Ph. D. thesis

PRODUCTION OF NANO-SIZE ELEMENTAL SELENIUM PARTICLES AND ITS INVESTIGATION IN THE SOIL-PLANT-ANIMAL SYSTEM

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1. BACKGROUND AND AIM OF THE PH.D. STUDY

Selenium (Se), an essential micronutrient with antioxidant effects, is an integral part of the proper and healthy functioning of humans, animals, archeas and other organisms. It occurs in rocks, soils, waters, but it has an uneven geographic distribution, even within the same country, depending on the natural substrates, climate and flora (*Hartill, 2004*). The low selenium content of soils in many parts of the world, such as in Hungary (*Bogye et al, 1998*), can cause serious health problems if supplementation is not used (*Reilly, 1998*).

After Schwartz and Foltz proved in 1957 that selenium is an essential micronutrient, the increased selenium research led to the recognition of its many health benefits, such as improving the mobility of spermiums, slowing the progression of AIDS (*Rayman, 2000*), preventing certain types of cancer (*Ip és Ganther 1992*), or its role in the immune system. Hypothyroidism, fatigue, obesity, infertility or serious diseases such as Keshan disease and Kashin-Beck disease may develop due to selenium deficiency.

Selenium is also known as "the essential poison" due to the very close necessary and toxic concentration limits (*Reilly, 2006*). It is no surprise that in selenium rich areas (eg. certain parts of China, Brasil) symptoms of selenosis can occur, such as garlic odor of breath, hair loss, sloughing of nails, fatigue. In nature selenium can be found in various species with significantly different availability and toxicity. Besides selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}) salts, organic selenium forms and selenides (Se^{2-}), elemental selenium occurring in sedimentary rocks in reduced, anaerob conditions (e. g. Keshan province, China) has lower toxicity than the other selenium forms (*Wang et al, 2007*). Lactic acid bacteria can also convert selenite ions to red elemental selenium as part of their defence mechanism, and store them as nanospheres inside their cell.

Due to these reasons selenium is one of the most investigated element nowadays, and its research have advanced with many new approaches and insights into the molecular, genetic, biochemical and health areas (*De Filippis, 2010*). Selenium supplementation also became important, selenium deficient countries looked for new methods besides selenium enriched fertilizers (eg. Finland), and it did not take long for selenium enriched medications and supplements to appear, which contain mainly seleniomethionin produced by yeast. The next logical step was to produce selenium enriched functional foods, such as yogurt, egg or onion.

The aims of my study were the following:

- Develop a method to extract the nano-size red elemental particles produced by lactobacteria, and produce a purified sol suitable for laboratory experiments (NanoSel)
- Modify the method to produce selenium enriched yogurt powder and optimize it for industrial production (LactoMicroSel)
- Investigation of the produced nanoselenium in the soil-plant-animal system to examine its bioavailability and toxicity
- Develop a theoretical model that explains the behavior of nanoselenium in aqeous media

2. MATERIALS AND METHODS

My research can be divided into 5 parts:

1. Production and investigation of a purified nanoselenium sol, which contains nano-size elemental selenium particles (NanoSel)
2. Production of selenium enriched yoghurt powder, which contains nano-size elemental selenium particles (LactoMicroSel)
3. Investigation of nanoselenium in soils
4. Investigation of nanoselenium in plants
5. Investigation of nanoselenium in animals

2.1. Production and investigation of nano-sized elemental selenium particles

Our primary goal was the development of a purified elemental selenium sol (NanoSel) for experimental use, and to modify and optimize the method for industrial production of a selenium-enriched yoghurt powder (LactoMicroSel) which can be used as a food and feed supplement. Although the developed production methods are part of our results, we present them in the Materials and Methods chapter in the interest of clarity and rationality.

2.1.1. Production of the NanoSel monodisperse selenium

The purified elemental selenium sol, called NanoSel is primarily used in laboratory experiments to investigate the behavior of bacteria-produced nano-size red elemental selenium particles, not as feed or food supplementation. We use 1 liter sterilized MRS broth as culture media *(de Man, Rogosa, Sharpe, 1960)* enriched with 10 ml sodium-hydrogen-selenite stock solution of 10.000 mg/l selenium content to produce the standard 200 mg/l elemental selenium sol. After inoculation with *Lactobacteria casei*, which was chosen due to its conversion efficiency, use in food industry and the size of the produced selenium particles,

we fermented the mixture at 37°C for 48 hours with 70 rpm shaking. During the fermentation the sol turns into red color due to the produced elemental selenium spheres. The first step of recovering these particles is the digestion of the cell wall, done by hydrolysis with 37 %(m/m) hydrochloric acid for 5 days at room temperature. Then, after repeated centrifuging at 6000 rpm and washing with distilled water, the final step is removing the cell fragments by filtering through 2 layers of 65g/m^2 density filtering paper and 1 layer of 3.3 μm pore-size teflon filter. The obtained NanoSel sol is showing the qualities of nanoparticle-containing suspensions as its color changes depending on the location of the light source, and it scatters laser light (Fig. 1).

Figure 1. NanoSel sol in direct light, backlight, and in laser light

2.1.2. Production of LactoMicroSel selenium enriched yoghurt powder

If we use the production process presented for NanoSel, but we do not extract the elemental selenium spheres from the bacterial cells, and use milk for culture media instead of MRS broth, we can produce selenium enriched yoghurt that can be used dried and ground as food or feed supplement. This product was named LactoMicroSel.

When deciding the culture media for the production of selenium supplementation it was necessary to choose something which is permitted to use in food production and is more economical than the MRS broth used for NanoSel. First, we chose milk that worked fine, but due to economic reasons the final decision was to use a mixture of 75% whey and 25% skimmed milk as culture media.

The choice of bacterial strain was based on its conversion ability, permission to use in food production and the filtration quality of the final product. The chosen strain was a commercially available *Streptococcus thermophilust* and *Lactobacillus delbrueckii subsp. bulgaricust* mix, the Yo-Mix 401 which is specifically optimised for yoghurt production.

The 11-day-long, 42-44 °C fermentation procedure resulted in selenium enriched yoghurt which was needed to be dried and ground. We compared air drying and lyophilisation. With lyophilisation or freeze-drying we get an easily ground, soft powder (Fig. 2), but this procedure is expensive and the product rapidly absorbs water from the air.

On the other hand, air drying at 50°C resulted in a very hard powder which was very difficult to grind to an acceptable homogeneity and size, but was finally done with an expensive grinder. Comparing the results and the price of the two methods, air drying was chosen (Fig. 2).

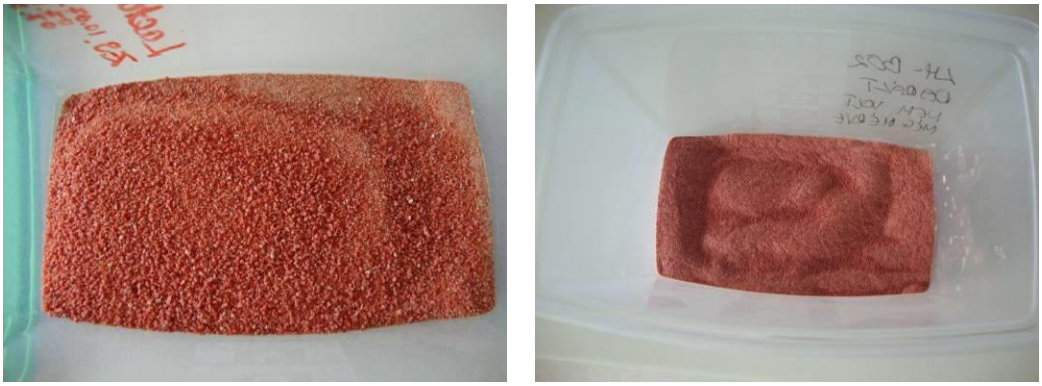

Figure 2.
Freeze-dried and ground LactoMicroSel (left)
Air-dried and ground LactoMicroSel (right)

With the development of the production method done, we modified it for industrial use, got the necessary permits and started the production in Instantpack Kft's factory in Berettyóújfalu. The final product contains 3000 mg/kg selenium, 93.88% of which is in elemental form. Using this LactoMicroSel a selenium enriched instant milk product (No.42) and a selenium containing food supplement product (LactoMicroSel supplement) was produced (Fig. 3).

Figure 3. LactoMicroSel selenium enriched yoghurt powder (left), selenium enriched instant milk (mid) and selenium containing food supplement (right)

2.2. Investigation of the purified nanoselenium sol (NanoSel)

Using the purified nanoselenium sol (NanoSel) the following measurements were performed:

- Total selenium content measurement using atomflourescent method (AFS). The sample preparation was performed according to *Kovács et al. (2003).*
- Selenium speciation by HPLC-AFS.
- SEM photos of the elemental selenium nanospheres.
- Particle size distribution using a laser diffraction particle size analyser (LDPSA).

We also investigated the nanoselenium particles in aqueous media. During this research we made 2 liter 200 mg/kg NanoSel sol, and sampled its supernatant every day for a month. In these samples we separated the nanospheres from the soluble selenium forms with a 200 nm membrane filter, and then we constructed a theoretical conversion model based on the results.

2.3. Investigation of nanoselenium in soils

For the investigation of nanoselenium in soils we used calcareous chernozem from Látókép (Hu%=3.02; K_A=42; pH($CaCl_2$)=7.18) and humic sandy soil from Pallag (Hu%=0.67; K_A=26; pH($CaCl_2$)=4.41) in 2 kg pots, without plants. The air-dried soils were treated with 200 mg/kg NanoSelenium sol and set to 0, 1.00 and 10.00 mg/kg selenium concentrations. The water content of the soils was set to 60% field capacity, and was maintained during the 8 weeks of the experiment, using deionised water. The following measurements were carried out using 10 samples from each group:

- Determination of total selenium content using AFS,
- Determination of water soluble selenium content using AFS,
- Determination of acid soluble selenium content using AFS.

2.4. Investigation of nanoselenium in plants

Our previous experiments with nanoselenium using tobacco plants has already proven that NanoSel treatment can result in higher root and shoot selenium content, faster root growth and higher root biomass, slower aging and improved resistance against vitrification in callus cultures *(Domokos-Szabolcsy, 2012)*. The goal of our present research was to determine if the nanoselenium successfully gets into the cell and chloroplast through the cell wall. For the experiment we used tobacco (*Nicotinia tabacum* L. cv. Ottawa) plants grown on selenium treated MS basal medium (*Murashige & Skoog, 1962*), with treatments of 0, 1, 10, 100 mg/kg selenate and 100 mg/kg NanoSel.

The experiment was performed for 4 weeks, in ten repetitions, and the following was measured:

- Root and shoot biomass production,
- Total selenium content of isolated protoplast *(Nagy and Maliga, 1976),*
- Total selenium content of isolated thylakoid membranes *(Jajoo et al., 2012),*
- Lipid peroxidation in intact leaves and isolated thylakoid membranes, using TBARS assay *(Zhang és Huang, 2013).*

2.5. Investigation of nanoselenium in animals

We used LactoMicroSel selenium enriched yoghurt powder in these two experiments conducted to investigate the nanoselenium in animals.

Our first animal experiment investigated the use of nanoselenium as feed supplement and its effect on physiological parameters, and was done with 120 Cobb 500 broiler chicken, in 1:1 gender ratio, for 42 days, with the following selenium treatment groups:

- Control: 0.2 mg/kg total selenium content in the base feed
- SelPlex: selenomethionin-containing feed supplement, 0.425 mg/kg total selenium content
- LactoMicroSel 1x (LMS 1): 0.425 mg/kg total selenium content
- LactoMicroSel 10x (LMS 10): 4.25 mg/kg total selenium content
- Crab/Fish meal + LactoMicroSel 1x (CF+LMS 1): 0.425 mg/kg total selenium content, supplemented with 40 g/kg 1:1 mix of crab/fish meal
- Crab/Fish meal + LactoMicroSel 10x (CF+LMS 10): 4.25 mg/kg total selenium content, supplemented with 40 g/kg 1:1 mix of crab/fish meal

We measured the total weight, weight gain, average feed consumption, feed conversion efficiency, relative liver, breast and leg weight, and total selenium content of liver, muscle and feather.

Our second 56-day-long animal experiment investigated the effect of nanoselenium on egg quality and quantity, and was conducted with 60 Bovans Goldline hybrid laying hens, in two rounds, with the following treatment groups: control, SelPlex, 1x LMS, 10x LMS in the first round, and control, crab/fish meal without selenium, CF+LMS 1x, CF+LMS 10x in the second round. We measured the egg production, egg quality index, egg weight, yolk weight and color, dry weight and thickness of the egg shells, and the total selenium content of egg yolks and whites.

3. RESULTS

3.1. Production and investigation of the NanoSel purified sol

The production of both the NanoSel monodisperse selenium sol and the LactoMicroSel selenium enriched yoghurt powder can be found in the Materials and Methods chapter.

3.1.1. Electronmicroscopy

Examining the NanoSel sol with a scanning electron microscope we concluded that the elemental selenium nanosphere produded by *Lactobacillus casei* have an average diameter of 250 nm (Fig. 4), and while homogenous, they tend to aggregate into larger clusters (Fig. 5). These results are very important, since with the use of a 200 nm pore-size filter we can easily separate the elemental selenium from the soluble selenium forms, for example during the investigation of the NanoSel supernatant. Furthermore, the images clearly show the successful extraction of the nanospheres and the removal of the bacteria cell fragments. The x-ray fluorescence spectroscopy proves that the examined material is indeed selenium (Fig. 6).

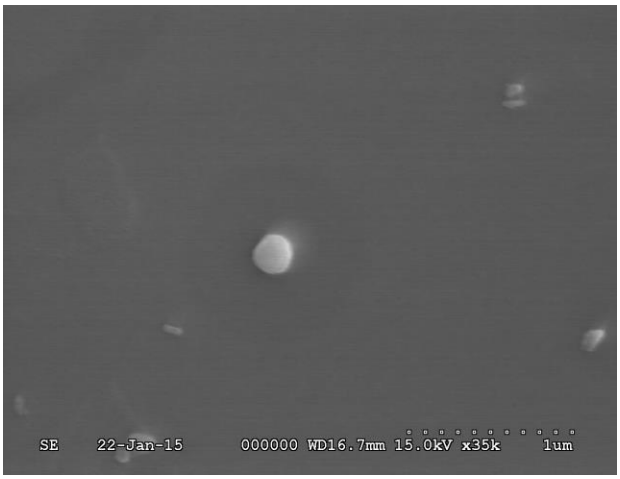

Figure 4. SEM image of a purified elemental selenium nanosphere (Hitachi SEM)

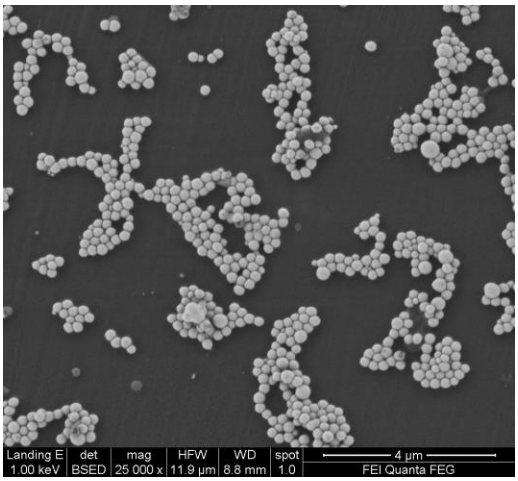

Figure 5. Nanosphere aggregation (QFEG)

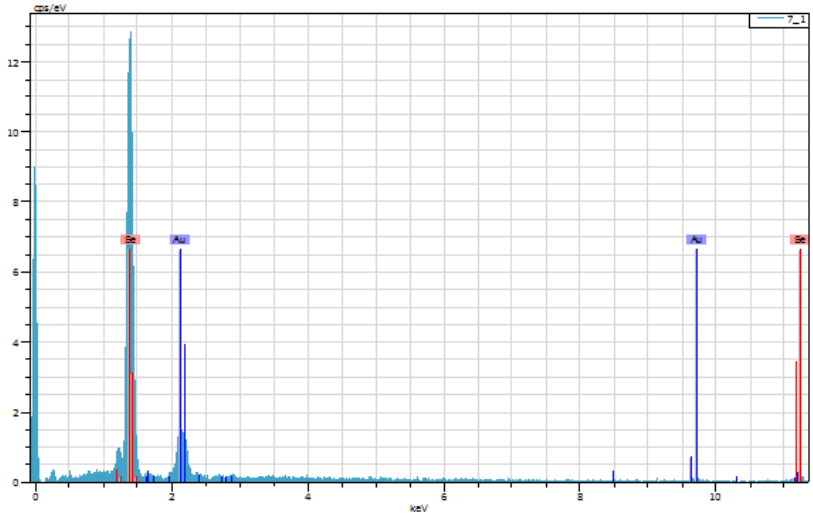

Figure 6. XRF spectrum of the selenium sol (Bruker SPECTRA EDX)

3.1.2. Laser diffraction particle size analysis

Since the aggregation of the particles was observable when we examined the elemental selenium nanospheres with SEM imaging, we measured our sol with a laser diffraction particle size analyser (Malvern Mastersizer 2000). It is an ideal method to measure the change of average particle size due to its rapidity. In figure 7 we can see that after using ultrasound bath to separate the particles the average particle size increased to 3.5 µm in just 1.5 minutes. The average particle size changes rapidly in the first 10 minutes, therefore it is presented in a separate figure (Fig. 8). In figure 8 we can see that after the rapid increase in the first few minutes the change in particle size slows down significantly until it reaches its maximum of 75 µm at 260 minutes.

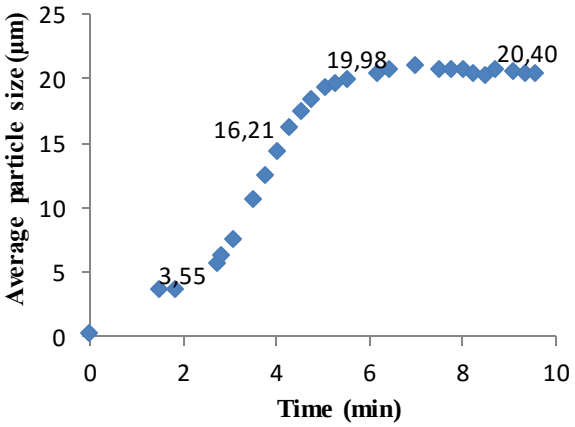

Figure 7. Change of the average particle size of the NanoSel sol, against time since ultrasound bath. First 10 minutes.

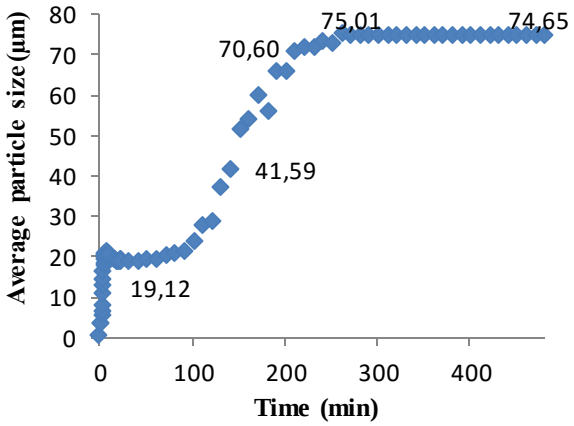

Figure 8. Change of the average particle size of the NanoSel sol, against time since ultrasound bath. First 6 hours.

3.1.3. Investigation of nanoselenium in aqueous media

Both the SEM imaging and laser diffraction size analysis indicated that the elemental selenium spheres in the NanoSel sol are 250 nm diameter and aggregate into much larger clusters. Since particles of this size cannot pass through the cell wall, there must be some kind of transformation mechanism going on. Our theory is that in aqueous media the elemental selenium nanospheres transform into dissolved selenite and hydrogen-selenide which can be used by organisms. The transformation can be characterized by the following equilibratory process which is strongly skewed toward the elemental form:

$$3\,Se + 3\,H_2O \rightleftarrows H_2SeO_3 + 2\,H_2Se.$$

In the experiment to prove our hypothesis we changed the supernatant of the NanoSel sol to fresh distilled water, then measured the change in dissolved selenium concentrations for a month. If we plot the concentration against time, we can see that a saturation curve can be fit on the results (Fig. 9), with a maximum concentration (C_{max}) of 1.219 mg/l, which is significantly lower than the 200 mg/l elemental selenium concentration of our sol. Using this saturation curve we can conclude that the change in dissolved selenium concentrations is very slow, it takes 2.5 month to reach the maximum value.

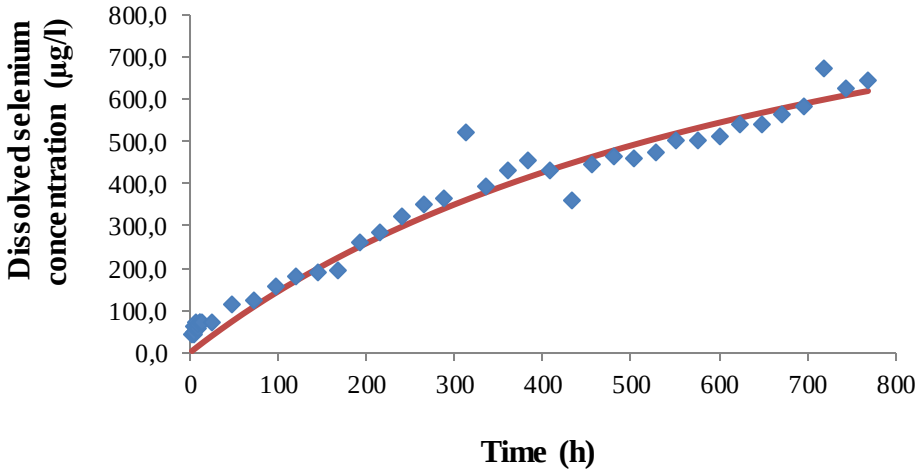

Figure 9. Dissolved selenium concentration of the NanoSel sol supernatant, against time, with a fitted saturation curve (1/C = 1/Cmax + k/Cmax*t)

The selenium speciation measurement of the supernatant showed that after filtration it contained selenite and selenate (Fig. 10). Dissolved selenide concentrations were under the detection limit, only the characteristic smell indicated its presence. We can see a small selenate peak that indicates the oxidation of the selenite during the 30-day experiment.

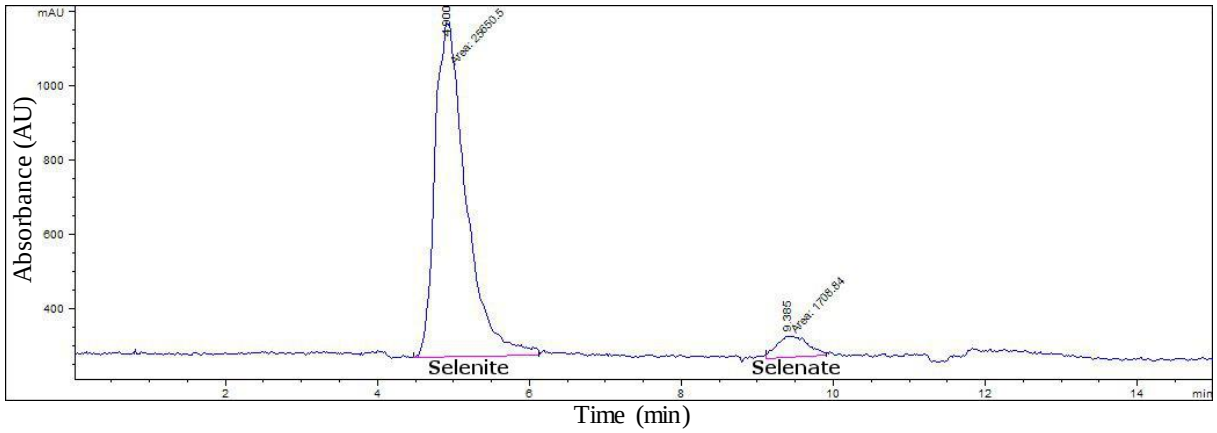

Figure 10. Selenium speciation of the NanoSel sols 1 month old supernatant (Millenium Merlin HPLC-HG-AFS)

We developed a theoretical model which explains the results and the equilibratory conversion of elemental selenium nanospheres into dissolved selenite and selenide:

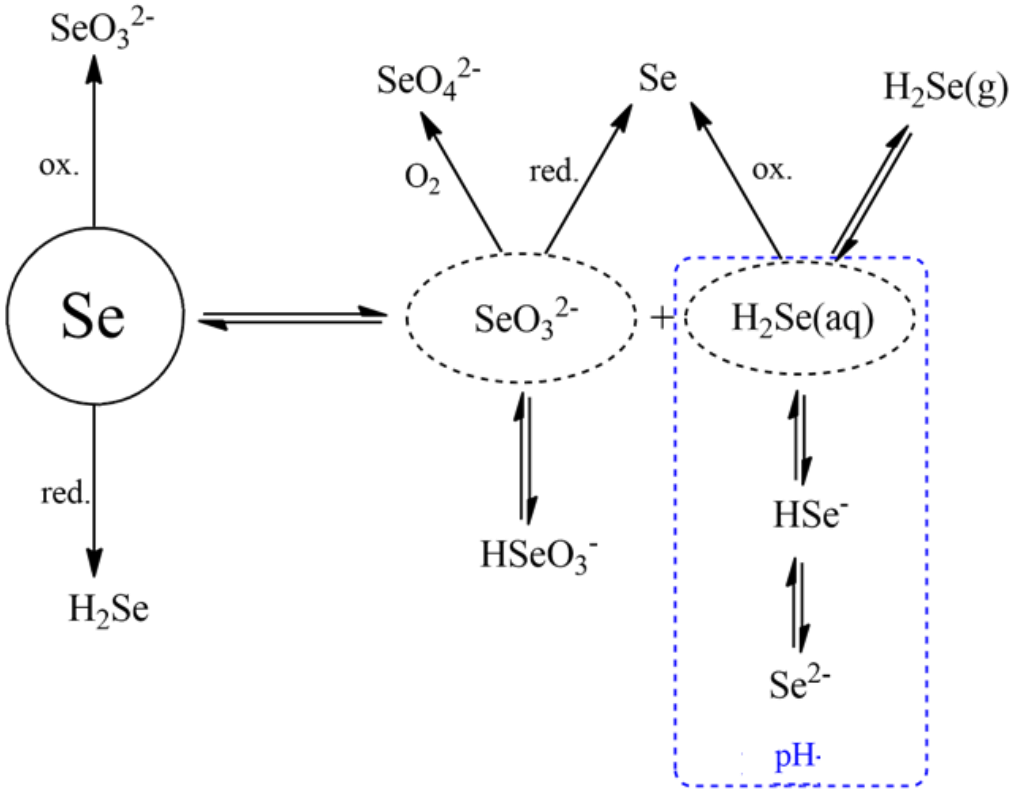

Figure 11. Transformation of the elemental selenium nanospheres in distilled water

Figure 11 presents this model of elemental selenium nanosphere disproportionation into dissolved hydrogen-selenide and selenite in distilled water. This is a very slow equilibratory process which is significantly skewed toward the elemental selenium form. The experiment was done in a closed system, but during the sampling periods when we removed the cap, we could smell the characteristic garlic smell of the hydrogen-selenide which indicates desorption. At the end of the experiment we measured the total selenium content of the sol to see how much selenium was lost due to opening the bottle every day. The result was 199.83 mg/l. This 1% loss implies that the desorption of hydrogen-selenide is a very slow process and its concentration is very low compared to the other forms. We can see in the model that the $H_2Se(aq)$ deprotonates in multiple steps and there is a pH-dependent equilibrium among these forms, which is also applicable for the protonated $SeO3^{2-}$. It is important to note that while this process is equilibratory and the dissolved forms transform back to elemental selenium, they are not red elemental spheres like the ones produced by lactobacteria, but hexagonal crystals stuck to the aforementoined spheres (Fig.12).

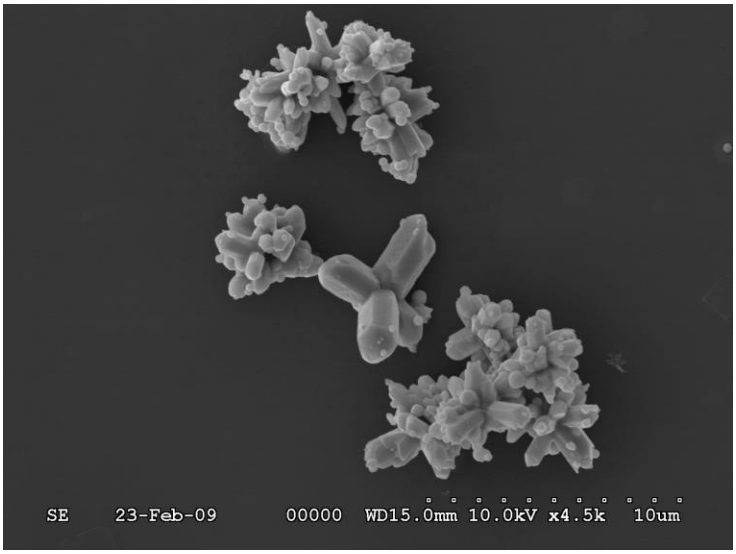

Figure 12. Electronmicroscopic image of the hexagonal selenium crystals transformed from dissolved selenite and selenide (Hitachi SEM)

3.2. Investigation of nanoselenium in soils

3.2.1. Total selenium content

While elemental selenium is strongly adsorbed to soil particles, dissolved hydrogen-selenide volatizes producing a characteristic garlic smell. This process can be seen in the change of total selenium content, shown in figure 13. In 8 weeks 20-28% of the initial 1.00 mg/kg and 10.0 mg/kg selenium is lost due to the conversion to gaseous hydrogen-selenide.

There was no significant difference between the two soil types, except for the control group, where there was 230 µg/kg selenium in the chernozem soil and only 75 µg/kg in the sandy soil. This can be explained with the higher organic content of chernozem soils.

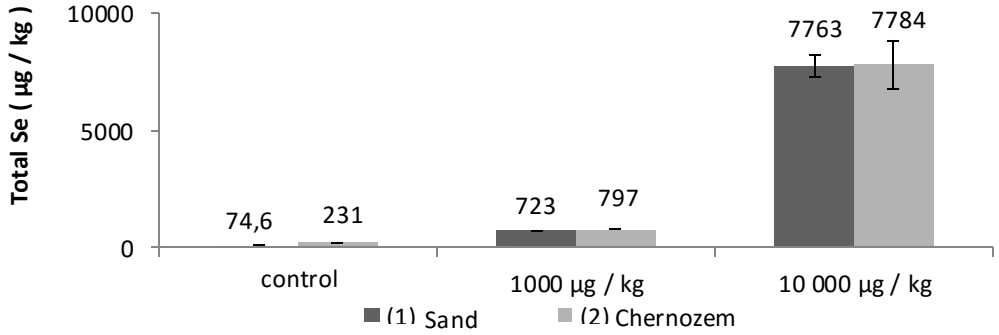

Figure 13. Total selenium content of soils, after 8 weeks (Pot experiment, Debrecen, 2012)

3.2.2. Water soluble selenium content

We tried to measure the water soluble content of the soils, but it was lower than the detection limit (0.1 µg/kg). This indicates that selenite is strongly adsorbed to soil particles, and 8 weeks were not enough for the oxidation of selenite to selenate since this process normally

takes several months, even years. Both claims are proven by numerous experiments *(Kádár, 1999; Kádár és Németh, 2003a; Kádár és Németh, 2003b; Széles, 2007).*

3.2.3. Acid soluble selenium content

Examining the acid soluble selenium content (Fig. 14), we can see that it is significantly lower than the elemental selenium content: out of 7800 µg/kg total selenium it is only 120 µg/kg. This proves that a significant part of the elemental selenium spheres used for treatment remained in elemental form and, besides the 20-28% loss as hydrogen-selenide, only a small amount transformed to selenite. The difference between the chernozem and the sandy soil can be due to the higher bacterial biomass of chernozem soils since the bacteria can take up the dissolved selenite and incorporate it into their proteins *(Mao, 1999)*.

In figure 15 we can see that the acid soluble selenium content is not proportional to the treatment concentrations. This correlates with the previously presented model as the disproportionation of the elemental selenium nanospheres to selenite and selenide is a slow process, the concentration of the elemental form only slightly affects the soluble forms in the starting period.

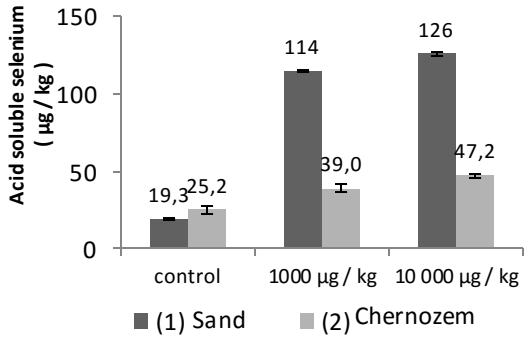

Figure 14. Acid soluble content of the soils (Pot experiment, Debrecen, 2012)

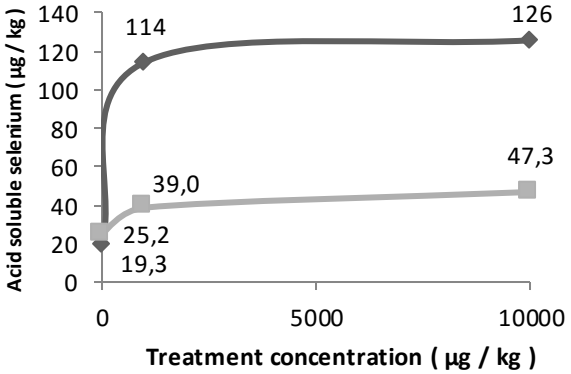

Figure 15. Acid soluble content of the soils against treatment concentration (Pot experiment, Debrecen, 2012)

3.3. Investigation of nanoselenium in plants

3.3.1. Root and shoot biomass production

In figure 16 we can see that the low concentration (1 mg/kg) selenate treatment had no effect. The higher 10 mg/kg selenate treatment resulted in significantly reduced biomass both in the root and shoot, and the highest 100 mg/kg selenate treatment was so toxic that the plants did not survive. The 100 mg/kg nanoselenium treatment reduced the shoot biomass by 31% but increased the root biomass by 13%, this correlates to our previous experiences.

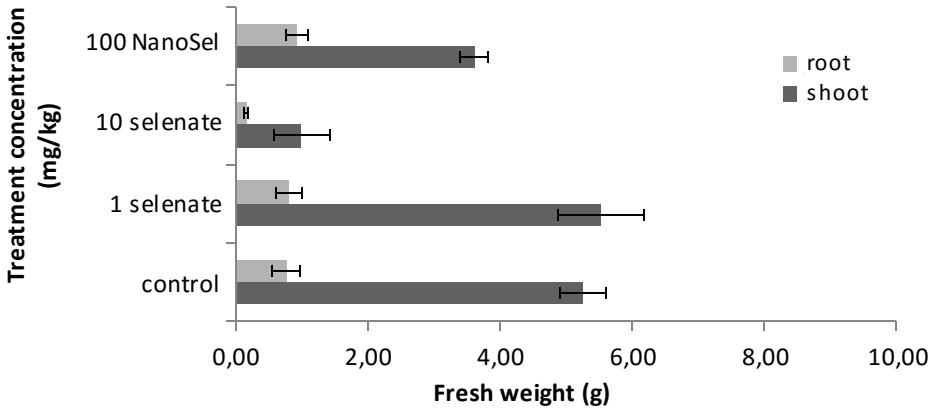

Figure 16. Change of root and shoot biomass of tobaco plant due to different selenium treatments
(Plants grown on medium, Debrecen, 2015)

3.3.2. Total selenium content of isolated protoplast and thylakoid membrane

The results shown in Table 1 are as expected: the 1 mg/kg and 10 mg/kg selenate treatments resulted in higher selenium concentrations in the cell wall-deprived plant cells (protoplasts). NanoSel treatment also resulted in higher concentrations which are lower compared to selenate treatment, but significantly increased compared to the control group.

As transport and communication between plant cells are done through the plasmodesma that is 2-2.5 μm in diameter (*Christensen et al., 2009)*, the 250 nm sized elemental selenium nanoparticles had to transfrom into soluble selenium forms, according to our model.

Table 1: Total selenium content of isolated protoplasts
(Plants grown on medium, Debrecen, 2015)

Treatment (mg/kg)	**ng/10^5 cell selenium content**
control	0.194
1 selenate	14.04
10 selenate	55.11
100 NanoSel	1.141

Using the thylakoid isolation method of *Jajoo et al. (2012)* we isolated membranes directly from plant leaves to see if the nanoselenium treatment results in higher selenium concentrations in the cell organelles. We also measured the selenium content in the cell leftovers. The results of thylakoid membrane showed the same result as the protoplast did: both nanoselenium and selenate treatment resulted in significantly higher selenium concentrations in not just the protoplast but in the thylakoid membrane, too (Fig. 17). This proves that the selenium from NanoSel treatment can get into not just the cells but into the

chloroplast, too. The selenium content of the cell leftovers shows the same results due to the intact cells and separated cell walls where the selenium accumulates.

Comparing the two different selenium forms we can conclude that selenate treatment results in higher selenium accumulation, even in lower (1-10 mg/kg) concentrations, compared to nanoselenium.

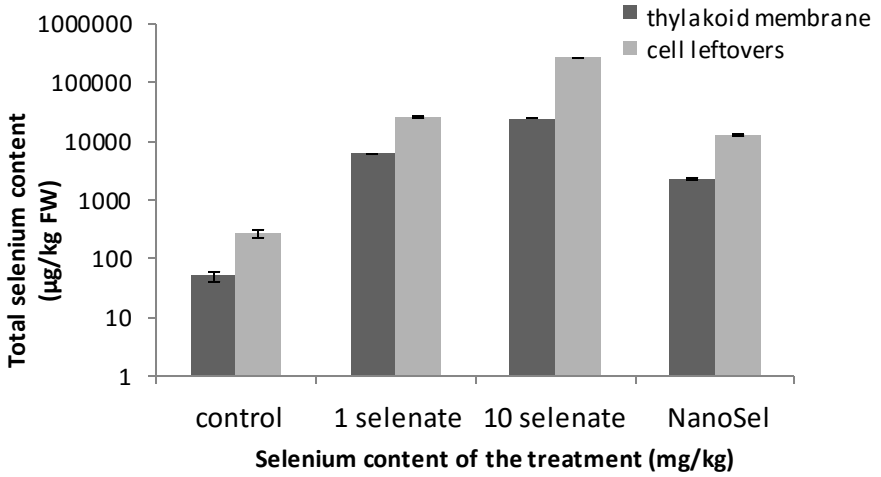

Figure 17. Total selenium content of isolated thylakoid membranes and cell leftovers (Plants grown on medium, Debrecen, 2015)

3.3.3. Lipid peroxidation of the thylakoid membranes

We measured the MDA content of intact leaves and isolated thylakoid membranes. The results show that while the nanoselenium treatment did not cause any significant change compared to the control group, the selenate treatment resulted in significantly higher MDA levels that indicate membrane damage (Table 2).

Table 2. MDA content of intact leaves and isolated thylakoid membranes (Plants grown on medium, Debrecen, 2015)

Treatment (mg/kg)	MDA nmol/g intact leaf	SD	MDA nmol/g isolated thylakoid	SD
control	**29.2^{A}**	3.29	**236^{A}**	30.7
1 selenate	**82.6^{B}**	4.90	**233^{A}**	25.8
10 selenate	**146^{C}**	28.6	**391^{B}**	51.6
100 NanoSel	**39.1^{A}**	9.08	**228^{A}**	6.14

The different letters in the same column indicate significant difference ($P<0.05$) (ANOVA)

3.4. Investigation of nanoselenium in animals – Broiler chicken experiment

Examining the average weight (Table 3) we can see that while the SelPlex and LactoMicroSel treatment did not have any significant effect, the crab/fish meal supplementation resulted in a positive change in both groups, which was significant in the first 4 weeks ($P<0.05$).

Table 3. Average weight of broiler chicken
(Broiler chicken experiement Gödöllő, 2012)

		0. day	7. day	14. day	21. day	28. day	36. day	42. day
Control	AVG	42	**113^{A}**	284^{AB}	599^{AB}	1064^{AB}	1524^{A}	1942^{AB}
	SD	2	11	59	124	202	303	347
SelPlex	AVG	40	109^{AB}	299^{AB}	**578^{A}**	1040^{AB}	1557^{A}	**1890^{A}**
	SD	2	15	34	88	154	202	232
LactoMicroSel 1x	AVG	40	**104^{B}**	**272^{A}**	**573^{A}**	**1026^{A}**	1526^{A}	1918^{AB}
(LMS 1)	SD	1	14	57	110	172	236	260
LactoMicroSel 10x	AVG	41	109^{AB}	292^{AB}	579^{AB}	1012^{AB}	1572^{A}	1958^{AB}
(LMS 10)	SD	2	7	31	78	137	188	213
Crab/Fish meal 1x	AVG	41	**118^{A}**	**311^{B}**	**657^{B}**	**1146^{B}**	1665^{A}	2023^{AB}
(CF+LMS 1)	SD	2	19	56	104	165	261	248
Crab/Fish meal 10x	AVG	41	115^{AB}	307^{AB}	623^{AB}	1087^{AB}	1648^{A}	**2033^{B}**
(CF+LMS 10)	SD	2	17	31	77	120	167	160

The different letters in the same column indicate significant difference ($P<0.05$) (ANOVA)

Using the average weight gain and feed consumption data we concluded that while all four groups of LactoMicroSel-containing treatments consumed less, their weight increased compared to the control, so LMS supplementation had a positive effect on their feed conversion efficiency. In the crab/fish meal supplemented groups this positive effect was even stronger (Fig. 18).

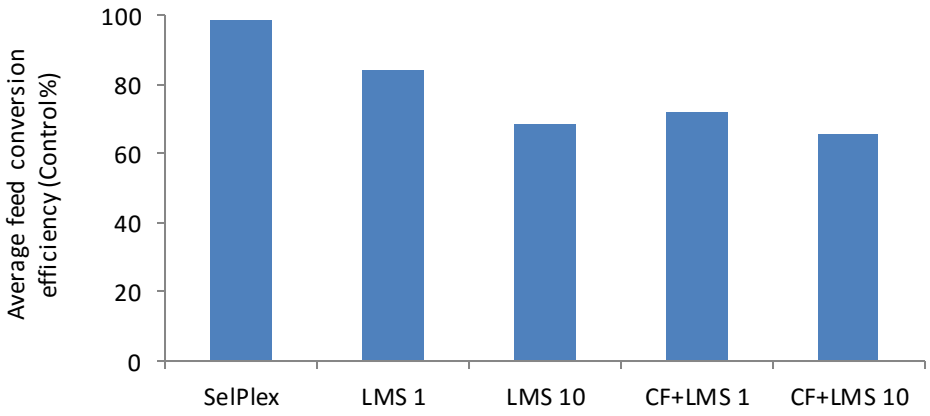

Figure 18. Average feed conversion efficiency of the different treatment groups
(Broiler chicken experiement Gödöllő, 2012)

After the experimental slaughter on day 42 we measured the relative weight of liver, breast and legs. The results show that every selenium treatment had a positive effect (Table 4). Examining the total selenium content of liver and muscle we can see that only the two 10x LMS treatments had any effect on the liver selenium content, and only the SelPlex and LMS 10x supplementation affected the muscle selenium content (Table 5).

Table 4. Relative liver, breast and leg weight (g/100 g body weight)
(Broiler chicken experiement Gödöllő, 2012)

		Liver	Breast	Leg
CONTROL	AVG	1.5	8.1	8.8
	SDs	0.2	0.8	0.4
SELPLEX	AVG	2.0	8.5	9.6
	SDs	0.3	0.9	0.6
LMS 1	AVG	1.8	8.4	9.6
	SDs	0.2	1.1	0.6
LMS 10	AVG	1.5a	8.2	9.4
	SDs	0.2	0.9	0.7
CF+LMS 1	AVG	2.0	8.6	9.2
	SDs	0.2	1.0	0.6
CF+LMS 10	AVG	2.0	8.2	9.4
	SDs	0.3	0.6	0.7

Table 5. Total selenium content in liver and muscle
(Broiler chicken experiement Gödöllő, 2012)

	Liver	Muscle
CONTROL	**185 ± 80**	**42.0 ± 5.9**
SELPLEX	300 ± 262	107.5 ± 12.8
LMS 1	160 ± 98	41.8 ± 5.2
LMS 10	**890 ± 461**	**68.3 ± 11.0**
CF+LMS 1	160 ± 129	45.4 ± 8.2
CF+LMS 10	517 ± 340	65.1 ± 17.5

We also measured the total selenium content of the feathers as it is a good indicator of selenium treatments. We can see that the selenium from the LactoMicroSel supplementation is successfully absorbed from the feed and used by the birds (Fig. 19).

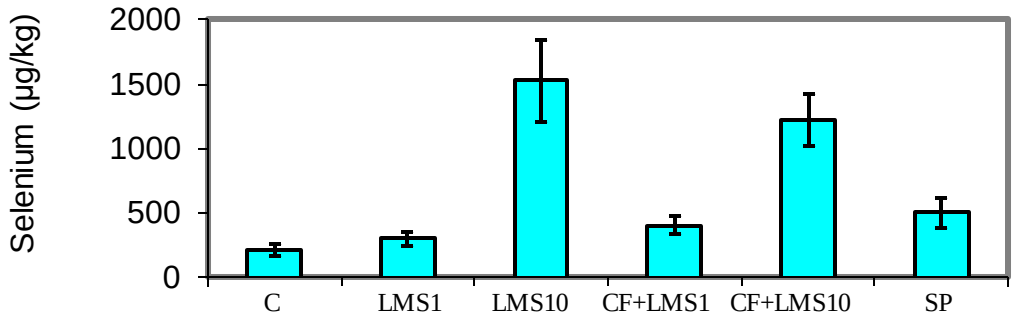

Figure 19. Total selenium content of feathers

3.5. Investigation of nanoselenium in animals – Laying hen experiment

Table 6 and 7 show that LactoMicroSel supplementation had a positive effect on egg production, in the crab/fish meal 10x group the difference is almost +10%.

Table 6. Egg production in phase I.
(Laying hen experiment, Gödöllő, 2012)

	Egg production	
	eggs	%
Control	13.9	92.4
SelPlex	13.3	88.9
LMS 1	13.4	95.6
LMS 10	14.2	94.9

Table 7. Egg production in phase II.
(Laying hen experiment, Gödöllő, 2012)

	Egg production	
	eggs	%
Control	12.8	85.1
CF-Control	14.0	93.0
CF + LMS 1	13.6	90.8
CF + LMS 10	14.1	94.3

Examining the yolk weight we can see that while every selenium supplementation resulted in lower weights, the added crab/fish meal compensated this effect (Table 8). The dry weight and thickness of the egg shells significantly increased in the case of 10x LMS treatment. The added crab/fish meal increased it even further, possibly due to the surplus Ca (Table 9).

Table 8. Yolk weight

	YOLK WEIGHT	
	AVG	SD
	Phase I.	
Control	16.88^{A}	0.64
SelPlex	15.96^{B**}	0.88
LMS 1	15.45^{B**}	1.16
LMS 10	16.04^{B*}	1.65
	Phase II.	
Control	14.82^{A}	1.04
CF-Control	14.95^{A}	1.12
CF + LMS 1	14.84^{A}	0.91
CF + LMS 10	14.79^{A}	1.09

Table 9. Weight and thickness of the egg shell

EGG SHELL	THICKNESS		WEIGHT	
	AVG	SD	AVG	SD
	Phase I.			
Control	0.37^{AB}	0.02	6.01^{A}	0.32
SelPlex	0.37^{AB}	0.03	5.96^{A}	0.46
LMS 1	0.36^{A}	0.02	5.67^{B}	0.28
LMS 10	0.38^{B}	0.02	6.06^{A}	0.53
	Phase II.			
Control	0.34^{A}	0.03	5.76^{A}	0.56
CF-Control	0.37^{B}	0.02	6.18^{B}	0.27
CF + LMS 1	0.37^{BC}	0.03	5.88^{A}	0.49
CF + LMS 10	0.35^{C}	0.03	5.95^{AB}	0.42

The different letters in the same column indicate significant difference
(One asterisk: P<0,05, Two asterisk: P<0.01)
(ANOVA)

Examining the color of the yolk we saw that the yellow color was more intensive with SelPlex treatments, while LMS treatments had the opposite effect. In the second phase the crab/fish meal supplementation increased the red hue, which is due to the astaxanthin pigment.

Figure 20 and 21 presents the total selenium content of the egg yolks and whites. We can see that the elemental selenium in the LactoMicroSel selenium enriched yoghurt powder increased the selenium concentration significantly more than SelPlex and can be used to produce selenium enriched eggs as functional food.

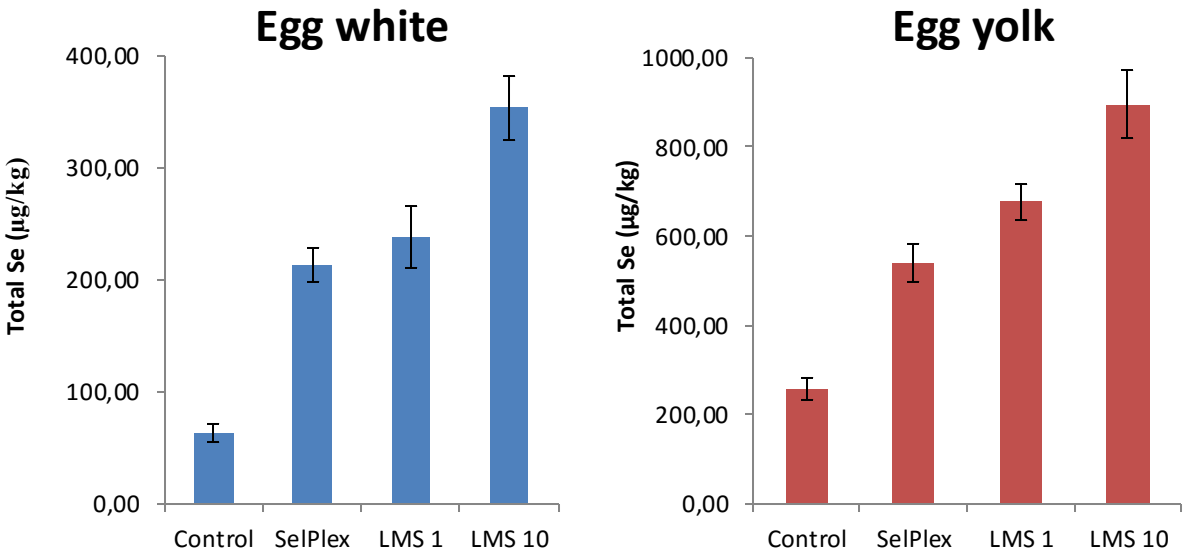

Figure 20. Total selenium content of egg yolk and white in phase I.

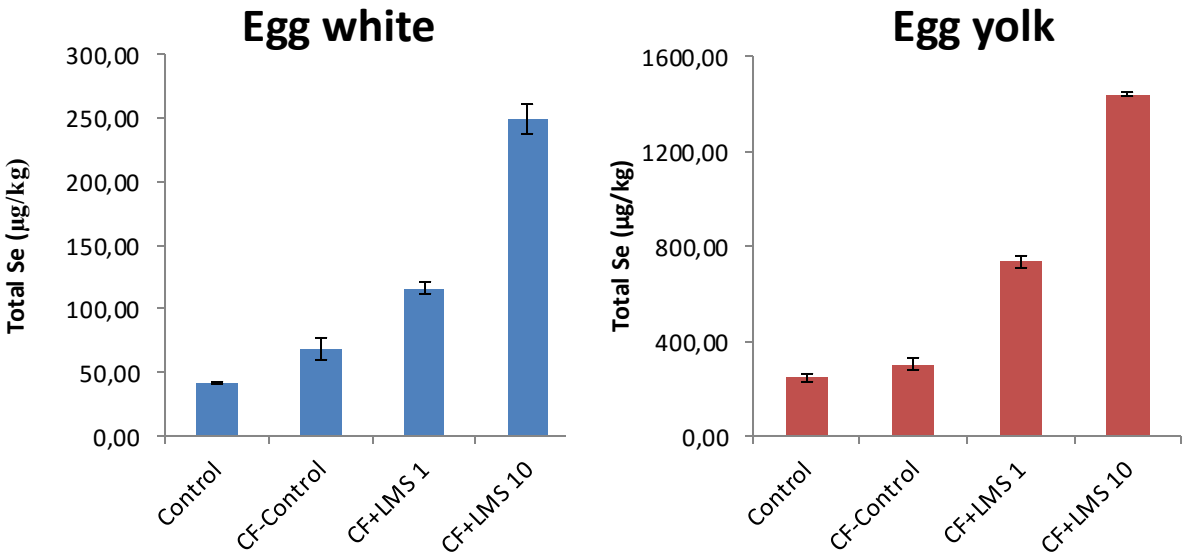

Figure 21. Total selenium content of egg yolk and white in phase II.

4. NEW AND NOVEL SCIENTIFIC RESULTS

1. We developed a method to produce a purified sol for experimental use, called NanoSel, which contains 250 nm sized red elemental selenium nanospheres produced by fermentation with *Lactobacillus casei* in selenite enriched MRS broth. The nanospheres are extracted and separated from the bacteria cells so the sol contains only distilled water and nanoselenium. With the modification of this production process and its optimization for food industrial production, we developed a method to produce selenium enriched yoghurt powder, called LactoMicroSel. Numerous products incorporate this yoghurt powder, such as the selenium enriched powdered milk *No. 42*, the dairy dessert *Milx*® or the food supplements *LactoMicroSel*®, *Pajzskomplex*® and *Cardio komplex*®. Investigating the nano-sized elemental selenium particles in aqueous media we constructed a model that demonstrates the disproportionation of elemental selenium to selenite and hydrogen-selenide, a slow equilibratory conversion process skewed toward the elemental form.

2. In our experiment with nanoselenium treated calcareous chernozem and humic sandy soils we demonstrated that elemental selenium is converted into selenite and hydrogen-selenide, the latter causing 20-28% loss in total selenium concentration after 8 weeks by desorption. The concentration of the dissolved selenium forms in this narrow timeframe depends only slightly on the elemental selenium concentration: in case of sandy soil it is only 114 μg/kg for the 1000 μg/kg treatment, and 126 μg/kg for the 10.000 μg/kg treatment. In the case of chernozem soil the same values are 39 μg/kg and 47.3 μg/kg.

3. Our experiment with tobaco plants showed that nanoselenium treatment results in higher protoplast and thylakoid membrane selenium content, which proves that the selenite transformed from the elemental selenium nanospheres can get into the cells and even into the chloroplast without any toxicity, even in 100 mg/kg treatment concentrations.

4. Experiments with broiler and layer chicken showed that the selenium enriched yoghurt powder is suitable to be used as feed supplement, and by adding crab/fish meal it has a positive effect on the production indexes, relative liver, breast and leg weight. It improves egg production and quality, and results in increased egg shell weight and thickness which can provide better mechanical resistance. Nanoselenium was successfully absorbed and used by the birds, that is indicated by the increased selenium concentration in liver, muscle and feathers. Furthermore, the LactoMicroSel treatments resulted in significantly higher egg yolk and white selenium content, which can lead to the production of selenium enriched eggs as functional food.

5. PRACTICAL APPLICABILITY OF THE RESULTS

1. Using the method presented in the thesis book a purified selenium sol can be produced, which contains elemental selenium nanoparticles. With the modification of this production process, and using whey and yoghurt bacteria, the selenium enriched yoghurt powder, called LactoMicroSel is acquired. Numerous products incorporate this powder, such as the selenium enriched powdered milk *No. 42*, the dairy dessert *Milx*® or the food supplements *LactoMicroSel*®, *Pajzskomplex*® and *Cardio komplex*®.

2. Nanoselenium produced by lactic acid bacteria can be used to supplement soils since the transformation of elemental selenium nanospheres produce a readily available, low concentration of selenite for an extended time, thus providing an ideal level of selenium for plants.

3. Plants can uptake the nanoselenium from the soil, and the selenite transformed from the elemental selenium nanospheres can get into the cells and even into the chloroplast providing the necessary selenium level without any toxicity.

4. The LactoMicroSel selenium enriched yoghurt powder is suitable to be used as feed supplement, and by adding crab/fish meal it has a positive effect on the production indexes, relative liver, breast and leg weight. It improves egg production and quality, and results in increased egg shell weight and thickness which can provide better mechanical resistance. Nanoselenium is successfully absorbed and used by the animals, resulting in increased selenium concentration in liver, muscle and eggs, and can be used to selenium enriched eggs as functional food.

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7. LIST OF PUBLICATIONS RELATED TO THE DISSERTATION

Registry number: DEENK/253/2015.PL
Subject: Ph.D. List of Publications

Candidate: Attila Sztrik
Neptun ID: I8T0GN
Doctoral School: Hankóczy Jenő Doctoral School of Crop Production, Horticulture and Food Sciences
MTMT ID: 10037687

List of publications related to the dissertation

Foreign language international book chapter(s) (1)

1. El-Ramady, H., Abdalla, N., Alshaal, T., El-Henawy, A., Faizy, S.E.A., Shams, M.S., Shalaby, T., Bayoumi, Y., Elhawat, N., Shehata, S., **Sztrik, A.**, Prokisch, J., Fári, M., Pilon-Smits, E.A., Domokos-Szabolcsy, É.: Selenium and its role in higher plants.
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19. El-Ramady, H., Abd Alla, N., Alshaal, T., Elhawat, N., Domokos-Szabolcsy, É., Prokisch, J., **Sztrik, A.**, Fári, M.: Selenium: Its Implications to Human Health through Soils under Climate Changes.
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The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

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