Theses of Doctoral (PhD) Dissertation

The production of alfalfa leaf protein concentrate and its potential for value addition by selenium fortification

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1. BACKGROUND AND OBJECTIVES OF THE DOCTORAL THESIS

In this PhD thesis, I summarise our research on the potential of linking green biorefining and agronomic selenium fortification using the perennial green protein crop, alfalfa, as an example. Our experiments involved soil treatments with three different inorganic forms of selenium (selenate (Se(VI); selenite (Se(IV) and red elemental selenium (Se(0)) at different concentrations under pot experiment and field conditions. Following the selenium treatments, the re-grown fresh alfalfa biomass was harvested several times in the season and processed using green biorefinery technology to obtain press fibre, leaf protein concentrate (LPC) and brown juice fractions. The effect of different forms of selenium and their incorporation into the biorefinery fractions was investigated.

Objectives of the doctoral research:

- Depending on the chemical form and dose, what is the effect of selenium fortification on the growth of the perennial forage crop, alfalfa, and does it affect the rate and yield of extracted fractions following the green biorefinery steps?
- Which inorganic form and concentration of selenium is optimal from a crop production point of view and therefore from a utilization point of view?
- During fortification, in what species does inorganic selenium accumulate in the alfalfa, and how are these species distributed in the processed alfalfa fractions?
- How do the form and concentration of selenium used affect its incorporation into organic species in alfalfa?
- Does fortification of selenium affect the protein content and phytochemical composition of fractions/product candidates from alfalfa?
- Considering the results of the pot experiment, under field conditions in small plots, what are the more practically relevant results?

2. MATERIAL AND METHOD

2.1 Experimental setup and selenium treatments

Pot and field alfalfa experiments were set up to carry out selenium treatments (Table 1). In both cases, soil treatments were applied at 5-10 leaf stage, followed by 4 harvests in the pot experiment and 3 harvests in the field experiment from harvestable crops at the appropriate stage of development. In the pot experiment, the entire fresh green biomass was processed. In open field small plot experiments, 1 kg samples were collected three times from each plot and the biomass was processed by green biorefining.

Pot experiment				
Form	Control ø	Sodium selenate Se(VI)	Sodium selenite Se(IV)	Red elemental selenium Se(0)
Applied concentration (mg/kg)	0	1 10 50	1 10 50	- 10 50
Field experiment				
Form	Control ø	Sodium selenate Se(VI)	Sodium selenite Se(IV)	Red elemental selenium Se(0)
Applied concentration (mg/m^2)	0	5 50	5 50	50 100

Table 1: Selenium forms and applied concentrations tested in the alfalfa experiment.

2.2 Harvesting and processing of green biomass from pot and small plot experiments

After harvesting, the alfalfa in the green budding stage was processed for green biorefining, the steps of which are illustrated in Figure 1.

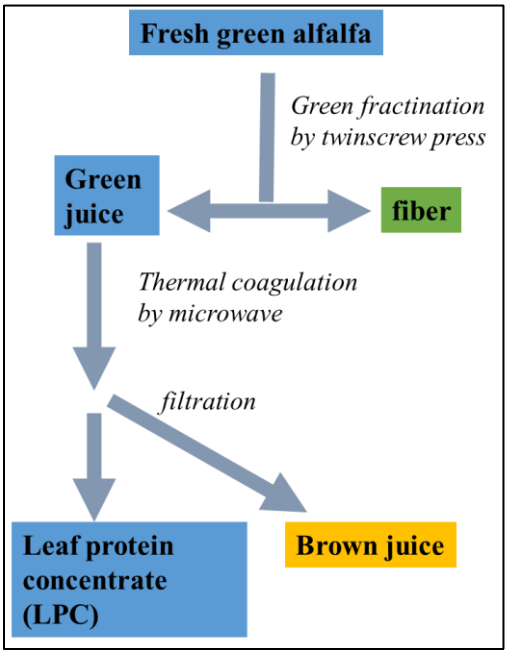

Figure 1: Flowchart of green biorefining.

The green juice from the pressed alfalfa was coagulated at 80±2 °C using a patented microwave heating technique (*Fári & Domokos-Szabolcsy*, 2018), resulting in leaf protein concentrate (LPC) and brown juice, which were separated by filtration through cotton textile (Figure 2).

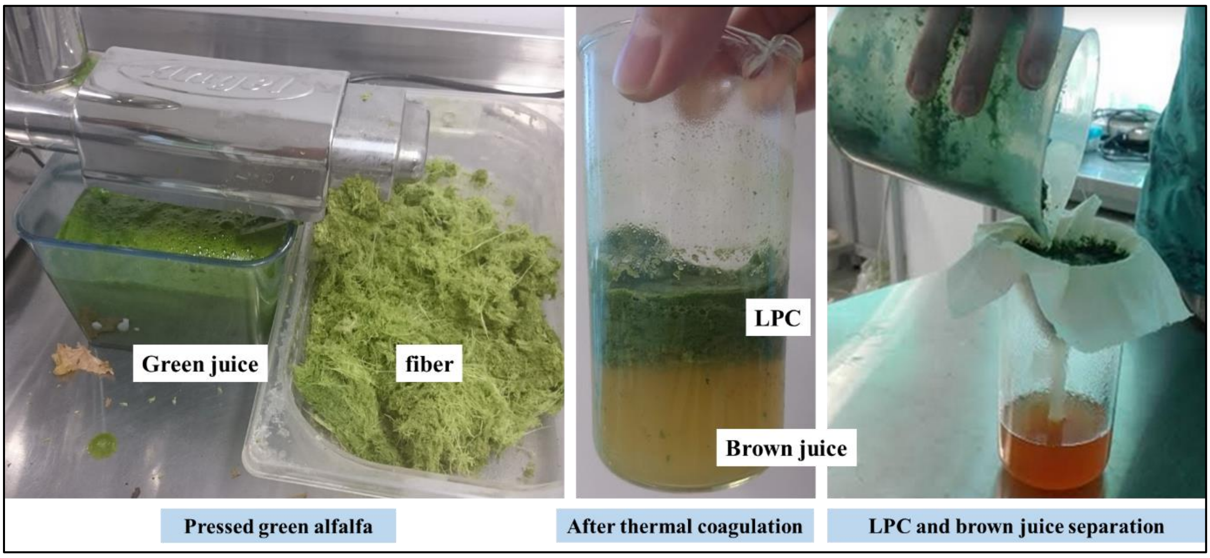

Figure 2: Green biorefinery process in pictures, with fractions at the end of the process, fibre, LPC and brown juice.

2.3 Determination of total selenium content

Nitric acid digestion was used to determine the total selenium content of complex plant samples. The total selenium content of the samples was determined using a hydride generation atomic fluorescence spectrometer (HG-AFS).

2.4 Selenium speciation measurements

For analytical studies of the speciation of selenium fortified alfalfa fractions, aqueous and enzymatic extracts were prepared according to *Dernovics et al*. (2002). Separation and measurement of selenium species were performed in an anion-exchange coupled chromatography system using a high-pressure liquid chromatograph-inductively coupled plasma mass spectrometer (HPLC-ICP-MS), and selenium isotopes with mass numbers ^{78}Se and ^{80}Se were detected.

2.5 Determination of crude protein and amino acid content

The crude protein content of selenium fortified alfalfa samples was determined by the Kjeldahl method. Measurements were performed under accredited conditions according to standard ISO 5983-2:2009. Protein constituent amino acids were measured by hydrochloric acid hydrolysis and ninhydrin derivatisation according to ISO 13903:2005.

2.6 Qualitative, quantitative determination of phytonutrients

For the phytonutrient components, methanolic extracts (70 (V/V)%) were prepared from the lyophilized powdered samples and liquid brown juice. The assays were performed on an ultra-high performance liquid chromatography-electrospray ionization mass spectrometry (UHPLC-ESI-MS) analytical system. Components were analysed and identified by software based on retention time and fragmentation pattern.

3. RESULTS

3.1 Harvestable biomass results of an open field alfalfa experiment

The amount of fresh biomass that can be harvested per square metre is illustrated in Figure 3. Most biomass was harvested in the first harvest after the treatments, with an average harvest of 1.9 kg/m^2. Subsequently, the second yielded 1.2; the third 1.3 kg/m^2 of harvestable fresh alfalfa biomass. Looking at selenium treatments within harvest, on average, the low concentration selenite and selenate (5Se(IV) and 5Se(VI)) treatments produced similar biomass results to the control alfalfa in all three harvests tested. However, at higher concentrations (50 g/m^2), treated plants yielded more fresh biomass per square meter than the control for both ionic forms. This positive difference was found to be highest at the first harvest, with the control value being 1899 g/m^2, 50Se(IV) 2015 g/m^2 and 50Se(VI) 2086 g/m^2.

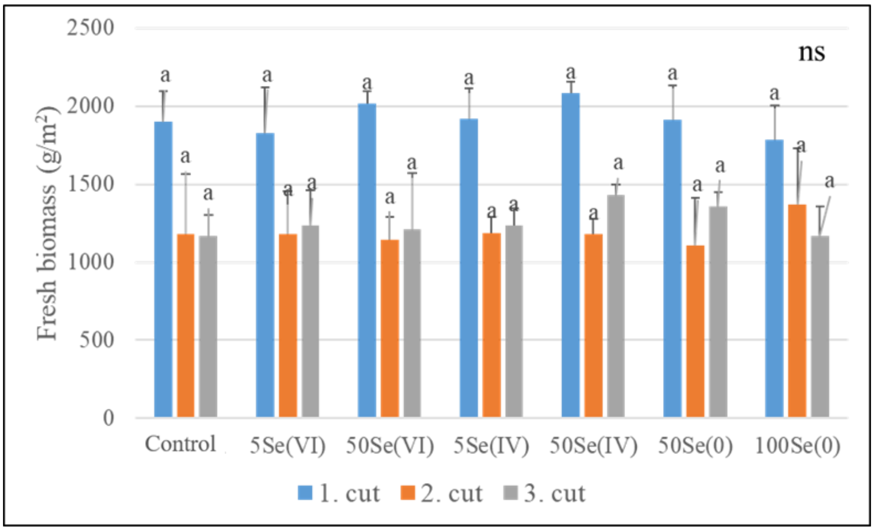

Figure 3: Amount of fresh green biomass harvestable from field plots, expressed in g/m^2, for three consecutive harvests.

3.2 Results of fortified alfalfa fractionation

For potted alfalfa, selenium treatment in several concentrations and forms did not affect the extraction rate of fibre and green juice fractions. Further, the proportion of LPC and brown juice fractions separated after microwave coagulation of proteins in green juice did not show any trend change with selenium treatments. On average, 40±3.6% fibre, 38±4.7% brown juice and 20±3.2% LPC were extracted in the 4 harvests studied. These rates agree with previous fractionation data from multiple replicates (*Kaszás et al*., 2020a; *Kaszás et al*., 2020b; *Domokos-Szabolcsy et al*., 2020; *Hansen et al*., 2022).

The relative proportions of alfalfa fractionations from field plot experiments are presented in Figure 4. We could not detect a correlation between selenium treatment and fractionation rate even for high concentration treatments (50, 100 g/m^2). However, the results obtained agree with the processing data of a previous field experiment comparing alfalfa varieties (*Kovács et al*., 2022), i.e. 30% of the press fibre, 17% of the LPC and 50% of the brown juice is produced at the end of the process. However, in the pot experiment the press fibre reached 40% compared to 30% in the open field experiment.

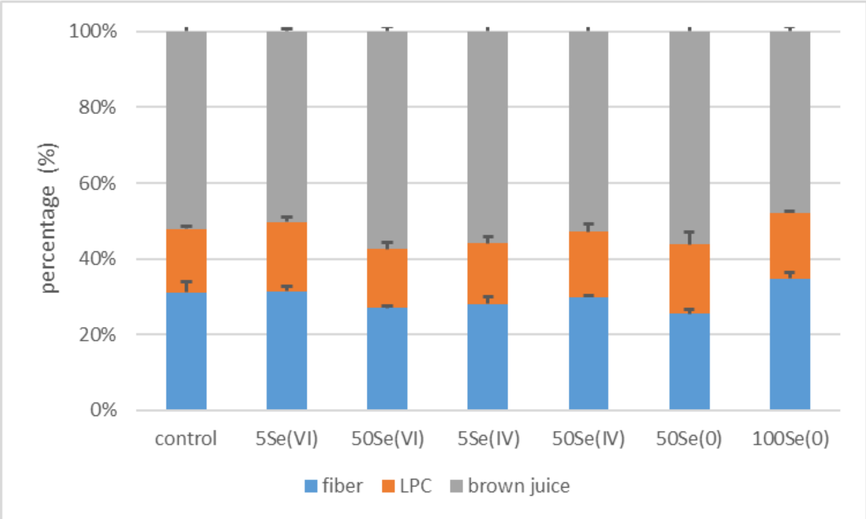

Figure 4: Percentage distribution of fractions from field alfalfa experiments at first harvest after selenium treatment.

3.3 Dry matter content of selenium in fortified alfalfa fractions

The dry matter content of the alfalfa grown in pot experiment varied between 16.5-31% over the four harvests, averaging 25.4%. Increasing the concentration of ionic Se forms significantly decreased the dry matter content of LPC, proportionally, the 10Se(VI) treatment LPC had 80% of the control and 50Se(IV) had 83%. The dry matter content of the fibre ranged from 35.2-51.5% in the treated and control alfalfa fractions. In the field experiment, both the fibre and LPC samples had a lower range of values compared to the pots experiment, which is attributed to the effect of lower doses of selenium treatments. The fibre had a dry matter content of 41-50% and LPC had a dry matter content of 23-27%. Selenium treatments had no clear negative effect on the dry matter content of fibre and LPC under field conditions.

3.4 Crude protein content and amino acid composition in selenium fortified alfalfa fractions

Coagulation of proteins in green juice resulted in the highest crude protein content of the LPC fractions (36.3±4.1 m/m%) followed by the fibre fractions (10.5±1.4 m/m%) and brown juice (1.1±0.3 m/m%). With respect to treatments, the highest crude protein content was measured in the control LPC, except for the third harvest. High concentrations of ionic forms, i.e. 10Se(VI) and 50Se(IV), caused a decrease in crude protein content of LPC as a characteristic of toxic selenium levels (*Kolbert et al.*, 2019). Protein content of pressed fibre showed a similar trend as LPC. The lowest values were 8.47 and 10.16 (m/m)% in the fourth harvest (Figure 5). The nitrogen content of the brown liquor varied between 0.58 and 1.59 (m/m)% (Figure 5). Compared to the LPC and fibre fractions, the low variance of brown juice means that the values do not show a strong trend related to selenium treatment.

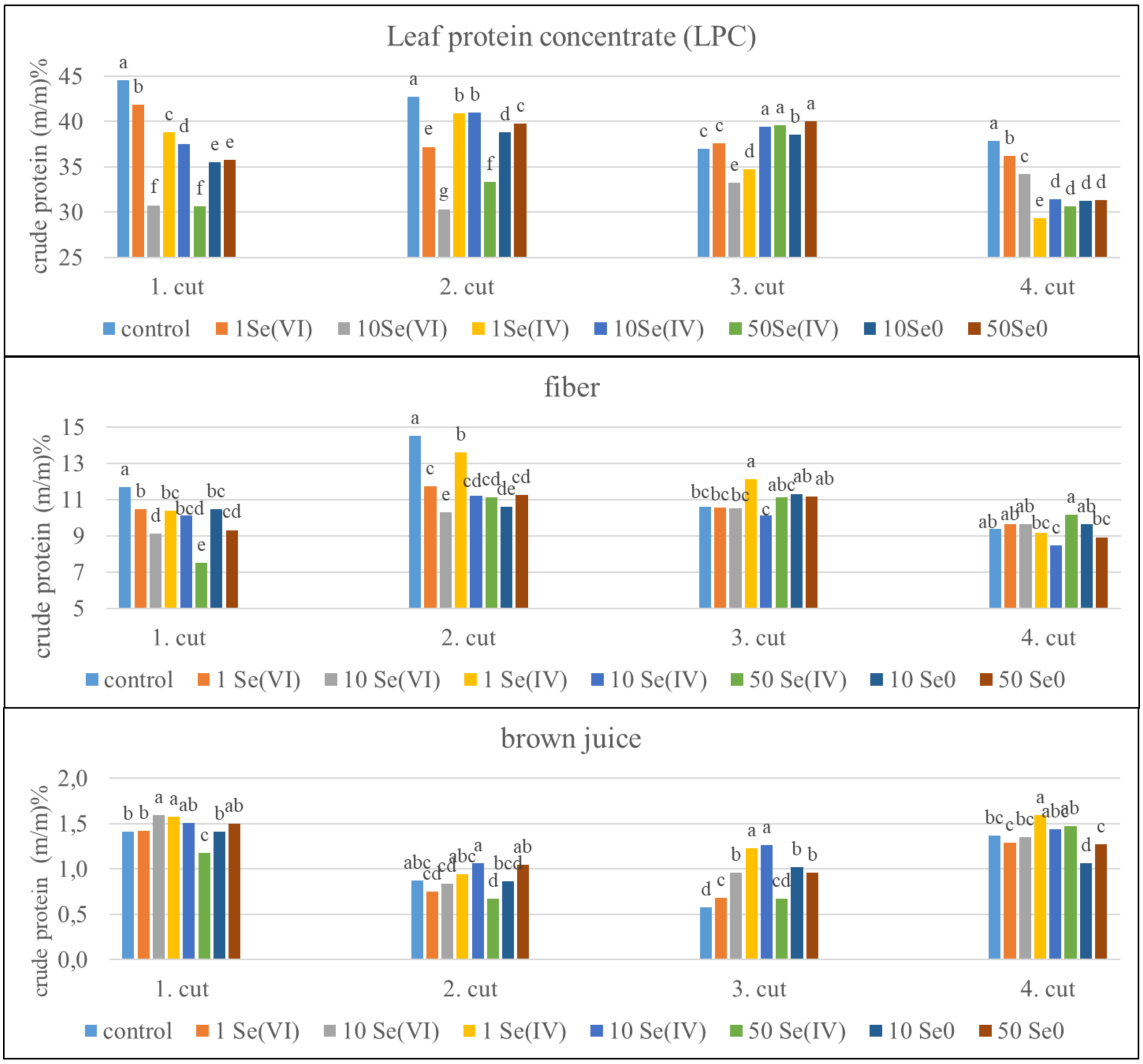

Figure 5: Results of the crude protein content of the LPC, fibre and brown juice fractions of the pot experiment for four consecutive harvests. Statistically significant differences between mean values (n=3) are indicated by the opposite letters above the columns ($p \leq 0.05$).

A negative correlation between selenium content and crude protein content was observed for treatments with high concentrations (10Se(VI), 50Se(IV), 10Se0) during harvest. At the first harvest, when the highest values of selenium content were measured, crude protein content was found to be low (Figure 6).

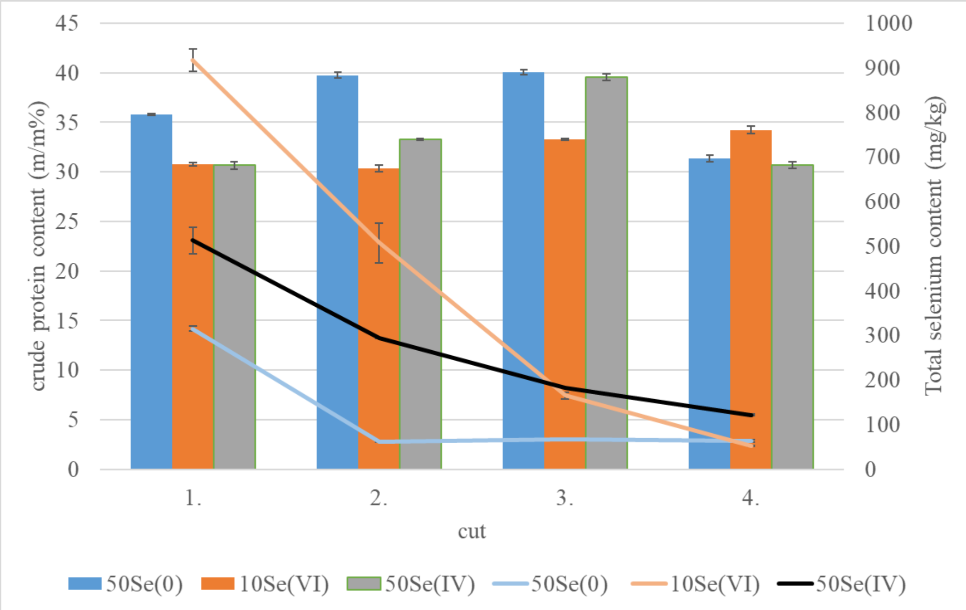

Figure 6: Correlations between crude protein content of LPC and total selenium content in the four harvests, only for the treatments with the highest concentrations of 50 mg/kg Se(0), 10 mg/kg selenate and 50 mg/kg selenite.

In the open field selenium fortification alfalfa experiment, three consecutive harvests were conducted after selenium treatments. Considering the average values for the sample types, similar results were obtained in the field and pot experiments, with the highest crude protein content of LPC being 38-39% in the field experiment (Figure 7.). In contrast to the pot experiment, lower doses were applied under field conditions. In this context, we did not observe such a large difference in the variation of protein content between treatments.

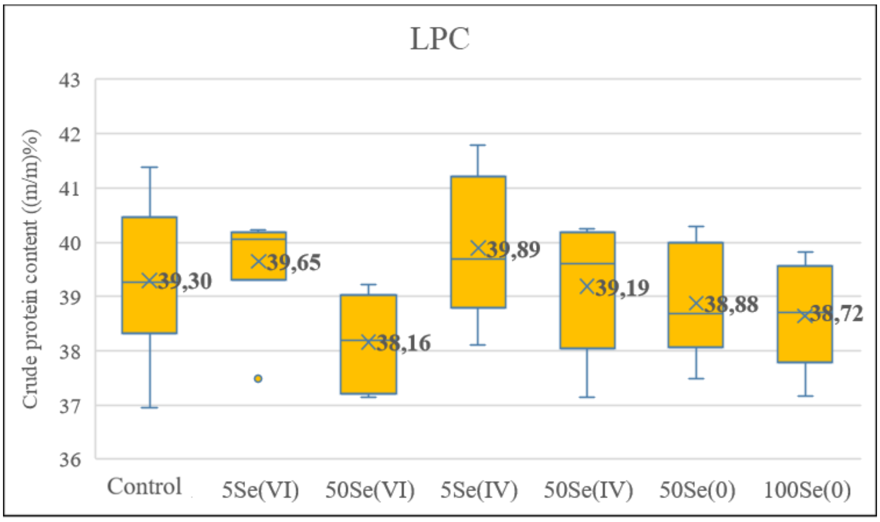

Figure 7: Crude protein content results from the LPC fraction, averaged over three consecutive harvests.

Proteinogenic amino acids were determined from the LPC and fibre fractions of 1. and 4. harvests. A decrease in the sum of amino acids was observed. However, 10Se(VI) and 50Se(IV) treatments increased the amount of amino acids in fibre and LPC samples of harvest 4. However, to different extents, the increase in amino acids was greater in the LPC for the 10Se(VI) treatment and in the fibre sample for the 50Se(IV) treatment.

3.5 Results of selenium content and selenium speciation measurements

The selenium content of the green biorefinery fractions, LPC, fibre and brown juice was determined in all four harvests. In addition, the selenium content of the fresh shoots used as raw material for green fractionation was also measured, separated into leaves and stems. The selenium treatment was applied once, after seed sowing, so that the plants took up the different forms of selenium applied from the soil during the growing period studied. The results of the four consecutive harvests show the dynamics of the uptake of the 3 tested selenium forms (Se(VI), Se(IV), Se0). When fresh shoots were examined, leaves tended to accumulate more selenium than stems for all selenium forms and concentrations. In the first harvest, 10 mg/kg selenate (Se(VI)) treatment resulted in 6.5 times more selenium in leaves (211.1±3.5 mg/kg) than in stems (32.1±4.1 mg/kg). In the case of 50 mg/kg selenite (Se(IV)) treatment, the selenium content was also high in both fractions (148.9±2.4 in leaves and 59.4±7.2 mg/kg in stems), but here the difference between them was only 2.5-fold (Fig. 8.). Typically, lower concentrations were measured in plants treated with red elemental selenium (10Se(0), 50Se(0)). When looking at the two ionic forms, the selenium content of the two treatments steadily decreased over time by the end of the 4. harvests. The red Se(0) application showed lower Se uptake compared to the Se(VI) and Se(IV) ionic forms; however, we observed less variability in the change in total Se content between the four harvests.

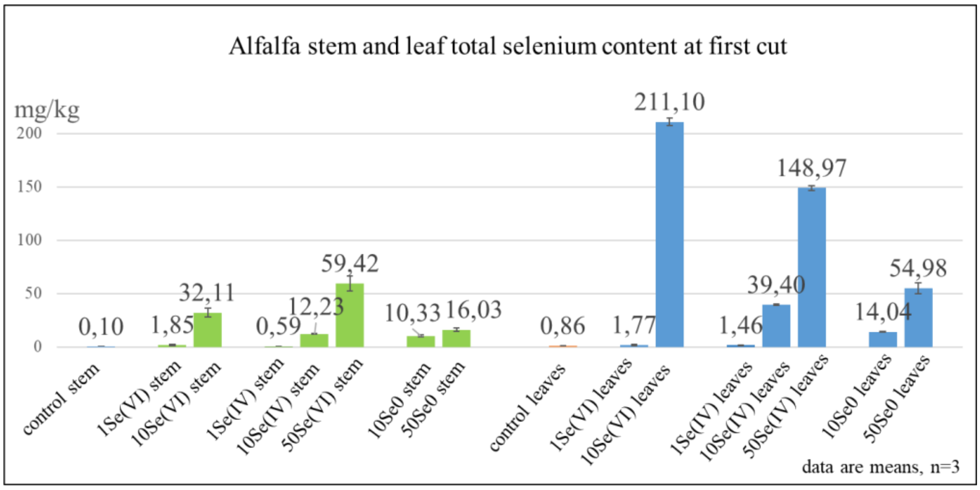

Figure 8.: Total selenium content of alfalfa leaf and stem samples at first harvest, 20 days after selenium treatment.

When comparing the selenium content of the processed alfalfa fractions, the highest values were measured in the LPC fractions followed by the fibre and brown juice fractions (Table 2.). The selenium content of fibre was approximately a quarter of the concentrations measured in LFK at all harvests. The selenium content of brown leaves ranged from 0.33 to 120.5 mg/l depending on the treatment and harvests, generally one tenth of the LFK from the same treatment.

Table 2.: Total selenium content of selenium fortified alfalfa products from green fractionation. Significant ($p \leq 0.05$) difference (Tukey's test) between the values indexed with different upper index letters within a harvest and sample type.

Total selenium content

Leaf protein concentrate (LPC) [μg/g]				
Harvest	**1.**	**2.**	**3.**	**4.**
0Se	0.7±0.04 ^{g}	1.1±0.13 ^{h}	0.8±0.02 ^{g}	0.7±0.01 ^{h}
1Se(VI)	93.0±1.95 ^{d}	46.7±0.45 ^{e}	11.4±0.18 ^{f}	6.6±0.01 ^{g}
10Se(VI)	916.7±24.66 ^{a}	507.4±31.79 ^{a}	165.1±6.66 ^{b}	52.7±0.41 ^{c}
1Se(IV)	18.6±0.33 ^{f}	12.6±0.79 ^{g}	4.0±0.05 ^{g}	9.3±0.18 ^{f}
10Se(IV)	92.3±3.58 ^{d}	64.9±0.20 ^{c}	29.0±0.04 ^{e}	34.4±0.40 ^{e}
50Se(IV)	512.7±29.06 ^{b}	294.1±0.36 ^{b}	183.3±0.06 ^{a}	122.1±2.46 ^{a}
10Se0	38.8±0.34 ^{e}	29.1±0.45 ^{f}	44.7±0.98 ^{d}	36.6±0.16 ^{d}
50Se0	126.3±5.26 ^{c}	62.1±1.67 ^{d}	66.6±2.20 ^{c}	63.8±3.20 ^{b}

Fiber [μg/g]				
Harvest	**1.**	**2.**	**3.**	**4.**
0Se	0.9±0.04 ^{h}	1.0±0.27 ^{h}	0.9±0.10 ^{h}	0.8±0.03 ^{h}
1Se(VI)	41.6±4.55 ^{d}	8.7±0.01 ^{e}	5.2±0.55 ^{f}	2.0±0.37 ^{g}
10Se(VI)	343.4±4.80 ^{a}	147.1±0.43 ^{a}	37.8±0.07 ^{b}	13.7±0.02 ^{c}
1Se(IV)	7.2±0.13 ^{g}	3.3±0.19 ^{g}	1.7±0.05 ^{g}	3.2±0.09 ^{f}
10Se(IV)	52.7±1.69 ^{c}	14.0±0.21 ^{d}	13.3±0.29 ^{d}	7.9±0.02 ^{e}
50Se(IV)	171.0±1.86 ^{b}	89.4±0.59 ^{b}	45.1±0.05 ^{a}	32.3±0.27 ^{a}
10Se0	10.6±0.18 ^{f}	7.8±0.04 ^{f}	10.5±0.95 ^{e}	11.5±0.03 ^{d}
50Se0	34.4±0.14 ^{e}	19.4±0.19 ^{c}	17.0±0.15 ^{c}	20.3±2.44 ^{b}

Brown juice [μg/ml]				
Harvest	**1.**	**2.**	**3.**	**4.**
0Se	0.2±0.01 ^{e}	0.2±0.02 ^{h}	0.1±0.00 ^{g}	0.1±0.01 ^{g}
1Se(VI)	17.8±0.02 ^{c}	2.6±0.02 ^{e}	0.6±0.00 ^{f}	0.3±0.07 ^{g}
10Se(VI)	120.5±7.95 ^{a}	62.0±0.07 ^{a}	10.6±0.31 ^{b}	3.9±0.04 ^{c}
1Se(IV)	2.1±0.01 ^{d}	0.9±0.06 ^{g}	0.5±0.23 ^{f}	0.7±0.02 ^{f}
10Se(IV)	18.3±0.13 ^{c}	7.2±0.05 ^{c}	1.6±0.03 ^{e}	2.2±0.25 ^{e}
50Se(IV)	78.5±1.29 ^{b}	34.2±0.23 ^{b}	11.6±0.03 ^{a}	9.1±0.04 ^{a}
10Se0	2.7±0.01 ^{d}	2.1±0.02 ^{f}	3.1±0.00 ^{d}	2.5±0.25 ^{d}
50Se0	17.7±0.23 ^{c}	6.6±0.05 ^{d}	3.8±0.02 ^{e}	4.9±0.30 ^{b}

means ± SD (n=3)

The chemical form applied strongly influenced the amount of selenium building up in the fractions. At the first harvest, the selenium content in LPC was 916.6 mg/kg (the highest value in LPCs) when treated with 10Se(VI), whereas when treated with 10 mg/kg selenite (10Se(IV)), only 92.3 mg/kg and with red elemental selenium (10 mg/l Se0), 38.7 mg/kg of selenium were present in LPC (Table 2.). The greatest reduction, about 17^{th}, was found with the 10Se(VI) treatment: 919.9 mg/kg at the first harvest, 507.4 mg/kg at the second, 165.1 mg/kg at the third and 52.7 mg/kg at the last fourth harvest (Table 8). In contrast, the application of red Se(0), regardless of its concentration, resulted in a minor reduction in the selenium content of LPC. When applied at the same concentration, selenate (Se(VI)) was taken up by the plants in higher amounts than selenite (Se(IV)).

Considering the high total selenium concentrations obtained at the selenium doses applied in the pot experiment, lower doses were designed to optimize the agronomic fortification of alfalfa under field conditions. Biomass yield and crude protein results indicated that these treatments did not cause inhibitory, negative stress to the plant or toxic accumulation.

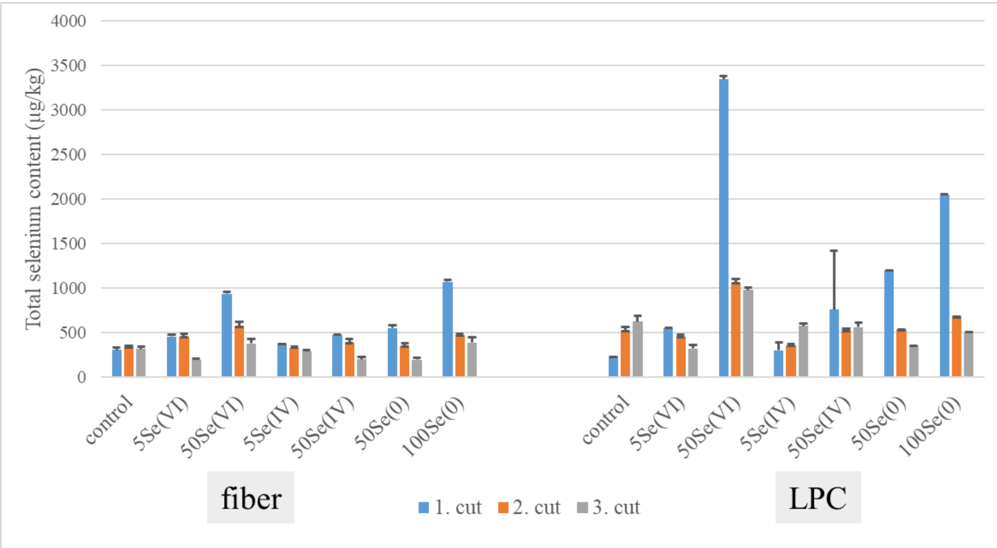

Figure 9: Results of total selenium content in fibre and LFK samples of alfalfa from three consecutive harvests.

In terms of selenium, the trend is similar between the fractions, i.e. brown alfalfa contains the least amount, followed by fibre, and LPC contains the most selenium, as was observed in the pot experiment. Figure 9 illustrates the total selenium content of the fibre and LPC samples, and the trend over the three consecutive harvests, i.e. a proportional decrease in the amount of selenium taken up and incorporated with time from treatment, can be traced in the values obtained, while no outliers were observed in most treatments. Exceptions to this were the 50 mg/m2 Se(VI) and 100 mg/m2 Se(0) treatments, which gave the highest selenium content results at first harvest for both fibre and LPC samples. For LPC, these values were significantly higher, 3349 µg/kg for the 50Se(VI) treatment and 4818 µg/kg for 100Se(0) (Figure 9). For brown juice, the highest values were also found in the first harvest samples of these two treatments, although the selenium content of brown juice from the field experiment is relatively low. The high values of 50Se(VI) in the first harvest can be explained by similar trends observed in the pot experiment.

As observed for selenium, higher values were measured in leaves compared to stems in the speciation measurements (Table 3). The general trend was that the inorganic form of selenate was dominant in the aqueous extracts, although selenomethionine (SeMet) was also found in the aqueous extracts, the predominant amount was only measured after enzymatic digestion. A large decrease in selenate content was found between the first harvest and the last harvest, specifically for the 10Se(VI) treatment, correlating with the results for total selenium content. In the case of stem, the selenate content in the aqueous extracts decreased from 79.5 to 4.3 mg/kg and from 901 to 10.7 mg/kg in leaf samples. It is an interesting result that even when selenite was applied at a concentration of 50 mg/kg Se(IV), no selenite could be detected in the samples tested.

Table 3: SeMet and Se(VI) content of aqueous and enzymatic extracts from alfalfa stem and leaf samples (*Kovács et al*., 2021).

Contents of selenomethionine (SeMet) and selenate (Se(VI)) in alfalfa stem and leaves													
	Water extracts												[mg/kg]
	stem						**leaves**						
	SeMet			**Se (VI)**			**SeMet**			**Se (VI)**			
Cut	**1.**	**2.**	**4.**	**1.**	**2.**	**4.**	**1.**	**2.**	**4.**	**1.**	**2.**	**4.**	**Cut**
10Se(VI)	2,2	0,9	0,5	79,5	32,3	4,3	8,2	1,5	1,2	901	141	10,7	**10Se(VI)**
50Se(IV)	1,7	1,4	1,1	89	22,4	5,2	2,3	1,9	4,2	336	65,8	20,5	**50Se(IV)**
50Se0	0,6	0,3	0,5	6,5	0,7	2,7	1,8	0,6	1,4	51,7	3,9	1,5	**50Se0**
	Enzyme extracts												[mg/kg]
	stem						**leaves**						
	SeMet			**Se (VI)**			**SeMet**			**Se (VI)**			
Cut	**1.**	**2.**	**4.**	**1.**	**2.**	**4.**	**1.**	**2.**	**4.**	**1.**	**2.**	**4.**	**Cut**
10Se(VI)	18,1	14,3	4,8	8,1	6	0,8	71,4	41	24,8	86,8	18,1	2,6	**10Se(VI)**
50Se(IV)	17,7	12,3	8,5	13	5,2	0,8	56,9	34,9	22,4	30,8	12,9	2,9	**50Se(IV)**
50Se0	7,1	4,1	11,9	1,1	*nd	nd	20,2	13,1	7,6	5,4	0,6	nd	**50Se0**

*non detected (>LOD)

The two most abundant forms of selenium found were inorganic selenate (SeO_4^{2-}) and organic selenomethionine (SeMet) in all fractions (LPC, fibre, brown juice) and extracts (water and enzymatic extracts).

The amount of SeMet varied between 0-246 mg/kg in the LPC samples (Table 4). The highest amount of selenate was found in the first harvest 10Se(VI) treated LPC sample, 451 mg/kg dry weight. The inorganic selenate form followed the same trend as the total selenium content for all sample types tested (LPC, fibre, brown juice).

Table 4: SeMet and selenate (SeVI) content of water and enzymatic extracts from LPC, fibre and brown juice samples (*Kovács et al*., 2023).

Selenomethionin (SeMet) and selenate Se(VI) content in leaf protein concentrate (LPC), fiber and brown juice

	LPC [mg/kg]						**fiber** [mg/kg]						**brown juice** [µg/ml]						
	SeMet			*Se(VI)*			*SeMet*			*Se(VI)*			*SeMet*			*Se(VI)*			
cut	1.	2.	4.	1.	2.	4.	1.	2.	4.	1.	2.	4.	1.	2.	4.	1.	2.	4.	**cut**
							Water extract												
10Se(VI)	1	nd*	nd	451	181	12	1,9	0,6	nd	237	68	2,6	0,3	0,1	0	111	52	2,5	**10Se(VI)**
50Se(IV)	nd	nd	nd	241	71	16	0,7	0,7	nd	98	34	3,6	0,1	0	0,2	74	16	5,5	**50Se(IV)**
50Se0	nd	nd	nd	41	3,9	6,2	nd	nd	nd	17	2,1	1,4	0,1	0	0	12	3,5	2,5	**50Se0**
							Enzyme extract												
10Se(VI)	246	164	38	30	14	0,7	48	27	6,5	29	13	nd	0,1	0,1	nd	3,5	0,7	0,1	**10Se(VI)**
50Se(IV)	158	128	68	13	4,6	1,9	32	22	15	18	6,9	1,2	0,1	0	0,1	2,4	0,5	0,2	**50Se(IV)**
50Se0	61	46	36	4,5	nd	nd	7,5	7,4	9	1,6	nd	nd	nd	nd	0,1	0,2	0,3	0,5	**50Se0**

* non detected (<LOD)

The SeMet ratio is important information, as the aim of fortification is to produce value-added organic selenium-rich product candidates/fractionations from alfalfa. The amount of SeMet in the enzymatically digested samples is higher in all cases tested, suggesting that the organic form of SeMet is incorporated into proteins in the alfalfa fractions. An inverse relationship in the distribution of SeMet was found when examining the SeMet content of the high protein LPC and lower fibre samples. Although, by fourth harvest, the total selenium content and the amount of selenate form decreased in the samples tested, especially for the ionic form treatments (10Se(VI), 50Se(IV)). However, the proportion of SeMet showed an increase. At the first harvest, 18% of the selenium content of the sample was SeMet in the fibre and 35% in the LPC. At the fourth harvest this increased to 45% in fibre and 60-70% in LPC (Figure 10).

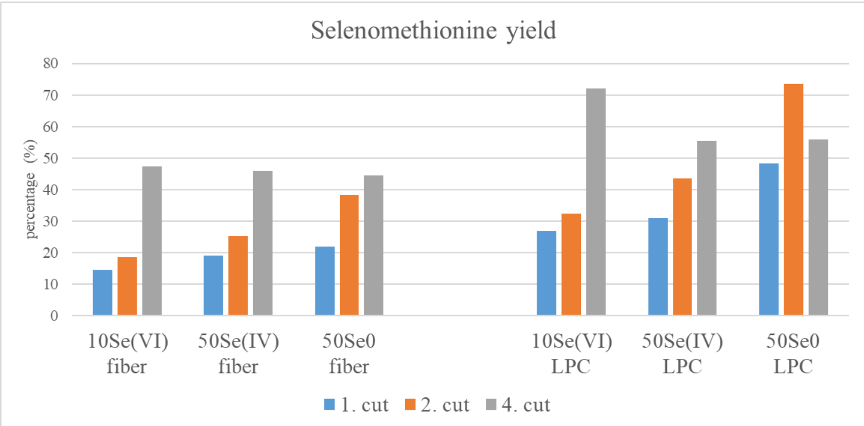

Figure 10: SeMet yield as a percentage of total selenium content. In the two fractions of LPC and fibre relevant for selenium and SeMet content in the first, second and fourth harvests.

3.6 Phytonutrients and phenolic components in selenium fortified alfalfa fractions

Overall, the profile of the phytochemical components was similar in the studied fractions. Consistent with literature sources (*Stochmal et al*., 2001a; *Stochmal et al*., 2001b), flavonoids were the most abundant phenolic components in all hydroalcoholic extracts (Figure 11). Of the 37 flavonoids identified, aglycones and glycosyl derivatives were found with flavanone, flavonol, flavone, flavone, isoflavone and chalcone skeletons. The most typical backbones were apigenin, tricin, chrysoeriol. The sugar chains of the flavonoids were not exclusively glucuronic acid; in apigenin, naringenin and quercetin, glucose and xylose were also found. The other large group was the saponins, of which several compounds with unknown backbone were found, but also medicozide, medicagenic acid, azucisaponin and soy sapogenol were found and previously identified (*Rafińska et al*., 2017).

Among the pterocarpans, medicarpin (3-hydroxy-9-methoxy-methoxyterocarpane) ([M+H]+ ion m/z 271,09704) and methylnissoline (3-hydroxy-9,10-dimethoxyterocarpane) ([M+H]+ ion m/z 301,1076) were detectable in all alfalfa fractions.

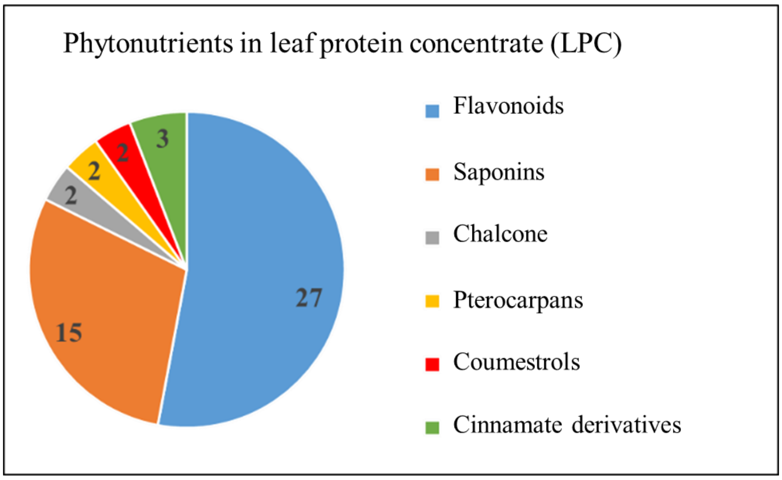

Figure 11: phytonutrients identified in the LPC samples separated into groups of compounds.

Based on the standards available from the qualitative test results, the amount of some phytocomponents with positive or negative physiological roles was also quantified per fraction as a function of selenium treatments. Quantitative analysis of the hydroalcoholic extracts revealed that apigenin glucuronide and apigenin (4'.5.7-trihydroxyflavone) aglycones were the most abundant flavonoids within the four harvests. The fibre fraction had the highest concentration of apigenin-7-O-glucuronide, ranging from 90.82 to 170.85 µg/g, while the LPC and brown juice had concentrations ranging from 33.31 to 68.10 µg/g and 376.13 to 898.88 ng/ml, respectively. Apigenin was the second most abundant flavonoid among the flavonoids selected for quantification, with values ranging from 7.97 to 72.93 µg/g. In contrast to apigenin-7-O-glucuronide, apigenin varied in the LPC and fibre fractions within a narrow range of 13-24 µg/g, except at fourth harvest, when an average of 67 µg/g was measured in the fibre fraction, irrespective of Se treatment.

The form and concentration of Se treatment influenced the apigenin (4'.5.7-trihydroxyflavone) and apigenin-7-O-glucuronide content in the fractions studied. Both flavone concentrations increased with Se(IV) and Se(VI) treatments, especially at first harvest, while red elemental selenium (Se(0)) showed lower values compared to the control, regardless of concentration. The other hydroxyflavones, luteolin (3'.4'.5.7-tetrahydroxyflavone) and its derivatives, luteolin-7-O-glucuronide, luteolin-di-O-glucuronide, luteolin-4'-O-glucuronide-7-O-[feruloyl(1→2)-glucuronyl(1→2)-glucuronide] were detectable in brown juice; however, some of them were absent in the fibre and LPC fractions. The concentration of luteolin aglycone was significantly lower (0.71 - 5.10 μg/g) than that of apigenin in all alfalfa fractions. Tricin (3',5'-Dimethoxy-4',5,5,7-trihydroxyflavone) was present at an average concentration of 14.5 μg/g in LPC and fibre samples and ~160 ng/ml in brown juice. Among the isoflavones, four compounds were identified, of which formononetin (7-hydroxy-4'-methoxy-4'-isoflavone) was found to be present in concentrations ranging from 0.34-8.39 μg/g in the LPC and fibre fractions and from 3.16-80.04 ng/ml in the brown juice. The 1 mg/kg Se(IV) and 10 mg/l red elemental Se(0) treatments showed formononetin contents similar to the control. In contrast, high concentration treatment of Se(VI), Se(IV) and red elemental Se0 resulted in lower formononetin content in the three fractions. Regardless of Se treatment, mediagenic acid, and its derivatives with short or long mono-, bi- or tri-desmosidic sugar chains were the most abundant saponins in all alfalfa fractions. The concentration of mediagenic acid ranged from 0.11 to 1.86 μg/g in the LPC and fibre fractions and from 0.72 to 32.72 ng/ml in the brown juice during four consecutive harvests of alfalfa.

4. NEW SCIENTIFIC RESULTS OF THE THESIS

1. Our results suggest that red elemental selenium sol can be successfully used for selenium enrichment of multiple harvested alfalfa (*Medicago sativa* L.).

2. Our results show that under closed culture conditions, the quantitative ratios of the fractionated products (leaf protein concentrate (LPC), fibre, brown juice) of alfalfa (*Medicago sativa* L.) obtained by green biorefining and microwave coagulation are not affected by selenium enrichment. However, in terms of dry matter, crude protein content, the increased selenium dose (10 mg/kg selenate and 50 mg/kg selenite) reduced the values of leaf protein concentrate and fibre fractions in a statistically detectable manner.

3. Among the LPC, fibre and brown juice fractions of alfalfa (*Medicago sativa* L.), a protein crop commonly used in green biorefining, LPC was the one with the highest accumulation of selenium regardless of the form of selenium applied (selenate, selenite, red elemental selenium) and the applied concentration.

4. We found the highest incorporation of organic selenium (in the form of selenomethionine) in LPC and fibre fractions in plants treated with red elemental selenium. When treated with high doses of ionic forms (10 mg/kg selenate and 50 mg/kg selenite), the amount of incorporated organic forms such as selenomethionine in alfalfa (*Medicago sativa* L.) shoots did not increase proportionally.

5. For the selenium enrichment of LPC as an alternative forage protein, red elemental selenium results in a more balanced selenium accumulation compared to ionic forms (selenate, selenite) over consecutive multiple harvests.

6. Qualitative and quantitative analysis of phytonutrients demonstrated that selenium did not cause qualitative or significant quantitative changes in the secondary metabolite composition of alfalfa derived fractions (LPC, fibre, brown juice) over the concentration range and experimental settings used.

5. THE PRACTICAL USE OF THE RESULTS

In this alfalfa selenium fortification experiment, the selenium content of harvestable green alfalfa shoots was successfully increased by soil treatment. The experiment with different concentration of selenate, selenite and red elemental selenium gave us practical results for the agronomic selenium fortification of perennial crop as alfalfa on chernozem soil with calcareous loam.

Further technological development of chemically synthesized red elemental selenium sol can be used to produce long-acting selenium fertilizer, which can provide safer selenium fertilization in crop production. The slow conversion of red elemental selenium to ionic forms in the soil can provide a constant and low supply of selenium while avoiding toxic accumulation.

Based on this results, alfalfa-based green biorefineries can produce selenium fortified leaf protein concentrate (LPC) with yields like conventional LPC.

1. The basic fractionation step sequence known in green biorefining can be used in combination with agronomic selenium enrichment without significant changes in the proportions of the fractions.

2. It has been experimentally demonstrated that selenite (1 - 50 mg/kg), selenate (1 - 10 mg/kg), and red elemental selenium in the range of 10 - 50 mg/kg do not affect the yield of alfalfa leaf protein concentrate (LPC).

3. When comparing the processed fractions of alfalfa, the leaf protein concentrate (LPC) showed the highest accumulation of selenium. As a feed protein alternative for selenium fortification, the red elemental selenium was found to be more suitable for fortification, because it results in a more balanced selenium accumulation compared to ionic selenium forms. Significantly for practical use, the LPC in this way is therefore not only valuable as a source of feed protein but can also be enriched with more biologically beneficial organic forms of selenium.

4. The chemically synthesized red elemental selenium can be further developed to produce a long-acting selenium fertilizer, which can provide safer selenium fertilization in cultivation. The slow conversion of red elemental selenium into ionic forms in the soil can provide a constant and low supply of selenium while avoiding toxic accumulation.

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7. LIST OF PUBLICATIONS

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List of publications related to the dissertation

Foreign language scientific articles in international journals (2)

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DOI: https://doi.org/10.1016/j.nbt.2018.05.978

Total IF of journals (all publications): 27,385
Total IF of journals (publications related to the dissertation): 9,651

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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