# Summary of doctoral (Ph.D.) thesis

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**Szent István University**

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

Summary of the doctoral (Ph.D.) thesis of

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# 1. INTRODUCTION AND OBJECTIVES

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. 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 21$^{st}$ 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.

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.

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. In my doctoral study I intended to identify and quantify selenium species and investigate the Se related physiological properties of this 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.

## 2. MATERIALS AND METHODS

*Cardamine violifolia* was identified and registered by the Wuhan Botanical Garden (Chinese Academy of Sciences; Wuhan, China). Plant biomass and seeds were collected in the springtime of 2016 and 2017 in the natural seleniferous region Yutangba, Enshi (Hubei Province, China) and provided by the Academy of Agricultural Sciences of Enshi Tujia and Miao Autonomous Prefecture (Wuhan). Plant biomass was cleaned with deionised water, lyophilised and milled. *Cardamine pratensis* (cuckooflower) seeds were purchased from Seedaholic (Cloghbrack, Clonbur, Ireland).

Different sample preparation methods were applied to investigate the selenium distribution in *C. violifolia* sample including acidic digestion, enzymatic extraction, sequential extraction: water extraction, sulphite extraction, and $CS_2$ extraction.

Screening of selenium species was carried out by strong anion-, cation-exchange chromatography, and ion pairing reversed phase chromatography coupled to ICP-MS. Orthogonal chromatographic clean-up procedure ensured the elimination of matrix interferences and pre-concentration of the selenocompounds for high resolution ESI-MS analysis. Selenocompound containing fractions were introduced to the Unispray/ESI-QTOF-MS instruments by means of an RP-HPLC system. Screening of selenium species in high resolution MS spectra was achieved by different approaches: (i) database search, (ii) extracting diagnostic in-source fragments, (iii) manual pattern exploration, (iv) mass defect based Se species filtering. Chemical synthesis was carried out in order to confirm the identification of the main selenium species and post column isotope dilution analysis was applied for quantification without an authentic standard.

The Se related physiological characteristics of *C. violifolia* was investigated with the comparison of a related non-hyperaccumulator species *Cardamine pratensis*. The two *Cardamine* species were cultivated on agar medium supplied with different concentration of selenate under sterile conditions. Plants cultivated in gravel were treated with Hoagland's solution supplied with selenate, selenite, selenate+sulfate and selenite+phosphate. Root length and shoot dry weight were measured to investigate Se tolerance. Total Se and S accumulation was measured by ICP-OES. X-ray fluorescence (XRF) and X-ray absorbance near edge structure (XANES) analyses were performed to investigate Se distribution and speciation in intact tissues. Physiological conditions of plants under Se treatment were monitored by measuring chlorophyll fluorescence parameters, antioxidant capacity and total phenolic content.

## 3. RESULTS AND DISCUSSION

Total Se concentration of pooled stem and leaves of *C. violifolia* sample harvested in the natural seleniferous region in China was found to be 3.7 g Se $kg^{-1}$ dry weight (DW) which places the plant among hyperaccumulator species. Water extract contained ~60% of the total Se while the sum of Se in $Na_2SO_3$ extract (assigned as elemental selenium) and $CS_2$ (assigned as selenides) accounted for ~18% of the Se originally present in the sample. Water extract was analysed by anion exchange chromatography-ICP-MS but only a negligible amount of selenite was detected; selenate was not detected above the detection limit and most of the selenocompounds eluted in the void volume. Proteolytic extract contained only negligible amount of *Se*-(methyl)selenocysteine and traces of selenomethionine. The most abundant selenocompound in the water extract was purified by orthogonal strong cation exchange (SCX) and ion pairing reversed phase (IP-RP) chromatography prior to analysing with RP-HPLC-ESI-QTOF-MS. Visual seeking allowed the exploration of the characteristic selenium isotopic pattern at m/z 257.0032 and at its putative in-source fragment 167.9555 (Figure 1).

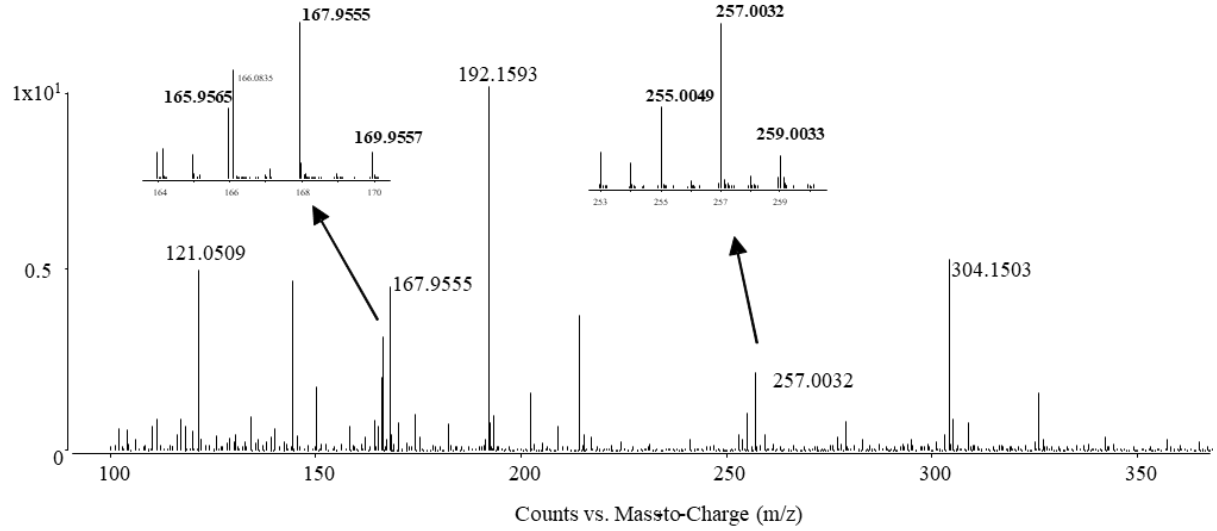


**Figure 1.** HPLC-ESI-QTOF-MS full scan spectrum recorded at 0.502 min of the most abundant selenium containing fraction of *C. violifolia*. The spectrum presents the isotopologue patterns of selenolanthionine and its in-source fragment in the insets. Masses highlighted in bold refer to the $^{78}Se$, $^{80}Se$ and $^{82}Se$ isotopologues. The mass with *m/z* 166.0835 arrives from an interference on the $^{78}Se$ isotopologue of the in-source fragment.

The only one possible elemental composition found for this molecule mass within 5 ppm was $C_6H_{13}N_2O_4Se^+$ with the theoretical m/z 257.0035 ($\Delta$=1.16 ppm). The composition indicated this molecule as selenolanthionine (SeLan), a non-protein building amino acid that has never been unambiguously identified before in any Se containing sample (Figure 3a).

The identification of the compound was confirmed by the chemical synthesis of SeLan, followed by retention time matching (Figure 2) with a spiking procedure and checking high resolution ESI-MS and MS/MS spectra.

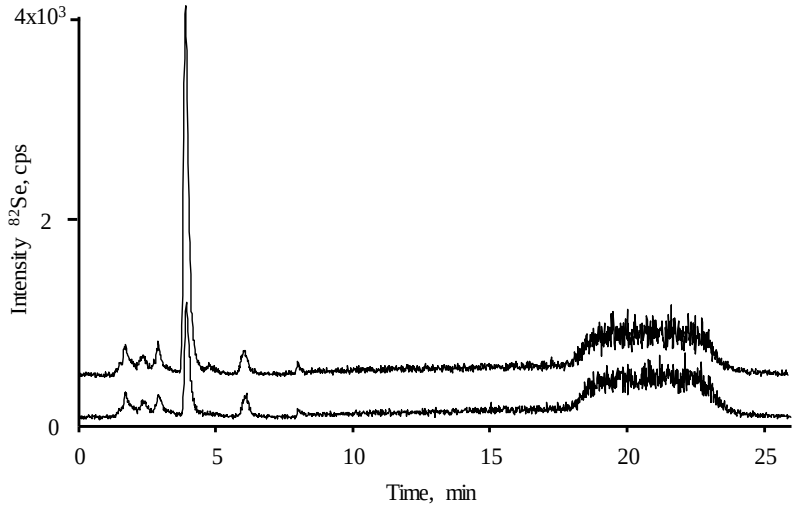


**Figure 2.** SCX – ICP-MS chromatograms of the *C. violifolia* water extract (lower chromatogram) and that of the water extract spiked with the synthesised selenolanthionine standard (upper chromatogram).

The quantification of SeLan was carried out with post column isotope dilution analysis, indicating that SeLan accounts for ~30% of total Se in the *C. violifolia* leaf sample (total Se 261 mg Se $kg^{-1}$ DW).

Less intense Se peaks of SCX-ICP-MS chromatogram were also set for high resolution MS analysis. Four known and 31 unknown selenocompounds could be detected in the fractions. Eleven species (presumed Se-containing sugars) showed the characteristic loss of hexoses (162 Da) in MS/MS fragmentation. The species detected at *m/z* 419.05681 and 581.10958 showed one and two hexose(s) loss(es), series of -18 Da losses, common fragments at *m/z* 257.00 and 167.95, which indicates that these molecules can be assigned as the mono or di-*N*-glycosides of SeLan (Figure 3b, c).

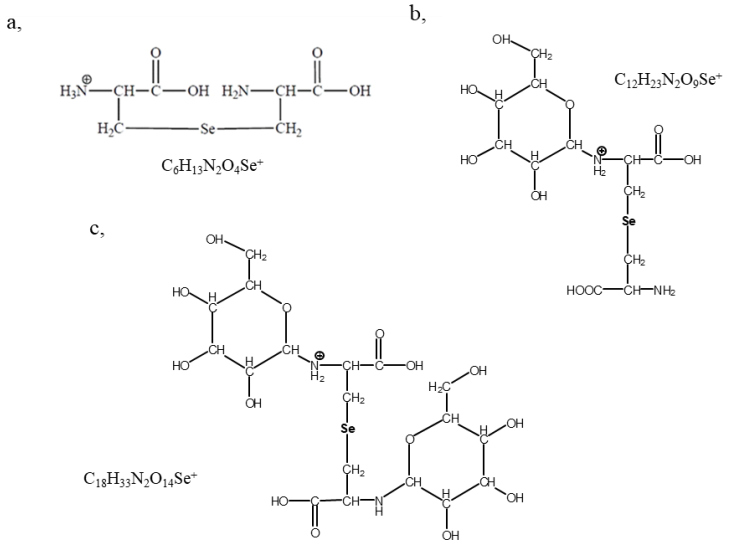


**Figure 3.** Structures of (a) selenolanthionine, (b) mono-*N*-glycoside of selenolanthionine, (c) di-*N*-glycoside of selenolanthionine

Selenium tolerance experiment of *Cardamine* species revealed that 50 µM selenate concentration inhibited the root growth of the control species that was not able to germinate at 200 µM concentration. *C. violifolia* root growth was significantly inhibited only at 200 µM selenate concentration (Figure 4a). At the smallest selenate concentration (50 µM) both species could accumulate Se in the shoots around the same level but only *C. violifolia* showed increasing accumulation with the increasing concentration (Figure 4b). *C. violifolia* did not show growth stimulation by Se at the levels supplied. With increasing Se uptake, *C. violifolia* showed increasing S uptake while opposite pattern was shown by *C. pratensis* indicating different Se-S interactions between the two species. Overall, *C. violifolia* was found to be more tolerant to Se and had higher accumulation capability compared to the control species.

The uptake study of the different inorganic forms of Se showed that shoot accumulation in both species was higher from selenate than from selenite (~10-fold) similarly to other plants. Increased sulphate ion concentration inhibited selenate uptake in both species indicating that they are taken up by the same group of sulphate transporters. The behaviour of the hyperaccumulator *C. violifolia* in this respect is in contrast to other Se hyperaccumulator plants where selenate uptake was shown to be less inhibited by sulphate. Contrary to expectations phosphate did not inhibit selenite uptake in either species and thus it seems that this process is not mediated by phosphate transporters in these plants.

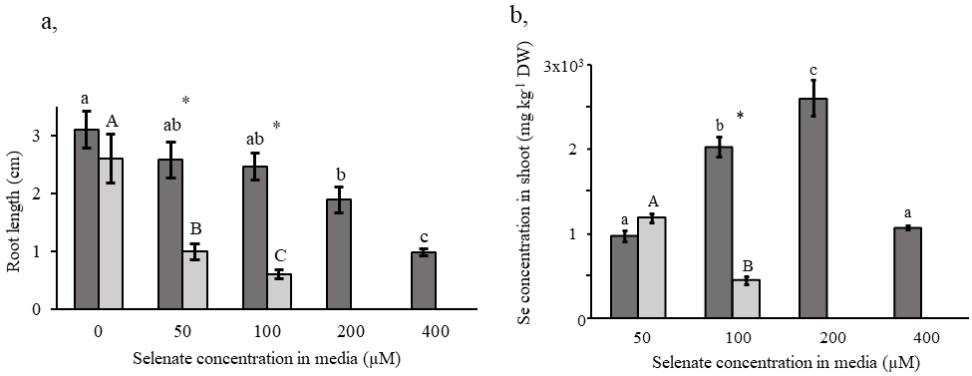


**Figure 4.** (a) Root length and (b) selenium concentration in shoots of *C. violifolia* and *C. pratensis* grown on agar media supplied with different concentrations of $Na_2SeO_4$. Values shown are the means ± SEM. Different letters indicate statistically different means among treatments within species ($P < 0.05$). 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. In the case of the control treatment, Se concentrations were below the quantification limit.

X-ray fluorescence maps of selenate supplied seedlings of the two species showed some differences in terms of Se localization. *C. violifolia* showed pronounced concentration of Se in the tips of the shoot and roots, at the apical meristems, while the control species *C. pratensis* tended to concentrate Se in its vasculature. In case of the leaves from selenate treated four-week old plants *C. violifolia* showed more pronounced Se signal along the leaf edges. The XANES spectra of the synthesised selenolanthionine was recorded which showed a similar curve compared to other organic Se compounds containing "C-Se-C" configuration. The μXANES analysis revealed that seedlings of both species and the selenate supplied leaf of the mature plant contained mainly (85-90%) selenocompounds with "C-Se-C" bonds, while the leaf of selenate supplied mature control species contained considerable amount of inorganic selenate as well (~44%). X-ray based Se distribution and speciation data showed similarities to other formerly reported hyperaccumulator and non-hyperaccumulator species. As several conflicting studies have been published reporting the main Se compound in hyperaccumulator *Cardamine* species as selenocystine (based only on SAX-ICP-MS), the presence of selenocystine in the samples was investigated by SCX-ICP-MS. The water extracts of plant materials were spiked with standard selenocystine and the results showed that no selenocystine was originally present in considerable amount in the extracts.

# 4. 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.

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 hyperaccunulators 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 bioinformatics 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.

# 5. 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.

# 6. RELATED PUBLICATIONS

## Articles in international journals with impact factors

**Both, EB** ; Shao, S ; Xiang, J ; Jókai, Z ; Yin, H ; Liu, Y ; Magyar, A ; Dernovics, M (2018): Selenolanthionine is the major water-soluble selenium compound in the selenium tolerant plant *Cardamine violifolia*. **BIOCHIMICA ET BIOPHYSICA ACTA-GENERAL SUBJECTS** 1862 pp. 2354-2362.
**IF:3.681**

**Both, EB** ; Stonehouse, GC ; Lima, LW ; Fakra, SC ; Aguirre, B ; Wangeline, AL ; Xiang, J ; Yin, H ; Jókai, Z ; Soós, Á ; Dernovics, M ; Pilon-Smits EAH (2020):
Selenium tolerance, accumulation, localization and speciation in a *Cardamine* hyperaccumulator and a non-hyperaccumulator. **SCIENCE OF THE TOTAL ENVIRONMENT** 703: 135041.
**IF(2019):6.551**

Ouerdane, L ; **Both, EB** ; Xiang, J ; Yin, H ; Yu, K ; Shao, S ; Kiszelák, K ; Jókai, Zs ; Dernovics, M (2020): Water soluble selenometabolome of *Cardamine violifolia*. **METALLOMICS**, doi: 10.1039/D0MT00216J (2020)
**IF(2019): 3.796**

## Article in other journals

**Both Eszter Borbála**: Áttekintés a szelénbevitellel kapcsolatos ismereteink jelenlegi állásáról (in Hungarian). Táplálkozástudományi Morzsák Hírlevél I. évfolyam 3. szám 2018

## Conference abstracts

## International conferences

**Both, EB** ; Kiszelák, K ; Jókai, Zs ; Shao, S ; Xiang, J ; Dernovics, M: Exploring the pathway of selenolanthionine synthesis in the selenium tolerant plant *Cardamine violifolia* p. 40 In: Hungarian, Chemical Society (ed.) II. Young Researchers' International Conference on Chemistry and Chemical Engineering: Program and Book of Abstracts, Budapest, Hungary 2018.

**Both, EB** ; Shao, S ; Xiang, J ; Jókai, Zs ; Yin, H ; Liu, H ; Magyar, A ; Dernovics, M: Selenolanthionine is the main selenium species of the hyperaccumulator plant *Cardamine violifolia* Paper: Paper O33, In: Elias, Arnér (ed.) 11$^{th}$ International Symposium on Selenium in Biology and Medicine and the 5$^{th}$ International Conference on Selenium in the Environment and Human Health: Book of Abstracts. Stockholm, Sweden 2017.

Dernovics, M ; Xiang, J ; Pilon-Smits, EAH ; Yin, H ; Yang, Y-k ; Ming, J-j ; Shao, S ; **Both, EB**; Jókai, Zs : Unprecented novel Se species in an unprecedented plant genus: instructive lessons on comprehensive approaches. pp. 62. Paper: OM-12, In: Lena, Ruzik; Joanna, Szpunar (eds.) Book of Abstracts. Warsaw, Poland 2019.

Dernovics, M ; Soós, Á ; Domokos-Szabolcsy, É ; Kovács, B ; Kurtaj, S ; **Both, EB** : Screening of Allium cepa cultivars for the ability to produce selenosugars. pp. 82. Paper: MP-24, In: Lena, Ruzik; Joanna, Szpunar (eds.) Book of Abstracts. Warsaw, Poland 2019.

## Hungarian conferences

Dernovics, Mihály ; **Both, Eszter Borbála** ; Jókainé, Szatura Zsuzsanna ; Magyar, Anna: Gyakorlatba vont szelén-fitoremediációs rendszer bemutatása egy kínai példán keresztül Paper: PL04, In: Mihucz, V (ed.) 60. Magyar Spektrokémiai Vándorgyűlés és XIII. Környezetvédelmi Analitikai és Technológiai Konferencia. Budapest, Hungary 2017.

**Both, Eszter Borbála** ; Jókainé, Szatura Zsuzsanna ; Dernovics, Mihály: Új generációs szelénbevitel: lehetőségek, feladatok és kérdések az európai, amerikai és kínai megoldások tükrében. In: Gelencsér, Éva; Lugasi, Andrea (eds.) TÁPLÁLKOZÁSTUDOMÁNYI KUTATÁSOK VIII. PhD konferencia PROGRAM ÉS ELŐADÁS ÖSSZEFOGLALÓK. Budapest, Hungary 2018.