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Changes on Levels of Essential Trace Elements in Selenium Naturally Enriched Milk

Amelia González-Arrojo[1], Ana Soldado[1], Fernando Vicente[1], Mª Luisa Fernández Sánchez[2], Alfredo Sanz-Medel[2], Begoña de la Roza-Delgado[1,*]

[1]Department of Nutrition, Grasslands and Forages, Regional Institute for Research and Agro-Food Development, SERIDA, Villaviciosa, Spain
[2]Department of Physical and Analytical Chemistry, Faculty of Chemistry, University of Oviedo, Oviedo, Spain
*Corresponding author: broza@serida.org

Abstract Selenium (Se) enrichment improves milk functional nutrient content enhancing its nutritive value and providing health benefits. This Se enriched milk can be considered as "nutraceutical" or "functional food". However, this benefit should not affect negatively other milk properties, such as its trace element contents. Holstein-Friesian cows diets were supplemented with increasing Se dosages in order to obtain on-farm Se enriched milk, and trace metals content including Co, Cu, I, Se and Zn were determined in these milk samples. Our results showed that Se milk supplementation did not affect negatively other trace element levels in milk, obtaining a functional food designed to allow consumers to drink enriched milk close to their natural state. No effect was detected on Co, I and Zn at any Se supplementation dosages. However, Cu level decreased when Se concentration in milk was higher than 100 ng/g.

Keywords: *milk minerals content, functional foods, supplementation effect, nutraceuticals*

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1. Introduction

Nowadays, there are many strategies to improve the functional nutrient content in food to enhance its nutritive value and to provide health benefits, creating the so-called nutraceuticals or functional foods. Within this scenario, milk and dairy products are a versatile source of nutrients in human diet and have been the subject of great interest due to their relevance, since they are widely consumed from childhood to elder age. Milk is an excellent source of macronutrients (proteins, lipids and carbohydrates) and micronutrients (vitamins and minerals). The potential health benefits of milk protein-derived peptides have been a subject of growing commercial interest in the context of health-promoting functional foods [1], particularly in relation to cardiovascular diseases [2]. Milk is also an ideal source of macroelements such as Ca, K and P, and micro trace elements such as Fe, Cu, Se, Zn, Co and I. These essential trace elements are vital to support normal physiological functions in humans, their deficiency is related to several diseases and their overdose is related to toxicity [3,4,5]. Total trace element and their ratio levels in milk also become important in formulas for infant nutrition, due to the role they play in an extremely high number of processes. There are works detailing speciation analysis in cow-based vs. human-based milk formulas with the aim of formula improvement [6,7].

Several studies have reported the distribution and occurrence of metal essential components in various animal milks [8,9,10]. The reported data show that metal essential component contents of milks vary considerably and that their composition appears to be affected by genetics, physical and environmental factors [10,11,12]. Dietary composition and husbandry practices largely determine essential trace element status of livestock and, consequently, their concentrations in animal products [13].

Selenium plays an important antioxidant role in humans at trace concentrations. Moreover, epidemiological studies also provide evidence for Se as a chemopreventive agent for some types of human cancers (prostate, lung, and colon) and it has a beneficial effect on a number of other pathologies [14,15,16]. However, from a human nutrition standpoint, Se content in milk is generally too low to cover the recommended daily intake (RDI), 20 -100 µg/day. Nowadays, it is technically feasible to increase Se concentrations in milk supplying a significant amount of Se in livestock diets [17,18,19]. This offers an alternative to provide an extra Se milk content (a nutraceutical milk) to help alleviate Se deficiencies through such a common nourishment as milk and to benefit population health. There are also studies showing the effect of Se supplementation of dairy cows diet on both milk production and composition to obtain supra-nutritional concentrations of Se using different Se supplementation forms [20,21,22]. Other research works have studied the effect in Se speciation [23] in cow's blood, milk, and dairy

products like cheese or yoghurt produced with cow´s or goat´s milk [8,23,24]. Some of these studies showed the adverse effect of Se supplementation in Zn-citrate binding in human milk [25,26,27]. Nevertheless, the majority of experiments have focused to increase selenium content in milk and less attention has been paid to other trace elements. There are not many works about the impact of Se supplementation over total content of some trace elements such as Co, Cu, I and Zn in cow milk. Most of them are focused on some of these trace elements speciation in whey milk as Hoac *et al. [28]*, but not in whole milk. Therefore, the aim of the present work has been to study the relationship between selenium and essential trace elements (Zn, Co, Cu and I) in Se enriched raw cow milk, in order to establish preliminary results of this functional nutrient enhancement.

2. Material and methods

2.1. Se Supplementation Protocol

The experiment was carried out during the winter season, over nine weeks with eight multiparous and primiparous Holstein-Friesian cows, all of them in the second third of lactation stage. Daily milk production was 22±7 L per animal. Diet consisted on total mixed rations (TMR) and forage ad libitum (21.4 kg DM/day in total). These cows were randomly divided in four groups and subjected Se supplementation dosages as follow: group 1, feeding the basal diet without Se supplementation (control group); group 2, feeding the basal diet supplemented with Se at dosage 8 mg Se per day; group 3, feeding the basal diet supplemented with Se at dosage16 mg Se per day; and group 4, feeding the basal diet supplemented with Se at dosage 24 mg Se per day. Always according to the EU Regulation [29]. An organic source of Se, such as Selenium yeast (SY), with 63% of Se-Methionine and 36% as other organic Se components was used for Se supplementation.

Cows were milked at 07:30 and 19:30 each day and individual milk yields were recorded at each milking. A sample aliquot consisted of the combination of morning and afternoon milking aliquots for each individual cow during the experimental period. Milk production was measured and the milk samples were analyzed for fat and protein contents (MilkoScan FT 6000, Foss Electric, Hillerød, Denmark). Sampling was carried out at t=0, before any supplementation, t=7, t=28 and t=63 days (32 milk samples in total) and samples were stored at -40ºC until analysed. Non-iodized sanitizers were used in premilking management. This work is in accordance with the European Commission guidelines [30] concerning the protection of animals used for experimental and other scientific purposes.

2.2. Instrumentation

Microwave (MW) digestion unit Ethos One, Milestone (Milestone, Srl, Sorisole, Italy) equipped with a rotor for ten TFM (chemically modified PTFE) vessels was used for sample mineralization. The ICP-MS instrument used in this study was an Agilent 7500c Octopole Reaction System (ORS) (Agilent Technologies, Tokyo, Japan). The ICP-MS operating conditions were optimized for the simultaneous determinations of all elements under scrutiny. MW and ICP-MS conditions are summarized in Table 1.

Table 1. Experimental conditions for ICP- MS analysis and microwave digestion for the simultaneous determinations of trace elements in whole milk samples

Plasma parameters			
Rf power (L/min)	1500		
Plasmogen gas flow rate (L/min)	15		
Auxiliary gas flow rate (L/min)	1		
Carrier gas flow rate (L/min)	1.12		
Sample flow rate (rps)	0.1		
Isotopes measured			
Acidic Digestion	^{59}Co,^{63}Cu,^{64}Zn,^{65}Cu,^{66}Zn,^{67}Zn,^{68}Zn ,^{69}Ga,^{71}Ga, ^{74}Se,^{76}Se,^{77}Se,^{78}Se, ^{80}Se,^{82}Se, ^{103}Rh,^{113}In, ^{115}In		
Basic Digestion	^{69}Ga,^{71}Ga, ^{103}Rh, ^{113}In,^{115}In, ^{127}I		
Isotopes selected for quantification			
Acidic Digestion	^{59}Co,^{65}Cu,^{66}Zn (*I.S*: ^{71}Ga),^{78}Se (*I.S.*:^{103}Rh)		
Basic Digestion	^{127}I (*I.S.*: ^{103}Rh)		
Microwave programs			
Acidic digestion			
Step		Time (min)	Power (W)	T (ºC)
	1	00:03:00	900	95
	2	00:10:00	900	160
	3	00:03:00	900	185
	4	00:15:00	900	185
Basic digestion				
Step		Time (min)	Power (W)	T (ºC)
	1	00:20:00	900	180
	2	00:10:00	900	180

I.S.: internal standard.

2.1.1. Acidic Digestion

After sample defrosting and homogenisation, an aliquot of 0.5 g of whole milk (or 0.1g for skim milk powder, reference material) was accurately weighted in the TFM MW vessels and digested using 1.5 mL of HNO_3 65%, 1.5 mL H_2O_2 30% and 5 mL of ultrapure water. Blanks, consisting of ultrapure water and reagents were subjected to the same sample preparation and analytical procedure as samples. Samples were mineralized in the MW employing the programme detailed in Table 1. After mineralization, samples were quantitatively transferred to polypropylene containers and brought up to 20 g with HNO_3 1% (dilution 1:40). Co, Cu, Se and Zn were determined by ICP-MS in the digested milk samples using external calibration.

2.1.2. Basic Digestion

After defrosting and homogenization of sample an aliquot of 1.0 g of whole milk (0.5 g for skim milk powder, reference material) was accurately weighted in the TFM microwave vessels for I quantification and digested using 2 mL of TMAH 25% and 8 mL of ultrapure water. Blanks were prepared following the same premises as acidic digestion and MW programme is detailed in Table 1. After mineralization, samples were quantitatively transferred to polypropylene containers and brought up to 20g with ultrapure water (dilution 1:20). I was determined by ICP-MS in the digested milk samples using external calibration.

2.1.3. ICP-MS Measurements

Standard solutions of Co, Cu, I, Se and Zn were prepared daily by appropriate dilution of stock standard 1000 mg/L for each element in 1% v/v Suprapur HNO_3

and in TMAH 1% for iodine. An appropriate internal standard was also required for each analyte to correct physical and/or matrix interferences in ICP-MS. Since the analysed elements are spread over a wide range of atomic masses, three internal standards ^{69},^{71}Ga, ^{113},^{115}In and ^{103}Rh were selected. The digested cow's milk samples, a reference whole milk powder, blanks and calibration standards were spiked with the internal standard solution to obtain a final concentration of 10 ng/g in each internal standard. The correctness of the analytical procedure was tested by determining the analyzed elements in a skim milk powder Reference Material (CRM-063R).

2.1.4. Statistical Analysis

Differences in trace element contents with dosages of Se supplementation were examined using GLM procedure with the LS means statement provided by the SAS [31] statistical analysis according to the model: Yij= μ + Ai + Bj + Eij ; where Yij is the dependent variable, μ is the overall mean, Ai is the sampling day, Bj is the effect of Se dosage and Eij is the residual error.

3. Results

3.1. Milk Composition

Fat, protein and lactose contents on experimental cow´s milk under study were also analyzed in order to detect any anomalous values and evaluate their possible influence in trace element content (Table 2). Also, milk yield was not affected either. No statistical effects on these parameters were obtained neither for supplemented groups nor supplemented and control group.

Table 2. Milk production and composition for the three Se supplementation dosages

	Treatment					
	Group1 [1]	Group2[2]	Group3[2]	Group4[2]	s.e.m[3]	*P*- value[4]
Milk yield (kg/day)	22.2	26.3	20.4	18.7	1.43	ns
Milk composition g/100g						
Fat	3.64	3.51	4.08	3.53	0.095	ns
Protein	3.03	3.02	3.15	3.28	0.056	ns
Lactose	4.89	4.77	4.98	4.98	0.023	ns

[1]Group1 Control group: no Se supplementation.
[2]Se supplementation doses groups
[3]s.e.m. Standard error of means
[4]*P*-value: Statistical significance of effect of Se supplementation; ns: non-significant.

3.2. Effect of Se Supplementation on Trace Element Levels in Cow Milk

Se level remained constant along the experiment in control group 1 (16.7 ± 2.5 ng/g). The average Se milk content in groups 2, 3 and 4 was 13.6 ± 1.4 ng/g before starting the assay (t=0). It rose quickly with the introduction of supplementation, reaching on day 7,100 % on group 2 and 80% on group 3 of steady-state level as Table 3 reports. Nevertheless, for the highest Se supplementation (group 4) Se level increased tills the end of the experiment. For this dosage, the total Se content rose to 205 ng/g after 63 days of Se supplementation. Table 3 also shows the mean and standard error of mean of the results obtained for Co, Cu, I and Zn.

Table 3. Trace elements contents (ng/g) in whole milk with different supplementation dosages of Se in cow diets

	Treatment				s.e.m[3]	*P*-value[4]	
	Group1 [1]	Group2[2]	Group3[2]	Group4[2]		Dosage	Sampling day
Se	16.7[a]	23[ab]	77[b]	101[b]	14.7	*	ns
Zn	4794	4020	5100	3928	262.8	ns	ns
Cu	111[ab]	49[b]	58[ab]	60[a]	4.8	*	ns
Co	4.4	3.4	3.3	2.1	0.12	ns	ns
I	522.0	95.0	488.0	166	37.8	ns	ns

[a,b] Means within a row with different superscripts differ at P<0.05.
[1]Group 1(control group): no Se supplementation.
[2]Se supplementation doses groups
[3]s.e.m. Standard error of means
[4]*P*-value: Statistical significance of effect of Se supplementation dosage and sampling day; ns: non-significant; *:*P*<0.05. Dosage x sampling day interaction is non-significant.

A wide range on initial values due to animal variability was observed for all trace elements evaluated before starting Se supplementation (Zn= 4340 ± 795 ng/g; Cu= 71 ± 31 ng/g; Co= 3.3 ± 0.7 ng/g and I= 310 ± 248 ng/g). Table 3 values correspond to average trace element contents obtained at sampling days 7, 28 and 63, for each dosage. Focussing on Se, the concentration ranged from 23 ng/g in the group 2 dosage to 101 ng/g in the group 4 dosage (P<0.05). Related with Cu, the concentration ranged from 44 ng/g in the group 2 dosage to 60 ng/g in the group 4 dosage (P<0.05).

Figure 1 shows the effect of increasing Se on milk over total content of Cu, Co, Zn and I. As can be seen, for dosages groups 2 and 3 the ratios Se/X (X= Co, Cu, I or Zn) were constant from day 0 to day 63. However for dosage group 4 and day 63 (Se content higher than 200 ng/g), the ratio Se/X increased 14 times for Zn and I, and 20 times for Cu and Co.

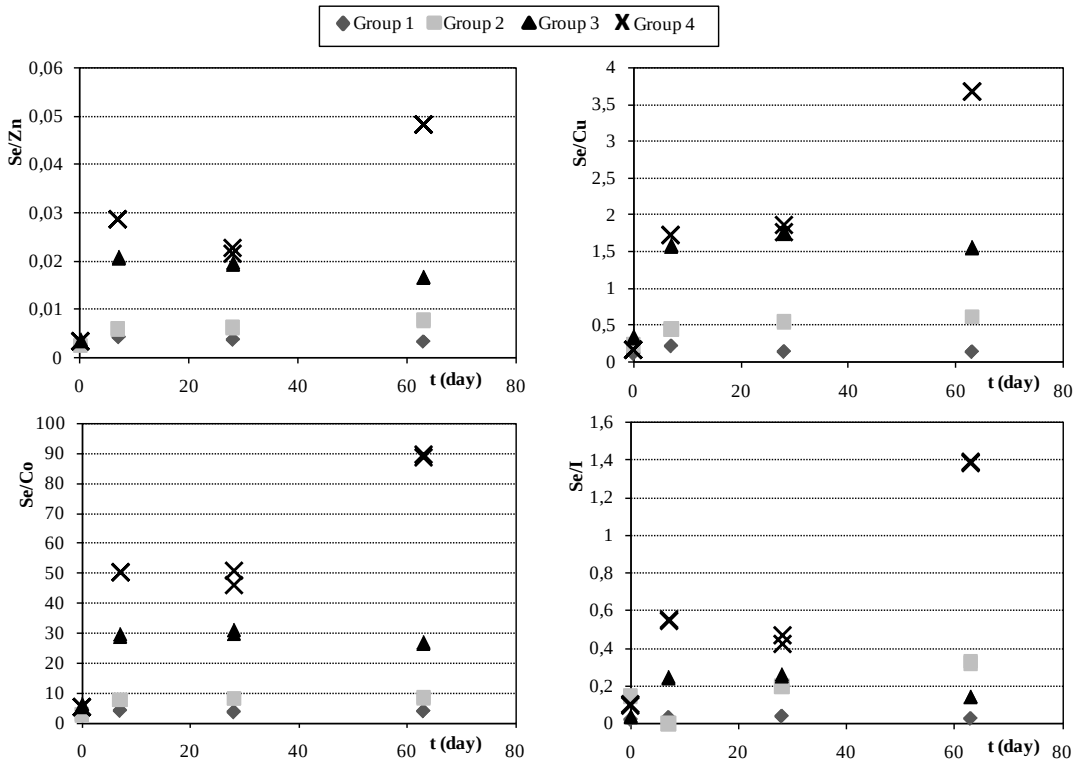

Figure 1. Ratio Se/trace element *vs.* days in whole milk coming from dairy cows with different supplementation dosages of Se

In order to avoid the incidence of animal variability and to achieve a better interpretation of the obtained results, these were expressed as relative values in base 100 respect to t0 and subsequent values were expressed on such a base 100 for 7, 28 and 63 days. These modified results are showed in Table 4 along with the statistical analysis. The interaction of dosage and sampling day effects were statistically studied. The effect of sampling day was not significant for the trace elements analysed. The dosage effect was significant for Se (as it was expected) and Cu concentrations on milk.

Table 4. Trace elements content in whole milk samples with different supplementation dosages of Se in cow diets, referred to average control level value as base 100

	Treatment				s.e.m[3]	*P*-value[4]	
	Group1 [1]	Group 2[2]	Group 3[2]	Group 4[2]		Dosage	Sampling day
Se	130[a]	291[ab]	656[b]	1011[b]	143.6	*	ns
Zn	94	116	101	107	12.1	ns	ns
Cu	98[ab]	131[b]	105[ab]	71[a]	11.4	*	ns
Co	106	99	105	82	6.2	ns	ns
I	93	118	98	137	32	ns	ns

[a,b] Means within a row with different superscripts differ at P<0.05.
[1]Group 1(control group): no Se supplementation.
[2]Se supplementation doses groups
[3]s.e.m. Standard error of means
[4]P-value: Statistical significance of effect of Se supplementation dosage and sampling day; ns: non-significant; *:P<0.05. Dosage x sampling day interaction is non-significant.

4. Discussion

In order to understand the results obtained in these experiments, it is necessary to point out that there are multiple factors involved in the process. The Se supplementation form (organic Se) was chosen as the best for animal absorption, however, dietary mineral supplements may not be properly absorbed due to interactions with other nutrients at rumen level [32]. Microbial digestion in the rumen and reticulum precedes mammalian digestion in the abomasum and small intestine [33] and may alter bioavailability of some trace minerals in ruminants. Trace elements concentration in milk is regulated by the mammary gland, which actively transports some elements, whereas mammary epithelium appears to inhibit the passage of others. Besides this active role, homeostatic mechanism regulating the serum concentration also play a role in determining the level of these elements in milk, through passive diffusion to varying extents [34]. These mechanisms interactions make difficult to predict the impact of Se supplementation in trace elements milk content.

Focusing specifically on Se, it was observed that there was a rapid effect of Se supplementation over milk composition and significant differences of dosage effect, as it was expected. Values obtained for group 1 agree with

the mineral contents described in other studies [13] and represented only 5% of the recommended daily value (RDV) for Se, established as 70 µg/day [35] and groups 3 and 4 have allowed reaching 15% of RDV.

Previous research works have shown that concentrations of Co, Cu and Zn in blood, tissues and milk are largely independent of their intake, as they relate to regulation of gut absorption and changing metabolic demands [36]. However, a potential risk of interactions between micronutrients affecting absorption and bioavailability must be considered in any supplementation strategy. High Se doses may hinder absorption of other essential nutrients. In this sense, Hoac *et al.* [28] found that distribution of Zn and Cu compounds in milk whey was essentially unaffected by Se supplementation. In this framework, our results showed that, specifically, total Zn content in milk seems not to be affected by Se supplementation diet. Nevertheless, total Cu levels in milk are affected at high Se supplementation (group 4), clearly decreasing from group 1 to group 4 at t= 63 days. It seems that a complex secretion mechanisms involved in Cu present in cow milk can be affected when animals are subjected to high dosage of Se supplementation. In ruminants, complex interactions can occur in the rumen environment affecting trace elements absorption. It is well documented that Cu requirements vary greatly depending on concentrations of other dietary components (e.g. sulphur) and high Se presence in the rumen could be an example. Compared to human, the concentration of Se in human milk whey is directly correlated with Zn level and high Se supplementation resulted in lower Zn content and no effect on Cu levels [27] and it seems that Se supplementation resulted in apparent increasing faecal losses of Cu in humans [37].

No research studies were found involving Se enriched milk and Co concentration. Our results providing functional milk enriched in Se, have shown no effect on Co content, a trace element essential to prevent the occurrence of vitamin B12 deficiency, especially on high risk populations, as elder people, vegetarians and people who have had gastric bypass surgery. Co is central component of vitamin B12 and naturally occurring cobalamins are only found in animal products such as meat, milk and dairy products [38]. Regarding I quantification, it is not a simple task [39,40]. A wide range of variation is reported for I concentration, not only in cow milk. The I level in milk is greatly influenced by its dietary intakes [5,33,41]. Results obtained in the present study on this trace element showed no significant effects of Se supplementation, with a carryover of 20%, calculated as the relationship between total I excreted in milk and total I intake. These results are in agreement with previous researches [42] that obtained an I carry over in milk calculated at plateau conditions of 15%.

5. Conclusions

Cow milk improvement as nutraceutical food has been achieved in this study by on-farm changes, avoiding the need for subsequent manipulations, through cattle diet supplementation with Se enriched yeast. After two weeks of treatment, milk Se level reached a plateau, increasing till fivefold control group content. These results showed that Se milk supplementation did not affect negatively other trace element levels in milk, obtaining a functional food designed to allow consumers to drink enriched milk close to their natural state. No effect was detected on Co, I and Zn at any Se supplementation dosages. However, Cu level decreased when Se concentration in milk was higher than 100 ng/g. Nevertheless, the intermediate dosage did not affect Cu levels and allowed reaching 15% of RDV for Se, through a common food as milk. Further studies are required for clear understanding these relationships and the complex mechanisms involved in trace elements absorption.

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Statement of Competing Interests

The authors of this research work declare that they have no conflict of interest and all institutional and national guidelines for the care and use of laboratory animals were followed.

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