COMPREHENSIVE SELENIUM SPECIATION OF A SELENIUM ACCUMULATOR PLANT, *CARDAMINE VIOLIFOLIA*

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<th>Full Form</th>
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<tbody>
<tr>
<td>1D</td>
<td>One Dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>Two Dimensional</td>
</tr>
<tr>
<td>AdoSeHCys</td>
<td>Se-adenosyl-homoselenocysteine</td>
</tr>
<tr>
<td>AdoSeMet</td>
<td>Selenoadenosylmethionine</td>
</tr>
<tr>
<td>AED</td>
<td>Atomic Emission Detection</td>
</tr>
<tr>
<td>AES</td>
<td>Atomic Emission Spectroscopy</td>
</tr>
<tr>
<td>APR</td>
<td>Adenosine 5’ Phosphosulphate Reductase</td>
</tr>
<tr>
<td>APSe</td>
<td>Adenosine 5’-Phosphoselenate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>ATPS</td>
<td>Adenosine Triphosphate Sulphurylase</td>
</tr>
<tr>
<td>CEC</td>
<td>Capillary Electrochromatography</td>
</tr>
<tr>
<td>CGS</td>
<td>Cystathionine- γ-synthase</td>
</tr>
<tr>
<td>Chl</td>
<td>Chlorophyll</td>
</tr>
<tr>
<td>CID</td>
<td>Collision Induced Dissociation</td>
</tr>
<tr>
<td>CoQ10</td>
<td>Coenzyme Q10</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary Zone Electrophoresis</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode-Array Detector</td>
</tr>
<tr>
<td>DHAA</td>
<td>Dehydroascorbic Acid</td>
</tr>
<tr>
<td>DMMDSe</td>
<td>Dimethyldiselenide</td>
</tr>
<tr>
<td>DMSe</td>
<td>Dimethylselenide</td>
</tr>
<tr>
<td>DMSeP</td>
<td>Dimethylselenopropionate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DW</td>
<td>Dry Weight</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Commission</td>
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<tr>
<td>EFSA</td>
<td>European Food Safety Authority</td>
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<tr>
<td>ESI</td>
<td>Electrospray Ionisation</td>
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<tr>
<td>FLD</td>
<td>Fluorescence Detection</td>
</tr>
<tr>
<td>FT ICR</td>
<td>Fourier-Transform Ion Cyclotron Resonance</td>
</tr>
<tr>
<td>GAE</td>
<td>Gallic Acid Equivalent</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GPS</td>
<td>Global Positioning System</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>HA</td>
<td>Hyperaccumulator</td>
</tr>
<tr>
<td>HFBA</td>
<td>Heptafluorobutyric Acid</td>
</tr>
<tr>
<td>HG-AFS</td>
<td>Hydride Generation Atomic Fluorescence Spectrometry</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophilic Interaction Chromatography</td>
</tr>
<tr>
<td>HMT</td>
<td>Homocysteine $S$-Methyltransferase</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HR</td>
<td>High Resolution</td>
</tr>
<tr>
<td>ICP</td>
<td>Inductively Coupled Plasma</td>
</tr>
<tr>
<td>IDA</td>
<td>Isotope Dilution Analysis</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric Focusing Electrophoresis</td>
</tr>
<tr>
<td>IP-RP</td>
<td>Ion Pairing Reversed Phase (Chromatography)</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union for Pure and Applied Chemistry</td>
</tr>
<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genoms</td>
</tr>
<tr>
<td>LA</td>
<td>Laser Ablation</td>
</tr>
<tr>
<td>Lan</td>
<td>Lanthionine</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid Chromatography</td>
</tr>
<tr>
<td>LSF</td>
<td>Least-Squares Fitting</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to Charge ratio</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix Assisted Laser Desorption Ionisation</td>
</tr>
<tr>
<td>MC</td>
<td>Multicollector</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar Electrokinetic Chromatography</td>
</tr>
<tr>
<td>MeSeCys</td>
<td>$Se$-(Methyl)Selenocysteine</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>MMT</td>
<td>Methylmethionine Methyltransferase, $S$-adenosyl-$L$-methionine:$L$-methionine $S$-methyltransferase</td>
</tr>
<tr>
<td>MS agar</td>
<td>Murashige and Skoog agar</td>
</tr>
<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>OAS</td>
<td>$O$-acetylserine</td>
</tr>
<tr>
<td>OAS-TL</td>
<td>$O$-acetylserine (Thiol)-Lyase</td>
</tr>
<tr>
<td>OPH</td>
<td>$O$-Phosphohomoserine</td>
</tr>
<tr>
<td>PAGE</td>
<td>Poliacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per Million</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluorethylene</td>
</tr>
<tr>
<td>Q</td>
<td>Quadrupole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RGB</td>
<td>Red-Green-Blue</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed Phase (Chromatography)</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise</td>
</tr>
<tr>
<td>SAT</td>
<td>Serine Acetyltransferase</td>
</tr>
<tr>
<td>SAX</td>
<td>Strong Anion Exchange (Chromatography)</td>
</tr>
<tr>
<td>SCX</td>
<td>Strong Cation Exchange (Chromatography)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size Exclusion Chromatography</td>
</tr>
<tr>
<td>SeCys</td>
<td>Selenocysteine</td>
</tr>
<tr>
<td>SeCys$_2$</td>
<td>Selenocystine</td>
</tr>
<tr>
<td>SeCysta</td>
<td>Selenocystathionine</td>
</tr>
<tr>
<td>SeHCys</td>
<td>Selenohomocysteine</td>
</tr>
<tr>
<td>SeHLan</td>
<td>Selenohomolanthionine</td>
</tr>
<tr>
<td>SeLan</td>
<td>Selenolanthionine</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SeMet</td>
<td>Selenomethionine</td>
</tr>
<tr>
<td>SeMetO</td>
<td>Selenomethionine-selenoxide</td>
</tr>
<tr>
<td>SeMM</td>
<td>Se-methyl-selenomethionine</td>
</tr>
<tr>
<td>SF</td>
<td>Sector Field</td>
</tr>
<tr>
<td>SiR</td>
<td>Sulphite Reductase</td>
</tr>
<tr>
<td>SMT</td>
<td>Selenocysteine Methyltransferase</td>
</tr>
<tr>
<td>SULTR</td>
<td>Sulphate Transporters</td>
</tr>
<tr>
<td>TEAC</td>
<td>Trolox-Equivalent Antioxidant Capacity</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of Flight</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic Acid</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>XANES</td>
<td>X-ray Absorption Near-Edge Structure</td>
</tr>
<tr>
<td>XAS</td>
<td>X-ray Absorption Spectroscopy</td>
</tr>
<tr>
<td>XRF</td>
<td>X-ray Fluorescence</td>
</tr>
<tr>
<td>$\gamma$-glu</td>
<td>$\gamma$-glutamyl</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Trace elements possess important roles in complex biological systems, such as active centres of enzymes or as structural components of bioactive substances. Besides essential or beneficial elements there are some notorious metals causing acute (e.g., Hg) or chronic (e.g., Pb) toxicity. However, several elements cannot be unambiguously classified due to their dual characteristics, that is, essential in one oxidation state and toxic in the other state or toxic in most forms, but essential or harmless in a particular organic form (Szpunar et al. 2003). Besides quality aspects, the quantity of a particular element can also define its effect on the biological system.

Among essential trace elements selenium (Se) shows one of the narrowest optimal daily intake, therefore both selenium deficiency and toxicity are global issues. Selenocysteine, the 21st amino acid incorporates into several antioxidant enzymes and therefore plays an important role in the protection of cells against oxidative stress. Se deficiency in humans can cause muscle weakness and inflammation, hypothyroidism, heart muscle dysfunction, weakness of the immune system, Keshan and Kashin-Beck diseases and others, whereas symptoms of Se toxicity include liver and kidney damage, necrosis of heart and liver, hair and nail loss. The selenium level in the European population is generally below the optimal, an Adequate Intake of 70 µg/day for adults and 85 µg/day for lactating women was determined by European Food Safety Authority (EFSA) in 2014 which can be provided by Se-rich/enriched foodstuff and food supplements (EFSA 2014).

There is a growing attention that not only the total intake of dietary Se is important to health, but the forms of the ingested Se can be also relevant due to the fact that different Se species can differ in bioavailability and can go together with beneficial or toxic health effects. The first step for making correct conclusions on health effects of different Se species is the adequate Se speciation in the food chain, especially in plants as being the main dietary sources of Se (Rayman et al. 2008).

Contrary to animals and humans, Se is not essential for higher plants but even toxic for most of them. A special group of plant species living on seleniferous soils evolved different metabolic pathways to cope with Se toxicity and adapted not only to tolerate but even to accumulate selenium in their tissues at high concentration level.

Selenium deficiency and toxicity are present side by side in China due to the high variances in soil Se content throughout the country. The controlled cultivation and
production of selenium enriched food on seleniferous areas can contribute to solve this health related issue of millions of people living in Se poor areas.

*Cardamine violifolia*, a selenium hyperaccumulator plant species originates from the naturally seleniferous region Yutangba, China and has been cultivated for phytoremediation purposes. The plant is also used for soil reclamation and food supplement production however the selenium species and metabolic pathways responsible for high Se content have not been elucidated yet. The work described here addressed the identification and quantification of selenium species and the investigation of Se related physiological properties of this selenium hyperaccumulator plant species.
2. OBJECTIVES

In my doctoral study I intended to investigate the selenium related characteristics of *Cardamine violifolia* selenium hyperaccumulator plant species. The experiments can be interpreted along two lanes.

For investigation of selenometabolites in *Cardamine violifolia* I intended:

- to purify the selenium containing fractions of selenium enriched *C. violifolia* plant extract with an ICP-MS assisted orthogonal chromatographic separation procedure;
- to detect selenium compounds by HPLC-ESI-QTOF-MS in the fractions;
- to putatively identify selenometabolites based on their accurate mass, chromatographic behaviour and MS/MS fragmentation pattern;
- to confirm the identification of the main selenocompound through synthesis;
- to quantify the main selenocompound without an authentic standard.

Comprehensive experiment was conducted to study the physiological effect of selenium on *Cardamine violifolia* with the comparison of the related non-accumulator *Cardamine pratensis* regarding:

- tolerance and accumulation of selenate;
- uptake capacity of different forms of selenium: selenate and selenite;
- interactions of selenate and sulphate, selenite and phosphate for selenium and sulphur uptake;
- effect of selenium treatment on chlorophyll fluorescence properties and antioxidant capacity;
- selenium localisation and speciation in intact plant tissues based on X-ray microprobe analysis.
3. REVIEW OF LITERATURE

3.1. SELENIUM IN PLANTS

3.1.1. Relation of selenium and plants

Selenium (Se) has not been shown essential for higher (vascular) plants in contrast to some microalgae, bacteria, animals and humans (Araie and Shiraiwa 2009, Guignardi and Schiavon 2017). Although not essential, Se can provide beneficial effects for plants (Pilon-Smits et al. 2009). Several studies have shown that proper levels of Se have a growth enhancing effect (Xue et al. 2001, Hartikainen 2005, Djanaguiraman et al. 2010) that can be associated with the enhanced accumulation of starch and sugar (Turakainen et al. 2004). Some plant species grown in Se-enriched media have shown increased tolerance against abiotic stresses like drought (Kuznetsov et al. 2003), cold stress (Chu et al. 2010), high temperature (Djanaguiraman et al. 2010), high ultraviolet-B radiation (Hartikainen and Xue 1999), salinity (Kong et al. 2005) and toxic metals (Fargasová et al. 2006). These physiological advantages may be related to the enhanced antioxidant capacity induced by Se against oxidative damage and lipid peroxidation (Xue et al. 2001, Hartikainen 2005); however, the exact molecular mechanisms that underlie these positive effects have not been elucidated yet (Feng et al. 2013).

Besides physiological advantages, even low Se levels (~10 µg Se g⁻¹ dry weight (DW)) in tissues provide ecological advantage as well, such as protection against certain herbivores (Hanson et al. 2004). At higher tissue levels, Se protects not only against other herbivores but also some microbial pathogens. Also, Se provides allelopathic benefits as well because of the negative effects on Se sensitive plants (El Mehdawi and Pilon-Smits 2012).

All plants can take up and metabolise Se due to its chemical similarity to the essential macroelement sulphur (S) (Figure 1). On the other hand, there are huge differences in the degree to which different plant species can tolerate and accumulate Se.
Figure 1. Accumulation and transformation of Se (red circles) in plants and the related effects on local Se cycling (Pilon-Smits 2019).

Based on the accumulated selenium level in plant tissues, three classes are most frequently distinguished in the literature. Species called Se hyperaccumulators can concentrate Se in the range of 1000-15000 µg Se g\(^{-1}\) DW. Non-accumulator species contain less than 100 µg Se g\(^{-1}\) DW. The third, distinct group of plants is named secondary accumulators (or simply accumulators) that accumulates Se in the range of 100-1000 µg Se g\(^{-1}\) DW while growing on seleniferous soils (Anderson 1993, Läuchli 1993). Additionally, several studies mention Se indicator plants that can colonise both non-seleniferous and seleniferous soils and can tolerate tissue Se concentration up to 1000 µg g\(^{-1}\) DW (White 2016). Different Se accumulation and tolerance capacity may be associated with the differences in Se metabolism and in tissue accumulation patterns (Pilon-Smits 2019). An overview about the biochemical and physiological characteristics used to differentiate these plant classes is given in Table 1. Secondary accumulator and non-accumulator plants take up Se and S proportionally to their abundance in the growth medium, which indicates that they are unable to distinguish these elements. Hyperaccumulator species appear to take up Se preferentially over S, which results in higher Se/S ratios in their tissues compared to growth media and surrounding vegetation (White et al. 2007, Harris et al. 2014). Mechanism of this qualitative difference is not known and there is a quantitative difference as well. Based on different studies it is assumed that the major genes involved in root sulphate/selenate uptake and assimilation are constitutively overexpressed in hyperaccumulators compared to non-hyperaccumulators (Pilon Smits 2017, Freeman et al. 2010, Schiavon et al. 2015, Cabannes et al. 2011).
Hyperaccumulators differ from other plants regarding the Se-S interactions as well. Sulphate uptake is more effectively inhibited by selenate while sulphate is less capable of inhibiting selenate uptake (Harris et al. 2014). Non-hyperaccumulator species tend to contain relatively more inorganic Se than hyperaccumulators do due to slower rate of selenate assimilation. *Brassica juncea* accumulator and *Arabidopsis thaliana* non-accumulator species were found to accumulate predominantly selenate while hyperaccumulator *Astragalus bisulcatus, Symphyotrichum ericoides, Stanleya pinnata* contain mostly organic Se species (Van Hoewyk et al. 2005, Freeman et al. 2006a, El Mehdawi et al. 2014). Hyperaccumulators show enhanced conversion of inorganic Se to organic Se and high activity to produce less toxic Se species from selenocysteine (SeCys) to avoid its non-specific incorporation into proteins. This explanation is often cited as “malformed protein theory”, introduced in 1980 by Brown and Shrift. For example, methylation of SeCys to Se-(methyl)selenocysteine (MeSeCys) or producing volatile Se forms like dimethyldiselenide (DMDSe) offer ways for Se depletion (Pilon-Smits 2017).

Further differences have also been described in the translocation of Se between species. Hyperaccumulators translocate more Se in the xylem (from root to shoot), show higher phloem remobilisation (from leaves to reproductive organs) and often sequester Se in specialised tissues such as leaf hairs or epidermis (Pilon-Smits 2019, Quinn et al. 2011, Cappa et al. 2014).

**Table 1.** Classification of angiosperm species into ecological types according to their ability to accumulate Se in their tissues (White 2017, Moreno Rodriguez et al. 2005).

<table>
<thead>
<tr>
<th>Classification</th>
<th>Leaf Se concentration (µg g⁻¹ DW)</th>
<th>Soil ecology</th>
<th>Biochemical and physiological characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-accumulator e.g., <em>Arabidopsis thaliana</em></td>
<td>&lt;10 - 100</td>
<td>non-seleniferous</td>
<td>Se uptake induced by S-starvation</td>
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<td></td>
<td></td>
<td></td>
<td>High S/Se selectivity for uptake</td>
</tr>
<tr>
<td>Se indicator e.g., <em>Atriplex halimus</em></td>
<td>&lt;1000</td>
<td>seleniferous &amp; non-seleniferous</td>
<td>Se transport to shoot induced by S-starvation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Se volatilisation as dimethylselenide</td>
</tr>
<tr>
<td>Se accumulator e.g., <em>Brassica juncea</em></td>
<td>&gt;100</td>
<td>seleniferous</td>
<td>selenate as the main Se species (sometimes selenomethionine)</td>
</tr>
<tr>
<td>Se hyperaccumulator e.g., <em>Stanleya pinnata</em></td>
<td>&gt;1000</td>
<td>seleniferous</td>
<td>Constitutive, S-independent Se uptake</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low S/Se selectivity for uptake</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Constitutively large Se transport to shoot</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Constitutively large Se metabolic flux</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Large Se volatilisation as DMDSe</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sequestration highest in reproductive organs</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MeSeCys is a common Se species</td>
</tr>
</tbody>
</table>
Selenium hyperaccumulation was discovered by Orville Beath and his coworkers in the 1930’s (Rosenfeld and Beath 1964). Since then, hyperaccumulation has been reported in 45 taxa from 14 genera and from six eudicot orders: Fabales, Brassicales, Caryophyllales, Gentianales, Lamiales and Asterales (White 2016, Figure 2). It is assumed that Se hyperaccumulation has evolved independently in different clades possibly due to similar ecological and physiological circumstances (Pilon-Smits 2017).

**Figure 2.** Distribution of proposed Se hyperaccumulating species among angiosperm orders. The number of Se hyperaccumulating genera and Se hyperaccumulating species in each order are given in parentheses, respectively (White 2016).

### 3.1.2. Seleniferous regions worldwide

Selenium is usually present in soils at trace level but high concentrations even above 1000 mg kg\(^{-1}\) have been reported for some regions (Fleming 1962, Zhu *et al.* 2008). There are several parts of the world where soils contain elevated levels of Se, which causes serious health problems due to Se accumulation in the food chain (Zhu *et al.* 2008). Soils with Se level > 0.1 mg kg\(^{-1}\) are considered seleniferous because
forages grown on such soils contain > 4 mg Se kg$^{-1}$ – the maximum permissible level for animal consumption (Dhillon et al. 1992). Seleniferous regions are known in the United States, Ireland, Australia, Brazil, China, and India (Dhillon and Dhillon 2003). An overview of Se tolerant species collected in nature from these areas is presented in Table 2.

Similarly to normal soils, natural seleniferous soils have developed as a result of so-called active and passive factors of soil formation, and toxic Se levels are a direct consequence of geological processes. Toxic Se content of soils is determined by slight modifications in formations or rock outcrops, formations lying beneath the soil mantle, decomposition of parent rocks by wind and water and subsequent transport by ground or surface water, indicator plants (Dhillon and Dhillon 2003, Rosenfeld and Beath 1964). Besides natural processes industrial activities contribute to significant amounts of Se liberated to aquatic and terrestrial ecosystems, such as coal combustion, crude oil processing, metal refining, mining, and crop irrigation (Staicu et al. 2017a).

In Queensland (Australia) the high Se level of the soil is associated with limestone shale. Vegetation grown on these soils are known to cause selenosis in livestock. Selenium accumulator plants, *Morinda reticulata* and *Neptunia amplexicaulis* have been identified in Central Queensland (Knott and McCray 1959).

In Ireland the locations of toxic seleniferous soils are sporadic. Some of the highest soil Se concentration in the world are found in Limerick, Tipperary and Meath Counties. Level of total Se ranges between 3.2 – 132 mg kg$^{-1}$ but can reach even 1200 mg Se kg$^{-1}$ (Fleming and Walsh 1957, Fleming 1962, Rogers et al. 1990). Based on Fleming’s descriptions (1962) these toxic soils have developed under highly humid conditions and air depression once occupied by old lakes.

Seleniferous soils are widespread in the Rocky Mountains and the Great Plains of Western United States of America (USA). Grazing on forages grown on these soils has been known since the 1930’s to cause toxicity in livestock termed as blind staggers and alkali disease (Wilber 1980). The toxic soils are concentrated especially in the states of Wyoming, South Dakota, North Dakota, Montana, Nebraska, Kansas, Colorado, New Mexico, Utah, Arizona and the San Joaquin valley in California. Volcanic eruptions in Cretaceous age are thought to be the primary source of Se through deposition in Cretaceous seas that have flooded these areas in the USA. Toxic soils have developed from disintegrating these deposits (Dhillon and Dhillon 2003). Several Se hyperaccumulators have been reported occurring in these areas: 25 taxa within the genus *Astragalus* (Fabaceae), *Stanleya pinnata*, *Stanleya bipinnata*
(Brassicaceae) and genera from Asteraceae (*Oonopsis*, *Xylorhiza* and *Symphyotrichum*; Rosenfeld and Beath 1964, El Mehdawi *et al.* 2014).

Soils with toxic Se content in India are found in different states, the largest affected area (1000 ha) was located in Punjab (Dhillon and Dhillon 2003). Investigations of Dey *et al.* (1999) in northeast India found wild animal species suffering from Se toxicity with Se concentration exceeding toxic limits in their bones and hair. Soil and water Se concentration up to 17 µg g⁻¹ and 1.4 µg g⁻¹ were recorded, respectively, at certain living area of these animals.

Carbonaceous shale and carbonaceous chert strata are the major source of Se in Enshi, located in Hubei province, central China. Mining Se-rich rocks and weathering have caused Se release into the environment. In the 1960’s high Se intake over short time caused Se poisoning in the local population (Zhu *et al.* 2004). Soils from this area were characterised with very high Se content in terms of total and water-soluble Se as well (up to 59 mg Se kg⁻¹ soil and 2.4 mg Se kg⁻¹ soil, respectively; Chang *et al.* 2019, Fordyce *et al.* 2000, Qin *et al.* 2012, Zhu *et al.* 2008, Zhu *et al.* 2004). Soil samples collected from croplands and discarded coal spoils were found to contain extremely high Se up to 2000 mg Se kg⁻¹ soil (Zhu *et al.* 2008). Besides Enshi, selenium toxicity in human population has been recorded in Ziyang County, Shanxi Province as well (Fordyce *et al.* 2000).

Several studies reported high Se concentration in soil samples from the Amazon regions in Brazil up to 0.6 mg kg⁻¹ and in Minas Gerais state even at 1.7 mg kg⁻¹ (Shaltout *et al.* 2011, Silva Junior *et al.* 2017). Endemic Brazil nuts grown in Amazon regions are known for their high Se content. In some cases the consumption of one single piece of nuts would cover or even exceed the recommended dietary intake for an adult per day (Silva Junior *et al.* 2017, Lima *et al.* 2019).

In contrast to seleniferous areas there are several countries in the world where the soil is poor in Se therefore the Se content of foods and feeds is also extremely low, which causes Se deficiency in local population. One of the concerned countries is Finland where this issue was handled in a unique and efficient way: in 1984, the Ministry of Agriculture and Forestry started to regulate the supplementation of fertilisers with selenium in parallel with monitoring Se status through the whole food chain. Quantity of the allowed sodium selenite was 16 mg kg⁻¹ in fertilisers for cereals and 6 mg kg⁻¹ for grasses. This value has been continuously modified according to the actual monitoring results (Pirjo 2005). After 27 years, Se concentration of spring cereals has increased approximately 15-fold, while the mean increase in beef, pork and
milk was six-, two- and three-fold, respectively. Finally, the average dietary human intake has reached the actual recommendation (Alfthan et al. 2015).

**Table 2.** Plant species identified as Se (hyper)accumulators from natural environment (partly adopted from White, 2016).

<table>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Asteraceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dieteria canescens</em></td>
<td>Midwest USA</td>
<td>Beath <em>et al.</em> (1939a)</td>
</tr>
<tr>
<td><em>Grindelia squarrosa</em></td>
<td>Lower Brule Reservation, SD, USA</td>
<td>Lakin and Byers (1941)</td>
</tr>
<tr>
<td><em>Gutierrezia microcephala</em></td>
<td>Thompson, UT, USA</td>
<td>Beath (1943)</td>
</tr>
<tr>
<td><em>Oonopsis foliosa</em></td>
<td>Lascar, CO, USA</td>
<td>Beath <em>et al.</em> (1939b)</td>
</tr>
<tr>
<td><em>Oonopsis wardii</em></td>
<td>Albany County, WY, USA</td>
<td>Byers (1935)</td>
</tr>
<tr>
<td><em>Symphyotrichum ascendens</em></td>
<td>Soda Springs, ID, USA</td>
<td>Pfister <em>et al.</em> (2013)</td>
</tr>
<tr>
<td><em>Symphyotrichum lateriflorum</em></td>
<td>SD, USA</td>
<td>Moxton <em>et al.</em> (1939)</td>
</tr>
<tr>
<td><em>Xylorhiza glabriuscula</em></td>
<td>Huerfano County, CO, USA</td>
<td>Byers <em>et al.</em> (1938)</td>
</tr>
<tr>
<td><em>Xylorhiza parryi</em></td>
<td>Albany County, WY, USA</td>
<td>Byers (1935)</td>
</tr>
<tr>
<td><em>Xylorhiza venusta</em></td>
<td>Midwest USA</td>
<td>Rosenfeld and Beath (1964)</td>
</tr>
<tr>
<td><strong>Fabaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acacia cana</em></td>
<td>NW Queensland, Australia</td>
<td>McCray and Hurwood (1963)</td>
</tr>
<tr>
<td><em>Astragalus albus</em></td>
<td>La Ventana, NM, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td><em>Astragalus beathii</em></td>
<td>Cameron, AZ, USA</td>
<td>Beath <em>et al.</em> (1940)</td>
</tr>
<tr>
<td><em>Astragalus beckwithii var. purpureus</em></td>
<td>Clark County, NE, USA</td>
<td>Lakin and Byers (1941)</td>
</tr>
<tr>
<td><em>Astragalus canadensis</em></td>
<td>Las Vegas, NE, USA</td>
<td>Byers <em>et al.</em> (1938)</td>
</tr>
<tr>
<td><em>Astragalus crotalariae</em></td>
<td>Truckhaven, CA, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td><em>Astragalus eastwoodiae</em></td>
<td>Utah, USA</td>
<td>Beath (1943)</td>
</tr>
<tr>
<td><em>Astragalus flavus</em></td>
<td>Aztec, NM, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td><em>Astragalus flavus var. argillosus</em></td>
<td>Greenriver, UT, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td><em>Astragalus flavus var. candidans</em></td>
<td>Thompson, UT, USA</td>
<td>Beath (1943)</td>
</tr>
<tr>
<td><em>Astragalus grayi</em></td>
<td>Carbon County, WY, USA</td>
<td>Byers (1935)</td>
</tr>
<tr>
<td><em>Astragalus osterhoutii</em></td>
<td>Kremmling, CO, USA</td>
<td>Beath <em>et al.</em> (1940)</td>
</tr>
<tr>
<td><em>Astragalus pattersoni</em></td>
<td>Thompson, UT, USA</td>
<td>Beath (1943)</td>
</tr>
<tr>
<td><em>Astragalus pectinatus</em></td>
<td>Teton County, MT, USA</td>
<td>Williams <em>et al.</em> (1940)</td>
</tr>
<tr>
<td><em>Astragalus praelongus</em></td>
<td>Leupp, AZ, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td>Scientific Name</td>
<td>Location</td>
<td>Reference</td>
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<tr>
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</tr>
<tr>
<td>Astragalus preussii</td>
<td>Thompson, UT, USA</td>
<td>Beath (1943)</td>
</tr>
<tr>
<td>Astragalus racemosus</td>
<td>WY, USA</td>
<td>Knight and Beath (1937)</td>
</tr>
<tr>
<td>Astragalus rafaelensis</td>
<td>Jensen, TX, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td>Astragalus sabulosus</td>
<td>Thomspson, UT, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td>Astragalus toanus</td>
<td>ID, USA</td>
<td>Lakin and Byers (1948)</td>
</tr>
<tr>
<td>Neptunia amplexicaulis</td>
<td>Richmond, Queensland, Australia</td>
<td>Knott and McCray (1959)</td>
</tr>
<tr>
<td><strong>Brassicaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brassica juncea</td>
<td>Punjab, India</td>
<td>Eiche <em>et al.</em> (2015)</td>
</tr>
<tr>
<td>Cardamine hupingshanensis</td>
<td>Yutangba, Enshi, China</td>
<td>Yuan <em>et al.</em> (2013)</td>
</tr>
<tr>
<td>Stanleya bipinnata</td>
<td>Laramie, WY, USA</td>
<td>Beath <em>et al.</em> (1940)</td>
</tr>
<tr>
<td><strong>Amaranthaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artiplex confertifolia</td>
<td>Thompson, UT, USA</td>
<td>Beath (1943)</td>
</tr>
<tr>
<td>Artiplex nuttallii</td>
<td>WY, USA</td>
<td>Beath <em>et al.</em> (1937)</td>
</tr>
<tr>
<td><strong>Rubiaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morinda reticulata</td>
<td>Cape York, Peninsula, Queensland, Australia</td>
<td>Knott and McCray (1959)</td>
</tr>
<tr>
<td><strong>Orobanchaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Castilleja angustifolia var. dubia</td>
<td>Lysite, WY, USA</td>
<td>Beath <em>et al.</em> (1941)</td>
</tr>
<tr>
<td>Lecythidaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lecythis ollaria</td>
<td>San Juan de los Morros, Venezuela</td>
<td>Ferri <em>et al.</em> (2004)</td>
</tr>
<tr>
<td>Bertholletia excelsa</td>
<td>Amazon, Brazil</td>
<td>Silva Junior <em>et al.</em> (2017)</td>
</tr>
<tr>
<td><strong>Lemnaceae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lemna minor</td>
<td>West Bengal, India</td>
<td>Singh <em>et al.</em> (2016)</td>
</tr>
<tr>
<td>Spirodea polyrhiza</td>
<td>West Bengal, India</td>
<td>Singh <em>et al.</em> (2016)</td>
</tr>
</tbody>
</table>
3.1.3. Biochemistry of selenium assimilation in plants

Plants take up Se from soil mainly in the form of selenate (SeO$_4^{2-}$) which is the prevalent bioavailable form in alkaline and well oxidised soils. The main bioavailable form of Se in anaerobic soils and wetlands is selenite (SeO$_3^{2-}$) that can be taken up by plants as well (Terry *et al.* 2000, Sors *et al.* 2005a). Plants are able to take up organic Se compounds but seem not to have the means to directly take up elemental Se or metal selenide compounds (White and Broadley 2009). Not only the concentration and chemical form (speciation) of Se available in the soil influence the rate and form of the Se uptake but rhizosphere conditions such as pH and the presence of sulphate and phosphate as well (Sors *et al.* 2005a). Due to its chemical similarity to sulphate, selenate is absorbed through sulphate transporters while selenite through phosphate transporters or anion channels (Shibagaki *et al.* 2002, Zhang *et al.* 2014). Sulphate transporters were characterised in *Arabidopsis thaliana* selenate resistant mutants (Shibagaki *et al.* 2002). Selenate enters the roots through the high affinity sulphate transporters SULTR1;1 and SULTR1;2 across the plasma membrane of root epidermal cells against their electrochemical gradients (Lass and Ulrich-Eberius 1984, Hawkesford *et al.* 1993). In *Stanleya pinnata* hyperaccumulator species selenate uptake and translocation rates were found to be constitutively elevated and not significantly inhibited by sulphate in contrast to *Stanleya elata* (non-accumulator) and *Brassica juncea* (secondary accumulator) species (El Mehdawi *et al.* 2018). Differences in the sulphate transporters were observed as well: the hyperaccumulator had an order of magnitude higher transcript levels of SULTR1;2 which may have enhanced Se:S specificity.

Selenite is taken up through a different pathway. Several studies showed reduced selenite uptake in the presence of higher phosphate concentration, e.g., in perennial ryegrass (*Lolium perenne*) and strawberry clover (*Trifolium fragferrum*) (Hopper and Parker 1999). These results indicate competition between these ions thus selenite uptake is assumed to be mediated by root phosphate transporters (Guignardi and Schiavon 2017). A silicon (Si) influx transporter (a nodulin 26-like intrinsic membrane protein subfamily of aquaporins) has been also shown to be permeable to selenite (Zhao *et al.* 2010).

Kikkert and Berkelaar (2013) investigated the uptake of organic Se compounds selenomethionine (SeMet), selenocystine (SeCys$_2$) in durum wheat (*Triticum turgidum*) and spring canola (*Brassica napus*). Their results showed higher rate in the
uptake of organic Se compound than of selenate or selenite. Organic Se compounds are assumed taken up via amino acid permeases (Guignardi and Schiavon 2017).

After uptake into the roots, selenate is converted into biologically active form for assimilation (Figure 3) through coupling to adenosine triphosphate (ATP) by the activity of ATP sulphurylase (ATPS, Enzyme Commission (EC) number 2.7.7.4), forming adenosine 5’-phosphoselenate (APSe). This reaction is thought to take place in the cytosol and plastids as well and was found to be rate limiting in Se assimilation (Pilon-Smits and Le Duc 2009). Four isoforms of ATPS have been identified in A. thaliana (APS1-4). Overexpression of APS1 from A. thaliana in transgenic Indian mustard (B. juncea) resulted in enhanced Se assimilation, accumulation and tolerance (Pilon Smits et al. 1999). Jiang et al. (2018a) investigated the role of ATPS in hyperaccumulator S. pinnata compared to closely related non-accumulator S. elata. In roots of S. pinnata ATPS enzyme activity and ATPS2 transcript levels were found to be two- and six-fold higher, respectively. ATPS2 in S. pinnata showed purely cytosolic localisation while ATPS2 from S. elata was found to be localised in plastids and cytosol. Based on the activity studies the enzyme in S. pinnata likely acts on both selenate and sulphate as substrates too.

APSe is converted to selenite by the enzyme adenosine 5’phosphosulphate reductase (APR). This step is reported to be another rate limiting step in selenate assimilation and occurs exclusively in plastids (Sors et al. 2005a). Transgenic A. thaliana overexpressing APR showed significantly enhanced selenate reduction (Sors et al. 2005b) while the reduced activity in the loss-of-function allele of APR2 in Arabidopsis accessions led to decreased reduction of sulphate supporting the role of the enzyme in S and Se assimilation in plants (Chao et al. 2014). The next step is the reduction of selenite to selenide which may run either enzymatically or non-enzymatically. Sulphite reductase (SiR) is responsible for the conversion of sulphite to sulfide so it may catalyze the reduction of selenite as well while the non-enzymatic way is assumed to occur through an interaction between selenite and reduced glutathione (GSH) (Pilon-Smits 2012). Cysteine synthase enzyme complex (serine acetyltransferase (SAT) and O-acetylsereine (thiol)-lyase (OAS-TL)) catalyses the formation of SeCys from O-acetylsereine (OAS) and selenide (White 2016). N.b., the official three letter amino acid code for selenocysteine is SeC; however, the abbreviation “SeCys” is also widely used in the literature. The reduction of selenite to selenide and SeCys formation are suggested to occur in the chloroplasts of cells, but
the latter step might take place in the cytosol and mitochondria as well (Ng and Anderson 1979).

SeCys can be incorporated non-specifically into proteins which then lose their function. Plants have evolved several ways to cope with Se-toxicity like producing non-protein building (i.e., non-proteinaceous) Se species or volatile compounds. SeCys methyltransferase (SMT) enzyme localised in the chloroplasts is responsible for the methylation of SeCys to form MeSeCys which is a key mechanism to reduce the amount of SeCys (Guignardi and Schiavon 2017). Overexpression of SMT enzyme in B. juncea resulted in the increased accumulation of MeSeCys compared to the wild-type plant (LeDuc et al. 2006). In tobacco and tomato non-accumulator species the overexpression of the enzyme resulted in turning the plants into Se accumulators (Wiesner-Reinhold et al. 2017).

It is to highlight that only the Se hyperaccumulator species derived isoform of SMT enzyme from an Astragalus ssp. had the ability to produce MeSeCys in contrast with the isoform obtained from a non-accumulator species. Therefore, this enzyme is assumed to have an essential role in the toleration and accumulation of high levels of Se (Sors et al. 2009). MeSeCys is oxidised to methyl-selenocysteine-selenideoxide which can be further converted to methaneselenol via the action of the enzyme Cys sulfoxide lyase and volatilised in the form of DMDSe (Sors et al. 2005a). DMDSe production was detected in the Se hyperaccumulator Astragalus racemosus (Evans et al. 1968).

Condensation of SeCys and O-phosphohomoserine (OPH) results in selenocystathionine (SeCysta) by the activity of cystathionine-γ-synthase (CGS). Several hyperaccumulator species are known to contain Se in the form of SeCysta, e.g., S. pinnata and Astragalus praelongus (Sors et al. 2005a). Overexpression of CGS from A. thaliana in Brassica juncea resulted two- to three-fold higher Se volatilisation rates compared to wild-type plants indicating that CGS is a rate-limiting enzyme for Se volatilisation (Van Huysen et al. 2003). Cystathionine beta lyase catalyses the reaction between SeCysta and water that ends up in selenohomocysteine (SeHCys) (Läuchli 1993). Conversion of SeHCys to SeMet may be catalyzed by the enzyme methionine synthase using methyl-tetrahydrofolate as a carbon donor (Cossins and Chen 1997). Methionine (Met) is synthesised in plants from S-methylmethionine and homocysteine via the activity of homocysteine S-methyltransferase (HMT) enzyme in the SMM cycle. In broccoli (Brassica oleracea var. italica) the gene BoSMT encodes an enzyme with high substrate specificity for the conversion of SeCys to MeSeCys
The gene showed HMT activity as well and was upregulated in case of treating with selenate indicating remarkable role in selenium metabolism (Lyi et al. 2007).

In hyperaccumulators, SeMet is usually further metabolised to avoid its incorporation into proteins. SeMet can be methylated and converted to volatile dimethylselenide (DMSe). Tagmount and coworkers (2002) proved that the first step in the synthesis of DMSe is the methylation of SeMet which forms Se-methyl SeMet (SeMM). This process is catalyzed by the enzyme S-adenosyl-l-methionine: L-methionine S-methyltransferase (MMT). SeMM can be converted to DMSe by two pathways. It may first be converted into the intermediate molecule 3-dimethylselenonopropionate (DMSeP) or directly into DMSe via the enzyme methylmethionine hydrolase (Sors et al. 2005a). While DMDSe is primarily produced in hyperaccumulators, DMSe is the main volatile Se compound isolated from non-accumulator plants (Pilon Smits and Le Duc 2009).

A gene encoding C-methyltransferases involved in the biosynthesis of ubiquinone or coenzyme Q was isolated from broccoli (named BoCOQ5-2). Interestingly, its expression in bacteria and in A. thaliana resulted in increased Se volatilisation, which indicates that proteins outside of the Se metabolism pathway also have significant effects on Se volatilisation (Zhou et al. 2009).

**Figure 3.** Schematic model of Se assimilation and metabolism in plant mesophyll cells. Red text and arrows indicate Se hyperaccumulator processes (Guignardi and Schiavon 2017). Asterisks indicate enzymes overexpressed via genetic engineering. Sultr sulphate/selenite cotransporters, APSε adenosine phosphoselenolate, GSH glutathione, SAT serine acetyltransferase, OAS O-acetylserine, (Se)cys (seleno)cysteine, OPH O-phosphohomoserine, (Se)Met seleno(methionine), MMT methylmethionine methyltransferase, DMSeP dimethylselenopropionate, DM(D)Se dimethyl- (di)selenide, SMT selenocysteine methyltransferase.
Se can be incorporated into glucosinolates which conversion is suggested to proceed from SeMet as precursor. Selenoglucosinolates and their volatile hydrolysis products (methylselenoalkyl nitriles and isothiocyanates) were identified in *Brassica* species: broccoli, cauliflower, forage rape (Matich *et al.* 2012) and black mustard (Ouerdane *et al.* 2013).

Another mechanism in plants to avoid Se toxicity is the breakdown of SeCys into elemental Se and alanine by the enzyme selenocysteine lyase (EC 4.4.1.16), which is a way to reduce the amount of SeCys before its misincorporation into proteins (Van Hoewyk *et al.* 2005).

In the case when plant metabolism cannot detoxify excess Se, toxicity symptoms like chlorosis, withering, stunted shoot and root growth can be detected. Se toxicity induces damages in non-proteomic processes (*e.g.*, in lipid peroxidation and altered redox status) and in the proteome as well. Besides the malformed, selenium containing proteins, proteomic impairments include the protein damage associated with general abiotic stress (oxidised or nitrated proteins) as well (Kolbert *et al.* 2019).

Hypothesis of malformed selenoproteins asserts that Se toxicity occurs when a transfer ribonucleic acid^cys^ (tRNA) inadvertently binds to SeCys instead of Cys during translation, resulting in a Cys-to-SeCys substitution in a protein which might lose its specificity and activity. Several transgenic studies support the theory that diverting SeCys away from non-specific protein incorporation increases Se tolerance in plants (Van Hoewyk 2013). Indeed, Cys residues have critical roles in protein structure and function like catalysis, redox regulation, formation of disulfide bridges and metal binding sites. Despite the similarity in their structure, the physical and chemical differences between Cys and SeCys can be harmful in case of substitution in the protein (Van Hoewyk 2013). The different size of SeCys causes the alteration of the protein structure: a diselenide bond is 0.2 Å longer than a disulfide bond. Moreover, SeCys possesses a lower redox potential, therefore its substitution can considerably alter enzyme kinetics: as cysteines are often found in the active site of enzymes, the higher reactivity of a SeCys residue dramatically influences all the enzymatic processes. In case of a protein residue that binds cofactors or ions, the related enzyme functions can be endangered due to the substitution. Additionally, Fe-Se clusters can also substitute for an Fe-S cluster in chloroplastic and mitochondrial proteins that participate in electron transport, which causes altered activity (Kolbert *et al.* 2019).
SeMet can also be accumulated non-specifically in selenium containing proteins (Eustice et al. 1981). However, it is not considered deleterious in plant proteins due to the lower reactivity of Met (Van Hoewyk 2013) and due to the quasi-specific accumulation of SeMet in Met-rich storage proteins (Németh and Dernovics 2015).

3.1.4. Selenium species identified in the Brassicaceae family

Se metabolism of Brassicaceae species has been in focus for a while due to their applicability for phytoremediation of seleniferous soils (White et al. 2007) and their evident presence in human diet. The actual known metabolic pathways of Se in *B. oleracea* based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database is shown in Figure 4. Green boxes refer to genes encoded in the organism genome: methyonil-tRNA synthetase (EC 6.1.1.10), homocysteine methyltransferase (MET), cystathionine β-lyase (CBL), cystathionine γ-synthase (CGS), methionine S-methyltransferase (EC 2.1.1.12), methionine γ-lyase (EC 4.4.1.11), thioredoxin reductase (EC 1.8.1.9), selenocysteine lyase (EC 4.4.1.16), and ATP sulphurylase (EC 2.7.7.4). Accordingly, the discovery of Se species that are either the substrate or the products of these enzymes has always been expected. The detection of SeMM, SeCysta, MeSeCys selenoxide, selenohomocystine, MeSeCys and SeMet with thin layer chromatography (TLC) in *B. oleracea* was published by Hamilton (1975) however not all of these species have been confirmed since then with the spreading of more reliable analytical methods as detailed below. SeMet and MeSeCys were identified in different *Brassica* species, and the pathway of SeMet has been underlined by deoxyribonucleic acid (DNA) coded enzymes in contrast to MeSeCys.

![Figure 4. Metabolism of Se in *B. oleracea* (Kanehisa and Goto 2000).](image-url)
Identification of Se species in plant tissues is an inevitable step on the way of exploring the pathways in Se metabolism. The most widespread analytical techniques for Se speciation are high performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) which is complemented with electrospray ionisation mass spectrometry (ESI-MS) measurements and X-ray fluorescence (XRF) and X-ray absorption near-edge structure (XANES) spectroscopy. Latter technique provides information about the Se-related covalent bonds in the analysed compounds. Detailed applicability of the techniques is to be discussed in Chapter 3.2. In this actual chapter, an overview of Se speciation results in Brassicaceae species is presented that considers plants both from natural habitat and from artificial Se supplementation experiments as well. It is to underline that critical evaluation of the upcoming results, especially on the reliability of species identification, is also addressed later.

Protease and lipase digested enzymatic extracts of A. thaliana plant tissues were tested for Se speciation using strong anion exchange (SAX)-ICP-MS instrumentation. Identification of Se species was based on retention time matching with standards. When treated with selenate or selenite, the most abundant organic Se species was SeMet both in leaf and root samples. Inorganic selenate could be also identified and a small peak was supposed to be MeSeCys. Several peaks did not show any retention time matching with the available standards and remained unidentified, which called attention to the need for complementary – organic mass spectrometry derived – solutions (Wang 2010).

The main Se compound in the proteolytic extracts of B. rapa and B. napus seeds and meal was found to be SeMet after selenate or selenite treatment in the soil or with foliar spraying. SeMet accounted for 80% of the total Se while traces of inorganic selenate was detected as well (Seppänen et al. 2010). SAX chromatography was complemented with size exclusion chromatography (SEC), similarly to the experiment where the dynamics of organic Se species production in B. napus (Seppänen et al. 2010, Seppänen et al. 2018) was in focus. Results showed that 81% of the Se content in the leaves was in the form of selenate six hours after selenate application to the soil; this concentration decreased to 60% by 14 days. In the case of selenite treatment, the applied inorganic form was detected in the leaves even after 7 days and substantial after 14 days. SeMet was found to account for 15% of total Se after 6 hours, and this species increased to 33% after 14 days. After 6 hours, MeSeCys could be identified as well; however, it could not be detected after 14 days.
Broccoli (*B. oleracea* L. var. *Marathon*) accumulated mainly SeMet in the florets in an experiment where the plants were grown in soils that had been amended with ground shoots of the Se hyperaccumulator *S. pinnata*. Besides the main form SeMet (58% of total Se), SeCys$_2$ (15%), MeSeCys (7.4%), selenate (6%) and selenite (3.1%) were identified as well. These SAX-ICP-MS data of the protease enzymatic extracts were complemented with XANES measurements of finely ground broccoli florets, revealing the following composition: 55% of total Se was found in “C-Se-C” forms (that include both SeMet and MeSeCys), 23% as SeCys$_2$, 18% in oxidised SeMet and 4% as selenate (Banuelos et al. 2015). Indeed, several studies identified the main Se species in broccoli as MeSeCys: Cai *et al.* (1995) analysed broccoli samples with gas chromatography atomic emission spectroscopy (GC-AES), Sugihara *et al.* (2004) tested broccoli sprouts with reversed phase (RP)-ICP-MS, while Lyi *et al.* (2005) studied leaves and florets of broccoli with RP-fluorescence detection (FLD) setup. Extraction procedures in these experiments were carried out with different concentration of HCl (0.05 M-0.2 M) without any enzyme in contrast to the experiment of Banuelos *et al.* (2015). Actually, HCl is considered a good extraction solvent for free amino acids, as demonstrated by Cankur *et al.* (2006).

In another study where high Se environment was provided by sodium selenite, MeSeCys was found to be the main Se compound in several other vegetables sprouts belonging to Brassicaceae family, such as *B. campestris* (qing gin cai, Chinese cabbage, turnip, nozawana), *Lactuca sativa* (red-tip lettuce) and *Raphanus sativus* (daikon – Japanese radish, Kaiware daikon) (Sugihara *et al.* 2004). Pedrero *et al.* (2006) revealed differences in Se speciation regarding selenate and selenite treated radish (*R. sativus*), covering fresh and dried samples as well. Samples were extracted with a protease enzyme and analysed with SAX-ICP-MS. In plants grown in the selenite containing media, the main species was found to be MeSeCys, followed by SeMet and SeCys$_2$; selenate could be identified too. After drying at 50°C a clear decline in MeSeCys content was observed, together with the increase of selenate concentration and the appearance of an unknown compound. In the fresh samples of the selenate treated radish the main species was found to be selenate, while SeMet, SeCys$_2$ and MeSeCys were present to a lesser extent. After drying, the decrease of organic selenium compounds and the increase of inorganic selenate were detectable.

A pioneering and demanding study was published in 2007 by Ogra *et al.*: selenohomolanthionine (SeHLan) was identified in the water extract of a Se-enriched radish variant (*R. sativus* L. cv. ’Yukibijin”), accounting for ~5% of total Se. This was
the first time that a Se compound without any former assumption on a possible sulphur analogue was unambiguously identified in a Brassicaceae plant. Ten years later the same research group detected SeHLan in *Brassica juncea* with retention time matching of their in-house synthesised standard, but no traces of the compound could be detected in garlic (Ogra et al. 2017). Interestingly, the presence of SeHLan in garlic was reported by Ruszcynska et al. (2017) based on exact mass analysis. The MS/MS fragmentation pattern and spiking procedure could clarify the identity of the compound. The detection of SeHLan in several species indicates this compound is an important metabolite in selenium tolerance and depletion processes.

Se metabolism of nozawana (*B. rapa* var. *hakabura*) and komatsuna (*B. rapa* var. *peruviridis*), two popularly consumed leafy vegetables in Japan, were parallel analysed with the well-known Se accumulator *B. juncea* (Indian mustard). After selenate treatment, the main selenium form in the water extract of the four-week old plants was identified as selenate with SEC-ICP-MS. Traces of SeMet and MeSeCys could be detected as well (Yawata et al. 2010).

Several other studies focused on the Se speciation in the secondary accumulator *B. juncea*. Plants were grown under hydroponic conditions in the presence of sodium selenite. Proteolytic extraction resulted in a higher signal for SeMet in the RP chromatogram compared to the acidic extraction, but the main Se compounds remained unidentified (Montes-Bayón et al. 2002). Similarly, enzymatic extracts were analysed by Kahakachchi et al. (2004) with ion pairing reversed phase (IP-RP)-ICP-MS and GC-AED. In selenate treated *B. juncea* plants MeSeCys, SeMet and *S*-methylseleno)cysteine were identified, while in case of selenite treatment SeMet and *S*-methylseleno)cysteine were identified with both detection techniques. The Se-S amino acid *S*-methylseleno)cysteine (*C*₄*O*₂*H*₉*NSeS) was detected for the first time in plant shoots and roots. The main forms of Se in *B. juncea* plants were detected only with IP-RP-HPLC-ICP-MS: inorganic selenate and selenomethionine Se-oxide hydrate in case of selenate and selenite treatment, respectively.

Production of volatile Se compounds like DMSe and DMDSe was detected with GC-MS from *B. juncea* seedlings grown hydroponically in closed vials. Different enrichment forms (selenite, selenate, SeMet, selenocyanate) did not provide significant difference in terms of the species released by the plants (Meija et al. 2002). Overexpression of ATPS and SMT enzymes in *B. juncea* resulted shift in the volatilisation pathways towards the form of DMDSe compared to wild-type plants (Kubachka et al. 2007). The genetic modification allowed the identification of minor
volatile Se compounds: dimethyl selenosulfenate (CH$_3$-Se-S-CH$_3$), dimethyl triselenide (CH$_3$-Se-Se-Se-CH$_3$), dimethyl diselenyl sulfide (CH$_3$-Se-S-Se-CH$_3$), and ethyl methyl selenide (CH$_3$-Se-CH$_2$-CH$_3$).

The natural presence of selenoglucosinolates has been called into question due to their absence in $S$. $pinnata$ grown at low or moderate Se concentration. Indeed, only high Se environment resulted in traces of detectable selenoglucosinolates (Bertelsen $et$ $al$. 1988). Selenoglucosinolates were unambiguously identified first in $Brassica$ species. Ethanolic extract of selenate treated forage rape ($B$. $napus$) and broccoli ($B$. $oleracea$ $L$. var. $italica$) were analysed by RP-HPLC-MS. Glucoselenoiberverin and glucoselenoerucin were identified in both samples while forage rape contained glucoselenoberteroin as well (Matich $et$ $al$. 2012). $R$. $sativus$ was found to contain a different glucosinolate compound: 4-(methylseleno)-but-3-enyl glucosinolate (McKenzie $et$ $al$. 2019).

Indeed, $S$. $pinnata$ is one of the most studied Se hyperaccumulator plants. XANES spectra of $S$. $pinnata$ leaves after selenate treatment revealed that the most prevalent form of Se is a ”C-Se-C” type species. LC-MS measurements of extracts from the leaf edges and tips revealed that the main organic Se forms were MeSeCys (88%) and SeCysta (12%) (Freeman $et$ $al$. 2006a). In comparison, the secondary accumulator $S$. $albescens$ leaf spectra were different and showed the best fitting with a composition of 75% of ”C-Se-C”, 20% SeCys and 5% selenate. Only SeCysta could be detected as organic Se compound with LC-MS (Freeman $et$ $al$. 2010). As supported by an upcoming study, several taxa in the Stanleya genus accumulate predominantly organic Se with the ”C-Se-C” configuration (Cappa $et$ $al$. 2015).

$Cardamine$ $hupingshanensis$ hyperaccumulator plants collected in a Se-mine drainage area in China were analysed with SAX-hydride generation atomic fluorescence spectrometry (HG-AFS) after Tris-HCl and enzymatic extractions. Authors denoted SeCys$_2$ as the main accumulated Se species in the plant (Yuan $et$ $al$. 2013).

3.1.5. Selenium metabolism and the plant microbiome

Generally, Se uptake of plants depends on total Se concentration in soils, Se speciation, competing ions present in soils and soil physicochemical factors: CaCO$_3$, silt content, organic matter, clay content, pH, iron, exchangeable quantity, and redox potential among others (Zhao $et$ $al$. 2005, Terry $et$ $al$. 2000). On the other hand, several studies have recently investigated the effect of plant related soil microbiome on Se
metabolism, providing a novel approach in the interpretation of Se metabolism. Eventually, the interaction of microbes with definite selenium requirements with plants, the kingdom without direct selenium needs, must be ecologically active in sharing this microelement. Here, an overview of the results is presented with a main focus on rhizosphere.

Microbiomes include bacteria, archaea, fungi and some protists attending a particular environment. All tissues of a plant host a microbial community which often benefits its host via plant growth promoting properties, while the host offers protection and nutrients to the microbial symbiont. Plant microbiome can be divided into three parts: rhizosphere, endosphere and phyllosphere that all are unique and have intra- and intercommunity interactions (Figure 5). Phyllosphere consists of the group of microbes that occur on the surface of plant shoots and endophytes are microbes that live inside plant tissues. Rhizosphere is the underground area within 5 mm proximity of the roots and it possesses the highest abundance of microbes among the three spheres (Turner et al. 2013, Cochran 2017).

Rhizosphere microbiomes of Se hyperaccumulators were found to be significantly different from non-accumulators and to harbour a higher rhizobacterial species richness (Cochran et al. 2018).

Figure 5. Schematic representation of the major sources of microbes that compose plant-associated communities (Andreote et al. 2014).

Bacteria in general seem to be tolerant to Se. Some bacteria are even selenium dependent, some synthesise selenoenzymes, or use selenium for respiration (Nancharaiah and Lens 2015). Most bacteria have capacity to assimilate inorganic Se
into organic forms and producing organic volatile forms similarly to plants’ metabolism (Staicu et al. 2017b). Not all plant-associated microbes are resistant to Se; e.g., fungi (for those selenium is ab ovo unessential) are much more sensitive than bacteria (Cochran 2017).

Contribution of soil microorganism to plant Se volatilisation was already studied decades ago. De-topped roots of broccoli were treated with antibiotics resulting in 95% inhibition of the Se volatilisation activity in case of selenate supplementation (Zayed and Terry 1994). Several studies showed that the presence of rhizosphere microbes can contribute to plant growth and enhanced Se accumulation. Experiments with ampicillin and selenate treatment showed that ampicillin inhibited Se volatilisation by about 35% of and tissue Se accumulation by about 70% of in B. juncea. Axenic plants inoculated with rhizosphere bacteria had 5-fold higher Se concentration in roots and 4-fold higher rates of Se volatilisation (de Souza et al. 1999). Inoculation with the rhizosphere extract of S. ericoides hyperaccumulator population from a seleniferous area stimulated growth and Se accumulation in the same species when it was grown upon surface-sterilised seed on autoclaved naturally seleniferous or non-seleniferous soils. The same inoculation enhanced shoot Se accumulation even in S. ericoides population originating from a non-seleniferous area grown on autoclaved seleniferous soil (El Mehdawi et al. 2015). Inoculation with a selenium tolerant environmental bacterium strain resulted in increased selenium uptake in wheat (Yasin et al. 2015).

Besides bacteria, rhizosphere fungi showed effects on plant Se accumulation as well. Alternaria seleniiphila and Aspergillus leporis from the rhizosphere of the hyperaccumulator S. pinnata were used to inoculate S. pinnata and the related non-hyperaccumulator S. elata. Reduced translocation of Se was detected in the hyperaccumulator species and increased root Se accumulation in the case of A. seleniiphila – S. pinnata and A. leporis – S. elata inoculation combinations; this observation indicated that the functional significance of fungal inoculation was host dependent. Similarly, in the case of comparing hyperaccumulator and non-accumulator Astragalus species, the hyperaccumulator plants showed decreased translocation of Se due to the inoculation with a Fusarium species previously isolated from the hyperaccumulator rhizosphere while the non-accumulator plant species was not influenced. Se speciation in roots was not affected by inoculation in these studies (Lindblom et al. 2013a, 2014). Only Alternaria astragali out of the four studied
rhizoplane fungi affected Se speciation in roots of A. bisulcatus and S. pinnata and gave rise to some elemental Se accumulation (Lindblom et al. 2013b).

Alford and coworkers (2014) investigated the nitrogen-Se relationship with the comparison of nodulated and non-nodulated Astragalus species. Enhanced nitrogen acquisition capacity associated with root nodulation was found to enhance Se accumulation in the form of selenoamino acids in hyperaccumulator A. bisulcatus. Microbial supply particularly seemed to provide glutamate for the formation of γ-glutamyl-(methyl)selenocysteine (γ-glu-MeSeCys).

Besides the rhizosphere microbiome, hyperaccumulator endophytes from S. pinnata and A. bisulcatus were also characterised with high Se resistance, high capacity to produce elemental Se and plant growth promoting properties (Sura-de Jong et al. 2015).

Besides plant-microbes, plant-plant interactions have been also in the focus of several studies. Soil collected nearby Se hyperaccumulators was found to be Se-enriched (and presumed to be) due to deposition of hyperaccumulator plant biomass (litter) and root exudation mainly in the form of organic Se compounds; this fact hypothesises an elemental allelopathic effect (El Mehdawi et al. 2011). The study of relative abundance of species occurring in seleniferous sites revealed that some species co-occurred either positively or negatively with hyperaccumulators compared to the overall vegetation. Neighbours of Se hyperaccumulators had higher tissue Se concentration compared to the cases the same species grew elsewhere in the area (Reynolds et al. 2020). The soil surrounding hyperaccumulators can easily become toxic to Se-sensitive species that leads to reduced germination and growth, while it can be beneficial for Se-tolerant species (Pilon-Smits 2019). It must be noted that the exact mechanisms of hyperaccumulators influencing their surrounding vegetation has not yet been fully elucidated.

3.1.6. Cultivation methods for selenium metabolism studies

Plant Se metabolism can be studied using different approaches in terms of the origin of analysed plant individuals. In case of field studies, plants are collected from their natural (usually seleniferous) habitat and soil samples are to be also analysed (Eiche et al. 2015). Even a particular individual can be analysed through several seasons, as global positioning system (GPS) coordinates can help to identify the exact location of individuals.
In contrast to field studies the cultivation of plants provides higher control over the growing environment. Glass- or greenhouses have usually transparent roof and/or walls that enable the light to enter during the day, which also contributes to upwarming. Greenhouses can be also equipped with several types of grow lights as sole light sources in growth chambers and growth rooms (Choi et al. 2015). Besides the intensity, the quality and the duration of light period, other factors can be monitored and/or controlled, such as temperature, irrigation, nutrient application and humidity.

Different media can be applied for plant cultivation (Figure 6). Plant seeds are often surface sterilised, e.g., with ethanol, diluted bleach or HgCl₂ (Lindblom et al. 2013a, b, 2014, Cappa et al. 2015, Yasin et al. 2015). In most cases seeds are germinated and sometimes grown until sprout size on moistened material like paper, cotton, rock wool, block wool, sheet of plywood or even coconut fiber before potting in the growing media or being analysed as sprouts (Montes-Bayón et al. 2002, Kahakachchi et al. 2004, Pedrero et al. 2006, Cappa et al. 2015, Sugihara et al. 2004, Finley et al. 2001, Yawata et al. 2010). In case of hydroponic set-ups, plants are grown until a certain size, evolving roots and shoots, then they are transplanted to float on a nutrient solution (Montes-Bayón et al. 2002, White et al. 2007). Main advantage of sand and gravel (Van Huysen et al. 2003, Freeman et al. 2010, Lindblom et al. 2013a, b, 2014, Harris et al. 2014, Schiavon et al. 2015) is their easier removal from the root in contrast to usual soil covering. The most widespread nutrient solution for watering and floating plants is Hoagland’s solution that can be applied at different strength (Hoagland and Arnon 1950, de Souza et al. 1998, Kahakachchi et al. 2004, Lindblom et al. 2013a, b, 2014, Schiavon et al. 2015). Most commonly, plants can be cultivated under sterile conditions on agar media using Murashige-Skoog agar, which excludes any kind of microbial effects (Murashige and Skoog 1962, Freeman et al. 2006, Cappa et al. 2015, El Mehdawi et al. 2015).

For Se metabolism investigations Se is usually provided through the growing media, but surface foliar spraying was also addressed (Seppänen et al. 2010). Different forms of Se can be added: selenate, selenite, organic Se like SeMet, SeCys₂ (Montes-Bayón et al. 2002, Freeman et al. 2006, Kikkert and Berkelaar 2013, Cappa et al. 2015). Tolerance and accumulation experiments require Se supplementation at different concentrations (Harris et al. 2014, El Mehdawi et al. 2014, Jiang et al. 2018b). As indicated in Chapter 3.1.4, even grounded tissue of Se accumulating species can be used by mixing it with soil to provide available Se for plant uptake (Banuelos et al. 2015). Se metabolism should be studied in the known
(or hypothetical) accumulator species in comparison with a closely related non-accumulator species (if available).

**Figure 6.** Overview of the most common cultivation set-ups used for Se metabolism studies.
3.2. Analytical Approaches in Selenium Speciation

Recognition of the fact that effects of an element on living systems depend on its chemical form lead to increasing number of studies employing the term „speciation” in a number of different ways. The clarification of the terminology was found necessary by the International Union for Pure and Applied Chemistry (IUPAC). According to their recommendations (Templeton et al. 2000) speciation analysis is the analytical activity of identifying and/or measuring the quantities of one or more individual chemical species in a sample. Chemical species are specific forms of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure. The speciation of an element is the distribution of an element amongst defined chemical species in a system.

Similarly to some trace elements, different chemical forms of Se show different bioavailability, toxicity, and biochemical properties. Identification of Se species in plants is getting more attention as plants are the main Se source in human diet (Rayman et al. 2008). Analytical speciation contributes to the exploring of Se mobility and Se-specific metabolic pathways, and molecular and cell biological approaches are complementary needed to understand the whole process (Ogra and Anan 2009).

The two major challenges in element speciation in biological systems are the low concentration of trace elements and the complexity of the matrix (Szpunar et al. 2003). Several analytical methods have been used for speciation studies; the applied technique depends on among others the purpose of the study, the issue addressed by the researcher and the sample matrix.

3.2.1. Direct methods in selenium speciation

Analytical approaches for speciation studies can be direct methods where separation techniques are not required in the form of extraction or column separation (Kroukamp et al. 2016).

Synchotron based techniques are widespread in studying metal(loid)s in plants. The most common used approaches include X-ray fluorescence (XRF) and X-ray absorption spectroscopy (XAS). This latter technique makes possible to determine the chemical form of an element while XRF can be used for imaging its distribution in tissues and cells and also for its quantification (Sarret et al. 2013).

During XRF the energy of the X-ray excites core electrons in the elements, creating a photoelectron and a core hole. The hole is filled by dipole-allowed decay of higher
bound electrons with concomitant emission of an X-ray fluorescence photon or with the emission of an Auger electron (Pushie et al. 2014). In case of Se, K-edge absorption corresponds to the excitation of the most tightly bound 1s electrons, and occurs at energies close to 12658 eV (Dolgova et al. 2018). Schematic diagram of the underlying physics and the fluorescence emission spectra of a selenium-containing sample exposed to X-rays above and below the Se K-absorption edge are shown in Figure 7. The spectrum with the incident X-ray energy below the Se K-edge shows only scattered X-rays, whereas peaks due to the Se Kα and Se Kβ X-ray fluorescence are clearly detected when the incident X-ray energy is above the Se K-edge (Pushie et al. 2014).

**Figure 7.** Physics of X-ray absorption and X-ray fluorescence. (a) Schematic diagram of the physics. (b) Experimental X-ray emission spectra collected with incident X-ray energy above and below the selenium K-absorption edge of a biological sample (Pushie et al. 2014).

A basic XRF experimental apparatus consists of a magnet source, a crystal monochromator, slits and focusing optics, a beam intensity monitor, a motorised sample stage and a fluorescence detector (Sarret et al. 2013). A schematic figure of a set-up is shown in Figure 8.

**Figure 8.** Schematic plan view of basic X-ray fluorescence microprobe (Pushie et al. 2014).
Due to the minimal required pretreatment, wide spectra of biological samples can be analysed in situ, including purified biomolecules, cell cultures, tissues, intact small organs or organisms, soil, sediment, dust or water (Dolgova et al. 2018). Synchrotron radiation microprobe can reach the spatial resolution of 0.1 – 1 μm (Lobinski et al. 2006a). To avoid the alteration of elemental distributions and chemical speciation in the sample careful fixation protocol is preferred. In case of some sample types chemical fixation of tissues can cause inaccuracies in the studied parameters. For hydrated tissue analysis, thin tissues can be cryo-preserved with immersion in liquid nitrogen, while in case of thick tissues high-pressure freezing provides homogeneous vitrification to yield the closest results to the in vivo state. Thin sections can be also prepared from frozen hydrated samples using a cryomicrotome. The samples must be kept in frozen state until analysis which is also carried out on a cryostage. Air-dried and dehydrated samples can be analysed at room temperature. In case of experiments carried out in vivo, radiation damage should be monitored carefully and exposure time should be reduced as much as possible (Sarret et al. 2013, Pushie et al. 2014).

Emission of characteristic X-rays from sample spots is recorded for μXRF imaging. High resolution optics focus X-rays into a small spot and the sample is scanned through the focal spot. Different visualisation approaches can be applied like plotting a gray-scale image for elements separately, scatterplotting, or making a red-green-blue (RGB) image where the RGB values of each pixel are proportional to the amounts of the elements they represent. μXRF images can be evaluated for quantification as well using standards for each element of interest or an internal standard element if the bulk composition, sample density and thickness are estimated reasonably (Sarret et al. 2013).

During XAS experiment the absorption of a monochromatic beam of X-rays is monitored as a function of the incident energy. Fingerprint information can be found in the X-ray absorption near edge structure (XANES) region of the XAS spectrum. This energy region lasts from 50 eV below the element absorption edge to 200 eV above it. The spectra depend on the electronic structure, thus they provide information about valence state and local structure. This means that XANES based speciation determines the chemical form of Se in the biological sample rather than the individual Se-containing molecules. Evaluation is done by least-squares fitting (LSF) of the linear combinations of spectra of known Se standards (Sarret et al. 2013, Dolgova et al. 2018).
In Se research the most common application of X-ray based methods is the investigation of the spatial distribution and chemical speciation in plant tissues. In some studies ecological partners of plants have been analysed as well. Se can be present in the oxidation states of +6, +4, 0, -2 and bound to different neighboring atoms, mostly to C, S, O, Se and H. Different selenocompounds show different XANES spectra, however the organic forms with "C-Se-C" bonds are indistinguishable and their speciation requires complementation with further methods.

XANES analysis of leaf and root tissues of non-accumulator A. thaliana species showed that Se was accumulated primarily in the form of inorganic selenate while hyperaccumulator A. bisulcatus and S. pinnata tissues contained predominantly organic selenocompounds with “C-Se-C” configuration. Further LC-MS analysis identified the compounds responsible for “C-Se-C” signal as MeSeCys and γ-glu-MeSeCys in A. bisulcatus and MeSeCys and SeCysta in S. pinnata. Differences in the localisation of Se in leaves of hyperaccumulator and non-accumulator species were also revealed. Hyperaccumulator A. bisulcatus accumulated Se mainly in the leaf hairs, S. pinnata did so in its epidermis (particularly along the leaf edge), while non-hyperaccumulator B. juncea and A. thaliana showed higher Se concentration in the vasculature (Freeman et al. 2006a, Van Hoewyk et al. 2005). Differences in moth populations originating from seleniferous and non-seleniferous areas were revealed with μXANES and LC-MS. The Se-tolerant moth accumulated MeSeCys similarly to its parasitic wasp while the Se-sensitive moth population accumulated SeCys that is toxic due to its non-specific incorporation into proteins (Freeman et al. 2006b).

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has been also employed to map the distribution of Se in biological tissues as a direct method, e.g., in thin sections of Se-treated slugs and sunflower leaves (Becker et al. 2007, da Silva and Arruda 2013, Cruz et al. 2018). The method could be indirectly used to detect selenium-containing proteins after extraction and gel electrophoresis. Selenoproteins in wheat were analysed after two dimensional poliacrylamide gel electrophoresis (2D-PAGE) using one dimensional isoelectric focusing electrophoresis (1D - IEF) and 1D sodium dodecyl sulphate PAGE (SDS PAGE) to shorten the time required to locate the position of proteins (Bianga et al. 2013). LA-ICP-MS technique allows for isotope specific analyses and ensures lower detection limit (down to 0.01 µg Se g⁻¹) compared to μXRF (0.1-1 µg g⁻¹) however its resolution is generally insufficient to map sub-cellular distribution (Lobinski et al. 2006a).
3.2.2. Orthogonal LC-MS techniques

Indirect methods require the separation of species prior to analysis, which can be carried out in the form of sequential extractions and/or column separation (Kroukamp et al. 2016). Speciation studies require the combination of separation techniques with element-specific detectors and often a molecule-specific detector as well. The set-up is expressed with the term “hyphenated” and the choice among the several possibilities available depends primarily on the objective of the research (Szpunar and Lobinski 2003). Schematic overview about hyphenated techniques for general bio-inorganic speciation analysis is shown in Figure 9. However, ICP-MS cannot be used for some elements (e.g., F), its role is unquestionable for quantification and species localisation purposes.

Figure 9. Hyphenated techniques for the speciation analysis of non-volatile species (Lobinski et al. 2006b).

1D one dimensional, 2D two dimensional, HPLC high performance liquid chromatography, CZE capillary zone electrophoresis, CEC capillary electrophromatography, MEKC micellar electrokinetic chromatography, Q quadrupole, TOF time of flight, SF sector field, MC multicollector, ICP MS inductively coupled plasma mass spectrometry, MALDI MS matrix assisted laser desorption ionisation mass spectrometry, FT ICR Fourier-transform ion cyclotron resonance

Volatile Se species and some selenoamino acids after derivatisation can be analysed with gas chromatography, but the most widespread approach in plant Se speciation is HPLC separation of plant extracts and monitoring Se with an element specific detector (mostly ICP-MS).
Steps of sample pretreatment/preparation can influence the accuracy and quality of the final result, therefore the chosen extraction methods should fit for the purpose depending on the matrix, the expected chemical form of selenium and the selected instrumentation. One should also keep in mind the quantitative extraction of selenium species and avoiding their interconversion/decomposition (Pedrero and Madrid 2009, Dernovics et al. 2006). Besides choosing a given extraction method, sequential extraction procedures can also be applied as developed for the characterisation of different classes of selenium species in garlic including water extraction, enzymatic cell wall lysis, proteolysis, and extractions with HCl, sulphite and CS₂ (Monicou et al. 2009). Recently, nanomaterial based extraction and preconcentration techniques have also been investigated for Se speciation studies (Clough et al. 2017).

One of the challenges in Se speciation is that most biological samples contain Se at low concentration levels and it is distributed among a wide variety of Se species (Pedrero and Madrid 2009). Due to the very low detection limit achievable, ICP-MS is the most frequently used detection method, however structural information of the compounds cannot be directly gained. During the so-called hard ionisation the covalent and non-covalent bonds are completely broken in molecules and monoatomic positive ions are guided to the mass analyser. Molecular (structural) identification is mostly based on retention time matching if standards are available (Lobinski et al. 2006b). As the majority of species have not been purified/synthesised yet for bioinorganic trace element speciation analysis, the parallel application of molecule specific detectors (ESI-MS, MALDI-MS) became inevitable for the standardless identification in Se speciation studies (Szpunar and Lobinski 2003).

Different types of liquid chromatography have been used for Se speciation analysis such as size exclusion, reversed phase, strong anion/cation exchange (SAX/SCX) or hydrophilic interaction (HILIC). Among these, SAX is applied in a considerable part of speciation studies because several important species (e.g., inorganic Se oxyanions) can be targeted. However, some species are hardly retained in SAX and co-elute close to the chromatographic void volume, e.g., SeCys₂, SeHLan and selenomethionine-selenoxide (SeMetO). Interestingly, this aspect is not always taken into account and the presence of SeCys₂ has been continuously published without any further confirmation by other separation or structural assignment techniques (Clough et al. 2019). Indeed, SAX usually enables the identification of SeMet, selenite, selenate, γ-glu-MeSeCys and MeSeCys on the basis of retention time matching in vegetable samples (Michalska-Kacmyrov et al. 2014, Ruszcynska et al. 2017).
RP chromatography is also used for the analysis of Se species, although selenoamino acids are too hydrophilic to be retained on typical non-polar stationary phases (C8 or C18). Their retention can be increased by using ion pairing reagents (for example, perfluorocarboxylic acids, alkyl sulfonic salts or tetraalkylammonium salts) in the mobile phase (Pryzynska et al. 2019).

Hydrophilic interaction chromatography is an alternative to RP separations of highly polar selenocompounds. In comparison with RP, HILIC mode showed higher and better separated signals, particularly for inorganic forms of Se and a shorter run when analysing onion extracts (Sentkowska and Pyrzynska 2018). The main advantage of HILIC in contrast to ion exchange separations is that the used mobile phases are compatible with the electrospray ion source therefore direct hyphenation can be carried out. This approach was first applied for yeast and yeast based food supplements analysis in the field of Se speciation (Dernovics and Lobinski 2008a, 2009).

SEC enables the separation of the compounds according to their hydrodynamic volume that is more or less proportional to their molecular mass and it can be tuned for the separation of low molecular mass Se species when low ionic strength SEC is addressed (Szpunar and Lobinski 2002, Encinar et al. 2003a). SEC usually is followed by a different separation, preferably orthogonally: even three-dimensional chromatography purifications have been used by the sequential application of SEC, anion and cation exchange separations for selenocompounds (McSheehy et al. 2002, Dernovic et al. 2007). Multidimensional HPLC separations are necessary not only because of the high number of selenocompounds present at low concentration in the samples but also to decrease matrix complexity (Szpunar and Lobinski 2002).

Molecular mass spectrometry based methods apply soft ionisation. In electrospray ion sources covalent and non-covalent bonds are usually preserved while during matrix-assisted laser desorption ionisation (MALDI) the covalent bonds are preserved and non-covalent bonds are often destroyed. Structural analysis and standardless identification of low molecular mass Se compounds is enabled by ESI (up to ~1000 Da) (Lobinski et al. 2006b). Casiot and coworkers (1999) were the first to successfully apply a generic approach where the purification of selenocompounds was monitored with ICP-MS prior to ESI-MS(/MS) identification.

The parallel coupling of ICP-MS and ESI-MS to chromatographic separation requires careful harmonisation, especially in terms of the solvent eluting into ion sources (Szpunar and Lobinski 2003). The purification and concentration of the fraction of a selenocompound detected by the ICP-MS may be necessary before ESI-
MS analysis, because of its high exposure to signal suppression events (McSheehy et al. 2002). The application of orthogonal separations and detections is mostly related to selenised yeast Se speciation in the literature (Lobinski et al. 2006a), but some examples for the application related to plants can also be found (Table 3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Instrumentation</th>
<th>Identified compound(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassica juncea roots</td>
<td>IP-RP-HPLC-ICP-MS, SCX-HPLC-ICP-MS, ESI-QTOF-MS</td>
<td>Se-methyl selenomethionine</td>
<td>Grant et al. 2004</td>
</tr>
<tr>
<td>Allium fistulosum (green onion)</td>
<td>SEC-ICP-MS, IP-RP-HPLC-ICP-MS, ESI-Iontrap-MS</td>
<td>γ -glu-Se-(methylseleno)cysteine</td>
<td>Shah et al. 2004</td>
</tr>
<tr>
<td>Allium sativum (garlic)</td>
<td>SEC-ICP-MS, RP-HPLC-ICP-MS, ESI-QTOF-MS</td>
<td>γ -glu-Se-(methylseleno)cysteine</td>
<td>McSheehy et al. 2000</td>
</tr>
<tr>
<td>Phaseolus vulgaris vulgaris (green bean)</td>
<td>SAX-HPLC-ICP-MS, RP-HPLC-ICP-MS, RP-HPLC-ESI-TOF-MS, RP-HPLC-ESI-Orbitrap-MS</td>
<td>Se-(methyl)selenocysteine, γ -glu-Se-(methyl)selenocysteine</td>
<td>Shao et al. 2014</td>
</tr>
<tr>
<td>Allium sativum (garlic) Helianthus annuus sprouts (sunflower) Raphanus sativus sprouts (radish)</td>
<td>SAX-HPLC-ICP-MS, RP-HPLC-Orbitrap-MS</td>
<td>SeHLan, Se-(methylseleno)cysteine, SeMet, SeMetO, deaminohydroxy-SeHLan, N-acetylCys-SeMet, γ -glu-Se-(methylseleno)cysteine, methylseleno-Se-pentose-hexose, Se-methyl-selenoglutathione, 2,3-dihydroxy-propionyl-SeCys-Cys, methylthio-selenoglutathione and 2,3-dihydroxypropionyl-SeLan, 2,3-dihydroxypropionyl-SeCys-Cys-alanine</td>
<td>Ruszcynska et al. 2017</td>
</tr>
<tr>
<td>Lecythis minor (monkeypot nut)</td>
<td>SEC-ICP-MS, SCX-HPLC-ICP-MS, IP-RP-HPLC-ICP-MS, nanoRP-HPLC-ESI-QTOF-MS</td>
<td>SeCysta, γ -glu-SeCysta</td>
<td>Demovics et al. 2007</td>
</tr>
</tbody>
</table>
3.2.3. Detection and basics of structural identification of selenocompounds by LC-ESI-MS and MS/MS

Apart from the evident option to search for previously reported selenium species with the help of a custom database, the detection of Se containing molecules in full scan ESI-MS spectra was first carried out by manual pattern exploration. Basically, the unique isotope distribution pattern of Se makes the detection of Se containing molecule possible (Figure 10).

**Figure 10.** The characteristic isotopic distribution of Se containing compounds. The green isotopologues correspond to the monoisotopic mass (Németh 2015).

Successful visual seeking not only requires sufficient signal-to-noise (S/N) ratio (Dernovics *et al.* 2007) but adequate resolution to be able to unambiguously recognise the isotopic pattern of Se. In the case of low resolution (single or triple quadrupole) instruments the spotting of Se pattern was a difficult task, especially when real world samples were faced, as evidenced by a series of studies by McSheehy *et al.* (2000, 2001, 2002). Indeed, usually the characteristic \(^{78}\text{Se} - ^{80}\text{Se}\) isotope ratio could only be spotted and a subsequent MS/MS step was required to prove the presence of a formerly reported selenium specific fragment, *e.g.*, selenohomocysteine. With the introduction of high resolution (HR) MS instruments, the possibility to look for the specific mass defect of selenium (for \(^{80}\text{Se}L: 79.9165 - 80 = -0.0835\) amu) was an evident tool to detect Se containing molecules (García-Reyes *et al.* 2007). The mass defect feature, together with the specific isotopologue pattern, unambiguously pinpoints selenium carrying molecules – however, manual screening (“walking the chromatogram”) is extremely time consuming and must be assisted with some kind of pre-filtering. However, when no software assistance is provided, such an approach is a must and calls for a personal experience to spot the mass defect carrying molecules in each full scan spectrum.
Software based pre-filtering can be based on the extraction of “diagnostic” (characteristic) Se-containing in-source fragments. These fragments are highly stable, carry an evident mass defect and their structure is known, which also determines their position in the final molecule. The most important in-source (or, as sometimes referred, ion-source) fragments on $^{80}$Se are the two different SeHCys residue derived forms ($\text{C}_4\text{H}_6\text{NO}_2\text{Se}^+$ at $m/z$ 181.97148 and $\text{C}_4\text{H}_{10}\text{NO}_2\text{Se}^+$ at $m/z$ 183.98713), their derivatives after formic acid loss (especially from the first form, $\text{C}_4\text{H}_6\text{NSe}^+$ at $m/z$ 135.96600), the selenocysteine derived fragment ($\text{C}_3\text{H}_6\text{NO}_2\text{Se}^+$ at $m/z$ 167.95583), the NH$_3$-loss derived structure of Se-(methyl)selenocysteine ($\text{C}_4\text{H}_7\text{O}_2\text{Se}^+$ at $m/z$ 166.96058) and the methyl-seleno group ($\text{CH}_3\text{Se}^+$ at $m/z$ 94.93945) (Németh et al. 2013; Dernovics and Lobinski 2008b). Among these, the $\text{C}_4\text{H}_{10}\text{NO}_2\text{Se}^+$ fragment is highly specific to selenohomolanthionine, Se-(methyl)selenocysteine and their derivatives (Ogra et al. 2007, Dernovics et al. 2009), $\text{C}_4\text{H}_8\text{NO}_2\text{Se}^+$ to selenocysteine and its derivatives (McSheehy et al. 2000, 2002), while $\text{C}_4\text{H}_8\text{NO}_2\text{Se}^+$ is the least specific and therefore the most useful stable fragment to locate any compound that is derived from the highly central selenocysteine – selenomethionine pathway in selenium metabolism. Once a peak is obtained in the EIC of an in-source fragment, the full scan spectrum at the apex should be opened, the in-source fragment must be checked (whether it has been a false positive hit or not), and the parent molecule(s) should be looked for. If no hypothetic parent is found, the relevant ion source voltage setting (e.g., ”declustering potential”, “cone voltage”, “fragmentor voltage”, etc.) should be decreased to give a rise to the potentially completely fragmented parent ion. Unfortunately, no useful selenised in-source fragment ions can be provided for negative ion mode measurements, which considerably hampers species spotting in the case of plant samples (Németh et al. 2013).

A semi-automated approach to detect selenium containing molecules, actually peptides, was published by McSheehy et al. (2005), taking advantage of automated peptide listing options of proteomic softwares. In case there is no ICP-MS assisted purification and enrichment of selenium containing peptides from the tryptic digest (cf. Encinar et al. 2003b), the sulphur and selenium analogues of the same peptides are present in the same sample to analyse with the RP-LC-ESI-MS proteomic setup. The search and fragmentation at + 48 amu masses related to automatically detected Met-containing peptides (regarding the + 48 amu as a variable /non-fixed/ modification of Met to arrive at SeMet) promote the detection and identification of peptides where SeMet was non-specifically built into the place of Met.
An attempt to automated screening for Se species was first developed by coupling an on-line 2D chromatographic system with parallel diode array UV, ICP-MS and linear trap-Orbitrap (ESI-MS\textsuperscript{3}) detection. The Metworks and Mass Frontier software (Thermo Fisher Scientific) enabled automatic isotopic pattern recognition in a full MS scan mode and structural identification based on the isotopic ratio, intra-isotope mass defect and the addition of a parent ion in the MS\textsuperscript{3} target list (Preud’homme \textit{et al.} 2012). Indeed, as no vendor independent (freeware) tools are available at the moment, analysts should explore the instrument derived software options whether they can be tuned to help to filter selenium containing species, \textit{e.g.}, by modifying the widespread tool intended for detecting chlorine containing molecules. In such cases, the intra-isotope mass defect based ($^{37}$Cl – $^{35}$Cl = 1.9970 amu) accurate mass filtering might be changed for $^{80}$Se – $^{78}$Se (= 1.9992 amu), which can provide a considerable decrease in hits if applied with highly stable accurate mass measurements, even without taking into account isotopologue ratios.

Before the spread of LC-ESI-MS techniques the structural characterisation of selenocompounds was only possible with time and reagent consuming procedures. For example, the isolation and identification of two isomers of $\gamma$-glu-SeCysta from seeds of \textit{Astragalus pectinatus} required long and solvent consuming extraction from more than 200 g sample, followed by resin chromatography, acid hydrolysis, amino acid analysis, derivatisation, paper electrophoresis, ninhydrin reaction, starch-iodide reaction, and Raney-Ni hydrogenolysis (Nigam and McConnell 1976). The application of LC-MS enables faster identification, however it must be noted that in modern organic chemistry, nuclear magnetic resonance (NMR) spectroscopy is the designated method for the definitive identification of compounds. The amount of the pure compound (~ 1-1.5 mg) required for NMR spectroscopy cannot often be extracted and purified from biological samples, therefore ESI-MS and ESI-MS/MS based identification might be adequate in selenium speciation in most cases.

Casiot \textit{et al.} (1999) published the first ESI-MS based standardless identification of Se-metabolites in a yeast sample. The principle of the sulphur-selenium analogy could be followed as Se can incorporate into compounds instead of S resulting a +48 amu difference in the molecular mass. The compound at $m/z$ 433 showing the characteristic Se isotope pattern was fragmented, resulting in two Se-containing fragments with masses of $m/z$ 182 and 298 and in Se-free fragments of $m/z$ 250 and 136. The hypothetic identity ($Se$-adenosyl-selenohomocysteine, AdoSeHCys) was confirmed through the fragmentation of its sulphur analogue standard ($S$-adenosyl-
homocysteine). Wrobel et al. (2002) assumed the S-Se analogy throughout the overall sulphur metabolic pathway and looked for selenoadenosylmethionine (AdoSeMet) as this compound should be the preceding compound leading to the synthesis of AdoSeHCys. Similarly, the identification was confirmed through the fragmentation of the sulphur analogue and also by a custom synthesised standard. Well, following the S metabolic pathway in the exploration of Se compounds can be successful mainly in case of stable compounds that are present at considerable concentration in the sample. For instance, cystathionine is another key compound in S metabolism and it is ubiquitous in all living organisms, however, its concentration is highly organism-dependent. The Se analogue SeCysta and its γ-glutamyl derivatives have been found in monkeypot nut following the S-Se analogy method (Dernovics et al. 2007).

The Met metabolism pathway towards S-methyl-5'-thioadenosine in the general Met salvage process has been also found to function in Se metabolism, as proven by the identification Se-methyl-5-selenoadenosine in Hericium erinaceus (lion’s mane mushroom; Egressy-Molnár et al. 2016). Species of Allium family are known to possess remarkable S metabolism; Se-rich garlic was found to contain mainly γ-glu-MeSeCys which has been graded as one of the main compound for Se deposition in Se accumulator plants (Larsen et al. 2006).

Active metabolites with disulfide bonds naturally play roles in the metabolism of cells in the glutathionine cycle. However, the ex vivo formation of compounds with Se-S bond has also been associated with oxidation processes due to, e.g., long storage (Uden et al. 2004). The presence of glutathionine S-conjugates with selenocompounds in yeast was reported first time by McSheehy et al. (2001). After the reduction of the detected selenocompounds glutathione and selenium-containing moieties could be partly identified with low mass resolution MS. HR MS data and MS/MS fragmentation allowed for the ultimate identification of conjugates of glutathionine with Se-glutathionine and glutathione with a methylseleno group through Se-S bridges (Infante et al. 2006). Similarly, the search for free thiol and selenol containing building block moieties (including glutamyl/seleno/cysteine or /seleno/cysteinylglycine among others) offers an effective tool in the exploration of Se-S(e) conjugates: the application of this strategy, together with the assignment of the m/z 167.96 in-source fragment referring to SeCys allowed for the identification of a series of conjugated (seleno)glutathione and 2,3-dihydroxy-propionyl-L-(seleno)cysteine derivatives (Dernovics and Lobinski 2008b). This latter, previously unreported moiety was confirmed by chemical synthesis as well (Egressy-Molnár et al. 2014). The same
“building block” identification methodology allowed for the detection of 19 Se-S(e) bond containing compounds in yeast samples by Gil Casal et al. (2010). Theoretical and experimental masses, isotopic patterns, empiric formulas and proposed structures of two newly detected selenocompounds are shown in Figure 11 as examples.

<table>
<thead>
<tr>
<th>Th mass</th>
<th>Exp mass</th>
<th>Error ppm</th>
<th>Se isotopic pattern</th>
<th>Empiric formula</th>
<th>Proposal structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>547.0075</td>
<td>547.0093</td>
<td>−0.30</td>
<td><img src="image1" alt="Se isotopic pattern" /></td>
<td>C₉H₁₄N₅O₆Se⁺</td>
<td><img src="image2" alt="Se-ethyl-5-selenoadenosine" /></td>
</tr>
<tr>
<td>553.9789</td>
<td>553.9789</td>
<td>−0.23</td>
<td><img src="image3" alt="Se isotopic pattern" /></td>
<td>C₉H₁₄N₅O₆S⁺</td>
<td><img src="image4" alt="Se-dimethyl-5-selenonium-adenosine" /></td>
</tr>
</tbody>
</table>

**Figure 11.** Two new selenocompounds detected by Gil Casal et al. (2010).

Taking into account the chromatographic behaviour of unknown selenium species might contribute to successful structural elucidation. MS/MS fragmentation of a Se compound detected at m/z 360.057 in *H. erinaceus* indicated the losses of adenosyl, adenine and ribose groups which fragments could relate to two possible structures: Se-ethyl-5-selenoadenosine and Se-dimethyl-5-selenonium-adenosine. Taking into account that the retention of the compound was close to the void on an RP column, an ethyl group containing compound should have eluted later, so this structure was excluded and the compound was assigned as Se-dimethyl-5-selenonium-adenosine (Egressy-Molnár et al. 2016). Similarly, the addition of a functional group to a detected compound appears not only in the m/z value but can considerably change the hydrophobicity of the compound as well. The addition of a hydroxyl group to geranyl-SeHCys contributed to a less hydrophobic character and therefore showed shorter retention time on an RP column while in the case of an additional carboxyl group the retention showed higher hydrophobicity compared to the hydroxyl group possessing compound (Németh et al. 2013).

Finally, when neither preconception nor additional information (e.g., glutathione related structure) is available on an unknown selenium species, the following general steps might be taken to arrive at a possible structure on the basis of LC-HR-MS/(MS) data:
elemental composition should be determined for the parent and for the at least medium intensity fragments as well, taking into account the number of participating selenium atoms (dedicated from the isotopologue pattern);

b) in-source and MS/MS fragments should be matched to decide whether co-eluting selenium species are present;

c) fragments arriving from co-fragmented isotopologues of the parent ion (e.g., from $^{78}$Se or $^{82}$Se) should be clearly assigned;

d) characteristic (diagnostic) fragments should be searched for, especially selenium containing ones, in the low abundance section of the MS/MS spectra;

e) characteristic losses (e.g., hexose $/\sim162.05$ Da/, formic acid $/\sim46.00$ Da/, water $/\sim18.01$ Da/, ammonia $/\sim17.03$ Da/, $^{80}$Se $/\sim79.92$ Da/) should be checked;

f) fragments completing each other to the parent mass should be looked for;

g) structure elucidation might be started at the characteristic selenium fragments with the following notes: (i) fragmentation often occurs at both sides of selenoethers that might stabilise either as R-Se$^+$ or R-SeH$_2^+$; (ii) free selenols (-R-SeH) should not be accounted for neither in MS, nor under MS/MS conditions; (iii) Se-S(e) bridges hardly dissociate in positive ion mode fragmentation;

h) in case of low abundance, looking for and fragmenting a surrogating higher intense sulphur analogue might provide additional information.

### 3.3. Quantification Without Authentic Standards

#### 3.3.1. Background

LC based quantification procedures usually require authentic standards that can be applied with different calibration protocols: external-, matrix matched calibration or standard addition. Unfortunately, lots of compounds are not commercially available in the form of standards due to their instability, low consumer demand, low natural concentration or when the extraction/purification from natural sources or the synthesis is expensive. Besides purchasing costs, there are several further problematic issues regarding the handling of standards: limited shelf life, cost of storage (e.g., -80 °C), administrative costs, introduction of standard deviation and biases during the
preparation of calibration solutions (e.g., components missing from a multi-component calibration), etc. Also, the separate handling of standards might be also required when they are incompatible (e.g., selenate oxidises selenite). Definitely, there are numerous cases in (bio)analytical studies when quantification must be carried out with a limited number of standards. In such situations, several analytical approaches might be addressed.

3.3.2. Making uniform analytes: general aspects

Adequate solution may be provided by the reduction of the number of target compounds. It is possible that the required information can be gained by summing the target compounds when their individual concentration is not important. For example, this is the case for vitamin C and dehydroascorbic acid (DHAA): they both have the same vitamin effect. A former (out-of-date) European Standard specifying a method for the determination of vitamin C in foodstuffs (EN 14130:2003) prescribed the reduction of DHAA, thus making a uniform analyte (vitamin C) from the two compounds. However, such protocol cannot be followed if the goal is the monitoring of degradation of vitamin C, e.g., in foodstuffs during heat treatments (Xanthakis et al. 2018). Anyhow, making uniform analytes is an established approach and it is presented with examples as follows.

3.3.2.1. Making uniform analytes for LC-ultraviolet and fluorescence detections through sample preparation and/or methodology

If a compound can exist both in reduced and oxidised forms, making one uniform analyte (one state) facilitates the quantification. In case of coenzyme Q10 (CoQ10) the application of an oxidizing agent drives the equilibrium to the fully oxidised state and total CoQ10 can be determined with LC-ultraviolet (UV) spectroscopy at 275 nm (Orozco et al. 2007).

Another situation is where the analytes differ in a functional group or in a moiety from each other. Steviol glycosides are often determined with UV absorbance with external calibration; however, there can be dozens of glycosides present in a sweetener mixture that are not available in the form of standards. This approach doesn’t take into account that principally non-UV chromophore aglycon side chains also influence the spectra besides the common aglycone moiety (JECFA 2010). Therefore, uniform
analytes can be prepared through the hydrolysis of the glycosyl chains, resulting in the common aglycone molecule, steviol (Bartholomees et al. 2016).

Quantification of vitamin B<sub>9</sub> content in foods also requires uniform analytes due to the presence of several vitamers. Enzymatic hydrolysis is applied for the conversion of folates and polyglutamate folates into monoglutamates, while 10-formyl-tetrahydrofolate (THF) is converted to 5-formyl-THF with a heat treatment to efficiently reduce the number of structures for analysis (Quinlivan et al. 2006). Similarly, acidic hydrolysis breaks the glycoside bonds in flavonoid glycosides which allows for their quantification in vegetables and fruits through their aglycones (Wach et al. 2007).

3.3.2.2. Making uniform analytes through LC-ESI-QQQ methodology

As indicated above, the quantification of flavonoids is a challenging task: with thousands of glycosylated flavonoids, there have not been and never will be standards for most compounds, which makes the identification very difficult as well. Although, the hyphenation of LC with a diode-array detector (DAD) and mass spectrometry (MS) also can provide a solution (Harnly et al. 2007). Retention time, wavelengths of maximal UV/VIS absorbance, molecular mass and fragmentation pattern allow for the structural characterisation of flavonoid compounds whereas the quantification is based on the UV signal from the structurally closest matching available standard (Lin et al. 2007). An elegant example of this comprehensive approach was published by Abrankó et al. (2015): the authors could quantify a novel, tentatively identified genisteine-hexoside in cherry samples with the calibration equation of genistin using the UV signal at 260 nm. To achieve this, the novel compound had to be proven a part of the genistein-derived flavonoids, which was made by in-source fragmentation and high resolution (TOF) mass spectrometric data as well.

3.3.3. Solutions with LC-ICP-MS; Part 1

While signal intensity is considered to be independent from the matrix in UV measurements, the analyte signal in MS is influenced by several factors, e.g., the eluent and the coeluting matrix components. When there is no authentic standard available, these effects should be considerably reduced or making compromises is needed.

In metallomics studies where some standards are available, unknowns might be quantified by using the closest eluting standard: Sloth et al. (2004) assumed that the
LC-ICP-MS setup would show similar matrix effects for the arsenic species eluting close to each other in a short retention time window (i.e., with similar HPLC eluent composition), therefore the instrumental sensitivity for these arsenicals should be quite similar. However sample derived matrix effects cannot be evidently monitored/ruled out (especially when non-UV chromophore compounds are eluting, e.g., sugars), but the monoisotopic feature of arsenic considerably decreases the number of quantification possibilities. Another possibility is the application of low flow rates on microbore (capillary, nano) columns together with isocratic separation: this combination increases the stability of the ICP-MS plasma in time, removes eluent-derived changes in sensitivity, and this way, calibration can be carried out with only one authentic standard (Far et al. 2010).

In case isocratic separation is not an option, and gradient separation is a must, different methods should be addressed. Indeed, constant elemental response can be a requirement for the absolute quantification with LC-ICP-MS but the increasing ratio of organic solvents in RP gradient separations influences the ionisation efficiency of certain elements in ICP either in negative (signal suppression) or in positive (carbon charge effect) manner. In the study of Pröfrock and Prange (2009), counter-current compensation was applied: the gradient separation was carried out on the analytical column, while a second matched reversed gradient was post-column mixed before entering the pooled flow into the ICP-MS. Another approach was published by Moller et al. (2005): the modification of the LC eluent prior to the ICP plasma was made by the use of a membrane desolvation system to remove a considerable part of the organic solvent from the eluent. However the desolvator system had to be individually optimised for each analysed element and the species independent sensitivity was lost for selenium compounds, the method was successfully validated for platinum compounds.

The most recent approach to achieve constant signal response factors has been published by Spanish scientists. Calderon-Celis et al. (2018, 2019) developed and optimised a method for six elements (S, P, As, Se, Br, I) with the help of CH₄ or CO₂ addition to the plasma following capillary LC (that is, low flow rate, < 5 µL min⁻¹) based separation. The so-called carbon saturation technique adds an adequately high amount of carbon in the plasma to reach special ICP conditions where neither the LC eluents, nor the matrix constituents, nor species dependent ionisation efficiency can significantly change elemental response factors. This approach also has the advantage that no additional instrumentation is needed.
3.3.4. Possibilities in LC-ICP-MS Part 2: isotope dilution

Isotope dilution analysis (IDA) has been widely used for quantification purposes both with standalone ICP-MS and together with LC hyphenation. The technique is based on the measurement of isotope ratios in samples where the isotopic composition has been altered by the addition of a known amount of an isotopically enriched standard. The method has excellent precision and accuracy because signal influencing factors equally affect isotope signals, therefore their ratios remain unchanged. Only a few disadvantages can be listed about this technique: it can be used only for stable multi-isotopic elements, additional equipment and software tools are required (however, none of them calls for extraordinary investments), and the isotopically enriched standards might not be easily available commercially (Fassett and Paulsen 1989).

When it comes to LC-IDA-ICP-MS, basically two approaches have been used. Species-specific IDA requires the isotopically labelled (molecular) entity of the compound to be analysed in the form of standards, which have far lower commercial availability than the related standards with normal isotope distribution. However this method can offer highly accurate and unbiased quantification due to the compensation of extraction efficiency losses, analyte decomposition and on-column analyte loss, only a few compounds can be purchased directly (that is, methyl-\(^{198/199/201}\)Hg, mono/di/tributyl-\(^{119}\)Sn and \(^{76}\)Se-selenomethionine), which acts as a real bottleneck in the application of this approach. On the other hand, the so-called species-unspecific spiking mode enables the quantification of structurally unknown compounds or when standard is not commercially available. The addition of the isotope spike is carried out after the separation in the form of post-column spiking and the spike with a known isotopic distribution exists in a chemical form that is different from that of the species. If the standard is in solution form, the isotope distribution is given on the certificate, while if the standard is solid the exact isotope distribution must be determined through isotope dilution, which is called reverse isotope dilution and it involves a standard with natural isotopic composition. This type of spiking mode does not correct for any error occurring during sample preparation or separation and corrects only for errors derived from instrumental instabilities and matrix effects (Rodríguez-González et al. 2005).

The first species-unspecific isotope dilution analysis was carried out in 1994 by Rottmann and Heumann; the schematic overview of their instrumental set-up is shown in Figure 12.
Figure 12. Schematic diagram of a species-unspecific isotope dilution analysis set-up by HPLC-ICP-MS (Rottmann and Heumann 1994, Rodríguez-González et al. 2005).

This on-line isotope dilution technique relies on the plotting of the mass flow vs. retention time. The mass flow is calculated by measuring the corresponding isotope ratio throughout the whole chromatographic run, then the peaks detected have to be integrated and normalised to the injection volume to obtain final concentration data.

Elemental isotope dilution analysis can be performed only if at least two isotopes can be measured free of spectral interferences. There are several ways to eliminate spectral interferences, like the use of double focusing HR-ICP-MS or addressing quadrupole analysers combined with collision and dynamic reaction cells (Reyes et al. 2003a). Isobaric interferences and polyatomic interferences from the sample matrix can be eliminated during the chromatographic separation or mathematical corrections can be applied. Moreover, the signal intensities must be corrected with the dead time of the detector, which is usually done automatically by the instrument, and the mass bias which is caused by the phenomenon that heavier isotopes are transmitted more efficiently than lighter isotopes into the MS part from the ICP (Rodríguez-González et al. 2005).

The application of IDA in Se studies is a powerful tool for quantification as the number of commercially available standard is especially limited. The most abundant Se isotopes are affected by the presence of Ar dimer ions in ICP-MS, which can be eliminated by the introduction of He, H₂, O₂, NH₃ or CH₄ in collision/reaction cells. In the case of H₂, the SeH⁺ and BrH⁺ formation requires mathematically correction as
well (Huerta et al. 2003). Examples about the application of IDA in Se studies are shown in Table 4.

Isotope pattern deconvolution (IPD) is a more recent method of calculation and it is based on the determination of the molar fractions for each pure isotope pattern (natural abundance or labeled) contributing to the isotope pattern observed in the mixture of natural (native) and labeled analytes (added) by multiple linear regression. This approach has been applied also for Se studies (Rodríguez-Castrillón et al. 2008, Castillo et al. 2013).

Table 4. IDA applications for quantifications in Se studies.

<table>
<thead>
<tr>
<th>Type of spiking</th>
<th>Instrumentation</th>
<th>Sample(s)</th>
<th>Quantified compounds</th>
<th>Enriched isotope in spike</th>
<th>Isotope ratio used for calculation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>species-specific</td>
<td>RP-HPLC-ICP-MS</td>
<td>human serum</td>
<td>SeMet, SeCys$_2$*</td>
<td>$^{77}$SeMet</td>
<td>77/80</td>
<td>Encinar et al. 2004</td>
</tr>
<tr>
<td>species-unspecific</td>
<td>Affinity HPLC-ICP-MS</td>
<td>human serum</td>
<td>selenoprotein P, albumin, glutathione peroxidase</td>
<td>$^{77}$Se</td>
<td>78/77 70/77</td>
<td>Reyes et al. 2003b</td>
</tr>
<tr>
<td>species-unspecific</td>
<td>IP-RP-HPLC-ICP-MS</td>
<td>wheat, triticale, flour, wheat biscuits</td>
<td>SeMet, SeMetO</td>
<td>$^{76}$Se</td>
<td>76/78</td>
<td>Kirby et al. 2008</td>
</tr>
<tr>
<td>species-unspecific</td>
<td>SAX-HPLC-ICP-MS</td>
<td>yeast, wheat flour</td>
<td>yeast: SeMet, Se(IV) wheat flour: SeMet</td>
<td>$^{77}$Se</td>
<td>78/77</td>
<td>Huerta et al. 2003</td>
</tr>
<tr>
<td>species-specific</td>
<td>SAX-HPLC-ICP-MS</td>
<td>certified reference materials, skimmed powdered milk, wheat flour, enriched yeast</td>
<td>SeMet</td>
<td>$^{76}$SeMet</td>
<td>77/76</td>
<td>Krata et al. 2008</td>
</tr>
<tr>
<td>species-specific</td>
<td>SAX-HPLC-ICP-MS</td>
<td>certified reference materials, skimmed powdered milk, wheat flour, enriched yeast</td>
<td>SeMet</td>
<td>$^{77}$Se</td>
<td>78/77</td>
<td>Krata et al. 2008</td>
</tr>
<tr>
<td>species-unspecific</td>
<td>RP-HPLC-ICP-MS</td>
<td>garlic</td>
<td>γ-glu-MeSeCys</td>
<td>$^{77}$γ-glu-MeSeCys</td>
<td>78/77 82/77</td>
<td>Infante et al. 2008</td>
</tr>
<tr>
<td>species-unspecific</td>
<td>2D SEC-AF-HPLC-ICP-MS</td>
<td>mouse plasma</td>
<td>selenoprotein P, extracellular glutathione peroxidase, selenoalbumin, seleno-metabolites</td>
<td>$^{74}$Se</td>
<td>78/74</td>
<td>García-Sevillano et al. 2014</td>
</tr>
</tbody>
</table>

* The accurately determined SeMet was used as an internal standard for SeCys$_2$ quantification.
4. EXPERIMENTAL

4.1. INVESTIGATION OF SELENOMETABOLITES IN *Cardamine violifolia*

4.1.1. Reagents and samples

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, UPLC-MS grade), and sodium sulphite (98%) and formic acid (~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) 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), pyridine (99.5%) 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). Certified ^82^Se isotopic abundance solution (10 µg mL⁻¹ ^82^Se in 5% nitric acid) was bought from Inorganic Ventures (Christiansburg, VA, USA).

*Cardamine violifolia* was identified and registered by the Wuhan Botanical Garden (Chinese Academy of Sciences; Wuhan, China). Pooled sample of stems and leaves was harvested in the springtime of 2016 in the natural seleniferous region Yutangba, Enshi (Hubei Province, China). Leaves and root samples of *C. violifolia* were collected separately in the springtime of 2017. Plant biomass was cleaned with deionised water, lyophilised and milled.
4.1.2. Selenium distribution in *C. violifolia*

4.1.2.1. Total selenium and sulphur determination

Total selenium and sulphur concentrations were determined after acidic digestion. Microwave digestion of the samples, the extraction residues and aliquots of extraction solutions was carried out in a CEM Mars-5 digestion system (CEM; Matthews, NC, USA). Samples (50 mg *C. violifolia*; entire extraction residues; 1.0–2.0 mL aliquots) were mixed with 5.0 mL HNO₃ in polytetrafluorethylene (PTFE) digestion tubes and after 24 h 3.0 mL H₂O₂ 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.

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.

4.1.2.2. Sequential extraction procedure

Sequential extraction protocol was adapted from Mounicou *et al.* (2009):

1. **Water extraction:** 200 mg of the *C. violifolia* sample was extracted with an ultrasonic probe (UP100H, Hielscher Ultrasound Technology, Teltow, Germany) at ambient temperature. Extraction was carried out with sonication in two steps: first in 6.0 mL deionised water for 2×1 min then with 3.0 mL deionised water for 2×1 min. 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. **Extraction with sulphite:** 5.0 mL of 1.0 M Na₂SO₃ was added to the water insoluble residue; the sample was vortexed and shaken in an orbital shaker at 180 1 min⁻¹ for 24 h at 37 °C. Supernatants and residues were separated by centrifugation (30 min at 4000 g). The residues were washed with 4.0 mL deionised water and the relevant supernatants (Na₂SO₃ and water) were pooled and made up to 10.0 mL with deionised water. This procedure was carried out in three replicates.

3. **CS₂ 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 that were afterwards vortexed, then 4.0 mL CS₂ was added, followed again 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 4000g) 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$_2$O$_2$ digestion procedure.

4.1.3. Identification of selenocompounds in *C. violifolia*

4.1.3.1. Extractions

Selenium speciation experiments were carried out from the water extract and from the enzymatic extract of the sample:

- 250 mg of lyophilised and pooled plant material (stems + leaves) was enzymatically digested according to the method described by Shao *et al.* (2014) with Pronase E in two subsequent steps in Tris-buffered medium. Supernatant arising from the treatments was 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.

- 250 mg of lyophilised and pooled plant material (stems + leaves) was extracted with an ultrasonic probe (UP100H) with 10.0 mL deionised water for 2x1 min. Supernatant was recovered by centrifugation (10 min 4000g) and 5 mL was lyophilised, dissolved, filtered through a 0.45 μm cellulose acetate syringe filter and diluted with eluent buffer applied in the corresponding chromatography.

- 50 mg of the *C. violifolia* leaf sample was extracted with an ultrasonic probe (UP100H) at ambient temperature with 10.0 mL deionised water for 1 min. Supernatant was recovered by centrifugation (10 min at 4000g, filtered (0.45 μm, cellulose acetate syringe filter) and lyophilised in 2.225 mL aliquots.
4.1.3.2. LC-ICP-MS set-ups (SAX, SCX and IP-RP)

All strong anion exchange, strong cation exchange and ion-pairing reversed phase chromatography set-ups were achieved with 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. The applied columns and chromatographic conditions are presented in Table 5.

Selenite was quantified with SAX, while Se-(methyl)selenocysteine was quantified with IP-RP, both through the method of three-point standard addition.

Table 5. Chromatographic conditions for LC-ICP-MS set-ups.

<table>
<thead>
<tr>
<th>Column</th>
<th>Flow rate</th>
<th>Eluent A</th>
<th>Eluent B</th>
<th>Gradient program</th>
<th>Injection volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAX: PRP-X100 (250mm×4.1mm×10 μm; Hamilton, Reno, NV, USA)</td>
<td>1.5 mL min$^{-1}$</td>
<td>10 mM ammonium acetate buffer pH=6</td>
<td>250 mM ammonium acetate buffer pH=6</td>
<td>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</td>
<td>100 μL</td>
</tr>
<tr>
<td>SCX: Zorbax 300 (150mm×4.6mm×5 μm, Agilent)</td>
<td>1.2 mL min$^{-1}$</td>
<td>1 mM pyridine formate buffer pH=2.2</td>
<td>40 mM pyridine formate buffer pH=2.2</td>
<td>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</td>
<td>5/10 μL</td>
</tr>
<tr>
<td>IP-RP: XTerra MS-C$_{18}$ (250mm×4.6mm×5 μm; Waters, Milford, MA, USA)</td>
<td>0.6 mL min$^{-1}$</td>
<td>deionised water with 0.05 v/v% HFBA</td>
<td>methanol with 0.05 v/v% HFBA</td>
<td>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</td>
<td>25 μL</td>
</tr>
</tbody>
</table>

4.1.3.3. 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$_{18}$ 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 of 0.35 mL min$^{-1}$. 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. Other related instrumental parameters are shown in the appendix (Table A1).
4.1.3.4. LC-Unispray-QTOF-MS set-up

A Vion ion mobility QTOF-MS (Waters) equipped with a UniSpray (Waters) ion spray source was applied coupled to an Acquity UPLC I-Class system (Waters) using a BEH-C\textsubscript{18} RP UPLC column (100 mm × 2.1 mm × 1.7 µm; Waters). Deionised water with 0.1 v/v% formic acid (A) and acetonitrile with 0.1 v/v% formic acid (B) were used as mobile phase at the flow rate of 0.4 mL min\textsuperscript{-1}. The column was kept at 25 °C. Gradient elution program was applied as follows: 0-1 min 10% B, 1-4 min up to 80% B, 4-4.5 min 80% B, 4.5-5 min down to 10% B, 5-7 min 10% B. UniSpray ion source was used both in positive and negative ionisation modes either in MS\textsuperscript{E} or MS\textsuperscript{E} → MSMS functions. Related instrumental parameters are described in the appendix (Table A2). Data evaluation was carried out with the help of the Unifi software (version 1.9.4; Waters).

Mass defect based Se species filtering was carried out in the frame of an iterative approach. Default settings (mass padding: 15 Da; defect padding: 40 mDa; isotope defect: $^{80}$Se-$^{78}$Se; minimal compound response: 2000 counts) were tested whether they allowed for the detection of known Se species (selenolanthionine and selenocystathionine among others) that had been detected in the fractions by database derived searching. Afterwards, the list of Se species was step-by-step completed with newly detected species resulting from the in-source selenohomocysteine fragment search ($m/z$ 135.9660 /C\textsubscript{3}H\textsubscript{6}NSe\textsuperscript{+} and 181.9715 /C\textsubscript{4}H\textsubscript{8}O\textsubscript{2}NSe\textsuperscript{+}) and from the manual Se isotopologue pattern searching processes; with these, the mass padding and defect padding settings were recursively tuned to include the newly introduced species in the result list. After each tuning step, the updated mass defect based Se species filtering was run on the LC-QTOF-MS data to look for still undiscovered selenium species that met the actual search settings. All positive hits were manually checked and individually validated to remove false positives.
4.1.4. Synthesis of selenolanthionine

4.1.4.1. Chemical synthesis

The chemical synthesis of selenolanthionine was based on a modified procedure originally described by Block et al. (2001). 200 mg L-selenocystine was suspended in 5 mL ethanol under argon atmosphere and 100 mg solid NaBH₄ was added. The solution was refluxed for 20 min then an additional volume of ethanol (5 mL) was added and the solution was refluxed for further 20 min. 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.

4.1.4.2. Chromatographic purification – analytical scale

Prior to SCX-ICP-MS analysis and purification the synthesised mixture was dissolved to a concentration 12.5 mg mL⁻¹ in MQ water with 10% HCOOH and 500-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 same chromatographic conditions were used as described in Table 5. 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.

4.1.4.3. Chromatographic purification – semi-preparative scale

Preparative chromatographic purification was needed to yield higher amount of pure selenolanthionine for recording XANES spectra. Synthesised mixture was dissolved in 0.1 M HCl to the concentration of 10 mg mL⁻¹. A Luna strong cation exchange column (250 mm × 10 mm × 10 μm; Phenomenex, Torrance, CA, USA) was used with gradient elution using pyridine formate eluent (pH 3.55; buffer A: 1 mM; buffer B: 40 mM). The optimised program was as follows: 0–15 min from 0% up to 30% B (2.5 mL min⁻¹), 15.1–16 min up to 100% B (2.5 mL min⁻¹), 16.1–17 min 100% B (3.0 mL min⁻¹), 17.1–17.5 min down to 0% B (3.0 mL min⁻¹), 17.6–25.9 min 0% B (3.0 mL min⁻¹), 25.9–26 min 0% B (2.5 mL min⁻¹). Injection volume was 10 μL. The peak corresponding to selenolanthionine was collected from 20 subsequent runs, pooled and lyophilised.
4.1.5. Quantification procedures

4.1.5.1. Quantification of selenolanthionine standard

In order to determine the approximate concentration of the synthesised selenolanthionine, 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. (2004) 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).

4.1.5.2. Quantification of lanthionine and selenolanthionine with LC-ESI-QQQ-MS

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) 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 optimised in positive MRM mode for lanthionine (Lan) and selenolanthionine by introducing a 2 mg mL⁻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 appendix (Table A3).

4.1.5.3. Quantification of selenolanthionine with isotope dilution analysis (IDA)

HPLC-ICP-MS set up was achieved by using an Agilent 1200 HPLC system connected to an Agilent 7500cs ICP-MS for the element-specific detection of $^{76}$Se,
$^{77}$Se, $^{78}$Se, $^{79}$Br, $^{80}$Se, $^{81}$Se and $^{83}$Kr. H$_2$ was used as collision/reaction gas in the flow rate of 2.5 mL min$^{-1}$ and oxygen (40 mL min$^{-1}$) as optional gas.

XTerra MS-C$_{18}$ (250 mm × 4.6 mm × 5 μm; Waters) column was used. The mobile phase consisted of deionised water (eluent A) and methanol (eluent B) both containing 0.1 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; 16–19 min 5% B. Injection volume was 40 µL.

The outlet of the RP column was connected through a T-piece where the $^{82}$Se standard solution was continuously added in the concentration of 20 ng g$^{-1}$ in 0.1% nitric acid with a flow rate of 0.1 mL min$^{-1}$ with the help of a peristaltic pump. Mass bias was determined with the repeated injections of 20 µL of Se-(methyl)selenocysteine standard solution (0.5 mg L$^{-1}$ as Se) and correction was carried out using the exponential approach (Reyes et al. 2003, Rodriguez-González et al. 2005). Dead time correction was automatically carried out by the instrument.

Second aliquot of the lyophilised water extract of C. violifolia leaf sample was dissolved in 2 mL deionised water, diluted 15x with deionised water containing 0.1 v/v% HFBA. Sample injection volume was 40 µL. IDA calculations were based on the equations published by Koellensperger et al. 2003:

\[ M_x(t) = M_y(t) \times \frac{R_y - R_b(t)}{R_b(t) - R_x} \times \frac{h_{iy}}{h_{ix}} - M_y(t) \times \frac{R_y - R_{b\text{blank}}(t)}{R_{b\text{blank}}(t) - R_x} \times \frac{h_{iy}}{h_{ix}} \]

where:

- $M_x$: mass flow of the analyte (ng min$^{-1}$);
- $M_y$: mass flow of the spike (ng min$^{-1}$);
- $R_y$: corresponding isotope ratio in the isotopically enriched spike standard solution (certificate);
- $R_x$: corresponding natural isotope ratio;
- $R_b$: measured isotope ratio of the sample blended with spike (corrected for dead time and mass bias);
- $R_{b\text{blank}}$: measured isotope ratio of the blank blended with spike (corrected for dead time and mass bias);
- $h_{iy}$: isotopic abundance of the reference isotope in the spike;
- $h_{ix}$: isotopic abundance of the reference isotope in the sample.
4.2. COMPARISON OF SELENIUM METABOLISM IN C. VIOLIFOLIA AND C. PRATENSIS

4.2.1. Reagents and biological materials

Murashige and Skoog agar (MS agar) medium, ethanol, sucrose (≥99%), sodium selenate (≥95%), L-selenocystine (95%), sodium selenite (≥98%), trolox (97%), Folin & Ciocalteu’s phenol reagent and salts used for nutrient solution: ammonium nitrate, calcium nitrate tetrahydrate, ethylenediaminetetraacetic acid ferric sodium salt, potassium nitrate, potassium hydroxide, magnesium sulphate heptahydrate, potassium phosphate monobasic, magnesium chloride hexahydrate, boric acid, manganese (II) sulphate monohydrate, zinc sulphate heptahydrate, copper (II) sulphate pentahydrate, molybdenum (VI) oxide were obtained from the Merck-Sigma group. Phytoagar was purchased from Research Products International (Mt. Prospect, IL, USA). Pyridine (a.r.) was obtained from Carlo Erba (Peypin, France), while formic acid (98–100%, used for liquid chromatographic purposes) was purchased from Scharlau. Standards for ICP-OES calibration were obtained from Elemental Scientific (Omaha, NE, USA).

*Cardamine violifolia* leaves and roots were collected in the springtime of 2017 in the natural seleniferous region Yutangba, Enshi (Hubei Province, China). Seeds were collected in the Yutangba region and were provided by the Academy of Agricultural Sciences of Enshi Tujia and Miao Autonomous Prefecture (Wuhan, China). *Cardamine pratensis* (cuckooflower) seeds were purchased from Seedaholic (Cloghbrack, Clonbur, Ireland).

4.2.2. Cultivation of plants

Different types of cultivation were applied to study several Se-related features of the two *Cardamine* species, which are summerised in Table 6. For cultivation on sterile half-strength MS agar medium (Murashige and Skoog 1962) the seeds were surface-sterilised by rinsing consecutively with 70% ethanol (1 min), sterilised distilled water (10 min), 10% household bleach (10 min) and finally five times (10 min each) with sterilised distilled water. The agar was sterilised by heating to 120 °C for 20 min and cooled to 55 °C before supplementation with Se and pouring in Petri dishes. For the study of root length, germinated seeds were carefully placed on the solidified medium along a line approximately 2 cm from one of the edges, with 1 cm distance (10 seeds per plate). Petri dishes were sealed with a double layer of parafilm and placed vertically in an incubator, with the seeds forming a horizontal line along the top rim of the Petri dishes. After 26 days, photos were taken and root lengths were determined using Image
J2 software (Rueden et al. 2017). Plants were then harvested, rinsed in distilled water, separated into shoots and roots and dried at 50 °C for 48 h. Dry weight data of shoots were determined (roots were too small to be weighed accurately), and the shoots from the different selenate treatments were used for elemental analysis as described below.

In case of the round Petri dishes using 25 μM selenate in the agar media, the plates were incubated horizontally and the seeds distributed across the entire surface.

In case of cultivation in gravels the strength of the Hoagland’s nutrient solution (Hoagland and Arnon 1950) was gradually increased. In order to investigate the uptake capacity of different forms of Se (selenate, selenite) and the interactions of sulphate and phosphate, five different treatments were applied (see Table 6). After the 10-day long treatments, chlorophyll (Chl) fluorescence measurements were carried out. Plants were then harvested, rinsed, shoots and roots were separated and dried (50 °C for 48 h) for elemental analysis as described below. For LC-ICP-MS analysis the leaves and stems of cultivated C. pratensis were rinsed, lyophilised and milled.
### Table 6. Cultivations of *Cardamine* species.

<table>
<thead>
<tr>
<th>Plant species</th>
<th>Seed treatment</th>
<th>Germination</th>
<th>Growth media</th>
<th>Circumstances</th>
<th>Dish/pot</th>
<th>Treatment</th>
<th>Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. violifolia</em></td>
<td>Surface sterilisation</td>
<td>Cold treatment in distilled water: 2 days at 4 °C</td>
<td>On wet filter paper</td>
<td>½ strength MS agar: 2.22 g L⁻¹ MS basalt salt mixture 10 g L⁻¹ sucrose 8 g L⁻¹ Phytoagar pH 5.6-5.8 (set with KOH)</td>
<td>Growth cabinet 23 °C 16 h: 8 h (light:dark photoperiod)</td>
<td>square petri dishes (12 × 12 cm)</td>
<td>Na₂SeO₄: 0, 50, 100, 200, 400 μM</td>
</tr>
<tr>
<td><em>C. pratensis</em></td>
<td>Cold treatment in distilled water: 2 days at 4 °C</td>
<td>In the growth media – water treatment</td>
<td>Turface® growth medium (pH 5.5) (PROFILE Products LLC; Buffalo Grove, IL, USA)</td>
<td>Growth room 23 °C 10 h: 14 h (light:dark photoperiod)</td>
<td>10 × 10 cm pots</td>
<td>After germination: 7 days: 1/8th Hoagland’s 7 days: 1/4th Hoagland’s 10 days: A: control 1/4th Hoagland’s B: 1/4th Hoagland’s + 20 μM Na₂SeO₄ C: 1/4th Hoagland’s + 20 μM Na₂SeO₄ and 5 mM MgSO₄ D: 1/4th Hoagland’s + 20 μM Na₂SeO₄ E: 1/4th Hoagland’s + 20 μM Na₂SeO₄ and 2.4 mM KH₂PO₄</td>
<td>Chlorophyll fluorescence Elemental analysis X-ray analysis</td>
</tr>
<tr>
<td><em>C. violifolia</em></td>
<td>Cold treatment in distilled water: 2 days at 4 °C</td>
<td>In sterilised garden soil – water treatment</td>
<td>Vermex vermiculite (Soprema; Strasbourg, France)</td>
<td>Growth room 21 °C 16 h: 8 h (light:dark photoperiod)</td>
<td>20 × 10 cm pots</td>
<td>After germination: 3 days: 1/16th Hoagland’s 7 days: 1/8th Hoagland’s 4 days: 1/4th Hoagland’s 9 days: 1/4th Hoagland’s + 20 μM Na₂SeO₄ and MgSO₄ changed for MgCl₂</td>
<td>LC-ICP-MS</td>
</tr>
<tr>
<td><em>C. pratensis</em></td>
<td>Cold treatment in distilled water: 2 days at 4 °C</td>
<td>In sterilised garden soil – water treatment</td>
<td>Vermex vermiculite (Soprema; Strasbourg, France)</td>
<td>Growth room 21 °C 16 h: 8 h (light:dark photoperiod)</td>
<td>20 × 10 cm pots</td>
<td>After germination: 3 days: 1/16th Hoagland’s 7 days: 1/8th Hoagland’s 4 days: 1/4th Hoagland’s 9 days: 1/4th Hoagland’s + 20 μM Na₂SeO₄ and MgSO₄ changed for MgCl₂</td>
<td>LC-ICP-MS</td>
</tr>
</tbody>
</table>
4.2.3. Elemental analysis

Se and S concentrations of the cultivated plants were determined with inductively coupled plasma optical emission spectrometry (ICP-OES) as described by Fassel (1978) using a Perkin Elmer Optima 7000 DV ICP-OES, at 196.026 nm (Se) and 181.975 nm (S), respectively after nitric acid digestion. The nitric acid digestion was carried out in glass tubes with heating for 2 h at 60°C and for 6 h at 125 °C. The limits of quantification for Se and S were ~1 mg kg\(^{-1}\) and ~0.2 mg kg\(^{-1}\) DW in plant samples, respectively.

Total Se content of *C. pratensis* grown for LC-ICP-MS analysis was determined with the method described in Chapter 4.1.2.1.

4.2.4. Selenium distribution and speciation analysis using X-ray microprobe analysis

X-ray microprobe analyses were performed at beamline 10.3.2 (X-Ray Fluorescence Microprobe) of the Advanced Light Source (ALS), at Lawrence Berkeley National Lab (Berkeley, CA, USA) using a Peltier cooling stage (-25 °C). Entire *C. violifolia* and *C. pratensis* seedlings grown on agar media containing 25 μM selenate were collected at harvest, rinsed, flash-frozen in liquid nitrogen and stored at -80 °C. Furthermore, young leaves from the plants grown on Turface® media (selenate and selenite treatment /treatment ‘B’ and ‘D’, respectively see Table 6/) were collected at harvest, flash-frozen and stored at -80 °C. Samples were shipped on dry ice for microprobe analyses. Selenium, calcium (Ca) and potassium (K) distribution were mapped using micro-focused X-ray fluorescence (μXRF), and Se speciation was investigated using X-ray absorption near-edge structure (μXANES) spectroscopy, essentially as described by Jiang *et al.* (2018b). XANES spectra of selenolanthionine standard was recorded from frozen droplet on the Peltier cooling stage (-25 °C). The μXRF maps were recorded at 13 keV incident energy, using 20 mm × 20 mm pixel size, a beam spot size of 7 mm × 7 mm, using 50 ms dwell time. Maps were deadtime-corrected and decontaminated. Selenium K-edge μXANES (in the range 12500–13070 eV) was used to analyse Se speciation on different spots, close to areas showing high Se concentration in the μXRF maps. Spectra were energy calibrated using a red amorphous Se standard, with the main peak set at 12660 eV.
Least-square linear combination fitting of the \( \mu \)XANES data was performed in the range of 12630–12850 eV using a library of 52 standard selenocompounds and procedures described by Fakra et al. (2018). All data were recorded in fluorescence mode using a seven-element Ge solid state detector (Canberra, ON, Canada) and processed using custom LabVIEW programs (National Instruments, Austin, TX, USA) available at the beamline.

4.2.5. Physiological parameters: chlorophyll fluorescence, antioxidant capacity analysis and total phenolic content

Chl fluorescence measurements were carried out on the leaves of plants cultivated on Turface® media before harvest, using a MultispeQ 2.0 instrument (Photosynq LLC, East Lansing, MI, USA) using the company’s recommended protocol.

Seedlings of \( C. \) violifolia and \( C. \) pratensis grown on agar media containing 25 \( \mu \)M selenate or without selenate were harvested and flash frozen. Samples were lyophilised, powdered and weighed. The freeze-dried material was extracted with 80% acetone at a ratio of 25 \( \mu \)L mg\(^{-1}\) tissue while rotated in the dark at 4 °C for 30 min. Supernatant was collected, diluted with additional acetone at 1:10 or 1:20 depending on the sample, and stored on ice until used. For the antioxidant capacity assay, the absorbancies of the diluted supernatants were recorded at \( \lambda = 734 \) nm with a UV–Vis spectrophotometer (PowerWaveXS2; BioTek Instruments, Winooski, VT) using the method of Miller and Rice-Evans (1996). Trolox (a water-soluble analogue of vitamin E) was used as standard for this assay, and results were expressed as micromoles of trolox-equivalent antioxidant capacity (TEAC) per gram dry weight (\( \mu \)mole TE g\(^{-1}\) DW). Diluted supernatant collected from extraction described above was used for total phenolics. Folin & Ciocalteu’s reagent was used as described by Singleton and Rossi (1965). All samples for this assay were recorded at \( \lambda = 765 \) nm using gallic acid as a standard with results expressed as milligrams of gallic acid equivalent (GAE) per gram of dry weight (mg GAE g\(^{-1}\) DW).

4.2.6. Strong cation exchange (SCX) – ICP MS analysis

0.5 g of \( C. \) violifolia leaf and root from China were extracted with 10.0 mL deionised water using an ultrasonic probe (UP100H) at ambient temperature. Supernatants were recovered by centrifugation (10 min at 4000g) and filtered through 0.45 \( \mu \)m cellulose acetate filters. 2.225 mL of the leaf extract and 3.0 mL of the root
extract were lyophilised and redissolved in 2.0 mL deionised water. 0.1 mL sample was diluted with 1.4 mL eluent buffer and 20 µL was injected onto the column. In case of spiking, the samples were spiked to 200 ng mL⁻¹ SeCys₂ concentration (calculated as Se). Same HPLC-ICP-MS set up was used as described in Chapter 4.1.3.2.

0.3 g of lyophilised C. pratensis biomass was extracted with 10.0 mL deionised water using a vortex shaking device for 5 min. The supernatant was recovered by centrifugation (10 min at 9000g), filtered through 0.45 μm PTFE filters and 20 µL was injected onto the column without any dilution. In case of spiking, the samples were spiked to 500 ng mL⁻¹ SeCys₂ (calculated as Se). The chromatographic set-up consisted of a Thermo Spectra System P4000 HPLC pump (Thermo Fisher Scientific) connected to the Thermo Scientific X-Series II ICP-MS for element specific detection of ⁷⁸Se and ⁸⁰Se, using 7% H₂-93% He as collision gas, introduced at 6.0 mL min⁻¹. A Luna SCX column (250 mm × 4.6 mm × 10 µm; Phenomenex) was used in gradient elution mode with pyridine formate (pH 3.0; buffer A: 1 mM; buffer B: 40 mM). The program was as follows: 0–1 min 0% B (1.7 mL min⁻¹), 1–15 min from 0% up to 30% B (1.7 mL min⁻¹), 15–16 min up to 100% B (1.9 mL min⁻¹), 16–21 min 100% B (1.9 mL min⁻¹), 21–21.5 min down to 0% B (1.7 mL min⁻¹), 21.5–26 min 0% B (1.7 mL min⁻¹).

4.2.7. Statistical analysis

Statistical analysis was performed with the IBM SPSS v.20 software (Armonk, NY, USA). Two-way ANOVA model was applied to compare the means of the measured parameters with the factors “species” and “treatment”. The normality of residuals and the homogeneity of variances were checked. In case of non-normality, data transformation was carried out. Tukey-Kramer’s (Tukey 1977) or Games-Howell’s post-hoc test (Games and Howell 1976) was used, depending on the fulfilment of homogeneity of variances assumption. Student’s t-test or Welch’s t-test was applied to compare two means, depending on the fulfilment of homogeneity of variances assumption (Jaccard et al. 1984).
5. RESULTS AND DISCUSSION

5.1. Selenium metabolites in *Cardamine violifolia*

5.1.1. Total selenium, sulphur contents and selenium distribution

Total Se concentration of the pooled stem and leaves *C. violifolia* sample harvested in the natural seleniferous region in China was found to be 3.7 ± 0.2 g Se kg\(^{-1}\) DW. This result places the plant among the hyperaccumulator species in agreement with other reports (Yuan *et al.* 2013, Cui *et al.* 2018). The sulphur concentration (15.2 g S kg\(^{-1}\) DW) falls in the range of sulphur accumulation of other Se (hyper)accumulator Brassicaceae species, *e.g.*, *Stanleya pinnata* var. *pinnata* (14.0-19.3 g S kg\(^{-1}\) DW, Cappa *et al.* 2014), *Sinapsis alba*, *Brassica arvense*, *B. oleracea* and *B. juncea* (12.9-21.8 g S kg\(^{-1}\) DW, White *et al.* 2007). The ratio of Se:S (1:10 molar ratio) is unusually high among the known Brassicaceae species; however, Se and S concentration data of the growing media is required to be taken into account when evaluating the Se enrichment factor. Figure 13 shows the distribution of selenium among different fractions. Water extract contained 60 ± 1.5 % of the total Se while the sum of Se in Na\(_2\)SO\(_3\) extract (assigned as elemental selenium) and CS\(_2\) extract (assigned as selenides) accounted for ~ 18% of the Se originally present in the sample. Different observations are found in the literature discussing the extraction efficiency and selectivity of Na\(_2\)SO\(_3\) and CS\(_2\) solvents in case of elemental selenium and selenides (Aborode *et al.* 2015, Chen *et al.* 2006, Gao *et al.* 2000, Velinsky and Cutter 1990). Considerable elemental selenium content is usually a feature of secondary and non-accumulator plants such as garlic (Mounicou *et al.* 2009) and Black-eyed Susan (Aborode *et al.* 2015), however some *Astragalus* and *Stanleya* hyperaccumulator species have been also shown to contain up to 35 % of total Se in this form (Lindblom *et al.* 2013b). Although in this latter case, the high elemental Se fraction was found in the roots and was attributed to the microbial activity of endophytic bacteria or fungi.

The overall Se 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 Se after the triple stage extraction process.
5.1.2. Chromatographic characterisations

High water-soluble Se content and considerable elemental Se concentration could indicate the accumulation of inorganic (anionic) Se species, selenate and selenite therefore strong anion exchange chromatography (SAX) was chosen for further characterisation. The aqueous extract of *C. violifolia* contained only a negligible amount of selenite (48 μg g$^{-1}$ as Se) that accounts for only 1.3% of total Se (Figure 14).

The SAX-ICP-MS chromatogram also indicates that most of the soluble Se compounds are either neutral or cationic at the pH of the separation (pH 6.0) and elute in the void volume.

Ion-pairing RP chromatography has also been chosen for characterisation as it is a well established method for the efficient separation of selenoamino acids and their derivatives arising from proteolytic extracts (Kotrebai *et al*. 2000). Figure 14 shows the elution of the proteolytic extract with the indication of the elution time of *Se*-(methyl)selenocysteine (MeSeCys) and selenomethionine (SeMet). SeMet was detected in traces, the amount of MeSeCys was quantified accounting for ~ 0.4% of total Se (13 μg g$^{-1}$ as Se). The two most abundant peaks could not be identified with any of the available standards with retention time matching; however, their retention at the acidic pH of the elution (pH < 3) with the actual anionic ion pairing agent (HFBA) provided useful information.
Figure 14. (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* enzymatic extract. The solid arrow indicates the retention time of Se-(methyl)selenocysteine, while the dashed arrow points at that of selenomethionine.

Water extract of the sample was analysed with strong cation-exchange (SCX) chromatography (Figure 15) showing one dominant peak at the retention time of 3.7 min and several minor peaks. Repeated fraction collection was carried out for the major one to obtain the compound in adequately pure form for ESI-MS based identifications. The collected and pooled fractions of the peak were re-analysed with IP-RP chromatography. The IP-RP chromatogram of the major peak indicated that the fraction of the major compound consists of mostly one Se compound however it was re-fractionated with IP-RP as well to eliminate further matrix compounds for ESI-MS analysis.

Figure 15. (a) Strong cation exchange (SCX)-ICP-MS chromatogram of the *C. violifolia* water extract. The dashed line indicates the major fraction. (b) Ion-pairing reversed phase (IP-RP)-ICP-MS chromatogram of the major fraction collected from the SCX chromatographic set-up.
5.1.3. Identification of the major selenocompound by HPLC-ESI-QTOF-MS and -MS/MS

The orthogonal sample clean-up procedure ensured the elimination of matrix interferences and the pre-concentration of the analytes required for the ESI-QTOF-MS analysis. The fraction of the major peak collected by IP-RP-HPLC was introduced into the ESI-QTOF-MS instrument by the means of an RP-HPLC system which provided an additional separation to achieve better S/N ratios. Selenium-containing molecules were searched by visual seeking for the characteristic selenium isotopic pattern. Two components with the mass to charge ratios \((m/z)\) 257.0032 and 167.9555 were found in the full scan spectrum showing the one Se atom containing isotopic pattern (Figure 16). The assumption that the ion at \(m/z\) 167.9555 is the in-source fragment of the compound with \(m/z\) 257.0032 was confirmed by matching their extracted ion chromatograms (EIC), as showed in Figure 16b and by decreasing the fragmentor potential of the ion source from 170 V to 150 V, which practically eliminated the in-source fragment.

![Figure 16](image)

**Figure 16.** (a) 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 \(^{78}\text{Se}\), \(^{80}\text{Se}\) and \(^{82}\text{Se}\) isotopologues. The mass with \(m/z\) 166.0835 arrives from an interference on the \(^{78}\text{Se}\) isotopologue of the in-source fragment. (b) Extracted ion chromatograms (EICs) of the ions \(m/z\) 257.003 (solid line) and \(m/z\) 167.955 (dashed line).

Accurate mass measurement offers the opportunity to calculate the elemental composition of the molecule. The calculating tool setting is shown in appendix (Table A4). Only one possible composition was found within 5 ppm: \(C_6H_{13}N_2O_4\text{Se}^+;\)
theoretical m/z 257.0035, Δ=−1.16 ppm. This composition and the note from Block et al. (2001) about the most significant fragment detected at m/z 168 indicated this molecule is selenolanthionine (SeLan). For further confirmation of the compound identity, MS/MS experiment was carried out on the $^{80}$Se and $^{77}$Se isotope containing isotopologues (Figure 17, Table 7).

![Figure 17](image)

**Figure 17.** (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 $^{80}$Se isotopologue and (c) on the $^{77}$Se isotopologue.
Table 7. MS/MS fragmentation data of selenolanthionine purified from *C. violifolia* water extract.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Theoretical mass, m/z</th>
<th>Experimental mass, m/z</th>
<th>Δ, ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆H₁₃N₂O₄[^80]Se⁺</td>
<td>257.0035</td>
<td>257.0037</td>
<td>0.78</td>
</tr>
<tr>
<td>C₆H₁₃N₂O₄[^77]Se⁺</td>
<td>254.0068</td>
<td>254.0081</td>
<td>5.12</td>
</tr>
<tr>
<td>C₃H₆NO[^80]Se⁺</td>
<td>167.9558</td>
<td>167.9551</td>
<td>-4.17</td>
</tr>
<tr>
<td>C₃H₆NO[^77]Se⁺</td>
<td>164.9592</td>
<td>164.9590</td>
<td>-1.21</td>
</tr>
<tr>
<td>C₂H₃NO[^80]Se⁺</td>
<td>139.9609</td>
<td>139.9606</td>
<td>-2.14</td>
</tr>
<tr>
<td>C₂H₃NO[^77]Se⁺</td>
<td>136.9642</td>
<td>136.9643</td>
<td>0.73</td>
</tr>
<tr>
<td>C₂H₄NO⁺</td>
<td>74.0236</td>
<td>74.0239</td>
<td>4.05</td>
</tr>
<tr>
<td>C₂H₄NO⁺</td>
<td>74.0236</td>
<td>74.0246</td>
<td>13.5</td>
</tr>
</tbody>
</table>

The possible structure of the fragments together with selenolanthionine structure is shown in Figure 18. The structures presented here are meant to present the origin of the fragments in the molecule, and they are not intended to show the actually formed fragment structure. Up to now only one record on SeLan has been published: Kotrebai *et al.* (2000) assigned this species in selenised yeast based on retention time matching in an HPLC-ICP-MS chromatogram on a shoulder of an eluting selenium containing peak (Figure 19).

![Figure 18. Structure of selenolanthionine and the possible fragments with their accurate masses.](image-url)
Figure 19. Selenolanthonine identification in selenised yeast (denoted with number “5”) based on retention time matching by Kotrebai et al. (2000).

Even though all the isotopic pattern, accurate mass data, fragmentation pattern and cationic chromatographic behaviour (Sykora 1984) confirmed the identity of this compound, it was found necessary to confirm the identification by the chemical synthesis of selenolanthonine as well especially in the light of the fact that only the derivatives of SeLan have been found so far in Se-containing plant (Aureli et al. 2012) and yeast (Far et al. 2010) samples.

5.1.4. Analytical and semi-preparative chromatographic purification of synthesised selenolanthonine

As the synthesis described by Block et al. (2001) could not be completely reproduced, the synthesised material was analysed by SCX-ICP-MS. Several selenium species could be found in the synthesised mixture (Figure 20). The highest peak eluting at 5.3 min was identified as residual selenocystine (SeCys₂) based on retention time matching. Its concentration indicated that about 70% of SeCys₂ did not react, which resulted in a yield of ≤30% for the SeLan synthesis. Repeated fraction collection was carried out for the compound eluting at 4.3 min that was supposed to be SeLan.
SCX – ICP-MS chromatogram of the synthesised selenolanthionine standard. The peak eluting at 5.3 min refers to residual selenocystine, while selenolanthionine eluted at 4.3 min.

Collected fractions were lyophilised for preconcentration and dissolved in 1.0 mL. Its concentration was estimated with an isocratic SCX method that ended up in a concentration of ~15.5 µg Se mL\(^{-1}\).

Semi-preparative purification was needed to yield higher amount of pure SeLan standard for recording its XANES spectra (Chapter 5.2.3). The separation of SeLan and SeCys\(_2\) required optimisation process as the method adaptation from Zorbax SCX (Agilent) analytical column did not provide adequate separation on the semi-preparative Luna SCX (Phenomenex) column (Figure 21a). Chromatographic parameters of the optimisation process are described in Table 8. However their experimental pKa values were unknown, SeLan (eluting before SeCys\(_2\)) seemed to be a stronger acid and the appropriate pH could be set up only through the approach called “trial and error”. The applied pH was increased stepwise (pH 2.15, 2.55, 3.05, 3.55) applying the chromatographic method #1 (Table 8); finally, pH 3.55 provided an acceptable separation of the two compounds (Figure 21).
Figure 21. Semi-preparative strong cation exchange chromatograms of the synthesised SeLan mixture at four different pH settings: (a) pH 2.15 (b) pH 2.55 (c) pH 3.05 (d) pH 3.55.

Isocratic approach could also be applied instead of a gradient separation but this case the two compounds partly overlapped, even after a careful optimisation of the isocratic method #2 (Table 8, Figure 22).

Figure 22. Separation of the synthesised SeLan mixture on the semi-preparative strong cation exchange column with isocratic method at pH=3.55.

The resolution and the retention factor (k’) of the compounds could be improved by cooling the column from 23 °C to 20 °C, flattening the gradient slope and decreasing the flow rate from 3.0 mL min⁻¹ to 2.5 mL min⁻¹, as presented in method #3 (Table 8, Figure 23). The k’ of SeLan was increased from 1.77 to 2.28, and the k’ of SeCys₂ reached 2.8 after the former 2.1 value. However adequate resolution (~1) was finally achieved, fraction collection was set to stop earlier to exclude the traces of
SeCys₂ and the purity was checked after lyophilisation. Injection volume of the synthesised mixture was 5 µL (dissolved at concentration 10 mg mL⁻¹) for optimisation and 10 µL for fraction collection; the injection of 25 µL resulted in saturated and tailing peaks.

The collected and pooled fraction was dissolved in 100 µL distilled water after the lyophilisation, its concentration was estimated with isocratic SCX method that indicated the concentration of ~ 120 µg Se mL⁻¹; this concentration was found high enough for recording the XANES spectra.

Table 8. Chromatographic parameters for the separation of selenolanthionine and selenocystine on a semi-preparative strong cation exchange column.

<table>
<thead>
<tr>
<th>Method</th>
<th>Eluents</th>
<th>Column temperature</th>
<th>Flow rate</th>
<th>Gradient</th>
</tr>
</thead>
</table>
| #1     | A: 1 mM pyridine formate pH: 2.15; 2.55; 3.05; 3.55
         B: 40 mM pyridine formate pH: 2.15; 2.55; 3.05; 3.55 | 23 °C | 3 mL min⁻¹ | 0-2 min 0% B
         2-20 min up to 100% B
         20-21 min 100% B
         21-21.5 min down to 0% B
         21.5-30 min 0% B |
| #2     | A: 1 mM pyridine formate pH 3.55
         B: 40 mM pyridine formate pH 3.55 | 23 °C | 3 mL min⁻¹ | 0-15 min 10% B
         15-15.5 min up to 100% B
         15.5-17 min 100% B
         17-17.5 min down to 10% B
         17.5-26 min 10% B |
| #3     | A: 1 mM pyridine formate pH: 3.55
         B: 40 mM pyridine formate pH: 3.55 | 20 °C | 2.5 mL min⁻¹
         (during gradient) | 0-15 min up to 30% B
         (2.5 mL min⁻¹)
         15-15.1 min 30% B
         15.1-16 min up to 100% B
         16-17 min 100% B
         17-17.5 min down to 0% B
         17.5-25.9 min 0% B
         25.9-26 min 0% B |

Figure 23. Separation of the synthesised mixture on the semi-preparative strong cation exchange column with optimised parameters.
5.1.5. Confirmation of selenolanthionine by chemical synthesis

The compound collected from analytical cation-exchange fraction collection (eluting at 4.3 min) was used for spiking the water extract of the *C. violifolia* sample. Exact retention time matching with the peak of the previously identified SeLan was detected with SCX-ICP-MS (Figure 24).

![SCX – ICP-MS chromatograms](image)

**Figure 24.** 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).

The synthesised compound and the spiked *C. violifolia* extract were analysed by RP-LC-ESI-QTOF-MS in MS and MS/MS experiments. Figure 25a shows the full-scan spectra where the *m/z* 257.0038 and *m/z* 167.9557 ions were both found with the characteristic Se isotopic pattern and also the MS/MS fragments showed matching with those detected in *C. violifolia* extract (Figure 25b).
Figure 25. (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.

The extracted ion chromatograms of SeLan and its in-source fragment showed matching in the spiked extract of *C. violifolia* which corresponds to another, orthogonal verification to the previous SCX chromatography (Figure 26).

Figure 26. 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.
It must be mentioned that seemingly contradictory speciation results have been reported for this plant under an alternative name *C. hupinshanesis*: selenocystine was identified as the main Se compound (Yuan *et al.* 2013). Indeed, this report was based on only one separation approach (SAX, pH=6.0) that cannot evidently separate SeCys₂ from SeLan.

5.1.6. Identification of minor selenocompounds in *C. violifolia*

5.1.6.1. Chromatographic characterisation

Water extract of *C. violifolia* leaf sample (261 mg total Se kg⁻¹ DW) was analysed by SCX-ICP-MS chromatography (Figure 27). The most intense selenolanthionine peak (eluting at 3.04 min) is surrounded by several less intense peaks, which were fraction collected for further analysis (indicated with Fr1 to Fr4). Figure 27b shows the respective and overlaid IP-RP-ICP-MS chromatograms of the fractions. Despite that the peaks could be slightly separated on SCX, three fractions (Fr1, 2 and 3) could not be clearly differentiated by IP-RP and eluted between 4.8-6.6 min; no adequate (baseline) separation could be achieved for the first three fraction eluting close to the void volume (k<2). Fr 4 was the only one fraction that showed an adequately retained and distinct peak at 6.9 min with no considerable overlapping. Each fraction consisted of several peaks indicating that numerous selenium species should be expected in the ESI-MS acquisitions. Fraction collection was carried out for each fraction that were afterwards concentrated by lyophilisation and subjected to LC-Unispray-QTOF-MS. No selenium species could be detected after the two dimensional (SCX+IP-RP) purification, which might be explained by unstable retention time parameters on IP-RP that hampered reproducible fraction collection. Therefore, the SCX-ICP-MS derived fractions were addressed for LC-Unispray-QTOF-MS analysis.
Figure 27. (a) Strong cation exchange (SCX) – ICP-MS chromatogram of water soluble extract of C. violifolia, recorded on the $^{82}$Se isotope. Fractions labelled #1 - #4 were collected for further characterisation on LC-Unispray-QTOFMS. (b) Ion-pairing reversed phase (IP-RP) – ICP-MS chromatograms of the fractions #1 - #4 collected from SCX – ICP-MS, recorded on the $^{77}$Se isotope.

5.1.6.2. Screening for selenium species in the LC-Unispray-QTOF-MS acquisitions

Intra-isotope mass defect based extraction, manual pattern exploration, extracting diagnostic ion source fragments and database dependent search allowed the detection of numerous selenium compound in the four fractions, which are listed in Table 9. As the first three fractions were co-eluting during fraction collection, and selenium species were often found in more than one fraction, they are positioned into the fraction where they showed the highest abundance. The most contributing technique to discover the given species is indicated, however most of them could be localised by more than one search method. Manual pattern exploration was crucial for discovering the higher ($m/z > 370$) molecular mass species. Full scan spectra under $m/z$ 300 are of relatively high density (“crowded”), the most visible mass spectral features of selenium containing molecules (i.e., characteristic /7-12 isotopologue wide/ base, unique isotope ratio of $^{80}$Se and $^{78}$Se, high /0.1-0.2 amu/ absolute mass defect compared to neighbouring ions) are difficult to explore. In contrast to the SeHCys in-source fragment, extracting the diagnostic ion source fragment at $m/z$ 183.9871 ($C_4H_{10}O_2NSE^+$) did not reveal any hit, which indicates no derivatives of SeHLan were present in the fractions at high concentration. The selenocysteinyl moiety ($C_3H_6O_2NSE^+$, $m/z$ 167.9558) could only be found in MS/MS spectra, possibly due to the fact that in-source and MS$^E$ fragmentation usually run at lower fragmentation voltage compared to MS/MS analysis.
Table 9. Summary of selenium species detected in the water soluble selenometabolome of *C. violifolia*. "n.a." denotes 'not available'; MS/MS quality scaling indicates high signal-to-noise ratio with good quality MS/MS spectrum (++;), moderate signal-to-noise ratio with moderate quality MS/MS spectrum (+), and low signal-to-noise ratio with missing or low quality MS/MS spectrum (+).
Optimisation of the intra-isotope mass defect filter settings was carried out with using all the 35 compounds detected. A compromise must be set as an increase in the mass padding or in the defect padding parameters increase the lists of both the true and false positive hits. After setting 200 Da for mass padding and 80 mDa for defect padding, the total number of compounds assigned for pattern validation as potentially Se carrying molecules ranged between 206-457, accounting for 4.5-6.3 % of the total number of detected compounds (Table A5). These data seem to cover too many false positive hits, however the practical implementation is reasonable. The outcome of some hundreds of compounds can be easily shortlisted on the basis of absolute mass defect and isotopologue redundancy. Finally, these settings allowed to recover 16 Se species out of the 35 involved in the optimisation process at signal intensity of 2000 counts (Table A6 and A7). Another benefit of this searching method is that several detected species eluted further from the void volume, where the generally hydrophilic Se species are less evidently occurring (e.g., m/z 197 and m/z 282).

Six accurate mass values matched with previously detected species but only four species could be marked as known in Table 9: selenocystathionine at m/z 271.02005 (Figure A2), selenohomocystine at m/z 364.95162 (Figure A3), a selenosugar at m/z 407.04499 (Figure A4) and Se-selenohomocysteinyl-diseleno-homocysteine at m/z 444.86739 (Figure A5). In two cases, at m/z 254.02899 and m/z 313.02972, the MS/MS fragments did not matched of the formerly reported species (that is, N-acetyl-Se-methylselenomethionine (Németh et al. 2013) and γ-glu-Se-(methyl)selenocysteine (McSheehy et al. 2000) or N-acetyl-selenocystathionine (Dernovics et al. 2009), respectively). This calls the attention to the fact that accurate mass data is necessary but not sufficient for the identification of Se species.

As an example, the MS and MS/MS spectra of one detected species is presented in the appendix (Figure A6).

5.1.6.3. Structural elucidation of novel selenocompounds: N-glycosylated selenoalanthionines

The characteristic loss of hexoses (162.05 Da) could be detected during MS/MS fragmentation of eleven species; three of them could be detected in negative ion mode as well ([M-H]-). These molecules accounting one-third of all Se species detected are presumed Se-containing sugars. Selenosugars in plants was first presented by Aureli et al. (2012). The species at m/z 407.04510 has been detected in several samples including crops and vegetables (Aureli et al. 2012, Ruszcynska et al. 2017) and may
be considered as ubiquitous Se species, still without structural elucidation. The structural identification of selenosugars generally requires NMR studies, however some properties can be noted on the basis of their MS/MS spectra.

The species at \( m/z \) 419 and \( m/z \) 581 share the selenium containing fragments at \( m/z \) 401.04, 257.00, and 167.95, and non-selenised fragments at \( m/z \) 214.07 and 232.08. The first fragment appears as an in-source fragment as well. The MS/MS spectra of both species show a series of -18 Da losses that is also characteristic to hexoses (sugars). The presence of the \( m/z \) 167.95 fragment is a reference to a selenocysteinyll moiety, which – together with the \( m/z \) 257.00 fragment – indicates that these molecules can be assigned as the mono or di-\( N \)-glycosides of selenolanthionine (Figure 28, A7, 29). The putative structures presented here are meant to present the origin of the fragments in the molecule, and they are not intended to show the actually formed fragment structure. Selenoamino acids and carbohydrates can form \( N \)-glycosyl conjugates by their direct reactions, or at alkaline pH, and through condensation in the Maillard reaction scheme when exposed to elevated temperature (Danehy and Pigman 1951). As selenium species are highly unstable under alkaline conditions (\( \text{pH} > 9.5 \)), this way of \( N \)-glycoside formation can be excluded in this actual case. To the best of my knowledge, \( N \)-glycosylated selenoamino acids have never been reported in any natural sample. However, it is unclear whether these species were formed \textit{in vivo} spontaneously or \textit{ex vivo} during the freeze-drying process.

The presumable mono- and di-glycosylated lanthionines (S analogues) could be also detected in the high resolution spectra at \( m/z \) 371.11250 (\( \text{C}_{12}\text{H}_{23}\text{N}_{2}\text{O}_{6}\text{S}^+ \), \( \Delta \text{ppm} = 1.67 \)) and at \( m/z \) 533.16335 (\( \text{C}_{18}\text{H}_{33}\text{N}_{2}\text{O}_{14}\text{S}^+ \), \( \Delta \text{ppm} = -2.53 \)), shown in appendix (Figure A8). The MS/MS fragmentation enabled the matching of several Se/S or non Se/S containing fragments of the analogues (Figure A8, Table 10, Table 11).
Figure 28. Putative mono- and di-N-glycosides of selenolanthionine.
(a) Full scan spectrum, (b) zoomed full scan spectrum, (c) MS/MS spectrum,
(d) structure of selenolanthionine-di-N-glycoside.
(e) Full scan spectrum, (f) zoomed full scan spectrum, (g) MS/MS spectrum
(h) structure of selenolanthionine-N-glycoside.
Additional MS/MS spectrum of selenolanthionine-di-N-glycoside can be found in
appendix (Figure A7).
Figure 29. Proposed structures of the MS/MS fragments of the m/z 419.0568 and 581.1092 compounds, together with mass accuracy data of the fragments.
Table 10. MS/MS fragment matching of di-N-glycoside selenolanthionine and lanthionine.

<table>
<thead>
<tr>
<th>Experimental m/z of detected fragment in the m/z 581 Se-analogue (elemental composition)</th>
<th>Experimental m/z of detected fragment in the m/z 533 S-analogue (elemental composition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>545.08614 (C_{18}H_{29}N_{2}O_{12}Se^{+})</td>
<td>497.13639 (C_{18}H_{29}N_{2}O_{13}S^{+})</td>
</tr>
<tr>
<td>401.04552 (C_{12}H_{15}N_{2}O_{8}Se^{+})</td>
<td>353.10216 (C_{12}H_{15}N_{2}O_{8}S^{+})</td>
</tr>
<tr>
<td>314.00981 (C_{9}H_{16}NO_{5}Se^{+})</td>
<td>266.06981 (C_{9}H_{12}NO_{5}S^{+})</td>
</tr>
<tr>
<td>296.00129 (C_{9}H_{14}NO_{6}Se^{+})</td>
<td>248.06214 (C_{9}H_{14}NO_{5}S^{+})</td>
</tr>
<tr>
<td>214.07215 (C_{9}H_{12}NO_{5}^{+})</td>
<td>214.06937 (C_{9}H_{12}NO_{5}^{+})</td>
</tr>
<tr>
<td>170.07906 (C_{9}H_{12}NO_{3}^{+})</td>
<td>170.08195 (C_{9}H_{12}NO_{3}^{+})</td>
</tr>
<tr>
<td>167.95580 (C_{9}H_{6}NO_{2}Se^{+})</td>
<td>119.18535 (C_{3}H_{6}NO_{2}S^{+})</td>
</tr>
</tbody>
</table>

Table 11. MS/MS fragment matching of N-glycoside selenolanthionine and lanthionine.

<table>
<thead>
<tr>
<th>Experimental m/z of detected fragment in the m/z 419 Se-analogue (elemental composition)</th>
<th>Experimental m/z of detected fragment in the m/z 371 S-analogue (elemental composition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>401.04805 (C_{12}H_{21}N_{2}O_{8}Se^{+})</td>
<td>353.10454 (C_{12}H_{21}N_{2}O_{8}S^{+})</td>
</tr>
<tr>
<td>257.00790 (C_{6}H_{13}N_{2}O_{4}Se^{+})</td>
<td>209.05841 (C_{6}H_{13}N_{2}O_{4}S^{+})</td>
</tr>
<tr>
<td>232.08104 (C_{9}H_{14}NO_{6}^{+})</td>
<td>232.08568 (C_{9}H_{14}NO_{6}^{+})</td>
</tr>
<tr>
<td>214.07029 (C_{9}H_{12}NO_{3}^{+})</td>
<td>214.07122 (C_{9}H_{12}NO_{3}^{+})</td>
</tr>
<tr>
<td>170.07906 (C_{9}H_{12}NO_{3}^{+})</td>
<td>170.08195 (C_{9}H_{12}NO_{3}^{+})</td>
</tr>
<tr>
<td>167.95580 (C_{9}H_{6}NO_{2}Se^{+})</td>
<td>120.01044 (C_{3}H_{6}NO_{2}S^{+})</td>
</tr>
</tbody>
</table>

The loss of a deoxyhexose moiety (146.06 Da) is also a specific event in the fragmentation of oligosaccharides. There are two unknown selenohexoses at m/z 391.09 and 377.07 (Table 9) which can be associated to a couple of molecules sharing the core molecules together with an additional +146.06 Da sized moiety (at m/z 537.15 and 523.13, respectively). The detection of isomers also support the affiliation of selenohexoses and selenohexose-deoxyhexose molecules. The discovery of selenium containing disaccharides opens a new class of molecules that can deposit Se even though their structure cannot be unambiguously assigned on the basis of MS and MS/MS data.
5.1.7. Quantification of selenolanthionine in *C. violifolia* samples

5.1.7.1. Quantification of lanthionine and selenolanthionine with HILIC-QQQ-MS

The ratio of sulphur and selenium analogue compounds together with total S and Se concentration can provide useful information on the accumulation process (Németh and Dernovics 2015). As lanthionine standard is commercially available and SeLan has been in-house synthesised, their quantification could be carried out with HPLC electrospray ionisation – triple quadrupole – mass spectrometry (ESI-QQQ-MS). Hydrophilic interaction chromatography has been chosen as the cationic amino acids show retention on HILIC and the eluents applied are compatible with the ESI ion source. No considerable interferences could be observed at the monitored MRM transitions of the two analytes (Figure 30). The concentration of SeLan was found 4.8 mg g\(^{-1}\) DW sample (equal to 1.5 mg Se g\(^{-1}\)) while Lan was quantified to be 0.5 mg g\(^{-1}\) DW. Accordingly, the molar ratio of Lan:SeLan is about 1:8, which exceeds by far the substitution rate for SeMet in the hyperaccumulator plant *Lecythis minor* seeds where this ratio for Met:SeMet was found to be 1:2 (Németh and Dernovics 2015). Also, the Lan:SeLan molar ratio highly exceeds the quantified total S: total Se molar ratio of 10:1.

Based on this result selenolanthionine accounts for approximately 68% of Se of the water-soluble fraction and about 40% of total selenium in the highly selenised (3.7 g kg\(^{-1}\) DW) stem + leaves sample of *C. violifolia*; however, the quantification might be biased due to the use of a non-commercial standard.

**Figure 30.** (a) HILIC-ESI-QQQ-MS chromatogram of selenolanthionine from the *C. violifolia* water extract (MRM transition 257→168; retention time: 6.77 min); (b) HILIC-ESI-QQQ-MS chromatogram of lanthionine from the *C. violifolia* water extract (MRM transition 209→120; retention time: 6.83 min).
5.1.7.2. Quantification of selenolanthionine with isotope dilution analysis (IDA)

The final dilution ratio of the water extract of lyophilised leaf sample of *C. violifolia* (261 mg total Se kg\(^{-1}\) DW; see Chapters 4.1.3.1 and 5.1.6.1) was 300 mL for 1.0 g lyophilised sample to achieve adequate resolution on the IP-RP setup for quantification through post-column IDA (Figure 31). The SeLan peak eluting at 6.8 min was evaluated and found at the concentration of 77.6 mg Se kg\(^{-1}\), accounting for approximately 30% of the total selenium content of the leaf. Minor selenium species could remain unresolved/undiscovered in the chromatographic peak which could slightly bias the actual value. This case the percentage contribution of SeLan to total Se content is 10% less than the value determined in the highly selenised (3.7 g total Se kg\(^{-1}\) DW) pooled (stem + leaves) *C. violifolia* biomass, which might indicate that the relative concentration of this non-proteinaceous selenoamino acid increases by the higher selenium accumulation rate. According to scientific databases (that is, Scopus and Web of Knowledge), this has been the first successful application of the post column isotope dilution LC-ICP-MS technique in Hungary.

**Figure 31.** IP-RP-IDA-ICP-MS based quantification of selenolanthionine (eluting at 6.8 min) extracted from *C. violifolia* leaves. (a) LC-ICP-MS chromatogram recorded on \(^{80}\)Se; (b) LC-ICP-MS chromatogram recorded on \(^{82}\)Se used for isotope dilution; (c) selenium mass flow calculated from the \(^{80}\)Se/\(^{82}\)Se data.
5.2. Selenium metabolism studies of *C. violifolia* in comparison with *C. pratensis*

5.2.1. Selenium tolerance and accumulation

Shoot dry weight and root length parameters were chosen for characterising Se tolerance of the plant species at different selenate concentration present in the agar media. *C. violifolia* hyperaccumulator (HA) and the non-HA *C. pratensis* showed similar root lengths (Figure 32a) grown on agar medium without selenate supply but *C. pratensis* shoot dry weight (DW) was 3.5-fold smaller (Figure 32b) likely reflecting different plant species characteristics. Clear differences in Se tolerance was detectable between the two species: the 50% inhibition point on root growth was below 50 µM selenate for *C. pratensis* and 100 µM selenate was its highest tolerated Se concentration where *C. violifolia* was still unaffected (Figure 32a). Root length of *C. violifolia* was 50% inhibited between 200 and 400 µM. Shoot dry weight was not significantly inhibited by Se in either species. Overall, *C. violifolia* is relatively more tolerant to selenate than *C. pratensis*.

![Figure 32](image-url)

**Figure 32.** (a) Root length and (b) shoot biomass of *C. violifolia* (Se hyperaccumulator) and *C. pratensis* (control) grown on agar media supplied with different concentrations of Na$_2$SeO$_4$. Values shown are the means ± SEM. Different letters indicate statistically different means among treatments within species (P < 0.05). Asterisks indicate statistically different means between species within treatments (P < 0.05). Note: there was no germination for *C. pratensis* on 200 and 400 µM Se.

Total selenium, sulphur content determined by ICP-OES and the Se:S molar ratio in seedlings at different selenate treatments are shown in Figure 33. Seedlings of both species accumulated ~ 1000 mg Se kg$^{-1}$ DW in shoots at 50 µM selenate supply. *C. pratensis* accumulation decreased to about 50% at 100 µM Se supply and could not survive at higher Se treatments. Se levels in *C. violifolia* shoots increased to 2000 mg kg$^{-1}$ DW and 2700 mg kg$^{-1}$ DW in case of 100 µM and 200 µM Se supply,
respectively, without showing any sign of toxicity while at 400 µM it decreased to a level of 1000 mg kg\(^{-1}\) DW similarly to the 50 µM supply. At the highest selenate concentration tested (400 µM) \(C.\) violifolia was still unaffected in terms of biomass but roots were significantly shorter than the control treatment indicating a Se tolerance ceiling for the HA.

![Figure 33](image.png)

**Figure 33.** (a) Selenium (b) Sulphur concentration and (c) Se:S molar ratio in shoots of \(C.\) violifolia and \(C.\) pratensis grown on agar media supplied with different concentrations of \(\text{Na}_2\text{SeO}_4\). Note: In the case of the control treatment, Se concentrations were below the quantification limit. Values shown are the means ± SEM. Different letters indicate statistically different means among treatments within species, and asterisks indicate statistically different means between species within treatment (\(P < 0.05\)).

The shorter roots at 400 µM may in part explain the decreased shoot Se accumulation at that level as well. It can be concluded that \(C.\) violifolia is capable of higher Se accumulation than \(C.\) pratensis however \(C.\) pratensis can also reach significant tissue Se levels but its accumulation breaks down at elevated selenate concentrations which is an indicative of a lack of Se tolerance. Neither species showed evidence of growth stimulation by Se at the levels supplied, which is in contrast to several Se HA species, e.g., *Symphyotrichum ericoides*, *Astragalus bisulcatus* and *Stanleya pinnata* (El Mehdawi et al. 2012, El Mehdawi et al. 2014). The Se:S molar
ratio in *C. violifolia* shoot increased with increasing selenate supply. At 400 µM Se treatment the ratio remained the same compared to 200 µM despite the lower Se concentration. With increasing Se uptake, *C. violifolia* showed increasing S uptake until 100 µM similarly to other Se HA *Astragalus bisulcatus* (El Mehdawi *et al.* 2014) and to secondary Se accumulator *Brassica juncea* (Harris *et al.* 2014). Opposite pattern was shown by *C. pratensis*: the S concentration decreased with higher selenate supply indicating different Se-S interactions between the HA and non-HA species.

The uptake capacity of different forms of Se (selenate, selenite) and the interactions of sulphate and phosphate were studied on mature plants grown in gravel, total Se and S concentrations in shoots and roots and their molar ratios were determined as shown in Figure 34.

Concerning the plants supplied with 20 µM selenate in gravel until the 4-week stage, both *Cardamine* species accumulated Se around 400 mg kg⁻¹ DW in their shoot. However, in case of selenite treatment (20 µM) Se accumulation was significantly (~2-fold) higher for HA *C. violifolia* in compared to the non-HA species. In both species shoot Se accumulation was higher from selenate than from selenite (~10-fold) which is consistent with earlier studies and reflects the different transport mechanisms (de Souza *et al.* 1998). The two *Cardamine* species did not show any difference in terms of the sulphate ion effect on selenate uptake. The raised sulphate ion concentration inhibited selenate uptake (~5-fold) as both are taken up by the same group of sulphate transporters (Schiavon and Pilon-Smits 2017). The behaviour of HA *C. violifolia* in this respect is in contrast to other Se HA plants such as *S. pinnata* which have a more selenate-specific uptake that is less inhibited by sulphate (El Mehdawi *et al.* 2018, Harris *et al.* 2014, White *et al.* 2007). The Se:S molar ratio was ~1.5-fold and ~2-fold higher in *C. pratensis* and in *C. violifolia* shoots, respectively, compared to that in the medium, when treated with 20 µM selenate (0.08 Se:S molar ratio in the treatment solution: 1/4th Hoagland’s solution contains 250 µM sulphate). The Se:S ratio in the shoot was ~ 70% compared to the ratio in the medium when the plants were supplied with 20 µM selenate + 5 mM sulphate (20-fold lower Se:S ratio in the treatment solution).

Phosphate did not inhibit selenite uptake in either species contrary to expectations and thus it seems that this process is not mediated by phosphate transporters in these plants and may involve anion channels as reported for some species (Schiavon and Pilon-Smits 2017).
Figure 34. (a) Selenium concentration in shoots (b) in roots, (c) the shoot:root Se ratio, (d) S concentration in shoots, (e) in roots, (f) Se:S molar ratio in shoots and (g) in roots of *C. violifolia* and *C. pratensis* grown on Turface® gravel media subjected to different treatments. Note: In the case of the control treatment, Se concentrations were below the quantification limit and in case of *C. violifolia* the root dry material was not enough for more replicates and was excluded from statistical evaluation. Values shown are the means ± SEM. Different letters indicate statistically different means among treatments within species, and asterisks indicate statistically different means between species within treatment (P < 0.05).

The shoot:root Se ratio differed between the two species in some respects. The ratio was higher for *C. pratensis* than for *C. violifolia* in case selenate plus sulphate treatment because of higher root Se level in the HA; no such difference was observed
when only selenate was supplied. In case both selenite treatments (with or without phosphate) the shoot:root Se concentration ratio was 2-3 fold higher in the HA species.

*C. violifolia* generally showed higher Se:S molar ratio in their shoots than *C. pratensis* while *C. pratensis* had higher Se:S ratio in roots however it should be noted that the differences were not found significant in all cases at the statistical evaluation (Figure 34). The difference in translocation between Se and S in HA may be caused by a Se-specific root-to-shoot transporter, or may point to the translocation of Se and S in different chemical forms that use different transporters (*e.g.*, organic Se vs. inorganic Se).

### 5.2.2. Chlorophyll fluorescence, antioxidant capacity and total phenolic content

Assessing the physiological conditions of the plants under Se treatment was carried out by measuring Chl fluorescence, antioxidant capacity and total phenolic content.

Chlorophyll fluorescence parameters were measured on 4-week old mature plants after 10 days of treatments and are shown in Figure 35. Only one parameter of the Chl fluorescence data differed significantly among the two plant species: *C. violifolia* had lower relative Chl content for the selenate treatment compared to *C. pratensis*. *C. violifolia* appeared to have overall lower relativ Chl content and higher non-photochemical quenching (NPQt), but this was not significant except for the above mentioned case. It may be interpreted to be indicative of less well functioning light reactions however no sign of enhanced stress is apparent from the other physiological measurements. Among treatments and within species the selenite plus phosphate treatment had a significant effect only for *C. violifolia*, causing higher NPQt and photochemical quenching (Phi2) compared to either selenite alone or control treatments. Similar but non-significant pattern could be seen for *C. pratensis*. It is possible that the high phosphate concentration caused salinity stress. The observed effects do not seem to be Se-related because the selenite-only treatment did not show a similar effect. Zhou *et al.* (2018) found that high selenite concentration suppressed the expression of photosynthetic genes in *C. hupingshanensis*; however in that study 50-fold higher selenite concentration was used than in our case.

25 µM selenate supply did not have any significant effect on total phenolic content or on total antioxidant activity of seedlings grown on agar media (Table 12). The two species showed a difference in total phenolic content for *C. pratensis* having ~30% higher levels.
Figure 35. Chlorophyll fluorescence parameters of *C. violifolia* and *C. pratensis* grown on gravel media with different treatments. (a) Phi2, the fraction of light energy quenched photochemically due to electron transport through photosystem 2; (b) NPQ, fraction of light energy quenched via non-photochemical processes; (c) relative chlorophyll content (all parameters are unitless). Values shown are the means ± SEM. Different letters indicate statistically different means among treatments within species, and asterisk indicates statistically different means between species within treatment (P < 0.05).

Table 12. Total phenolic content and antioxidant activity of *C. violifolia* and *C. pratensis* grown on agar media supplied with or without 25 µM Na₂SeO₄. Total phenolic content is expressed in Gallic Acid Equivalents. Antioxidant activity is expressed as Trolox Equivalent Antioxidant Capacity. Different uppercase letters indicate statistically different means (P < 0.05).

<table>
<thead>
<tr>
<th>Species</th>
<th>Se in media</th>
<th>Total phenolic content (mg GAE g⁻¹ DW)</th>
<th>Antioxidant activity (µmol TEAC g⁻¹ DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. violifolia</em> +</td>
<td>18 ± 1ᵃ</td>
<td>168 ± 12ᵃ</td>
<td></td>
</tr>
<tr>
<td><em>C. violifolia</em> -</td>
<td>17 ± 2ᵃ</td>
<td>179 ± 19ᵃ</td>
<td></td>
</tr>
<tr>
<td><em>C. pratensis</em> +</td>
<td>26 ± 5ᵇ</td>
<td>213 ± 24ᵃ</td>
<td></td>
</tr>
<tr>
<td><em>C. pratensis</em> -</td>
<td>24 ± 8ᵇ</td>
<td>197 ± 42ᵃ</td>
<td></td>
</tr>
</tbody>
</table>

5.2.3. Selenium localisation and speciation based on X-ray microprobe analysis

X-ray microprobe analysis was carried out on seedlings grown on agar media supplied with selenate and on leaves of selenate/selenite treated mature plants grown on gravel media. Besides, the XANES spectra of the synthesised selenolanthionine was also recorded.
Selenate supplied seedlings of the two species showed some differences in terms of Se localisation (Figure 36). The HA *C. violifolia* showed pronounced concentration of Se in the tips of the shoot and roots, at the apical meristems, while the non-HA *C. pratensis* tended to concentrate Se in its vasculature.

**Figure 36.** Micro focused X-ray fluorescence (µXRF) elemental maps of seedlings from *C. violifolia* (a, c, d) and *C. pratensis* (a, b, d) grown on agar media supplied with 25 µM selenate. Selenium is shown in red (or in white, b), calcium in green, zinc in blue. Yellow circles denote locations where XANES spectra were collected to determine Se speciation.
In case of the leaves from selenate treated 4-week old plants *C. violifolia* showed a more pronounced Se signal along the leaf edges and in undefined discrete locations along the outside of the petiole (Figure 37). Both species also showed relatively low concentration of Se in the vasculature. Leaf curling cannot be excluded as a reason for the higher Se signal in the leaf periphery, but it was detected only in the HA.

![Figure 37](image_url)

**Figure 37.** Micro focused X-ray fluorescence (XRF) elemental maps of leaves from 4-week old *C. violifolia* (a,c) and *C. pratensis* (b) grown on Turface® media supplied with 20 µM selenate (a,b) or selenite (c) for 10 days. Selenium is shown in white. Yellow circles denote locations where XANES spectra were collected to determine Se speciation. Note: *C. pratensis* treated with selenite is not shown because of lack of sufficient Se signal. Leaf a) broke during transfer of frozen leaf to the XRF cryostage.

*Cui et al.* (2018) concluded in an earlier µXRF study that in HA *Cardamine* species Se was primarily located in the cortex, endodermis and vascular cylinder in roots, while in the epidermis, the cortex and vascular bundle of stems; also, Se was concentrated in the leaf veins and the peripheral parts of leaves. These results are in agreement with our leaf data of *C. violifolia*. Other HA species also showed Se sequestration along the leaf periphery, e.g., *Astragalus bisulcatus* and *Stanleya pinnata* (Freeman *et al.* 2006a). The concentration of Se in the leaf periphery of the HA may be indicative of specialised Se sequestration mechanisms in areas where Se can be tolerated to the highest extent and where it may offer the most ecological benefits via herbivore or pathogen protection. Concentration of Se in the leaf vasculature, as seen particularly in non-HA *C. pratensis*, is similar to what was found in other non-HA Brassicaceae such as *Brassica juncea* (Freeman *et al.* 2006a); it may reflect a more limiting uptake rate from the xylem into the mesophyll cell.
The µXANES based Se speciation of the seedlings were similar for both species. Yellow circles on Figures 36 and 37 denote the locations where XANES spectra were collected. Both species accumulated predominantly (~85%) organic Se with a “C-Se-C” configuration (Table 13). As selenolanthionine showed similar XANES spectra compared to other organic Se compounds containing “C-Se-C” configuration such as selenomethionine or Se-(methyl)selenocysteine, it cannot be determined which compound(s) is/are responsible for the signal (Figure 38). In the root of C. violifolia almost all Se was in the same form; unfortunately XANES spectra of C. pratensis root were of insufficient quality for fitting, because of lower Se signal intensity.

Table 13. Selenium speciation in C. violifolia (C. v.) and C. pratensis (C. p.) as determined by XANES LSQ fitting. Values show the average of the XANES spectra within species and organ, for each of the two experimental setups. Corresponding XRF images for agar-grown plants are shown in Fig. 36; gravel-grown XRF images are shown in Fig. 37. Numbers for each form of Se represent the percentage of total Se. ND: not detected. C-Se-C may include SeMet, MeSeCys, SeLan, etc.

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth media</th>
<th>Supplied Se form</th>
<th>Organ</th>
<th>Se (VI) (%)</th>
<th>Se(IV) (%)</th>
<th>C-Se-C (%)</th>
<th>Se(0) (%)</th>
<th>Se(GSH)₂ (%)</th>
<th>SeCys₂ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. v.</td>
<td>gravel</td>
<td>SeO₃</td>
<td>leaf</td>
<td>ND</td>
<td>6</td>
<td>91</td>
<td>ND</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>C. v.</td>
<td>gravel</td>
<td>SeO₄</td>
<td>leaf</td>
<td>1</td>
<td>ND</td>
<td>87</td>
<td>ND</td>
<td>4.5</td>
<td>12</td>
</tr>
<tr>
<td>C. p.</td>
<td>gravel</td>
<td>SeO₄</td>
<td>leaf</td>
<td>44</td>
<td>ND</td>
<td>59</td>
<td>ND</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>C. v.</td>
<td>agar</td>
<td>SeO₄</td>
<td>shoot</td>
<td>2</td>
<td>4</td>
<td>86</td>
<td>5</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>C. p.</td>
<td>agar</td>
<td>SeO₄</td>
<td>shoot</td>
<td>8</td>
<td>6</td>
<td>84</td>
<td>ND</td>
<td>2</td>
<td>ND</td>
</tr>
<tr>
<td>C. v.</td>
<td>agar</td>
<td>SeO₄</td>
<td>root</td>
<td>2</td>
<td>ND</td>
<td>96</td>
<td>ND</td>
<td>1</td>
<td>ND</td>
</tr>
</tbody>
</table>

Se speciation showed differences between the two Cardamine species when sampled at the 4-week old stage. In case of selenate treatment the leaf of C. violifolia contained ~ 90% “C-Se-C” compounds while C. pratensis only contained ~56% and ~44% selenate, the form of Se supplied. Selenite supplied C. violifolia accumulated about 90% of Se in organic “C-Se-C” compounds and no adequate signal could be obtained from C. pratensis. The speciation differences between the mature stages can be possibly caused by different relative enzyme activities at developmental stages; it is also possible that the differences in selenate supply affected the speciation outcome.

The finding that at the mature plant stage the main form(s) of Se in the HA C. violifolia had “C-Se-C” configuration and the non-HA had relatively more selenate shows similarities to former studies where S. pinnata and A. bisulcatus had been compared with non-HA relatives (Alford et al. 2014, Freeman et al. 2006a).
The accumulation of relatively more organic Se indicates that the HA possesses a more active sulphate/selenate assimilation pathway, converting selenate to organic “C-Se-C” forms; however the “C-Se-C” form of Se in the different HA species covers different Se species and their identification is highly required. *A. bisulcatus* and *S. pinnata* were found to contain mostly Se-(methyl)selenocysteine according to LC-MS based data and it was shown that *S. pinnata* also contains selenocystathionine.

Figure 38. μXANES spectra of selenium standards (sodium selenate, selenocystine, selenomethionine, Se-(methyl)selenocysteine, selenolanthionine) and *C. violifolia* and *C. pratensis* seedlings (spots denoted in Figure 36).

The collected XANES data regarding *C. violifolia* contains mainly organic Se in “C-Se-C” form; this is in agreement with the above described selenolanthionine identification by LC-ICP-MS, LC-ESI-MS and chemical synthesis. Cui *et al.* (2018) investigated Se species in HA *Cardamine* by μXANES and found the majority of Se in the form of “C-Se-C” in roots and stems, but of “C-Se-“ in leaves using selenocystine as model compound. According to Table 13, LSQ fitting indicated 12% of Se in the form of “C-Se-Se-C” in the leaf of selenate supplied *C. violifolia* grown on gravel media. Therefore, SCX-ICP-MS analysis was found to be necessary to determine whether selenocystine might be responsible for this signal. It should be
noted that the lack of SeCys$_2$ in the water extracts doesn’t exclude the possible presence of minor water soluble “C-Se-Se-C” species such as selenohomocystine and its derivatives (Németh et al. 2013) or even some plant proteins (Cheajesadagul et al. 2014) where inherent “S(e)-S(e)” bonds can be present (e.g., like in the granule bound starch synthase in rice; Uniprot entry Q0DEV5).

5.2.4. Investigation of the presence of selenocystine by SCX-ICP-MS in Cardamine samples

As selenocystine is a commercially available standard, its presence or absence in a sample can be determined with LC-ICP-MS on the basis of retention time matching. Strong cation exchange chromatography of the water extract of C. violifolia root (157 mg total Se kg$^{-1}$ DW) and leaf samples (261 mg total Se kg$^{-1}$ DW) collected in the Yutangba region are shown in Figures 39a and c, respectively. The highest peaks eluting at ~3 min correspond to the previously identified selenolanthionine. Both leaf and root extracts were spiked to 200 ng mL$^{-1}$ selenocystine concentration, calculated for Se (Figures 39b and d). The peak of selenocystine eluted at 3.5 min between two peaks originating from the samples, which indicated that no selenocystine was present in considerable amount in the water extracts of the leaf and of the root. SCX chromatogram of the water extract of cultivated C. pratensis leaf sample (324 mg total Se kg$^{-1}$ DW) is shown in Figure 40. Inorganic and other anionic Se species eluted in the void volume at the retention time of 1.1 min while several low intensity Se compounds eluted later. The peaks eluting at 4.6 min and 7.2 min matched the retention times of Se-(methyl)selenocysteine and SeLan, respectively, however their intensity was too low for reliable identification. In order to check whether the most abundant organic Se compound eluting at 12.1 min might be selenocystine, the extract was spiked to 500 ng mL$^{-1}$ SeCys$_2$ (calculated for Se). The spiking procedure indicated no selenocystine was present in the leaf water extract in considerable amounts as a separate peak appeared at 11.9 min on the chromatogram.
Figure 39. Strong cation exchange (SCX)-ICP-MS chromatogram of *C. violifolia* (a) leaf, (c) root water extract and (b, d) each spiked to 200 ng mL\(^{-1}\) selenocystine (dashed line).

Figure 40. (a) Strong cation exchange (SCX)-ICP-MS chromatogram of selenium enriched non-HA *C. pratensis* leaf water extract and its spike to 500 ng mL\(^{-1}\) selenocystine (see the inset (b)). Signs of (i) and (ii) refer to the retention time of *Se*-(methyl)selenocysteine and selenolanthionine, respectively.
6. CONCLUSIONS AND SUGGESTIONS

*Cardamine violifolia* is a species from the genus *Cardamine*, which is one of the less studied genera of the Brassicaceae family. This latter has been in the focus of selenium speciation for decades due to the highly intense sulphur and selenium metabolism and to its evident importance in food industry. The basic instrumental approaches usually addressed for the selenium speciation of Brassicaceae plants, that is, one dimensional (mostly strong anion exchange) chromatographic purification followed by ICP-MS based identification has been referred / cited by so many research groups that analysts might believe it is not only a common but analytically well-established method.

This PhD thesis proves that such a general approach shouldn’t be followed without a critical assessment, even in the case of samples arriving from the same plant family. One dimensional anion exchange purification has been unable to provide an adequate basis for the direct identification of even an extremely abundant selenium species, selenolanthionine. Taking into account that XRF and XANES cannot distinguish this “C-Se-C” type species from well-known selenium species such as selenomethionine and Se-(methyl)selenocysteine, it might be assumed that this non-proteinaceous selenoamino acid (maybe not alone…) has already escaped several identification trials in laboratories dealing with plant selenium accumulation before it could be clearly assigned in our studies. Without identification, no conclusion can be drawn about any concerned plant metabolic pathway in any plant, therefore the responsibility of the analysts providing such data is crucial and definitely influences all the upcoming research strategies.

Clearly, numerous papers published in the 2000’s and 2010’s should be carefully re-evaluated in this term, as seemingly “closed” and “completely described” metabol(om)ic information might derail future efforts and studies in this field. Indeed, the complexity of selenium metabolism (as evidenced by the 35 water soluble selenium species that were extracted from naturally selenium enriched, wild-grown plant biomass) should indicate that no scientist should stop after spotting a few selenium compounds in the actual sample and claim that all the compounds required for the unambiguous description of a metabolomic pathway are reported.

Accordingly, selenium speciation oriented studies should follow a workflow that ensures a reliable identification strategy, possessing access to LC-ICP-MS and LC-
ESI-HR-MS facilities, under a strict – and sometimes quite basic – quality assurance control: well, the fact that no direct identification can be done for any compound in the chromatographic void volume cannot be regarded as a novel requirement. XRF and XANES studies (especially if feed or food industry related experiments are running) must be accompanied with comprehensive (sometimes ultimate) ESI-MS based identification processes to support/decline XRF / XANES derived hypotheses. Taking into account that IDA experiments can provide the quantification of species without commercially available standards, limits are more and more pushed apart in selenium speciation.

The identification of the main selenium species in the water extract of Cardamine violifolia as selenolanthionine, a non-protein building amino acid which has never been identified in any Se containing sample before, the detection of numerous unknown Se species (including several selenosugars) and the physiological differences in contrast to other hyperaccumulators indicate the function of a unique, still non-elucidated metabolic pathway for Se detoxification in C. violifolia.

Concerning the limits and future goals, three aspects must be listed. First, the successful spotting/seeking of selenium metabolites in ESI-MS data has still been a challenging task for almost a decade on, calling for a straightforward bioinformatic tool. Second, the ultimate identification of selenium species definitely demands NMR applications, which in turn, requires highly efficient and non-invasive sample preparation protocols. The identification of new Se species (such as selenoamino acids, their derivatives or selenosugars) reaching significant concentration in plants calls the attention to the need of their nutritional physiological evaluation.
7. THESIS STATEMENTS

1) I identified the main selenometabolite, selenolanthionine, in the water extract of selenised *Cardamine violifolia* with the help of high resolution mass spectrometry that was assisted with the in-house chemical synthesis of the selenolanthionine standard. This selenium species has never been unambiguously identified before in any selenium containing sample.

2) I quantified selenolanthonine (accounting for ~30% of the total Se) from *Cardamine violifolia* derived samples with the help of a post-column isotope dilution LC-ICP-MS analysis. This was the first successful application of this technique in Hungary.

3) I revealed the complexity of the water soluble selenometabolome of naturally grown and naturally selenium enriched *Cardamine violifolia* leaf sample. Besides the presentation of 35 selenium species (apart from selenolanthionine) I reported for the first time the presence of an N-glycosylated selenoamino acid, *i.e.*, mono- and di-N-glycosylated selenolanthionines in a natural sample.

4) By conducting a comprehensive experiment regarding selenium-related physiological and biochemical properties of *C. violifolia* in comparison with related species *C. pratensis*, I presented that *C. violifolia* shows clear selenium-related physiological and biochemical differences compared to *C. pratensis* and other Se hyperaccumulators: *C. violifolia* is capable of higher Se accumulation and more tolerant to Se than *C. pratensis* however its growth is not stimulated by Se.

5) I recorded for the first time the µXANES spectra of a synthesised selenolanthionine standard which showed identical spectra with other “C-Se-C” bond containing compounds. I analysed for the first time the selenium localisation and speciation in intact tissues of *C. violifolia* and *C. pratensis* by X-ray microprobe analysis. I concluded that the results show similarities to other hyperaccumulator and related non-accumulator species: *C. violifolia* concentrated Se along the leaf periphery while *C. pratensis* in the vasculature. Mainly organic Se in the “C-Se-C” bond was found in mature *C. violifolia* plant, according to µXANES results, while selenate-supplied mature *C. pratensis* contained approximately half selenate and half “C-Se-C” bond containing compounds.
**8. SUMMARY**

*Cardamine violifolia* (family Brassicaceae) is the first discovered selenium (Se) hyperaccumulator species from the genus *Cardamine*, originating from the naturally seleniferous region Yutangba, Enshi, China. The plant is cultivated for phytoremediation purposes, soil reclamation and food/feed supplement production.

In my study I intended to investigate the selenium related characteristics of *C. violifola* including selenium speciation and physiological experiments.

The identification of selenometabolites was addressed by different extraction procedures and the complementary use of liquid chromatography inductively coupled plasma (inorganic) and high resolution organic mass spectrometry setups. No considerable amount was found of the “usual” organic species (that is, selenomethionine and Se-(methyl)selenocysteine) that are often formed in hyperaccumulator plants; moreover, the inorganic selenium content including elemental Se accounted only for less than 20% of the total Se. In our study the application of an orthogonal chromatographic purification allowed for the identification of the main selenometabolite, selenolanthionine, in the water extract of *C. violifolia* selenium hyperaccumulator plant species. This non-protein building amino acid has never been identified before in any selenium containing sample unambiguously. It is of note that several conflicting LC-based chemical speciation reports have been published on the putatively same selenium compound from the putatively same *Cardamine* species (Yuan et al. 2013, Cui et al. 2018), but the identification of selenolanthionine was confirmed in our case with high resolution MS data and chemical synthesis as well. Our results warn that the application of at least two orthogonal separation mechanisms and the combination of inorganic (LC-ICP-MS) with high resolution organic mass spectrometry (ESI-MS) are usually required for adequate species assignment.

Species-unspecific (post-column) isotope dilution analysis was addressed for the quantification of selenolanthionine that accounted for ~ 30% of total Se in the *C. violifolia* sample. Apart from selenolanthionine, four previously known (e.g., selenocystathionine, selenohomocystine) and 31 unknown selenium containing molecules were additionally detected in the water extract including derivatives of selenohomocysteine and selenosugars.
A comprehensive experiment about the Se related physiological characteristics of *C. violifolia* was conducted with the comparison of the related non-hyperaccumulator *Cardamine pratensis* species covering Se tolerance and accumulation, chlorophyll fluorescence, spatial selenium/sulphur distribution patterns through synchrotron-based X-ray fluorescence (µXRF) and ICP-OES, speciation analysis through selenium K-edge micro X-ray absorption near-edge structure analysis (µXANES). The physiological study revealed clear differences between the hyperaccumulator *C. violifolia* and the related non-accumulator *C. pratensis* plant species; on the other hand, *C. violifolia* showed contrasting properties with other hyperaccumulator species even within the *Brassicaceae* family, compared to, *e.g.*, *S. pinnata*, like missing growth stimulation by Se. The hyperaccumulator and non-hyperaccumulator *Cardamine* species did not show any difference in terms that sulphate inhibited selenate uptake but selenite uptake was not inhibited by phosphate.

µXRF derived distribution maps and selenium/sulphur uptake characteristics showed similarities to other formerly reported hyperaccumulator and non-hyperaccumulator *Brassicaceae* species. Mainly organic Se in “C-Se-C” bond was found in the seedlings of both species and in mature *C. violifolia* plant analysed by µXANES while selenate-supplied mature *C. pratensis* contained approximately half selenate and half “C-Se-C” bond containing selenocompounds. The lack of selenocystine was proved by strong cation exchange chromatography-ICP-MS in the water extract of both species.

All the speciation and selenium-related physiological properties refer to the function of a unique, still unelucidated biochemical pathway for Se detoxification in this hyperaccumulator plant.
9. ÖSSZEFOGLALÁS

A *Cardamine* nemzetség (Brassicaceae család) első szelén hiperakkumulálóként azonosított faja a *Cardamine violifolia*, mely Kína természetes szelénben dús régiójából, Enshiból (Yutangba övezet, Hubei tartomány) származik. Ezen a területen a növényt fitoremediációs célokra, talajjavításra, valamint takarmány / táplálékkiegészítő gyártásban való felhasználásra termesztik ill. gyűjtkik.

Kutatásom során a *C. violifolia* növény szelénkel kapcsolatos jellemzőit vizsgáltam, beleértve szelénspeciációs analízist és fiziológiai kísérleteket.

A szelénmetabolitok azonosításához különböző extrakciós eljárásokat, valamint folyadékkromatográfiával kapcsolt induktív csatolású plazma (szervetlen) és nagy felbontású szerves tömegspektrometriás rendszereket alkalmaztam. A növény nem tartalmazott számottevő mennyiségben a hiperakkumulálókban gyakran azonosított szerves komponenseket (mint pl. szelenometionin, Se-(metil)selenocisztein), valamint a szervetlen és elemi szélén tartalom együtt is kevesebb, mint 20%-ot tett ki az összes szeléntartalomból. Ortogonális kromatográfiás tisztítás alkalmazása tette lehetővé a fő szélénmetabolit, a szelenolantionin azonosítását a *C. violifolia* vizes extraktumában. Ez a nem fehérjeépítő aminosav korábban még sosem került egyértelmű azonosításra szelén tartalmú mintában. Bár több ellentmondó, LC alapú speciációs eredmény került publikációba a feltehetően azonos szelénkomponens és *Cardamine* faj kapcsán (Yuan et al. 2013, Cui et al. 2018), azonban esetünkben a szelenolantionin azonosítása nagy felbontású MS adatok és kémiai szintézis útján is megerősítésre került. Az eredmények felhívják a figyelmet arra, hogy a megfelelő komponens azonosításához legalább két ortogonális elválasztási technika és szervetlen (LC-ICP-MS) valamint nagy felbontású szerves tömegspektrometria (ESI-MS) együttes alkalmazása szükséges.

Nem módosulat-specifikus (oszlop utáni) izotóphígítással került sor a szelenolantionin mennyiségi meghatározására, mely körülbül az összes szeléntartalom 30%-át tette ki a *C. violifolia* mintában. A vizes extraktumban a szelenolantioninon túl négy korábban azonosított (pl.: szelenocosztationin, szelenohomocisztein) és 31 ismeretlen szeléntartalmú komponenst detektáltunk, többek között szelenohomocisztein származékokat és szelenocukrokat.

Széleskörű kísérletet végeztem a *C. violifolia* szelénkel kapcsolatos fiziológiai tulajdonságainak vizsgálatára, összehasonlítva a rokon, nem hiperakkumuláló
Cardamine pratensis fajjal, a következő szempontok/célok szerint: szelén tolerancia ésakkumuláció, klorofill fluoreszcencia meghatározás, térbeli szelén/kén eloszlás vizsgálata szinkrotron alapú röntgen fluoreszcencia (µXRF) és induktív csatolású plazma optikai emissziós spektrometria (ICP-OES) révén, ill. speciációs analízis röntgen abszorpciós módszerrel (µXANES). A fiziológiai vizsgálatok egyértelmű különbségeket tártak fel a hiperakkumuláló C. violifolia és rokon nem hiperakkumuláló C. pratensis fajok között, valamint C. violifolia különbségeket mutatott más hiperakkumuláló fajokkal szemben, még a Brassicaceae családon belül is (pl. Stanleya pinnata), hiszen például a szelén jelenléte nem eredményezett növekedés stimulációt. A hiperakkumuláló és nem hiperakkumuláló Cardamine fajok nem különbözték abban a tekintetben, hogy szulfát jelenléte gátolta a szelenát felvételt, míg foszfátuk nem volt gátló hatása a selenit felvételre.

A röntgen alapú méréstechnikával rögzített szelén eloszlás és a szelén/kén felvétel jellemzői hasonlóságot mutattak más, korábban jellemzett hiperakkumuláló és nem hiperakkumuláló Brassicaceae fajokkal. Főként szerves „C-Se-C” kötést tartalmazó szelénkomponensek jellemzők mindkét Cardamine fajta hajtásait és az érett C. violifolia növényt, míg a szelenáttal kezelt érett C. pratensis mintát körülbélül 50-50%-ban szelenát, ill. „C-Se-C” kötősű szelén jellemzi. Kationcserés-ICP-MS technikával igazoltam mindkét faj esetén, hogy a növények vizes extraktuma nem tartalmaz szelenocisztint.

Mind a speciációs, mind a szelénnel kapcsolatos fiziológiai tulajdonságok egy egyedi, eddig fel nem tárt szelén detoxifikációs útvonal működésére utalnak a C. violifolia növényben.


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11. APPENDIX II: TABLES AND FIGURES

Table A1. Instrumental parameters of the HPLC-ESI-QTOF-MS set-up.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
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<td>ESI source</td>
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</tr>
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<td>Precursor ion selection in MS/MS mode</td>
<td>low (1.7 m/z)</td>
</tr>
<tr>
<td>Mass accuracy</td>
<td>&lt; 2 ppm</td>
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<tr>
<td>Mass resolution</td>
<td>&gt;10000</td>
</tr>
<tr>
<td>Detection frequency</td>
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<td>Curtain voltage</td>
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<tr>
<td>Drying gas</td>
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<tr>
<td>Capillary voltage</td>
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<td>Nebuliser pressure</td>
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<tr>
<td>Gas temperature</td>
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<tr>
<td>Data analysis software</td>
<td>Mass Hunter Acquisition B. 02.01 with SP3</td>
</tr>
</tbody>
</table>

6530 Accurate Mass QTOF LC-MS (Agilent)

Table A2. UPLC-Unispray-QTOFMS instrumental setup parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
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<tbody>
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<td>UPLC column</td>
<td>Acquity BEH C₁₈, 2.₁*100 mm; 1.₇ µm</td>
</tr>
<tr>
<td>Eluent “A”</td>
<td>water with 0.1 v/v% formic acid</td>
</tr>
<tr>
<td>Eluent “B”</td>
<td>acetonitrile with 0.1 v/v% formic acid</td>
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<tr>
<td>Flow rate</td>
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<tr>
<td>Column temperature</td>
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<tr>
<td>Gradient</td>
<td>0 - 1.0 min 10% „B“&lt;br&gt;1.0 - 4.0 min ↑ 80% „B“&lt;br&gt;4.0 - 4.5 min 80% „B“&lt;br&gt;4.5 - 5.0 min ↓ 10% „B“&lt;br&gt;5.0 - 7.0 min 10% „B“</td>
</tr>
<tr>
<td>Injection volume</td>
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<td>Source temperature</td>
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<td>MS scan</td>
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<tr>
<td>MS scan time</td>
<td>0.2 s</td>
</tr>
<tr>
<td>Lock mass</td>
<td>ON</td>
</tr>
<tr>
<td>MS/MS scan</td>
<td>50 – 1000 m/z</td>
</tr>
<tr>
<td>Low mass ramp</td>
<td>20 – 30 eV</td>
</tr>
<tr>
<td>High mass ramp</td>
<td>30 – 80 eV</td>
</tr>
</tbody>
</table>

Table A3. Optimal parameters and instrumental data for the LC-MS quantification of selenolanthionine and lanthionine.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Precursor (m/z)</th>
<th>Declustering potential (V)</th>
<th>Quantification peak (m/z)</th>
<th>Collision Energy (V)</th>
<th>Confirmation peak No. 1 (m/z)</th>
<th>Collision Energy (V)</th>
<th>Confirmation peak No. 2 (m/z)</th>
<th>Collision Energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanthionine</td>
<td>6.83</td>
<td>209.1</td>
<td>36</td>
<td>120.2</td>
<td>19</td>
<td>74.0</td>
<td>37</td>
<td>146.0</td>
<td>17</td>
</tr>
<tr>
<td>Selenolanthionine</td>
<td>6.77</td>
<td>256.9</td>
<td>31</td>
<td>167.9</td>
<td>19</td>
<td>140.2</td>
<td>27</td>
<td>74.2</td>
<td>37</td>
</tr>
</tbody>
</table>

Ion spray voltage: 5500 V; curtain gas (N₂): 10 psi; ion source gas: 40 psi; desolvation temperature: 350 °C; collision activated dissociation gas: 5 arbitrary units.
Table A4. Calculation settings for the elemental calculator tool.

<table>
<thead>
<tr>
<th>Element</th>
<th>Min.</th>
<th>Max.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>H</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>O</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>P</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>N</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Se</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table A5. Efficiency of automatic selenium pattern recognition. It is to note that the compounds with detected theoretical selenium mass defect include false positive hits and the different isotopologues of the same compound as well.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Number of compounds detected</th>
<th>Number of compounds with detected theoretical selenium mass defect</th>
<th>Relative amount of compounds detected with theoretical selenium mass defect</th>
<th>Number of compounds with detected theoretical selenium mass defect</th>
<th>Relative amount of compounds detected with theoretical selenium mass defect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Settings: defect padding, 60 mDa; mass padding, 200 Da; minimum intensity, 2000 cps</td>
<td>Settings: defect padding, 80 mDa; mass padding, 200 Da; minimum intensity, 2000 cps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#1</td>
<td>7293</td>
<td>350</td>
<td>4.8%</td>
<td>457</td>
<td>6.3%</td>
</tr>
<tr>
<td>#2</td>
<td>5062</td>
<td>227</td>
<td>4.5%</td>
<td>277</td>
<td>5.5%</td>
</tr>
<tr>
<td>#3</td>
<td>4838</td>
<td>229</td>
<td>4.7%</td>
<td>269</td>
<td>5.6%</td>
</tr>
<tr>
<td>#4</td>
<td>4572</td>
<td>178</td>
<td>3.9%</td>
<td>206</td>
<td>4.5%</td>
</tr>
</tbody>
</table>

Table A6. Effect of defect padding settings on the efficiency of automatic selenium pattern recognition.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>m/z</th>
<th>Minimally required mass padding (Da) for successful detection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>at 20 mDa defect padding</td>
</tr>
<tr>
<td>#1</td>
<td>282</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>284</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>407</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>242</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>391</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>391</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>#2</td>
<td>419</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>581</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>419</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>401</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>285</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>405</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>285</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>#3</td>
<td>441</td>
<td>391</td>
</tr>
<tr>
<td></td>
<td>419</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>446</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>268</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>397</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>215</td>
<td>40</td>
</tr>
<tr>
<td>#4</td>
<td>254</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>312</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>271</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>282</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>284</td>
<td>89</td>
</tr>
</tbody>
</table>
Table A7. Efficiency of automatic selenium pattern recognition at optimised settings (mass padding: 200 Da, defect padding: 80 mDa). Values in bold indicate successful detection.

<table>
<thead>
<tr>
<th>m/z</th>
<th>background of unsuccessful automatic detection</th>
<th>m/z</th>
<th>background of unsuccessful automatic detection</th>
<th>m/z</th>
<th>background of unsuccessful automatic detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>241.99302</td>
<td>-</td>
<td>285.05998</td>
<td>-</td>
<td>197.00725</td>
<td>254.02883</td>
</tr>
<tr>
<td>357.07037</td>
<td>isobaric interference</td>
<td>301.09063</td>
<td>isobaric interference</td>
<td>241.99302</td>
<td>271.02005</td>
</tr>
<tr>
<td>391.08672</td>
<td>isobaric interference</td>
<td>405.06603</td>
<td>low intensity</td>
<td>268.04462</td>
<td>282.06027</td>
</tr>
<tr>
<td>407.04499</td>
<td>low intensity</td>
<td>416.08220</td>
<td>low intensity</td>
<td>270.02341</td>
<td>284.03997</td>
</tr>
<tr>
<td>435.04004</td>
<td>low intensity</td>
<td>419.05681</td>
<td>isobaric interference</td>
<td>446.09233</td>
<td>312.03454</td>
</tr>
<tr>
<td>447.11604</td>
<td>low intensity</td>
<td>434.09228</td>
<td>low intensity</td>
<td>463.02100</td>
<td>313.02949</td>
</tr>
<tr>
<td>448.10670</td>
<td>low intensity</td>
<td>537.14451</td>
<td>low intensity</td>
<td>364.95162</td>
<td>-</td>
</tr>
<tr>
<td>460.10880</td>
<td>low intensity</td>
<td>581.10958</td>
<td>low mass padding</td>
<td>444.86739</td>
<td>low intensity + triple selenium</td>
</tr>
<tr>
<td>482.99120</td>
<td>low intensity</td>
<td></td>
<td></td>
<td>489.05794</td>
<td>low intensity</td>
</tr>
<tr>
<td>523.12784</td>
<td>low intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>552.06109</td>
<td>low intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>614.14680</td>
<td>low intensity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure A1. (a) HPLC-ESI-QTOF-MS full scan spectrum of the *C. violifolia* selenolanthionine fraction spiked with synthesised selenolanthionine prior to MS/MS analysis. (b) HPLC-ESI-QTOF-MS/MS collision induced dissociation (CID) spectrum of the *C. violifolia* selenolanthionine fraction spiked with synthesised selenolanthionine fragmented on the $^{77}$Se isotopologue.
**Figure A2.** Selenocystathionine (for details, see Table 9); (a) full scan spectrum; (b) full scan spectrum /zoomed/; (c) MS/MS spectrum.

**Figure A3.** Selenohomocystine (for details, see Table 9); (a) full scan spectrum; (b) full scan spectrum /zoomed/; (c) MS/MS spectrum.
Figure A4. Selenosugar at m/z 407 (for details, see Table 9); (a) full scan spectrum; (b) full scan spectrum /zoomed/; (c) MS/MS spectrum.

Figure A5. Se-selenohomocysteinyl-diseleno-homocysteine (for details, see Table 9); (a) full scan spectrum; (b) full scan spectrum /zoomed/; (c) MS/MS spectrum.
Figure A6. Compound at the experimental m/z 268.04482 (for details, see Table 9); (a) full scan spectrum; (b) full scan spectrum /zoomed/; (c) MS/MS spectrum.

Figure A7. Additional (zoomed) MS/MS spectrum of the m/z 581.1092 compound.
Figure A8. Full scan with zoomed view in the inset (a) and MS/MS (b, c) spectra of the sulphur analogues (#1, #2) of the putative N-glycosides of selenolanthionine (#1’, #2’). (b) presents the MS/MS spectrum of the molecule \( m/z \) 533.163 \((C_{18}H_{33}N_{2}O_{14}S^+)\), while (c) shows the MS/MS spectrum of the molecule \( m/z \) 371.112 \((C_{12}H_{23}N_{2}O_{9}S^+)\).
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