40 mg of Pronase enzyme per sample was dissolved in 1.0 ml TRIS buffer and added to the samples the following day. The samples were shaken for an additional six hours at 37°C. Samples were then centrifuged at 8000 × g for 10 minutes. Supernatants were removed, pooled, and filtered through a 0.22 µm pore-sized hydrophilic PTFE syringe filter. About 35 ml of enzymatically digested extract was obtained, which was afterwards lyophilized, then dissolved in 5.0 ml of the starting SAX eluent (see later).

The separation was done with the help of a Waters Alliance 2695 HPLC instrument (Waters Co., Milford, MA, USA) using strong anion exchange (SAX) and strong cation exchange (SCX) chromatographic setups. The analytical columns were thermostated at 25°C. The injection volume was 100 µl in all cases. The LC effluents were introduced into an X-Series II inductively coupled plasma mass spectrometer (ICP-MS; Thermo Scientific, Bremen, Germany) to monitor the elution of Se-containing compounds on the ⁷⁸Se isotope and to determine the retention time frames. For fraction collection purposes, the effluent was disconnected from the ICP-MS, and the fractions were collected manually. Retention time frames were checked on a daily basis during the whole fraction collection campaign.

At the first step of the purification, SAX chromatography was applied on a PRP-X100 column (250 mm × 4.1 mm, 10 µm; Hamilton, Reno, NV). Eluent A was 10 mM ammonium acetate (pH=5.5), while eluent B was 300 mM ammonium acetate (pH=5.5), prepared from ammonium acetate (ACS, Reag. Ph. Eur., ≥ 98 %, Merck-Sigma Group), and the pH was adjusted with acetic acid (glacial; ACS, Reag. Ph. Eur., VWR, Debrecen, Hungary). Gradient elution was applied as follows: 0 min: 0 % B, 0–8.5 min: linear gradient up to 85 % B, 8.5–8.6 min: linear gradient down to 0 % B, 8.6–11.5 min: 0 % B. The flow rate was 2.0 ml min⁻¹. About 50 injections were done for fraction collection from each sample.

After fraction collection, each fraction was separately lyophilized, and the residues were dissolved in 2.0 ml of 1 mM pyridine formate (pH=2.8). The solutions were filtered (0.22 µm hydrophilic PTFE), then the purification was continued with SCX chromatography on a PRP-X200 column (250 mm × 4.1 mm, 10 µm; Hamilton). Eluent A was 1 mM pyridine formate (pH=2.8), while eluent B was 40 mM pyridine formate (pH=2.8; the concentration refers to pyridine (Spectranal, Riedel-De Haën AG, Germany) and the pH was adjusted with formic acid (98–100 %, ACS, Reag. Ph. Eur.; Scharlab S.L. Sentmenat, Spain). The gradient program was as follows: 0–1 min: 0 % B at 1.7 ml min⁻¹; 1–15 min: linear gradient up to 30 % B at 1.7 ml min⁻¹; 15–16 min: linear gradient up to 100 % B at 1.9 ml min⁻¹; 16–21 min: 100 % B at 1.9 ml min⁻¹; 21–21.5 min: linear gradient down to 0 % B at 1.7 ml min⁻¹; 21.5–26 min: 0 % B at 1.7 ml min⁻¹.

Fractions were lyophilized and then dissolved in 0.25 ml of deionized water containing 0.1 % V/V formic acid for the UPLC-UniSpray-QTOF-MS analyses.

2.4. UPLC-UniSpray-QTOF-MS set-up

A Vion ion mobility quadrupole time-of-flight mass spectrometer (Waters) equipped with a UniSpray (Waters) ion spray source was applied. Chromatographic elution was provided by an Acquity UPLC I-Class system (Waters) using a BEH-C18 reversed phase (RP) UPLC column (100 mm × 2.1 mm, 1.7 µm; Waters). Chromatographic elution with eluent A (deionized water with 0.1 % V/V formic acid /LC-MS

grade; VWR/) and eluent B (acetonitrile /LC-MS grade, VWR/ with 0.1 % V/V formic acid) was carried out at 0.4 ml min⁻¹ with two different gradients (#1 and #2) to check the possible co-elution of species. The UniSpray ion source was used both in positive and negative ionization modes, either in MS^E or MS^E → MS/MS /data dependent acquisition, DDA/ functions. The related instrumental and gradient parameters are described in the Tables S1 and S2. Data evaluation was carried out with the help of the Unifi software (version 1.9.4; Waters). Selenium-containing species were screened in the full scan spectra according to the method developed by Ouerdane et al. [13] and they were subjected to MS/MS analysis with an individually optimized fragmentation energy setting.

3. Results and discussion

3.1. Purification through two-dimensional orthogonal chromatography

Two- or multidimensional chromatographic purification is not a definite must in selenium speciation [18], especially in the case of highly selenized (> 1000 mg Se kg⁻¹ dry weight) samples where even a single hydrophilic interaction chromatographic separation, assisted with automated selenospecies recognition can be addressed [19]. On the other hand, any additional purification, based on either a robust ultra-filtration process with a low molecular weight cut-off membrane, preparative size exclusion chromatography [20,21], or the application of orthogonal chromatographic setups [22,23] can considerably contribute to a higher species assignment rate. In this study, the depletion of the most concentrated selenospecies, selenate, and selenomethionine (together with a supposedly high proportion of sample matrix) was achieved with the help of SAX and SCX HPLC-ICP-MS setups.

As presented in Fig. 1, four fractions containing unknown seleno-compounds could be collected from the water extract of the 'BJ' fraction (BJ0, BJ1, BJ2, and BJ3) prior to the elution of selenite, and two fractions from the proteolytic digest of the 'F' fraction (F0 and F1) prior to the elution of selenomethionine (Fig. 2). The individual concentrations of total Se in these six fractions were estimated on the basis of our previous study [10], using the calibration curve of selenomethionine, the closest eluting selenocompound. For the BJ0–1–2–3 fractions, the estimated total Se concentrations were 1.14, 0.11, 0.72 and 0.13 mg Se kg⁻¹ fresh weight, respectively, which accounted for approximately 3 % of total Se content in the brown juice sample. For the F0 and F1 fractions, the estimated concentrations were 0.93 and 1.15 mg Se kg⁻¹ d.w., respectively, accounting for 1.2 % of total Se content in the fiber sample.

All these fractions from the SAX purification step were subjected to the SCX HPLC-ICP-MS experiment. According to Figs. 3 and 4, the BJ fractions 1, 2, 3 and the fiber fraction F1 showed adequate retention on the SCX column, while fractions B0 and F0 were eluted in the void volume of this column as well. The lyophilization of the fractions obtained from SCX revealed that the B0 and F0 fractions were still highly loaded with matrix and/or chromatographic mobile phase-derived constituents; therefore, they were not subjected to UPLC-ESI-MS analyses.

3.2. Assignment of selenometabolites with tentative identification

Taking into account the very short list of commercially available organic selenocompounds (e.g., selenomethionine and methyl-selenocysteine), the assignment of unknown analytes must rely on formerly reported MS/MS data. While focusing only on the positive ionization mode derived information, the most recent compilation of selenocompounds of selenized yeast origin [24] is still the most valuable database that must be addressed during the evaluation process.

The screening for selenometabolites in the four fractions (F1, BJ1, BJ2, and BJ3) selected for UPLC-ESI-MS analyses resulted in the detection of 30 compounds, including isomers. Five out of these 30 analytes could be characterized with high quality MS/MS information that

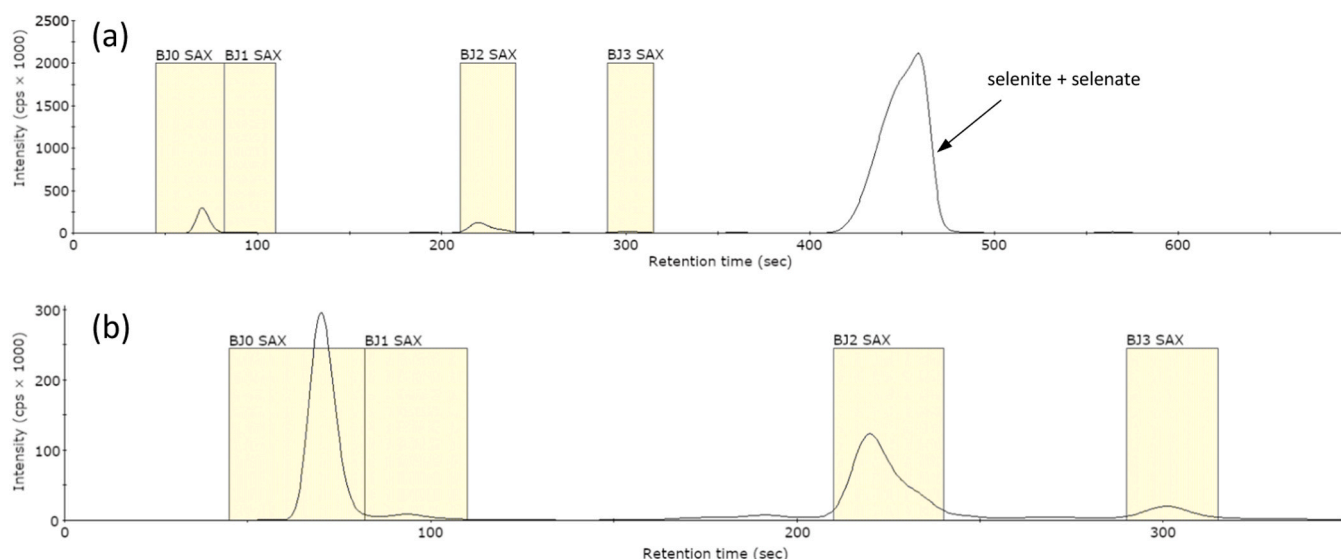


Fig. 1. SAX-ICP-MS chromatogram of brown juice (BJ), recorded on the ^{78}Se isotope in full view (a) and zoomed to the less abundant peaks eluting before the co-elution of selenite and selenate (b). Yellow squares mark the retention windows of the fractions: fraction BJ0, between 45 and 82 s; BJ1, between 82 and 110 s; BJ2, between 210 and 240 s; while for BJ3, between 290 and 315 s. The co-elution of selenite and selenate starts after 400 s.

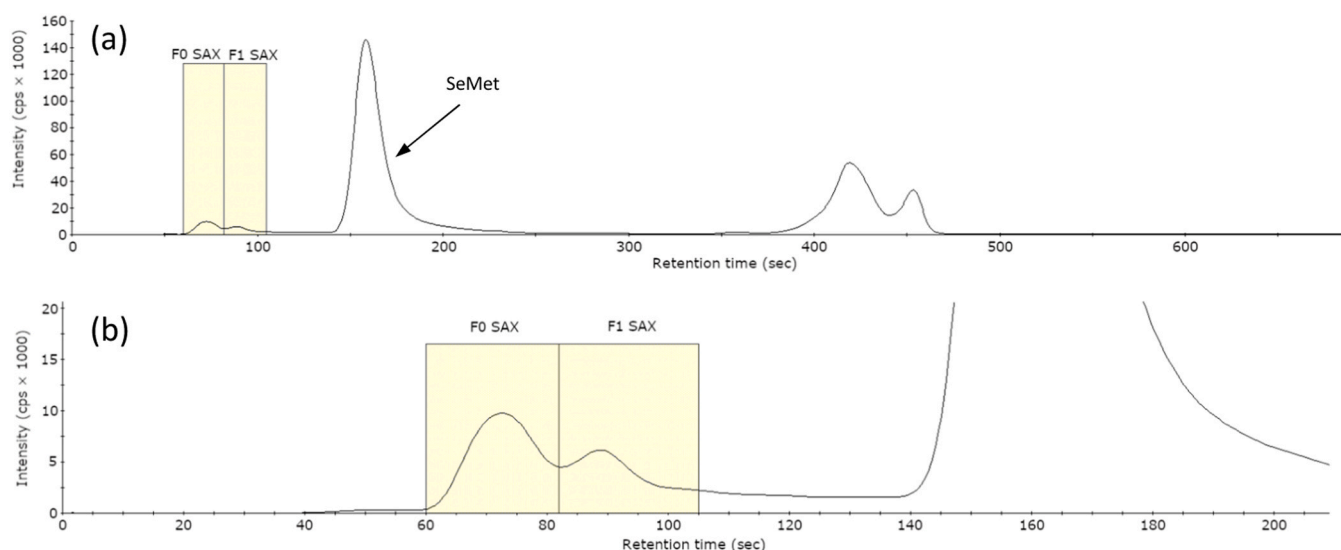


Fig. 2. SAX-ICP-MS chromatogram of enzymatic fiber extract, recorded on the ^{78}Se isotope in full view (a) and zoomed to the less abundant peaks (b). Yellow squares mark the retention windows of the fractions: fraction F0, between 60 and 82 s; F1, between 82 and 105 s. The elution of selenomethionine starts after 140 s.

allowed even for their tentative structural identification; these compounds are qualified with Level ‘2a’, ‘2b’ and ‘3’ scores in Table 1 and Table S3, according to the grading system introduced by Schymanski et al. [25]. Also, an additional set of nine species is presented in Table 1, where either the MS/MS information could provide matching with formerly reported selenocompounds or where important molecular features could be highlighted.

In the BJ1 fraction, the presence of selenosugars was a key feature. First, two isomers of the molecule at m/z 363.0553 (ESI ‘+’; m/z 361.0410 in ESI ‘-’) were detected (Figs. S1–S3). The MS/MS spectrum (Fig. S4) revealed the neutral loss of a hexose moiety, which could contribute to the suggestion of the elemental composition of $\text{C}_{11}\text{H}_{23}\text{O}_8\text{Se}^+$. This composition matches that of the selenium analogue of neoponkoranol, the de-*O*-sulfonated glycosidase inhibitor ponkoranol, which contains a constant cationic selenonium moiety [26]. No MS/MS spectra have been published either on this selenium analogue or the sulfur species to compare with the actual, alfalfa-derived molecule.

However, the ESI-triple quadrupole-MS/MS fragmentation of neokotalanol and neosalacinol, two other antidiabetic compounds of the genus *Salacia* (*Celastraceae* family) with a sulfonium moiety, results in a characteristic m/z 115 fragment that should arise from the sulfonium moiety [27]. Such a fragment could not be detected in our case, considering either the neat (non-selenized; m/z 115) or the possibly S→Se substituted (m/z 163) form. Accordingly, the two isomers of $\text{C}_{11}\text{H}_{23}\text{O}_8\text{Se}^+$ should be regarded as unknown selenosugars.

Another selenosugar was also detected in fraction BJ1, at m/z 407.0458 (ESI ‘+’; $\text{C}_{12}\text{H}_{23}\text{O}_{10}\text{Se}^+$; m/z 405.0309 in ESI ‘-’; Figs. S5–S6). This selenosugar was first reported in 2012 by Aureli et al. [28] with a tentative structure in a series of crop samples (rice, maize, and wheat) and has been found in other plant samples (*Brassica nigra* [12]; *Cardamine violifolia* [13]; *Astragalus bisulcatus* [29]) as well.

Fraction BJ2 provided the highest number of detected selenometabolites, including selenosugars and other molecules as well. The most abundant selenocompound was a tentative selenosugar at m/z 389.0359

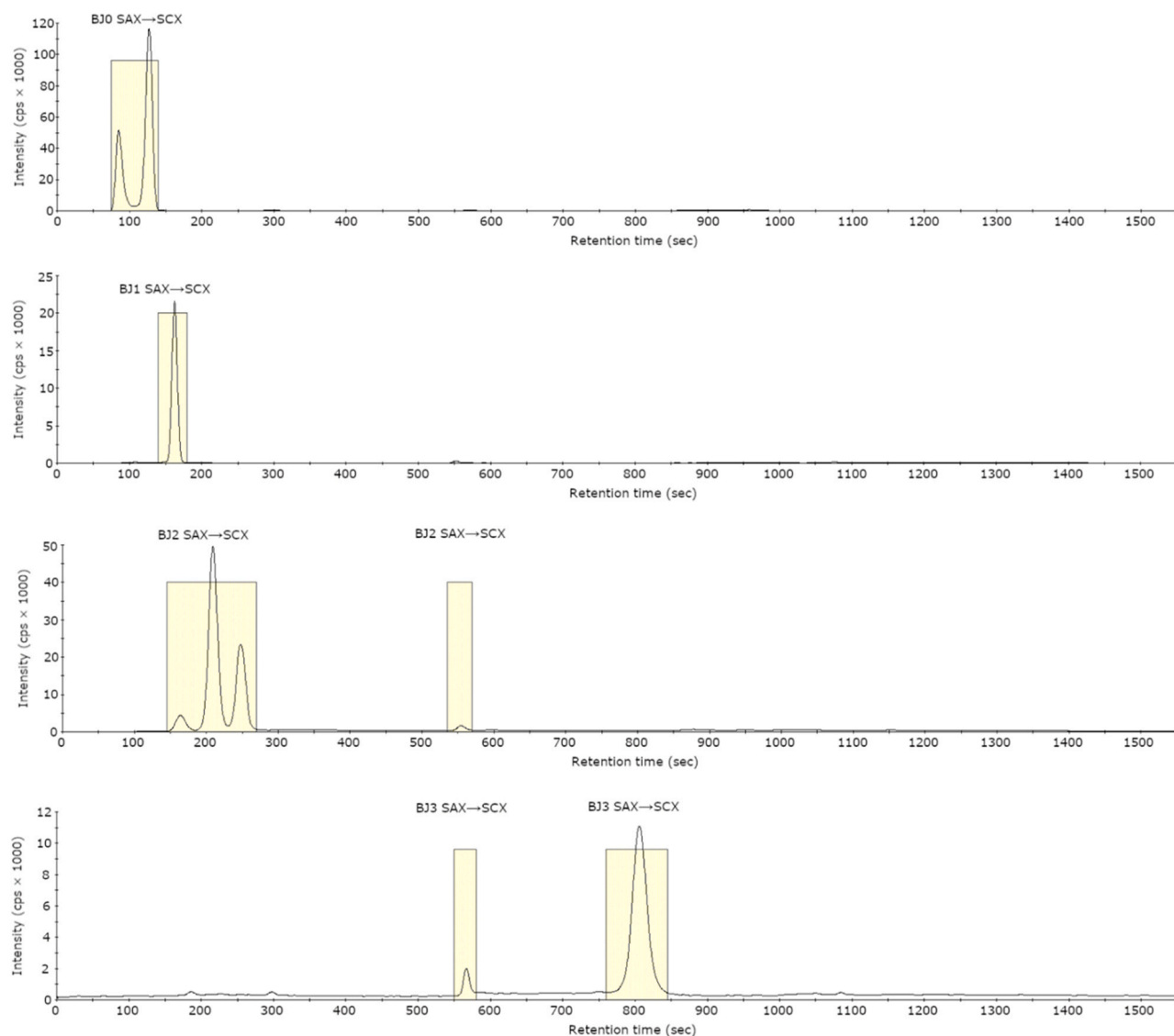


Fig. 3. SCX-ICP-MS chromatograms of the BJ0, BJ1, BJ2 and BJ3 fractions of brown juice after SAX purification, recorded on the ^{78}Se isotope. Yellow squares mark the retention windows of the fractions: BJ0, between 75 and 140 s; BJ1, between 140 and 180 s; BJ2, between 145 and 270 and 535–570 s; BJ3, between 550 and 580 and 760–845 s. In the case of BJ2 and BJ3, the related two-two fractions were pooled after collection to result in a single BJ2 and a single BJ3 fraction.

(ESI $^-$; RT=1.02 min; $\text{C}_{12}\text{H}_{21}\text{O}_9\text{Se}^-$) that was also detected in the form of Na^+ and K^+ adducts in the positive ion mode (Figs. S7–S8). The compound was so abundant that its $[2\text{M}-\text{H}]^-$ at m/z 779.0783 and its $[2\text{M}+\text{anhydrohexose neutral loss}-\text{H}]^-$ adducts at m/z 941.1308 were also detected. As presented in Fig. S9, the compound exhibits its hexose loss from the sodiated adduct to result in a $\text{C}_6\text{H}_{12}\text{O}_4\text{Se}+\text{Na}^+$ MS/MS fragment at m/z 250.9795. In turn, in the negative ion mode, selenium leaves in the form of a neutral loss ($\text{C}_2\text{H}_4\text{O}_2\text{Se}$; $\Delta = 139.937$ Da) during MS/MS fragmentation (Fig. S10) and the anhydrohexose is detected as an MS/MS fragment at m/z 161.0449. The neat neutral loss of $\text{C}_2\text{H}_4\text{O}_2$ can be indicative of a carboxylic acid group with one carbon attached [30]. The presence of a carboxylic group in the selenized moiety of this selenosugar can explain the relatively poor retention of this molecule on the reversed-phase column. The exact structure of the molecule cannot be determined without nuclear magnetic resonance (NMR) spectroscopy-based data; however, the fragment at m/z 94.9402 is characteristic of an end-chain methylseleno-species.

In this BJ2 fraction, there were at least three other selenium species that belong to this compound:

- (i) the molecule detected at m/z 551.0885 (ESI $^-$) exhibits the same selenized neutral loss ($\text{C}_2\text{H}_4\text{O}_2\text{Se}$), the same series of selenium-free fragments at m/z 101.02, 113.02, 149.04, 161.04, 179.05, and 249.09 during MS/MS fragmentation, and presents an extra hexose loss between the fragments at m/z 249.09 and 411.15. Accordingly, this molecule can be tentatively identified as $\text{C}_{18}\text{H}_{31}\text{O}_{14}\text{Se}^-$ (ESI $^-$), the diglycoside derivative of the compound m/z 389.0359 (Figs. S11–S13).
- (ii) the MS/MS spectrum of the molecule detected at m/z 649.0563 (ESI $^-$) shows a selenized fragment at m/z 551.0873 that differs from the precursor ion with the mass of 97.9689 Da and has a fragment at m/z 96.9607 – these masses refer to a sulfate loss, resulting in the tentative elemental composition of $\text{C}_{18}\text{H}_{33}\text{O}_{18}\text{Se}^-$. This molecule also shares several common fragments (m/z 161.04, 249.09, and 411.15 /as selenium-free/; m/z 389.04, and 457.01 /as selenized/) and the same neutral loss of $\text{C}_2\text{H}_4\text{O}_2\text{Se}$ (Figs. S14–S15).
- (iii) the ion at m/z 417.0309 (ESI $^-$; $\text{C}_{13}\text{H}_{21}\text{O}_{10}\text{Se}^-$) was detected at RT=2.01 min. This molecule could not be detected in the positive ion mode, and it showed the feature of on-column (or in-source) oxidation as its oxidized form (Fig. S16). This selenocompound

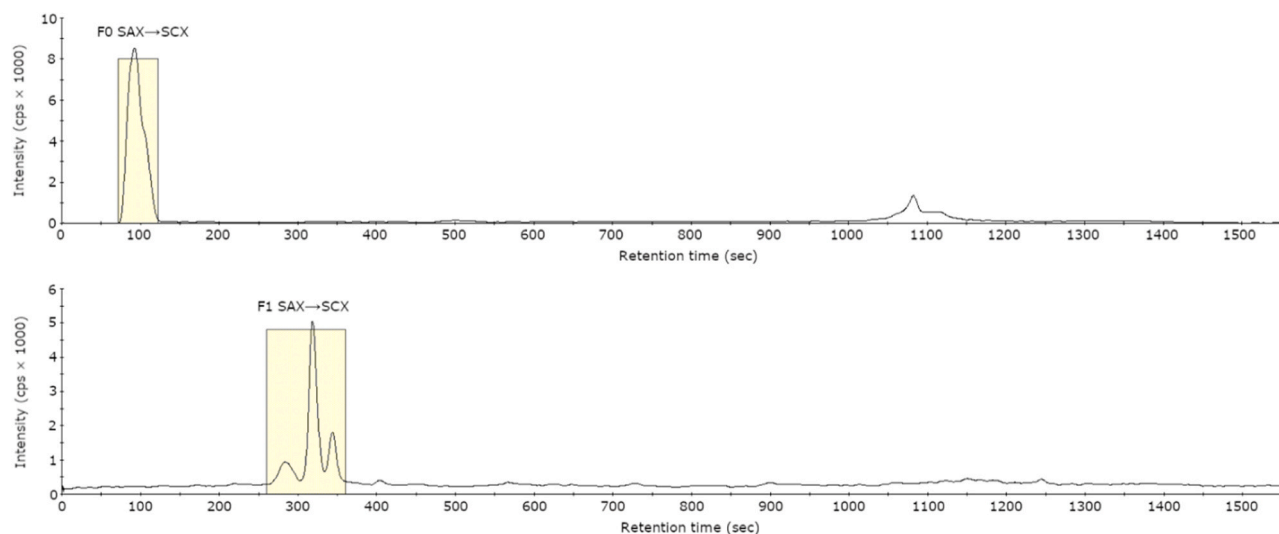


Fig. 4. SCX-ICP-MS chromatogram of the F0 and F1 fractions of fiber after SAX purification, recorded on the ^{78}Se isotope. Yellow squares mark the retention windows of the fractions: F0, between 72 and 122 s; F1, between 260 and 360 s.

possesses the same series of selenium-free fragments at m/z 101.02, 113.02, 161.04, 179.05, and 249.09 during MS/MS fragmentation, while the selenized neutral loss ($\text{C}_3\text{H}_4\text{O}_3\text{Se}$; $\Delta = 167.932$ Da) is different, resulting in the suggested elemental composition of $\text{C}_{13}\text{H}_{21}\text{O}_{10}\text{Se}^-$ (Fig. S17). This neutral loss can be regarded as an extended form of a neat ($\text{C}_3\text{H}_4\text{O}_3$) neutral loss that can refer to a recombination of small (i.e., free carboxyl and acetyl) group losses [30]. Accordingly, the elemental difference (CO) from the molecule at m/z 389.0359 can be associated with an additional acetyl group in the molecule; however, no structural information can be given without NMR experiments.

The tentative assignment of a sulfated selenosugar in this fraction is supported by the detection of another similar couple of molecule entities. The match of the extracted ion chromatograms (EICs) of the molecules detected at m/z 398.0004 and 495.9677 (ESI $^-$; Fig. S18) suggests the former one is the ion source fragment of the latter, revealing a mass difference of 97.9673 Da. At the same retention time, a molecule at m/z 400.0136 was detected together with its K^+ and Na^+ adducts in the ESI $^+$ mode (Fig. S19). MS/MS acquisition could be recorded only for the compound detected in the positive ion mode (Fig. 5), showing the presence of the characteristic selenohomocysteine fragment couple (m/z 135.9664 / $\text{C}_3\text{H}_6\text{NSe}^+$; 2.96 ppm/ and 181.9736 / $\text{C}_4\text{H}_8\text{NO}_2\text{Se}^+$; 11.7 ppm/). These fragment compositions allow for one elemental composition ($\text{C}_{12}\text{H}_{18}\text{NO}_9\text{Se}^+$) that does not match the species at m/z 398.0004 in the negative ion mode within 2 ppm mass accuracy. Moreover, it is not highly probable that a compound becomes simultaneously desulfated and sodiated/potassiated in the ion source. Accordingly, no elemental composition could be suggested for the tentative sulfated selenosugar species detected in the negative ion mode.

The compound at m/z 400.0136 with the suggested elemental composition of $\text{C}_{12}\text{H}_{18}\text{NO}_9\text{Se}^+$ has a selenized MS/MS fragment at m/z 269.9892 ($\text{C}_7\text{H}_{12}\text{NO}_5\text{Se}^+$) which is associated with its highly abundant selenium-free couple at m/z 190.0721 ($\text{C}_7\text{H}_{12}\text{NO}_5^+$). However the overall intensity of the MS/MS fragments is low and the mass accuracy is poor, the presence of the selenohomocysteine-related fragments, the characteristic neutral losses of formic acid (to result in the fragments of m/z 354.0092, m/z 308.0022, and m/z 144.0667) and water (to result in the fragments of m/z 282.1287, 336.0011, and 382.0018), the high Double Bond Equivalent (DBE) value (5) suggest this molecule is a selenoether that contains several hydroxyl groups, but not a selenosugar. Regarding the fact that the single nitrogen atom in the molecule takes part both in the selenohomocysteinyl fragment ($\text{C}_4\text{H}_8\text{NO}_2\text{Se}^+$) and in another, non-

selenized fragment ($\text{C}_7\text{H}_{12}\text{NO}_5^+$), the presence of an amide bond can be suggested to explain the formation of the $\text{C}_7\text{H}_{12}\text{NO}_5\text{Se}^+$ fragment. As a matter of fact, the accurate mass of the fragment of m/z 269.9875 was reported first by Dernovics and Lobinski [31] for the 2,3-dihydroxypropionyl-Se-homocysteine moiety. Indeed, the fragmentation of the amide bond in the positive ion mode releases the primary amine that appears in the two selenohomocysteine-derived fragments and renders them readily ionized. The other part of the molecule ($\text{C}_5\text{H}_6\text{O}_4$, responsible for a DBE value of 2) cannot be unambiguously determined; therefore, the structure presented in Fig. 5 is only tentative and based on the relatively abundant fragment at m/z 186.9669 ($\text{C}_7\text{H}_7\text{OSe}^+$): i.e., the fragmentation of the amide bond, accompanied by a series of neutral losses (two formic acid residues, one ammonia, and one water molecule) might arrive at the cationic fragments presented (Fig. S20). As the molecule is an asymmetric selenoether, it is unclear whether the resulting selenized fragments possess selenonium-type or selenol-type structures in case the nitrogen is not lost during the fragmentation process. Taking into account the postulated fragmentation pathways presented for the sulfur-containing amino acids by Zhang et al. [32], both structures have been shown in Fig. S20. Also, due to its high unsaturation value, the possible structure of the m/z 186.9669 fragment might contain either a ring or an allene moiety; however, according to the recent study of van Tetering et al. [33], the presence of a ring is more possible. Additionally, the non-selenized fragment at m/z 300.1458 ($\text{C}_{14}\text{H}_{22}\text{NO}_6^+$) can be the result of an in-source recombination event after the central selenium atom splits off.

The detection of the 2,3-dihydroxypropionyl-Se-homocysteine moiety is supported by the analysis of two other, doubly selenized compounds in this BJ2 fraction. The MS/MS spectrum of the compound detected at m/z 538.9691 ($\text{C}_{14}\text{H}_{23}\text{N}_2\text{O}_{10}\text{Se}_2$; RT=2.13 min; Figs. S21–S22; Fig. 6) shows the feature of a diselenide bond and a symmetric structure by the sequential loss of selenium atoms, the presence of the HSe_2 fragment (at m/z 160.8417) and the non-selenized fragment of m/z 188.0567 that is also detected in the form of a neutral loss ($\text{C}_7\text{H}_{11}\text{NO}_5$; Fig. S23). This molecule can be assigned as the dimer of 2,3-dihydroxypropionyl-Se-homocysteine. However this species has not been reported earlier, its homologue, the Se-Se conjugate of 2,3-dihydroxypropionyl-selenocysteine and 2,3-dihydroxypropionyl-selenohomocysteine was detected by Preud'Homme et al. [34] in a selenized yeast sample. Indeed, this latter species was the other doubly selenized compound in this BJ2 fraction, detected at RT=1.32 min ($\text{C}_{13}\text{H}_{22}\text{N}_2\text{O}_{10}\text{Se}_2$ for the neutral composition; Figs. S24–S25 and Fig. 7). It is to highlight that these two species could be detected in both

Table 1
Liquid chromatography – high resolution mass spectrometry data of the Se-enriched alfalfa derived selenometabolites detected in the study (part I). ‘#1’ and ‘#2’ refer to the applied LC gradient (for details, see [Supplementary Material /SM/ Tables S1 and S3](#)), while ‘?’ refers to uncertainty in the assessment due to the lack of characteristic neutral losses of monosaccharides and/or unambiguous elemental composition. Identification levels are assigned according to Schymanski et al. [25].

Sign	RT #1, min	RT #2, min	Ionization	Suggested elemental composition (neutral)	Tentative seleno-sugar	Name, note, or reference	Theoretical m/z	Experimental m/z	Δ, ppm	Identification level	Figure
BJ1	1.30	-	[M+H] ⁺	C ₁₁ H ₂₂ O ₈ Se	yes	hexose loss detected;	363.0553	363.0553	0.00	4	SM–1,4
			[M-H] ⁻			possible oxidation artefact	361.0407	361.0410	0.94		SM–3
	1.49	-	[M+H] ⁺			from the C ₁₁ H ₂₂ O ₇ Se species (Table 2)	363.0553	363.0553	0.00		SM–2
	1.02	-	[M+H] ⁺	C ₁₂ H ₂₂ O ₁₀ Se	yes	Aureli et al. [28]	407.0451	407.0458	1.72	3	SM–5
BJ2			[M-H] ⁻				405.0305	405.0309	0.99		SM–6
	0.99	0.90	[M+H] ⁺	C ₁₂ H ₁₇ NO ₉ Se	no	derivative of 2,3-dihydroxypropionyl-Se-homocysteine	400.0141	400.0136	-1.25	3	Fig. 5; SM–19,20
			[M-H] ⁻	-	?	sulphated derivative of the m/z 398.00044 species	-	495.9677	-	5	SM–18
			[M-H] ⁻	-	?	ion source fragment of the m/z 495.96771 species	-	398.0004	-	5	
	1.02	0.89	[M-H] ⁻	C ₁₂ H ₂₂ O ₉ Se	yes	hexose loss detected	389.0356	389.0359	0.77	4	SM–8,10
			[M+Na] ⁺				413.0321	413.0322	0.24		SM–7,9
	2.01	1.70	[M-H] ⁻	C ₁₃ H ₂₂ O ₁₀ Se	yes	derivative of the C ₁₂ H ₂₂ O ₉ Se species	417.0305	417.0309	0.96	4	SM–16,17
	2.13	1.89	[M-H] ⁻	C ₁₄ H ₂₄ N ₂ O ₁₀ Se ₂	no	dimer of 2,3-dihydroxypropionyl-Se-homocysteine	538.9689	538.9691	0.37	2b	Fig. 6; SM–21,23
			[M+H] ⁺				540.9834	540.9835	0.18		SM–22
	1.32	1.38	[M-H] ⁻	C ₁₃ H ₂₂ N ₂ O ₁₀ Se ₂	no	Se-Se conjugate of 2,3-dihydroxy-propionyl-selenocysteine and 2,3-dihydroxy-propionyl-selenohomocysteine; Preud'Homme et al. [34]	524.9532	524.9537	0.95	2a	Fig. 7; SM–24
			[M+H] ⁺				526.9678	526.9676	-0.38		SM–25
	1.04	1.03	[M-H] ⁻	C ₁₈ H ₃₂ O ₁₄ Se	yes	diglycoside derivative of the C ₁₂ H ₂₂ O ₉ Se species	551.0886	551.0879	-1.27	4	SM–11–13
	1.05	1.04	[M-H] ⁻	C ₁₈ H ₃₄ O ₁₈ SSe	yes	sulphated diglycoside derivative of the C ₁₂ H ₂₂ O ₉ Se species	649.0558	649.0563	0.77	4	SM–14,15
BJ3	0.75	0.43	[M+H] ⁺	C ₈ H ₁₅ NO ₅ Se	no	deamino-hydroxy-selenohomolanthionine; Ouerdane et al. [12]	286.0188	286.0189	0.35	2a	Fig. 8a; SM–26,28
			[M-H] ⁻				284.0043	284.0046	1.06		Fig. 8; SM–27,28
	0.99	0.85	[M+H] ⁺	C ₈ H ₁₃ NO ₅ Se	no	mono-deaminated selenohomolanthionine, oxidized into a ketone (deamino–2-oxo-selenohomolanthionine)	284.0032	284.0039	2.46	2b	Fig. 9; SM–29,30
	1.50	1.45	[M+H] ⁺	C ₁₂ H ₂₂ O ₁₀ Se	yes	hexose loss detected; Aureli et al. [28]	407.0451	407.0449	-0.49	3	SM–32,33
			[M-H] ⁻				405.0305	405.0299	-1.48		
			[M-H] ⁻	C ₁₂ H ₂₂ O ₉ Se			389.0356	389.035	-1.54	4	SM–31

ionization modes, but to a far lesser extent in the positive ion mode. This phenomenon calls attention to the need to address both ionization modes when carrying out untargeted selenometabolomics.

In the BJ3 fraction, several selenium species were detected in the lower molecular mass range (Table 1). Among these, the monoselenized compound at *m/z* 286.0189 (ESI ‘+’; *m/z* 284.0046 in ESI ‘-’; Figs. S26–S27) was the most abundant one. Taking into account its MS/MS analysis, which shows the characteristic neutral losses of amino acids, i.e., formic acid and ammonia (Fig. 8), the only possible elemental composition of C₈H₁₆NO₅Se⁺ could be assigned within 5 ppm mass accuracy. To set up the tentative structure of this compound, the MS/MS fragments at *m/z* 183.9885 (C₄H₁₀NO₂Se⁺) and at *m/z* 102.05531 (C₄H₈NO₂⁺) were of high importance, because they are characteristic fragments of selenohomolanthionine [15]. Indeed, this selenoamino acid (C₈H₁₆N₂O₄Se in neutral state) could be involved in the oxidative deamination process into an alcohol (R₂HC-NH₂ -> R₂HC-OH; Δ= +0.984 Da) of one of its two primary amine groups. Accordingly, the structure of the mono-deaminated and oxidized selenohomolanthionine is suggested for this molecule with the resulting composition, C₈H₁₅NO₅Se: Fig. S28 presents the possible structures of the fragments in both ionization modes, which supports the identification. This selenometabolite was

first reported as deamino-hydroxy-selenohomolanthionine by Ouerdane et al. [12] in mustard seeds (*Brassica nigra*; order: Brassicales) and later in garlic (*Allium sativum*; order: Asparagales) by Ruzszczyńska et al. [35] – our report is the first to detect it in a plant of the order Fabales.

What also reinforces the hypothesis of detecting this selenohomolanthionine derivative is that in this BJ3 fraction, another selenium species at *m/z* 284.0039 (ESI ‘+’) was detected (Fig. S29). The relevant MS/MS spectrum (Fig. 9) contains the two characteristic fragments of selenohomocysteine (*m/z* 135.9668 /C₃H₆NSe⁺/ and 181.9727 /C₄H₈NO₂Se⁺/), which indicates this molecule possesses the elemental composition of C₈H₁₄NO₅Se⁺. This composition was reported in the selenized *Torula* yeast [19], with the tentative origin of selenohomolanthionine after its possible oxidative (mono)deamination to ketone (R₂HC-NH₂ -> R₂C=O; Δ= -1.032 Da). Fig. S30 presents the suggested fragments of this selenoamino acid derivative, deamino-2-oxo-selenohomolanthionine. The oxidation to ketone, that is, the appearance of a more electrophilic moiety, can be responsible for the formation of the C₄H₈NO₂Se⁺ fragment instead of the C₄H₁₀NO₂Se⁺ fragment. Anyhow, it must be added that selenomethionine was formerly reported as the main selenometabolite in alfalfa [36]; accordingly, the involvement of selenomethionine in the formation of the *m/z* 284.0039

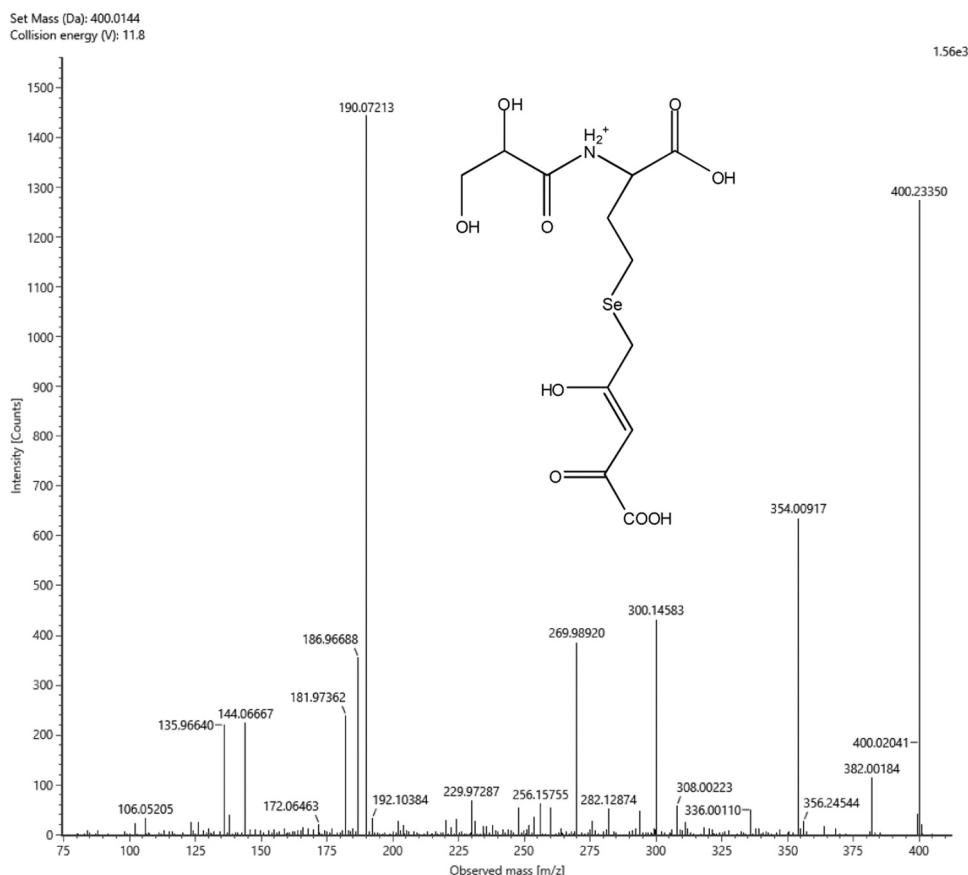


Fig. 5. MS/MS spectrum of the tentative $C_{12}H_{17}NO_9Se$ selenocompound (neutral composition), a derivative of 2,3-dihydroxypropionyl-Se-homocysteine in the ESI '+' mode at RT=0.99 min (Tables 1 and S3; collision energy: 11.8 eV). The inset shows one of the possible structures.

compound cannot be excluded.

No trace of selenohomolanthionine was detected in any of the fractions. One possibility is that this selenocompound was eluted in the void of both analytical columns used (SAX and SCX). Indeed, selenohomolanthionine could be successfully retained either on HILIC [19] or on mixed mode columns [15] and no reports on its analysis on SCX systems have been published. The other possibility is its degradation: the processing of brown juice can be regarded as a highly oxygen- and heat-exposed technique, which can explain the possibly complete transformation/decomposition of selenohomolanthionine into its oxidized derivatives. Regarding plant sources, selenohomolanthionine was identified first from a radish cultivar [15] to an unambiguous extent, i.e., with its chemical synthesis and the related ESI-(HR)-MS support. Its later detection in garlic was accompanied by the co-detection of deamino-hydroxy-selenohomolanthionine [35], while deamino-hydroxy-selenohomolanthionine was reported alone, i.e., without finding selenohomolanthionine, in mustard seeds [12]. On the other hand, all three selenometabolites (that is, selenohomolanthionine and its two oxidized derivatives) have been recently reported in selenized *Candida utilis* [19], the torula yeast in which selenium is accumulated mostly in the form of selenohomolanthionine. Therefore, the co-occurrence of genuine selenohomolanthionine and its oxidized forms can be sample processing and sample species-dependent.

In this fraction, the monoselenized species at m/z 389.0350 (ESI '-'; RT=1.50 min) and its tentatively in-source oxidized derivatives (m/z 407.0449, ESI '+'; m/z 405.0299 in ESI '-'; Figs. S31–S32) were also detected. As the related MS/MS spectrum (Fig. S33) clearly presents the neutral loss of a hexose unit, the elemental composition of $C_{12}H_{21}O_9Se^-$ (ESI '-') can be proposed with high mass accuracy. This selenosugar can be a more hydrophobic isomer of the selenosugar detected in the BJ1 and BJ2 fractions at RT=1.02 min.

3.3. List of selenometabolites without tentative identification

Sixteen out of the 30 detected selenocompounds could not be characterized either with an at least tentative structure or with a group level assignment (Tables 2 and S4). However, important characteristics can be highlighted that can contribute to their future identification processes.

Only one selenium-containing species could be detected in the fiber fraction, at m/z 615.0521 (ESI '-'; Fig. S34). Unfortunately, no characteristic fragments could be identified in the MS/MS spectrum (Fig. S35). In turn, this molecule has a relatively high mass defect that is step-by-step eliminated by the neutral losses, resulting in the product ions at m/z 483.1288 and 451.1570. Taking this into account, this analyte can contain an end-chain R-S-Se-CH₃ moiety, showing the neutral losses of HS-Se-CH₃ (between the fragments of m/z 579.0763 – m/z 451.1570; closest match at Δ –4.64 ppm) and HSe-CH₃ (between the fragments of m/z 579.0763 – m/z 483.1288; closest match at Δ –3.36 ppm), respectively. The R-S-Se-CH₃ conjugates are often reported in selenized samples, mostly in the form of (seleno)amino acid or glutathione derivatives [34,37,38]; however, this actual species was not detected in the positive ion mode, which indirectly indicates it is possibly not an amino acid derivative. Finally, no elemental composition can be proposed because there are too many possible options within the mass accuracy range of the instrument.

Showing close elution times, a selenium species at m/z 345.0462 (ESI '-') was detected in both ionization modes with different adducts (Figs. S36–S37). Its elemental composition, $C_{11}H_{21}O_7Se^-$, differs only by one oxygen atom from that of the previous selenosugar. It cannot be determined whether these two species are related, and a possible in-source (or on-column) oxidation takes place, resulting in the oxidized form and indicating that the analyte at m/z $C_{11}H_{21}O_7Se^-$ is the genuine (non-artifact) species, because of the lack of adequate quality MS/MS

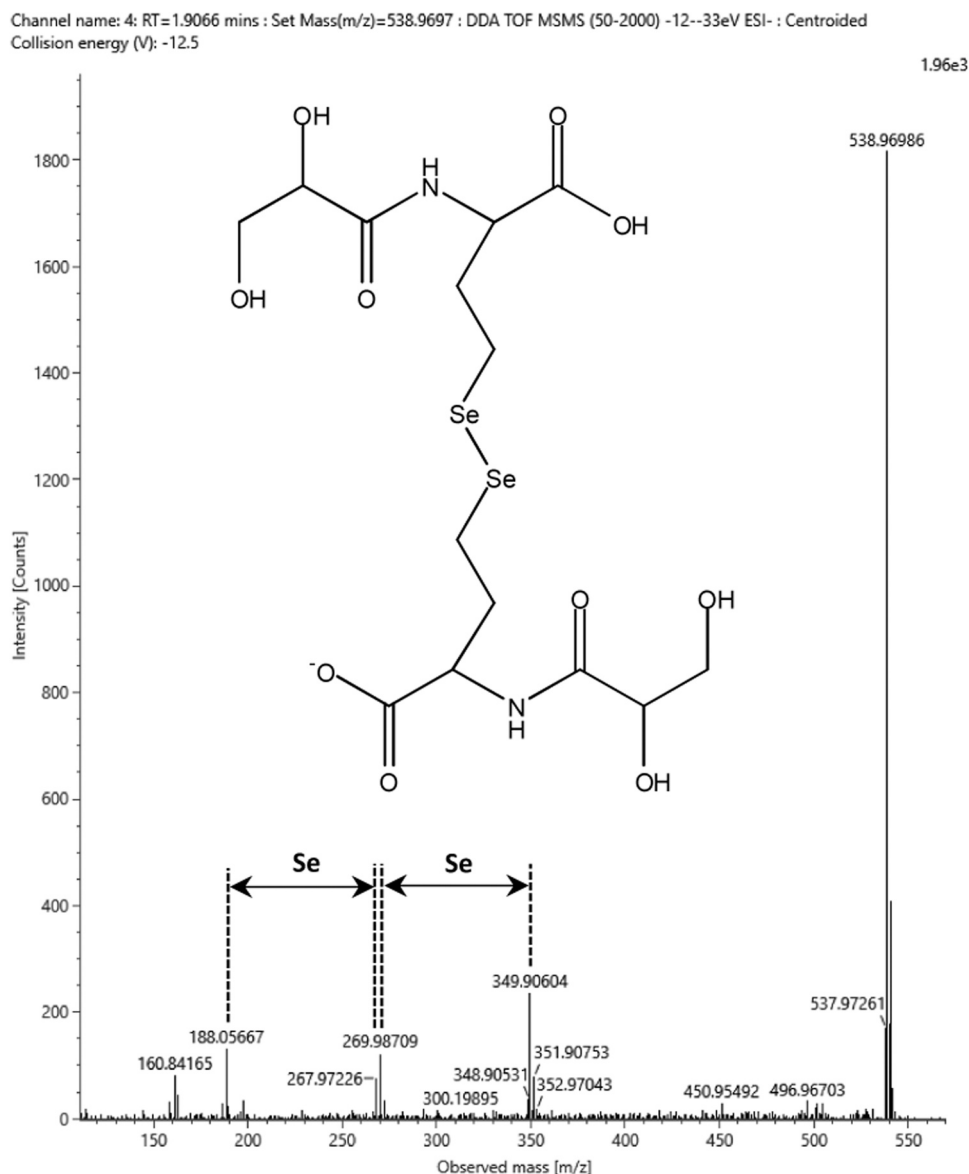


Fig. 6. MS/MS spectrum of the tentative $C_{14}H_{24}N_2O_{10}Se_2$ selenocompound (neutral composition), the dimer of 2,3-dihydroxypropionyl-Se-homocysteine in the ESI $^{-}$ mode (collision energy: -12.5 eV). The inset shows the suggested structure of this doubly selenized selenocompound.

spectra. Efforts to separate the two species with a different gradient were unsuccessful, which might support the idea that the oxidation event goes on either during the separation or in the ionization process.

The structural assignment of other, higher-mass selenocompounds in this BJ2 fraction was not successful. Still, some information can be provided due to the characteristic neutral losses. There is a selenized and presumably nitrogen-containing MS/MS fragment at m/z 415.03 whose exact elemental composition cannot be determined; this fragment is present in the three highly abundant isomers of the ion at m/z 547.0753 (RT=1.00 min, 1.19 min, 1.49 min, Figs. S38–S40). The MS/MS spectra of these isomers present clear evidence for the neutral loss of a pentose moiety ($C_5H_8O_4$; $\Delta=132.04$ Da) that also appears as an MS/MS fragment too ($C_5H_9O_5$; m/z 149.045; Figs. S41–S42). Accordingly, these three isomers, together with two homologue ($+CH_2$) analytes detected at m/z 561.0906 (RT=1.25 min, 1.61 min; Figs. S43–S44), with the potentially acetylated ($+CO$) isomers detected at m/z 575.0698 (RT=1.52 min, 1.63 min, 2.13 min; Figs. S45–S48), and finally, with the glycosylated ($+C_6H_{10}O_5$) isomers detected at m/z 709.1258 (RT=1.00 min, 1.56 min; Figs. S49–S50) belong to a selenosugar (selenopentose and selenopentose-hexose) family. It is to be noted that –

except for the selenized fragments of m/z 415.03 and m/z 94.94 – no lower-mass selenized fragments can be detected in the MS/MS spectra, which can refer to an end-chain R-Se- CH_3 moiety.

Noteworthy, these higher molecular mass species (m/z 547.07, m/z 561.09, m/z 575.07, and m/z 709.12) exhibited the visible feature of on-column (or in-source) oxidation in the order of their elution time: i.e., the later eluting isomers from all three species were always surrogated with a more abundant $+15.995$ Da species. Whether this is related to the formation of selenoxides or other oxidation events, it cannot be straightforwardly elucidated yet.

In the BJ3 fraction, four other metabolites, each containing one selenium atom, were detected for which the tentative decomposition process of selenohomolanthionine can be taken into account: (i) a couple of two isomers detected only in the positive ion mode at m/z 650.3283 (also doubly charged, at m/z 325.6676; ESI $^{+}$; RT=1.05 min and 1.31 min; Figs. S51–S52); (ii) another couple of two isomers simultaneously detected both in the positive and negative ionization modes at m/z 651.3124 (ESI $^{+}$) and m/z 649.2978 (ESI $^{-}$) at RT=2.00 min and 2.14 min (Figs. S53–S56). The difference between the two ($\Delta=+0.984$ Da) accurate masses in the positively charged molecules and the

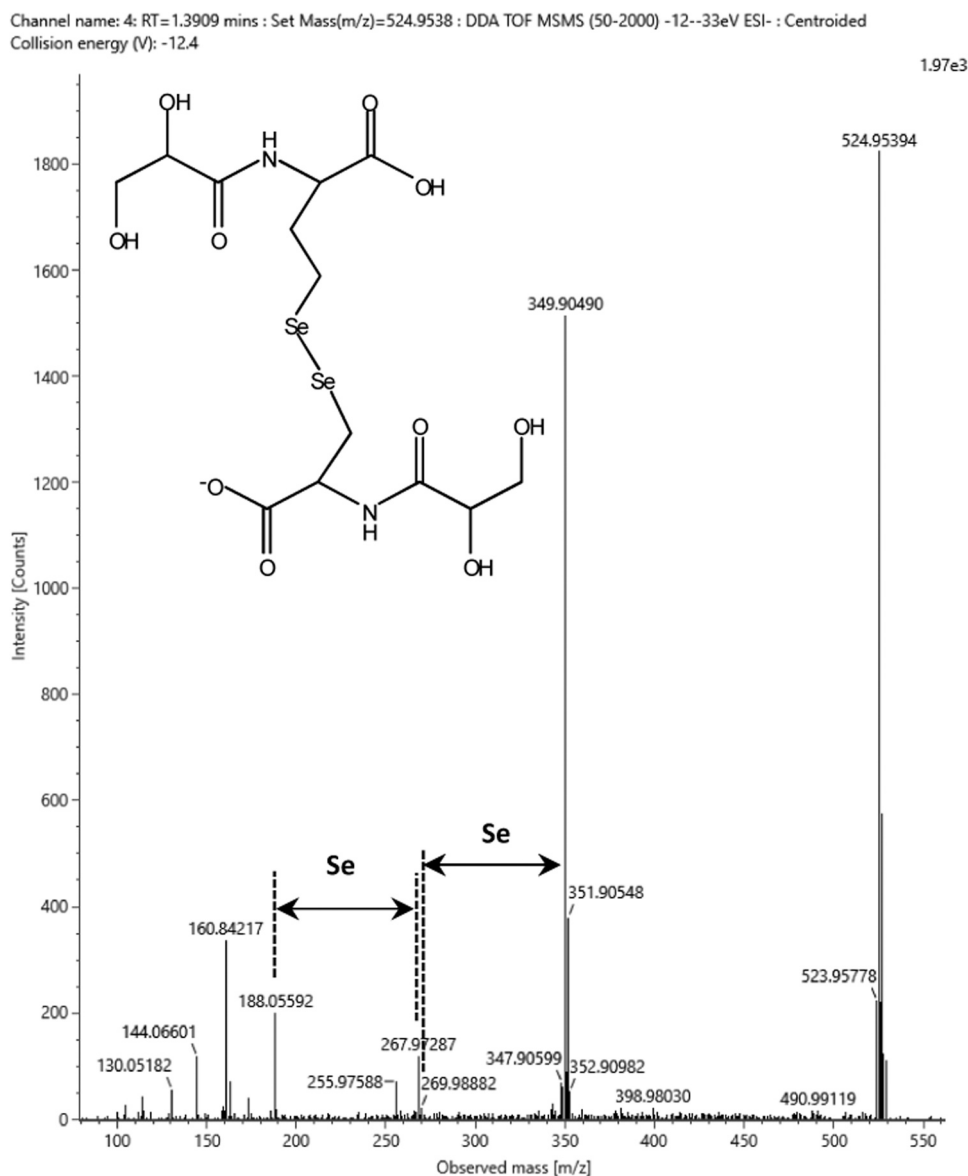


Fig. 7. MS/MS spectrum of the $C_{13}H_{22}N_2O_{10}Se_2$ selenocompound (neutral composition), the Se-Se conjugate of 2,3-dihydroxypropionyl-Se-homocysteine and 2,3-dihydroxypropionyl-Se-cysteine in the ESI $^-$ mode (collision energy: -12.4 eV). The inset shows the structure of this doubly selenized selenocompound.

match of the main, most abundant non-selenized fragment at m/z 441.332 indicate that these molecules could be formed with the involvement of the $C_6H_{11}NO_2Se$ moiety (for the m/z 650.3283; Fig. S57) and its oxidatively deaminated (into an alcohol) derivative of $C_6H_{10}O_3Se$ (for the m/z 651.3124; Fig. S58), respectively. Having one less nitrogen atom in the latter species is also supported by the lack of its doubly charged form in ESI '+' mode and its detection in the negative ion mode too, similarly to the behavior of the $C_8H_{16}NO_5Se^+$ compound. Also, all of the selenized fragments of the m/z 651.3124 species are nitrogen-free ($C_3H_5OSe^+$: m/z 136.9477; $C_4H_7O_3Se^+$: m/z 182.9542; $C_6H_{11}O_3Se^+$: m/z 210.9866; Fig. S58b), which possibly indicates the introduction of the deaminated derivative of selenohomolanthionine during the formation of this species.

In turn, the MS/MS spectrum of the compound at m/z 650.3283 (Fig. S57) contains the characteristic selenohomocysteine fragments (m/z 135.9667 and 181.9722). Actually, as selenohomolanthionine can be readily ionized in the negative ion mode, the MS/MS fragments of the m/z 650.3283 species (i) should contain the highly specific m/z 183.978 fragment if this molecule were a simple, e.g., N-glycosylated form of selenohomolanthionine, (ii) the molecule should be detected in the

negative ion mode, and additionally, (iii) the m/z 650.3283 species might be surrogated by its oxidative monodeaminated derivative to ketone ($\Delta = -1.032$ Da to arrive at m/z 649.2963; not detected in any of the fractions). Accordingly, the formation of the m/z 650.3283 species might be associated with the $C_8H_{14}NO_5Se^+$ compound.

Unfortunately, there are still too many options for the elemental composition of these two sets of compounds, and there are no hints about any possible glycosylation either; therefore, no structures can be suggested for these species, and no straightforward explanation can be provided for the formation of isomers.

As the identification of selenocompounds might be facilitated if their sulfur analogues are present in the samples, all the analytes listed in [Tables 1 and 2](#) were manually searched for their possible sulfur analogues by filtering for the $^{80}\text{Se}-^{32}\text{S} \rightarrow \Delta = -47.9445$ Da accurate masses. Unlike selenized yeast and some selenium accumulator plant samples, none of the experimental masses of selenized molecules were accompanied by a sulfur analogue. However, while the fraction collection aimed at focusing on the selenium-containing peaks, the applied anion exchange chromatographic system with 10 μm particle sized column package material hardly enables the separation of the sulfurized /

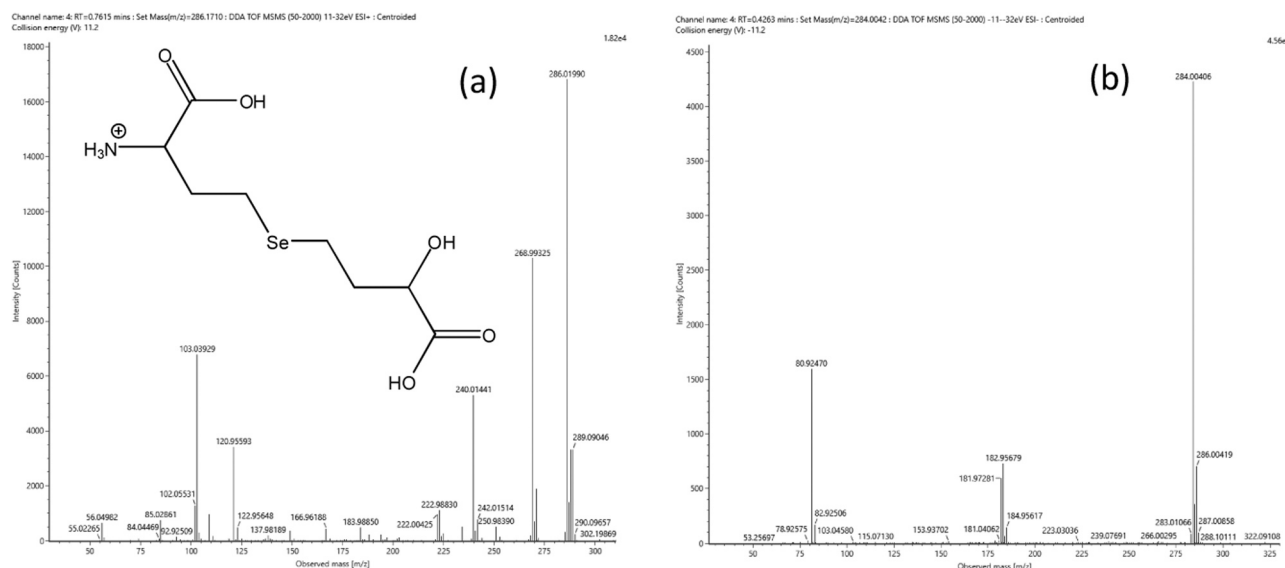


Fig. 8. MS/MS spectra of the tentative selenohomolanthionine derivative, deamino-hydroxy-selenohomolanthionine, $C_8H_{15}NO_5Se$ (neutral composition) (a) in the ESI '+' mode (collision energy: 11.2 eV; gradient #1) and (b) in the ESI '-' mode (collision energy: -11.2 eV; gradient #2). The inset shows the structure of this species.

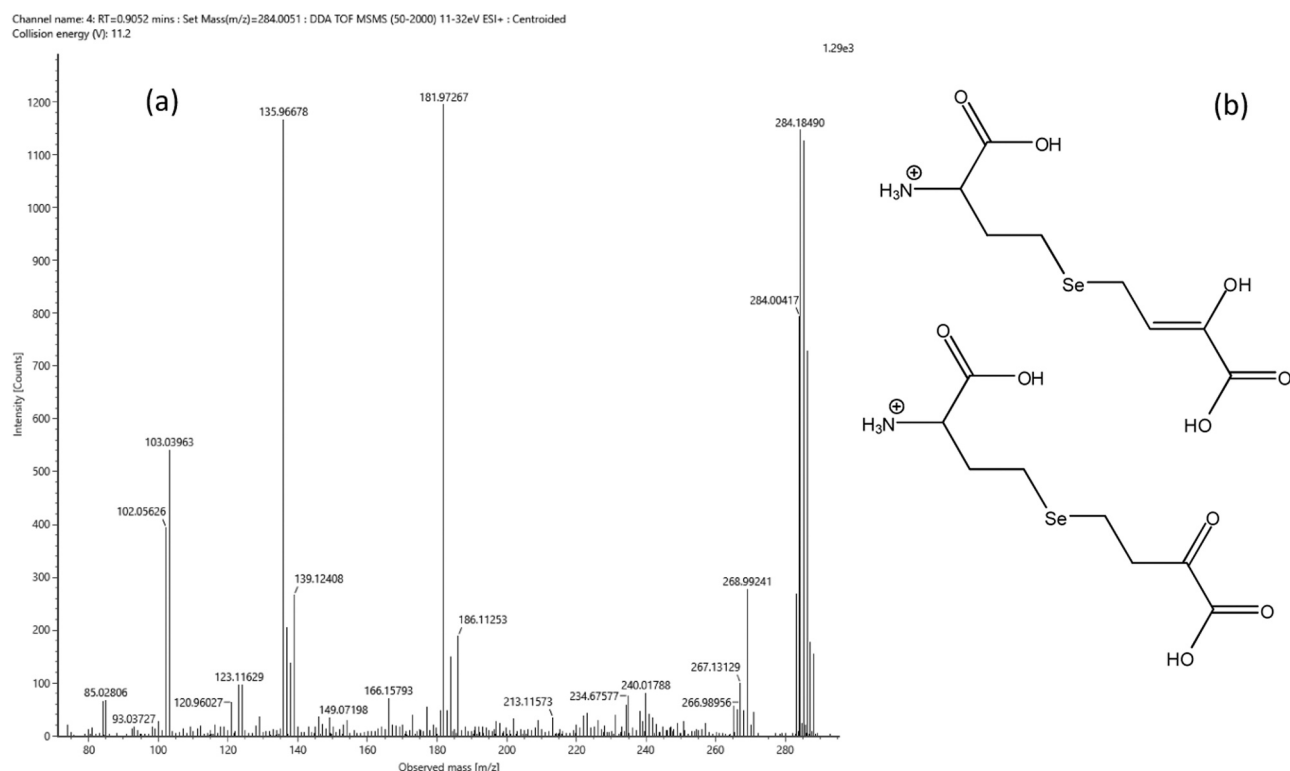


Fig. 9. (a) MS/MS spectra of the tentative selenohomolanthionine derivative, deamino-2-oxo-selenohomolanthionine, $C_8H_{13}NO_5Se$ (neutral composition) in the ESI '+' mode (collision energy: 11.2 eV); (b) suggested structures of the selenospecies.

selenized analogues. Accordingly, this feature of missing sulfur analogues can indicate the compounds listed in Tables 1 and 2 do not share a common sulfur/selenium metabolic pathway and they can be derived from either the higher reactivity of selenium or a selenium specific-metabolism.

4. Conclusions

However *M. sativa* is one of the most frequently grown forage

legumes and the high-yielding perennial forage crop, our study is the first to report on its selenocompounds with at least ESI-HR-MS based elemental composition. Apart from the most commonly reported selenospecies, selenomethionine, that has been identified with LC-ICP-MS on the basis of retention time matching and spiking procedures [10, 36], no other organic selenometabolite has been presented, while selenium-enriched alfalfa-derived hay proved beneficial for, e.g., dairy cows [39] and newborn calves [40]. Moreover, enriching selenium in alfalfa through foliar sodium selenate application was reported to be an

Table 2
Liquid chromatography – high resolution mass spectrometry data of the Se-enriched alfalfa derived selenometabolites detected in the study (part II). ‘#1’ and ‘#2’ refer to the applied LC gradient (for details, see [Supplementary Material /SM/ Tables S1 and S4](#)), while ‘?’ refers to uncertainty in the assessment due to the lack of characteristic neutral losses of monosaccharides and/or unambiguous elemental composition. Identification levels are assigned according to Schymanski et al. [25].

Sign	RT #1, min	RT #2, min	Ionization	Suggested elemental composition (neutral)	Tentative seleno-sugar	Name, note, or reference	Theoretical m/z	Experimental m/z	Δ, ppm	Identification level	Figure
F1	1.14	-	[M-H] ⁻	-	no	with an end-chain R-S-Se-CH ₃ moiety	-	615.0521	-	5	SM–34,35
BJ1	1.30	-	[M+Na] ⁺ [M-H] ⁻	C ₁₁ H ₂₂ O ₇ Se	?	possible origin of the C ₁₁ H ₂₁ O ₈ Se ⁻ species	369.0423 345.0458	369.0424 345.0462	0.27 1.25	4	SM–37 SM–36
BJ2	1.00 1.19 1.49 1.25 1.61 1.93 2.00 2.31 1.00 1.56	0.89 1.23 1.44 1.44 1.54 1.52 1.63 2.13 1.02 1.57	[M-H] ⁻ [M-H] ⁻ [M-H] ⁻ [M-H] ⁻ [M-H] ⁻ [M-H] ⁻ [M-H] ⁻ [M-H] ⁻ [M-H] ⁻ [M-H] ⁻	-	yes yes yes yes	pentose loss detected +CH ₂ homologue of the m/z 547.07 species potentially acetylated (+CO) derivative of the m/z 547.07 species hexoside derivative of the m/z 547.07 species	- - - - -	547.0753 547.0746 547.0745 561.0906 561.0901 575.0707 575.0689 575.0698 709.1258 709.1269	- - - - -	5 5 5 5	SM–38–42 SM–43,44 SM–45–48 SM–49,50
BJ3	1.05 1.30 2.13 2.19	1.42 1.56 2.00 2.14	[M+H] ⁺ [M+2H] ²⁺ [M+H] ⁺ [M-H] ⁻ [M+H] ⁺ [M-H] ⁻	-	? ? ? ?	derivative of either selenohomocysteine, or selenohomolanthionine or the C ₈ H ₁₃ NO ₅ Se species derivative of either selenohomocysteine, or selenohomolanthionine or the C ₈ H ₁₃ NO ₅ Se species mono-deaminated derivative of the m/z 650.32 compound, oxidized into an alcohol	- - - -	650.3277 650.3283 651.3123 649.2978 651.3124 649.2981	- - - -	5 5 5 5	SM–51 SM–52,57 SM–53–56,58

effective strategy to increase Se concentration in forage harvested from Se-depleted areas [41].

While most of the 30 selenocompounds listed in [Tables 1 and 2](#) have not been reported yet, even in the form of their neat elemental composition, some of them refer to the highly characterized yeast selenometabolism the general database of which has been recently published [24]. Especially the appearance of the 2,3-dihydroxypropionyl derivatives should be highlighted, as these compounds were first regarded as yeast-specific metabolites and have only been reported from plants in the *Asterids/Lamiales* (*Thunbergia allata*) and *Mono-cots/Commelinids* (*Oryza sativa*) clade/order [28,42], distinctly far from alfalfa (*Rosids/Fabales*). Similarly, the detection of selenosugars such as the C₁₂H₂₂O₁₀Se species (neutral composition; first described by Aureli et al. [28] in rice, wheat, and maize) indicates these compounds might share a relatively common selenium detoxification pathway the detailed steps of which have not yet been elaborated. The presence of such a high number of selenocompounds (besides the series of potentially missed or inherently undetected species eluting in the void volume of both purification systems, SAX and SCX) underlines that the selenium metabolism of this non-accumulator *Fabaceae* plant, alfalfa, is far more complex than would be expected solely based on the results of SAX HPLC-ICP-MS analyses that could identify and quantify the main selenium species only. It is, however, actually unclear whether the appearance of these formerly unreported organic selenospecies depends on the processing of brown juice and whether it is a function of processing technology or at least plant(alfalfa) specific. In terms of metabolism, the selenohomolanthionine-derived species (deamino-2-oxo-selenohomolanthionine and deamino-hydroxy-selenohomolanthionine) might share the metabolic pathway of selenohomolanthionine [43] – indeed, this is the only selenocompound that had been referred on its toxicity and which has a common moiety with the selenocompounds reported here from alfalfa. Basically, the lack of commercially available selenocompounds evidently hampers their toxicological and physiological investigation, while their in-laboratory synthesis cannot usually provide enough substance for animal or even human studies [44].

More than half of the selenospecies (16/30, including isomers) could only be detected in the negative ionization mode, while this ratio was only 5/30 for the selenocompounds solely detected in the positive ionization mode. However vendor-dependent parameters like the ESI ion source setups and ion lens settings evidently have an effect on these rates, this observation is a clear indication that no selenium speciation analyses should be conducted only in the positive ionization mode, as important speciation information can be dismissed.

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CRedit authorship contribution statement

Zoltán Kovács: Investigation. **Mihály Dernovics:** Writing – original draft, Visualization, Methodology, Investigation, Conceptualization. **Éva Domokos-Szabolcsy:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Áron Soós:** Visualization, Methodology, Investigation. **Béla Kovács:** Supervision, Resources.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used <https://quillbot.com/grammar-check> in order to correct grammatical errors. After

using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mihály Dernovics reports financial support was provided by National Research, Development and Innovation Fund of Hungary. Éva Domokos-Szabolcsy reports financial support was provided by Hungarian Academy of Sciences. Éva Domokos-Szabolcsy reports financial support was provided by National Research, Development and Innovation Fund of Hungary. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jtemb.2024.127545](https://doi.org/10.1016/j.jtemb.2024.127545).

Data availability

Data will be made available on request.

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