"Green and simple extraction of free seleno-amino acids from powdered and lyophilized milk samples with natural deep eutectic solvents"

Romina LÓPEZ[1], Roberto D´AMATO[2], Massimo TRABALZA-MARINUCCI[3], Luca REGNI[2], Primo PROETTI[2], Ariel MARATTA[4], Soledad CERUTTI[1], Pablo PACHECO[1*]

[1] Instituto de Química San Luis (INQUISAL-CONICET), Área de Química Analítica, Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, Argentina.

[2] DSA3, Department of Agricultural, Food, and Environmental Sciences, University of Perugia, 06121, Perugia, Italy.

[3] Department of Veterinary Medicine, University of Perugia, 06121, Perugia, Italy.

[4] Instituto de Investigaciones Mineras (CONICET-IIM), Facultad de Ingeniería, Universidad Nacional de San Juan, Av. Lib. San Martín 1109(O), San Juan 5400, Argentina.

Emails:

Romina López (romilopez19@gmail.com),

Roberto D´Amato (roberto.damato@unipg.it),

Massimo Trabalza-Marinucci (massimo.trabalzamarinucci@unipg.it)

Luca Regni (regni.luca.agr@gmail.com)

Primo Proetti (primo.proietti@unipg.it)

Ariel Maratta (arielmaratta@gmail.com)

Soledad Cerutti (ecerutti@gmail.com)

Abbreviated running title: Free seleno-amino acid extraction from milk with NADES.

*Corresponding author:

Pablo Pacheco (ppacheco@unsl.edu.ar)

Abstract.

Natural deep eutectic solvents (NADES) were introduced for the extraction of free seleno-amino acids from lyophilized and powdered milk samples. Different NADES were evaluated, and lactic acid:glucose (LGH) showed the highest selenium recoveries. Selenium analysis was performed by inductively coupled plasma mass spectrometry (ICP MS). Se-NADES analysis in ICP MS was optimized according to the radio frequency power and nebulization gas flow rate. Se-NADES extraction was optimized by an experimental design. LGH dilution, LGH volume, sample quantity, and ultrasound time were factors influencing the extraction. Seleno-amino acids were determined by liquid chromatography-ICP MS. After optimization, the limits of detection obtained were 7.37, 8.63, and 9.64 µg kg^{-1} for selenocysteine, selenomethionine, and seleno-methyl-selenocysteine, respectively. The NADES-extraction is a green procedure with 2 penalty points in the EcoScale. The method was applied to the analysis of powdered milk, lyophilized Se-fortified sheep milk, and ERM-BD151 skimmed milk powder.

Keywords: free seleno-amino acid; powder milk; lyophilized Se-biofortified sheep milk, NADES, LC-ICP MS, Eco-Scale

1. Introduction

Selenium (Se) is an important micronutrient, essential for animals, that exists ubiquitously in the environment (Ullah, Liu, Yousaf, Ali, Irshad, Abbas, et al., 2019). Selenium is an essential component of selenoproteins like glutathione peroxidase (GxP), which has antioxidant properties; thioredoxin reductases (TR) and desiodase, proteins that regulate the functioning of the thyroid gland; and selenoprotein P (SeP), a protein that participates in the transport of Se between different organs (Kuras, Reszka, Wieczorek, Jablonska, Gromadzinska, Malachowska, et al., 2018). As a result of different geological conditions, selenium is distributed in nature in a non-uniform way in animals and crops worldwide (D'Amato, De Feudis, Hasuoka, Regni, Pacheco, Onofri, et al., 2018).

Milk and milk products are foods recognized for their high nutritional value since they provide macronutrients like proteins and carbohydrates. They are also an important source of essential vitamins and minerals such as calcium, magnesium, and selenium (Kanwar, Kanwar, Sun, Punj, Matta, Morley, et al., 2009). In milk and its derivatives, most selenium is associated with proteins in the form of seleno-amino acids like selenomethionine (SeMet) or selenocysteine (SeCys) (Vacchina, Bierla, Szpunar, & Lobinski, 2018). The highest levels of Se are found in whey and casein, the lowest levels are in fat (Liu, Zhu, Lu, Wei, & Ren, 2015). The remaining selenium is present in the water-soluble fraction of milk in the form of free seleno-amino acids (Acosta, Torres, Mariño-Repizo, Martinez, & Gil, 2018; Dorea, 2002). In dairy farming, different amino acids are easily incorporated into the milk protein, and they may become a good source of Se for humans (Ling, Henno, Jõudu, Püssa, Jaakson, Kass, et al., 2017).

Currently, the development of selenium-fortified foods is promoted in order to reach the optimal levels of this micronutrient in the diet (Kieliszek & Błażejak, 2013) for populations with low selenium rates. Dietary supplements have been added to the feed of

dairy cows with the objective of fortifying milk with selenium. (Ceballos, Espíndola, Uslar, Neumann, Quiroz, Chihuailaf, et al., 2013). In the framework of a balanced diet, the ingestion of Se in organic form is recommended, mainly as amino acids present in food, because the human body assimilates organic forms of Se more easily than inorganic forms (Thiry, Ruttens, De Temmerman, Schneider, & Pussemier, 2012). It has been stated that fortified supplements like selenized yeast increased Se status to an extent similar to SeMet (Thiry, Ruttens, De Temmerman, Schneider, & Pussemier, 2012). However, the actual selenium bioavailability would depend on the digestion and biotransformation of selenium into free SeMet (Yang, Liu, & Zhou, 2017). In selenized yeast, the results showed that 89 ± 3% of the total Se was extracted after gastrointestinal digestion, but surprisingly only 34 ± 1% was quantified as free SeMet (Reyes, Encinar, Marchante-Gayón, Alonso, & Sanz-Medel, 2006). Foods with a higher concentration of free SeMet are more valuable in terms of selenium nutrition. In this sense, it is important to evaluate the form of selenium that is used to fortify milk. It is worth to mention that proteins containing SeMet are not regarded as selenoproteins due to the non-specific nature of Se utilization in these proteins (Lobanov, Hatfield, & Gladyshev, 2009).

Conventional chromatographic methods are usually used for the separation and identification of selenium species. Recently, free seleno-amino acids have been determined by enantioselective hydrophilic interaction liquid chromatography-tandem mass spectrometry (Piovesana, Montone, Antonelli, Cavaliere, La Barbera, Canepari, et al., 2019). Amino acids found in proteins are *L*-amino acids, and it has been reported that *D*- and *L*-amino acids have different intestinal absorption and metabolic pathways. More specifically, the absorption rate of *D*-isomers is slower than *L*-isomers. *L*-SeMet was determined in olive oils (Capriotti, Montone, Antonelli, Cavaliere, Gasparrini, La Barbera, et al., 2018). In wheat bran, the results showed that seleno-methyl-*L*-selenocysteine was

the major seleno-amino acid, while SeMet and SeCys were both minor species (Montone, Antonelli, Capriotti, Cavaliere, La Barbera, Piovesana, et al., 2019). These techniques have not been applied to free seleno-amino acid analysis in milk samples.

An extraction process must be performed before seleno-amino acids can be analysed in milk. Conventionally, the use of organic solvents is necessary in order to eliminate the fatty phase of milk. However, according to green chemistry principles (Gałuszka, Migaszewski, Konieczka, & Namieśnik, 2012), organic solvents should be avoided because they represent an environmental hazard. Recently, works have been published that apply natural deep eutectic solvents (NADES) to replace conventional solvents for the extraction of proteins in different foods (Lores, Romero, Costas, Bendicho, & Lavilla, 2017). Deep eutectic solvents (DES) are mixtures of substances that form a joint super-lattice that melts and freezes at a single temperature that is lower than the melting points of the separate constituents (Abbott, Capper, Davies, Rasheed, & Tambyrajah, 2003). NADES are mixtures formed by molecular constituents such as sugars, alcohols, amino acids, organic acids, and choline derivates (Fernández, Boiteux, Espino, Gomez, & Silva, 2018). They are considered as the third solvent in living cells, which explains their high solubilizing capacity for natural products. NADES in combination with ultrasonic energy is a green approach for proteins solubilisation (Lores, Romero, Costas, Bendicho, & Lavilla, 2017).

This research describes a new process for extraction of free seleno-amino acids from milk samples with NADES. To this end different NADES were tested and an experimental design was performed to define the optimized values of the extraction parameters. Seleno-amino acids in milk were determined by LC-ICP MS. The introduction of NADES to ICP MS was optimized. As a result, a green extraction procedure was obtained and applied to analyse commercial and selenium fortified milk samples.

Hypothesis statement. Free seleno-amino acids are extracted with NADES from powder and lyophilized milk samples in a simple green procedure.

2. Experimental

2.1. Reagents and Standards.

The reagents were used directly as purchased or purified according to standard procedures (Armarego, 2017). Certified multi-elemental standard solutions from Perkin Elmer Pure Plus-Atomic Spectroscopy Standards (Norwalk, USA) were used for calibration and recovery studies (St. Louis, MO, USA). SeMet, SeCys, and seleno-methyl-selenocysteine (Se-Met-SeCys) standards were purchased from Sigma Aldrich (St. Louis, MO). Standard solutions were prepared by dissolving the respective substances in 0.1 M hydrochloric acid, except for SeMet, which was prepared in 0.5% 2-mercaptoethanol (0.3 mg g^{-1}). Stock solutions were prepared once and stored at -20 °C. Dilutions were made with a 0.004% (w v^{-1}) aqueous solution of 2-mercaptoethanol to avoid oxidation of SeMet (Torres, Martínez, & Pacheco, 2018). Compounds for NADES preparation, including anhydrous glucose (99%), anhydrous citric acid (99%), D-(–)-fructose (99%), and L-(+)-lactic acid (85–90%) were purchased from Biopack (Bs. As., Argentina). Ultrapure water with a resistivity of 18.2 mΩ cm^{-1} was obtained from a Milli-Q system (Millipore, Billerica, MA, USA). Skimmed milk powder, (ERM® -BD151) certified by ERM European reference materials and the European Commission, was used as a reference material (Spain-Europe).

2.2. Preparation of NADES.

NADES were prepared following the recommendations described by different authors (Dai, van Spronsen, Witkamp, Verpoorte, & Choi, 2013; Pisano, Espino, de los

Ángeles Fernández, Silva, & Olivieri, 2018). The components LGH (lactic acid: glucose 5:1); CGH (citric acid: glucose, 1:1) and FCH (fructose: citric acid; 1:1) were mixed with 18% H_2O (v v^{-1}) in a glass beaker. The mixture was heated on a magnetic stirrer with temperature control (Decalab, Buenos Aires, Argentina) at 80 °C for approximately 60 minutes until a transparent and homogeneous mixture was obtained. The synthesized NADES were stored at 4 °C to ensure conservation until their use. Different dilutions were made in the molar ratios 1:9, 3:7, and 5:5 (NADE:H_2O) of these solvents for additional studies (Lores, Romero, Costas, Bendicho, & Lavilla, 2017). The prepared NADES were evaluated for selenium extraction from milk powder samples.

2.3. Instrumentation

The following laboratory equipment was used for sample treatment: ultrasound bath (Testlab, Buenos Aires, Argentina), magnetic stirrer with hot plate (Decalab, Buenos Aires, Argentina), ultracentrifuge U-320-R (Boeco-Germany), analytical balance (Ohaus, New Jersey).

Selenium was analysed by an ICP MS (ELAN DRC-e, Perkin-Elmer SCIEX, Thornhill, Canada). Air Liquide (Rio IV-Córdoba, Argentina) supplied argon gas with a purity of 99.996%. An HF-resistant and high performance perfluoracetate nebulizer, model PFA-ST, was used. Before changing to the microconcentric nebulizer, a performance check was carried out for sensitivity, oxide and doubly charged ion formation, using a conventional PTFE cross flow nebulizer and a Scott-type spray chamber. Peristaltic pump tubing, Tygon black/black 0.76 mm i.d. and 40 cm long, was used. The instrument conditions were as follows: autolens mode, peak hop scanning mode, dwell time of 500 ms in standard mode, 3 replicates, and dual mode detector. Nickel sampler and skimmer cones were used. Gas flow rates correspond to plasma, 13 L min^{-1}; auxiliary, 1.35 L min^{-1}; and

nebulizer, 0.87 L min^{-1}. The radio frequency power was optimized to 1200 W, and the sample flow rate corresponded to 400 µL min^{-1}.

At a later stage, seleno-amino acids were determined by liquid chromatography (LC) using a C8 column, Phenomenex, Luna (4.6 mm x 150mm x 5µ), under isocratic conditions. The mobile phase consisted of 10 mM trifluoroacetic buffer (pH 3.0) and 2% (v v^{-1}) methanol pumped at 2.0 mL min^{-1}. The sample volume injected was 200µL. The chromatographer used was a Perkin-Elmer 200 Series (Thornhill, Canada) coupled to an ICP MS equipment detailed above.

2.4. Samples.

Samples of sheep's milk were obtained from the AZD farm of the Department of Veterinary Medicine, University of Perugia (Italy). Twenty Sarda ewes in mid lactation (3rd – 4th month after parturition) were randomly divided in two groups of equal number. Both groups were fed with two isoenergetic and isonitrogenous pelleted concentrates. However, one received a control concentrate containing ground dehydrated olive leaves (202.9 g kg^{-1}), while the second was treated with an experimental concentrate that included the same amount of olive leaves from sodium selenate-fertilized trees (Se content in leaves: 7.83 ± 0.13 mg kg^{-1}).

In addition, samples of milk powder of bovine origin were obtained from various commercial brands produced in Argentina. Table 1 SM shows the characteristics of each of the milks studied.

2.5. Sample treatment.

For each experiment, 0.94 g of homogenized milk sample were accurately weighted in a 10 mL centrifuge tube. Subsequently, 3.09 mL of LGH (lactic acid-glucose-water)

25% (v v^{-1}) were added. The test tubes were shaken at 300 rpm for 30 seconds. Ultrasound assisted extraction was performed for 34 min using an ultrasound equipped with a digital timer. The mixture was centrifuged for 15 minutes (8335 g, 4 °C). After centrifugation, three phases were defined in the mixture: the upper layer corresponds to the fats present in milk, the medium phase represents the NADES extract where the concentrated free seleno-amino acids are present, and the final precipitate contains mainly high molecular weight protein. For comparison, ultrapure water and SDS-Tris pH 7.5 were also tested as extraction solvents.

Sample treatment for total selenium concentration analysis was performed by microwave-assisted acid digestion. For the digestion procedure, 0.5 g of milk sample were mixed with 7.0 mL of HNO_3 and 1.0 mL of H_2O_2 in PTFE flasks. Then they were introduced to an optimized MW temperature program. Finally, digests were diluted to 50 mL.

2.6. Optimization strategy for ICP MS for Se-NADES analysis.

NADES solutions with 50 µg L^{-1} selenium were prepared by dilution 20% (v v^{-1}) with LGH, CGH, and FCH solutions. HNO_3 1.0% (v v^{-1}) was added to Se-NADES solutions to favour nebulization and sustain plasma. The following ICP MS parameters were tested: selenium isotopes, Se^{77}, Se^{78}, and Se^{82}; nebulization gas flow rates (NGFR), 0.75, 0.80, 0.85, and 0.90 L min^{-1}; and the RF power, 900, 1000, 1100, 1200, and 1300 W. After the optimization process was set for all further determinations, the solutions were introduced into the plasma source at 400 µL min^{-1} applying 1200 W RF power and 0.87 mL min^{-1} nebulizer gas flow rate.

2.7. Application of the experimental design for optimization of the method.

After NADES evaluation to identify the extractant with the higher Se recovery from milk samples, an experimental design was performed to obtain accurate data on the most influential factors in the system. A Box-Behnken design was applied using a Design Expert® 7.0.0 software. The minimum and maximum ranges of each evaluated factor were as follows: NADES concentration, 10-50% v v^{-1}; NADES volume, 0.5-5 mL; extraction time, 15-45 minutes; sample amount, 0.01-1 g. The intensity of the selenium signal was selected as the response variable, which was measured for each of the design points.

3. Results and discussion

3.1. NADES selection according to selenium extraction from milk samples.

NADES represent a green alternative for the extraction of bioactive compounds from various complex matrices. These environmentally safe extractants have been used in ultrasound-assisted microextractions to determine different selenium species from water and food samples such as fruit juices, eggs, cow's milk, sheep's milk, yogurt, etc (Panhwar, Tuzen, & Kazi, 2017). Among the most studied mixtures are combinations of organic acids and sugars or choline chloride (Espino, de los Ángeles Fernández, Gomez, & Silva, 2016). The incorporation of water into the euctectic system is a factor that affects the physical-chemical properties and also influences the extraction performance. Although it has been reported that low water content is more suitable for low polarity compounds, satisfactory results are obtained with higher water content for polar compounds (Bosiljkov, Dujmić, Bubalo, Hribar, Vidrih, Brnčić, et al., 2017). Based on these observations, it was decided to

243 evaluate the extraction of organic selenium species by NADES formed from natural
244 organic compounds such as glucose and an 18% (v v^{-1}) NADE dilution (Shishov, Bulatov,
245 Locatelli, Carradori, & Andruch, 2017).

246 LGH, lactic acid: glucose 5:1; CGH, citric acid: glucose 1:1, and FCH, fructose:
247 citric acid 1:1 were evaluated for selenium extraction. The results obtained are shown in
248 Figure 1. Extraction is expressed as relative recovery, considering the recovery of the
249 NADES extractant with the higher efficiency as 100 %. Comparatively, a typical amino
250 acid extractant, SDS-Tris, was also evaluated (Huang, Feng, Chen, Wu, & Wang, 2018).
251 The highest recoveries were obtained with LGH, which showed higher extraction
252 efficiency than a typical amino acid extractant. Accordingly, LGH was selected for the
253 subsequent optimization of seleno-amino acid extraction.

254

255 ## 3.2. ICP MS optimization for Se-NADES solution analysis.

256 Thanks to its high sensitivity, selectivity, and its multi-elemental and isotopic
257 nature, one of the analytical techniques that is most often used for the determination of
258 trace elements and oligo-elements in complex food samples is ICP MS (Sola-Larrañaga &
259 Navarro-Blasco, 2009). NADES present a complex organic matrix representing a challenge
260 for the conventional sample introduction system of ICP MS, because NADES can generate
261 instability in the plasma and even alterations in the interface (Dubascoux, Andrey, Vigo,
262 Kastenmayer, & Poitevin, 2018). In addition, carbon resulting from NADES and the milk
263 matrix can generate deposits in the cones and lenses with the consequent loss of sensitivity
264 in the signal (Azcarate, Savio, Smichowski, Martinez, Camiña, & Gil, 2015). To maintain
265 a reproducible analysis without losing sensitivity, water dilution, along with NGFR and RF
266 optimization, is a strategy to overcome these difficulties. To evaluate NADES polyatomic
267 molecules' contribution to the ICP MS background signal, 50 µg L^{-1} Se - LGH solutions

(as described in section 2.6) were analysed by ICP MS monitoring Se^{77}, Se^{78} and Se^{82} under different NGFR and RF power levels. The results can be observed in Figure 2. Se^{77} may be interfered since an exaltation of the signal-noise ratio (S/N) is observed at an RF power of 1100 W. Se^{77}, Se^{78} and Se^{82} signals are exalted in the NGFR range of 0.85-0.9 mL min^{-1}. RF power analysis in the 950 – 1100 W range shows an improved signal stability for Se^{78} and Se^{82} isotopes. The Se isotopes studied are stable, and increasing the RF power enhances the degree of ionization and collisions, improving selenium atomization. Despite the fact that Se^{78} has a higher relative abundance, the S/N is low compared to Se^{82}; so, Se^{82} was selected for monitoring the selenium signal. The best condition found was at an Ar gas flow rate of 0.85 mL min^{-1} and an RF power of 1000 W.

3.3. Study of the factors influencing the Selenium extraction process.

A design of experiments is an application of the scientific method to generate knowledge about a system or process. It is a set of techniques that allows one to achieve maximum efficiency at the lowest cost. In addition, it is a useful tool to achieve improvements in established processes (Gutiérrez Pulido & Salazar, 2004). Accordingly, it was decided to propose an experimental design to improve the extraction of free selenium species with LGH from milk samples. After evaluation of the extraction process, the following factors were considered relevant and should be studied since they can influence the response: water percentage incorporated into the NADE (% LGH), volume of NADE (mL), sample quantity (g), and ultrasound time (min). The working ranges of each factor were detailed in section 2.7.

A multivariate strategy was adopted to study the influence of the selected variables and their interactions according to the experimental design shown in Table 2 SM. The final optimization of the proposed methodology and the expected response according to the

293 selected factors was carried out using the response surface method (RSM). The RSM has
294 been seen in other reports where multivariate optimization strategies are applied (Maratta,
295 Carrizo, Bazán, Villafañe, Martínez, & Pacheco, 2018). A Box-Behnken design was
296 exploited. This design is formed by combining factorial designs on two levels with
297 incomplete balanced block designs (IBBD). The most significant variables were
298 considered in order to determine the values for the best selenium signal intensity.

299 The response surfaces from the experimental design can be observed in Figure 3.
300 An improvement in the extraction performance of the selected seleno-amino acid was
301 observed in Figure 3a by increasing the volume of NADE extractant and increasing the
302 sample quantity. Despite the fact that increasing the extractant volume might decrease the
303 Se signal by dilution, this is compensated by the higher quantity of sample.

304 Figure 3b shows an analysis of the compromise between the best dilution
305 percentages of LGH and the volumes. The results showed that the extraction process is
306 considerably favoured by lower LGH percentage and higher volume. One of the variables
307 that significantly affects the extraction system is the percentage of water added to the
308 selected NADES. The super-molecular structure of NADES changes after dilution with
309 water because of the progressive rupture of hydrogen bonds. The physicochemical
310 properties such as viscosity, conductivity, density, water activity, and polarity vary to some
311 extent depending on the chemical nature of the components (Dai, Witkamp, Verpoorte, &
312 Choi, 2015). A compromise situation was found at an optimal dilution without NADE
313 losing its capacity as an extraction solvent, because at 10% (v v^{-1}) dilution, LGH loses
314 hydrogen bridge bonds which affect its characteristic as a DES (Pisano, Espino, de los
315 Ángeles Fernández, Silva, & Olivieri, 2018). Finally, from the analysis of Figure 3c, it is
316 observed that the selenium signal increases with increasing the ultrasound time, thus
317 improving the extraction process. The results showed that the optimal working conditions

were as follows: 21.94% of LGH, 33.26 minutes of ultrasound time, 0.94 g of sample, and an extractant volume of 3.09 mL.

3.4. Evaluation of the greenness of the extraction procedure.

The greenness of the extraction procedure was evaluated according to the penalty points of the Eco-Scale (Gałuszka, Migaszewski, Konieczka, & Namieśnik, 2012). An ideal green procedure has 100 points on the Eco-Scale; penalty points lower the total score. Penalty points are calculated by considering the amount of reagents, hazards, energy, occupational hazards, and waste. After NADES addition to milk samples, three layers are formed, an upper one containing fats, a NADES middle one, and proteins are separated at the bottom. Selenium concentration in the protein fraction corresponds to 4-28% of the total selenium concentration in the milk samples. This selenium percentage in the protein fraction is low compared to the 70% obtained when acetone ratio is used to precipitate proteins (Bierla, Szpunar, & Lobinski, 2008). NADES molecular structure increase free-seleno amino acids extraction avoiding co-precipitation with proteins.

Fats and proteins are separated from milk in one step. This avoids the use of hazardous reagents, saves energy, and decreases waste. A comparison of the penalty points of a NADES extraction with a reported milk sample treatment involving defatting and protein precipitation is presented in Table 1. Free seleno-amino acid extraction with NADES represents advantages compared to other techniques, because it avoids several extraction steps that are necessary with common solvents, such as dilution, defatting, and protein precipitation of lyophilized or powdered milk samples for seleno-amino acid analysis (Bierla, Szpunar, & Lobinski, 2008).

3.5. Validation of the proposed method

The proposed methodology was validated at 4 concentration levels for SeCys, SeMet, and Se-Met-SeCys with 3 replicates. The results can be observed in Table 2. Calibration curves were obtained by linear regression between the signal intensity for the isotope Se^{82} (cps) and the concentration of each seleno-amino acid, observing excellent linearity in the working range studied (25-200 µg L^{-1}); linearity coefficients (R^2) were 0.989-0.995. The tests are statistically similar to the *t*-test of paired samples ($\rho = 0.05$). The average results were used to represent the data. Microsoft Excel® was used to test unidirectional variance analysis (ANOVA) with 95% confidence. Additionally, the F test showed that the linear regression was statistically acceptable in the working range, and this model showed a good fit. The limit of detection (LoD) and the limit of quantification (LoQ) for SeCys were calculated according to the recommendations of the IUPAC (International Union of Pure and Applied Chemistry), (Uhrovčík, 2014), and they were 7.37 and 22.36 µg kg^{-1} respectively. The LoD and LoQ for SeMet and Se-Met-SeCys corresponded to 8.63, 26.25 and 9.64, 29.2 µg kg^{-1}, respectively. On the other hand, the percentage relative standard deviation (RSD %), was less than 7.08%.

As observed in Table 2, the extraction efficiency was evaluated in lyophylized Se-fortified sheep milk and cow milk powder samples by spiking SeCys, SeMet, and Se-Met-SeCys at 4 concentration levels. Recoveries were quantitative for free seleno-amino acids in the range of 90.44-109.44% after the application of the NADES extraction method. Figure 1 SM shows a chromatogram of seleno-amino acids analysis after NADES extraction of a commercial cow milk sample, and the same sample spiked at LoQ levels. Trifluoroacetic acid present in the mobile phase acts as an ionic pair with seleno-amino acids, being retained in the C8 column. This column allowed a faster analysis, since retention was lower compared with a C18 column; this is a desirable aspect because of the high running costs of ICP MS.

3.6. Application of the developed method.

To demonstrate the applicability of the system to real samples, the developed method was applied to free seleno-amino acid analysis in lyophilized samples of Se-enriched sheep milk and commercial samples of powdered cow's milk. The method could only be applied to free seleno-amino acids analysis, because selenium in milk samples is associated with proteins that are found in the high molecular weight protein fraction (Bierla, Szpunar, & Lobinski, 2008).

The free seleno-amino acids concentrations determined are shown in Table 3. The following ranges of concentrations were found: SeCys, 61.8-181.9 µg kg^{-1}; Se-Met-SeCys, 46.7-237.7 µg kg^{-1}, and finally, 46.07-180.94 µg kg^{-1} for SeMet. Total free seleno-amino acids in the analysed milk samples ranged from 4.06-5.38% compared to the total selenium concentration. The Certified Reference Material ERM®-BD150 was also analysed, and SeMet was detected with a concentrations of 46.07 µg kg^{-1}. These results are in good agreement with the results reported by Krata et al. who introduced dilution analysis (IDA) and species-unspecific isotope dilution analysis with LC-ICP MS (Krata, Wojciechowski, Karasinski, & Bulska, 2018). However, the results reported in this study refer to proteic and free SeMet in ERM®-BD150. Specific reports of free seleno-amino acid concentrations in milk samples were not found in the literature.

4. Conclusions

The proposed hypothesis statement was confirmed. This research describes a new methodology for free seleno-amino acids analysis in milk samples with selenium determination by LC-ICP MS. Free seleno-amino acid extraction is based on a simple and green analytical procedure employing NADES for solubilization and ultrasound assisted

393 extraction. In a first approach, Se determination in NADES solution was optimized in ICP
394 MS, since NADES viscosity affects Se atomization. RF power and gas carrier were
395 successfully adjusted to achieve an increase in sensitivity and an adequate quantification of
396 selenium.

397 Free seleno-amino acid extraction was performed with different NADES, and LGH
398 showed the best extraction performance. The extraction experimental conditions were
399 optimized by an experimental design. Water percentage in LGH, ultrasound time, sample
400 quantity, and LGH volume proved to be the most influential variables in the extraction, and
401 consequently these factors were optimized.

402 The optimized extraction of free seleno-amino acids with NADES showed
403 quantitative recoveries with good precision and sensitivity compatible with the SeCys,
404 SeMet and Se-Met-SeCys concentrations in the samples. The method was applied
405 successfully to real samples like cow's milk powder samples, freeze dried selenium-
406 biofortified sheep's milk, and a CRM ERM-BD150 skimmed milk powder.

407 The proposed extraction method is a green method and a one-step alternative to
408 traditional milk powder sample solubilization and extraction processes with organic
409 solvents for seleno-amino acid analysis. This study represents an important contribution to
410 assess the nutritional quality of milk samples according to the different bioavailability of
411 seleno-species to humans.

412

413 **Acknowledgement.**

414 Funding: This work was supported by the National Council of Scientific and
415 Technical Research (CONICET) [2 grants, Lopez, Maratta], National Agency for
416 Scientific and Research Promotion (ANPCyT) and the National University of San Luis
417 (UNSL).

418

References

Abbott, A. P., Capper, G., Davies, D. L., Rasheed, R. K., & Tambyrajah, V. (2003). Novel solvent properties of choline chloride/urea mixtures. *Chemical Communications*(1), 70-71.

Acosta, M., Torres, S., Mariño-Repizo, L., Martinez, L. D., & Gil, R. A. (2018). Novel method for metalloproteins determination in human breast milk by size exclusion chromatography coupled to inductively coupled plasma mass spectrometry. *Journal of pharmaceutical and biomedical analysis, 158*, 209-213.

Armarego, W. L. (2017). *Purification of laboratory chemicals*: Butterworth-Heinemann.

Azcarate, S. M., Savio, M., Smichowski, P., Martinez, L. D., Camiña, J. M., & Gil, R. A. (2015). Single-step solubilization of milk samples with N, N-dimethylformamide for inductively coupled plasma-mass spectrometry analysis and classification based on their elemental composition. *Talanta, 143*, 64-70.

Bierla, K., Szpunar, J., & Lobinski, R. (2008). Specific determination of selenoaminoacids in whole milk by 2D size-exclusion-ion-paring reversed phase high-performance liquid chromatography–inductively coupled plasma mass spectrometry (HPLC–ICP MS). *Analytica Chimica Acta, 624*(2), 195-202.

Bosiljkov, T., Dujmić, F., Bubalo, M. C., Hribar, J., Vidrih, R., Brnčić, M., Zlatic, E., Redovniković, I. R., & Jokić, S. (2017). Natural deep eutectic solvents and ultrasound-assisted extraction: Green approaches for extraction of wine lees anthocyanins. *Food and bioproducts processing, 102*, 195-203.

Capriotti, A. L., Montone, C. M., Antonelli, M., Cavaliere, C., Gasparrini, F., La Barbera, G., Piovesana, S., & Laganà, A. (2018). Simultaneous Preconcentration, Identification, and Quantitation of Selenoamino Acids in Oils by Enantioselective

High Performance Liquid Chromatography and Mass Spectrometry. Analytical Chemistry, 90(14), 8326-8330.

Ceballos, A., Espíndola, S., Uslar, D., Neumann, J., Quiroz, E., Chihuailaf, R., & Wittwer, F. (2013). Concentración de selenio en leche de estanques de rebaños lecheros del sur de Chile y su relación con indicadores productivos. *Archivos de medicina veterinaria, 45*(1), 33-39.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic solvents as new potential media for green technology. *Analytica chimica acta, 766*, 61-68.

Dai, Y., Witkamp, G.-J., Verpoorte, R., & Choi, Y. H. (2015). Tailoring properties of natural deep eutectic solvents with water to facilitate their applications. *Food chemistry, 187*, 14-19.

Dorea, J. G. (2002). Selenium and breast-feeding. *British Journal of Nutrition, 88*(5), 443-461.

Dubascoux, S., Andrey, D., Vigo, M., Kastenmayer, P., & Poitevin, E. (2018). Validation of a dilute and shoot method for quantification of 12 elements by inductively coupled plasma tandem mass spectrometry in human milk and in cow milk preparations. *Journal of Trace Elements in Medicine and Biology, 49*, 19-26.

D'Amato, R., De Feudis, M., Hasuoka, P. E., Regni, L., Pacheco, P. H., Onofri, A., Businelli, D., & Proietti, P. (2018). The Selenium Supplementation Influences Olive Tree Production and Oil Stability Against Oxidation and Can Alleviate the Water Deficiency Effects. Frontiers in Plant Science, 9(1191).

Espino, M., de los Ángeles Fernández, M., Gomez, F. J., & Silva, M. F. (2016). Natural designer solvents for greening analytical chemistry. *TrAC Trends in Analytical Chemistry, 76*, 126-136.

Fernández, M. d. l. Á., Boiteux, J., Espino, M., Gomez, F. J. V., & Silva, M. F. (2018). Natural deep eutectic solvents-mediated extractions: The way forward for sustainable analytical developments. *Analytica Chimica Acta, 1038*, 1-10.

Gałuszka, A., Migaszewski, Z. M., Konieczka, P., & Namieśnik, J. (2012). Analytical Eco-Scale for assessing the greenness of analytical procedures. *TrAC Trends in Analytical Chemistry, 37*, 61-72.

Gutiérrez Pulido, H., & Salazar, V. (2004). Análisis y diseño de experimentos.

Huang, Y., Feng, F., Chen, Z.-G., Wu, T., & Wang, Z.-H. (2018). Green and efficient removal of cadmium from rice flour using natural deep eutectic solvents. *Food chemistry, 244*, 260-265.

Kanwar, J. R., Kanwar, R. K., Sun, X., Punj, V., Matta, H., Morley, S. M., Parratt, A., Puri, M., & Sehgal, R. (2009). Molecular and biotechnological advances in milk proteins in relation to human health. *Current Protein and Peptide Science, 10*(4), 308-338.

Kieliszek, M., & Błażejak, S. (2013). Selenium: significance, and outlook for supplementation. *Nutrition, 29*(5), 713-718.

Krata, A. A., Wojciechowski, M., Karasinski, J., & Bulska, E. (2018). Comparative study of high performance liquid chromatography species-specific and species-unspecific isotope dilution inductively coupled plasma mass spectrometry. A case study of selenomethionine and the origin of its oxidized form. *Microchemical Journal, 143*, 416-422.

Kuras, R., Reszka, E., Wieczorek, E., Jablonska, E., Gromadzinska, J., Malachowska, B., Kozlowska, L., Stanislawska, M., Janasik, B., & Wasowicz, W. (2018). Biomarkers

of selenium status and antioxidant effect in workers occupationally exposed to mercury. *Journal of Trace Elements in Medicine and Biology, 49*, 43-50.

Ling, K., Henno, M., Jõudu, I., Püssa, T., Jaakson, H., Kass, M., Anton, D., & Ots, M. (2017). Selenium supplementation of diets of dairy cows to produce Se-enriched cheese. *International dairy journal, 71*, 76-81.

Liu, H., Zhu, W., Lu, B., Wei, Z., & Ren, D. (2015). Effect of feed selenium supplementation on milk selenium distribution and mozzarella quality. *Journal of dairy science, 98*(12), 8359-8367.

Lobanov, A. V., Hatfield, D. L., & Gladyshev V. N. (2009). Eukaryotic selenoproteins and selenoproteomes. *Biochimica et Biophysica Acta, 1790*, 1424-1428.

Lores, H., Romero, V., Costas, I., Bendicho, C., & Lavilla, I. (2017). Natural deep eutectic solvents in combination with ultrasonic energy as a green approach for solubilisation of proteins: application to gluten determination by immunoassay. *Talanta, 162*, 453-459.

Maratta, A., Carrizo, B., Bazán, V. L., Villafañe, G., Martínez, L. D., & Pacheco, P. (2018). Antimony speciation analysis by hydride trapping on hybrid nanoparticles packed in a needle trap device with electro-thermal atomic absorption spectrometry determination. *Journal of Analytical Atomic Spectrometry, 33*(12), 2195-2202.

Montone, C. M., Antonelli, M., Capriotti, A. L., Cavaliere, C., La Barbera, G., Piovesana, S., & Laganà, A. (2019). Investigation of free and conjugated seleno-amino acids in wheat bran by hydrophilic interaction liquid chromatography with tandem mass spectrometry. Journal of Separation Science, 42(10), 1938-1947.

Panhwar, A. H., Tuzen, M., & Kazi, T. G. (2017). Ultrasonic assisted dispersive liquid-liquid microextraction method based on deep eutectic solvent for speciation,

preconcentration and determination of selenium species (IV) and (VI) in water and food samples. *Talanta, 175*, 352-358.

Piovesana, S., Montone, C. M., Antonelli, M., Cavaliere, C., La Barbera, G., Canepari, S., Samperi, R., Laganà, A., & Capriotti, A. L. (2019). Investigation of free seleno-amino acids in extra-virgin olive oil by mixed mode solid phase extraction cleanup and enantioselective hydrophilic interaction liquid chromatography-tandem mass spectrometry. Food Chemistry, 278, 17-25.

Pisano, P. L., Espino, M., de los Ángeles Fernández, M., Silva, M. F., & Olivieri, A. C. (2018). Structural analysis of natural deep eutectic solvents. Theoretical and experimental study. *Microchemical Journal, 143*, 252-258.

Reyes, L. H., Encinar, J. R., Marchante-Gayón, J. M., Alonso, J. I. G., & Sanz-Medel, A. (2006). Selenium bioaccessibility assessment in selenized yeast after "in vitro" gastrointestinal digestion using two-dimensional chromatography and mass spectrometry. Journal of Chromatography A, 1110(1), 108-116.

Shishov, A., Bulatov, A., Locatelli, M., Carradori, S., & Andruch, V. (2017). Application of deep eutectic solvents in analytical chemistry. A review. *Microchemical Journal, 135*, 33-38.

Sola-Larrañaga, C., & Navarro-Blasco, I. (2009). Chemometric analysis of minerals and trace elements in raw cow milk from the community of Navarra, Spain. *Food Chemistry, 112*(1), 189-196.

Thiry, C., Ruttens, A., De Temmerman, L., Schneider, Y.-J., & Pussemier, L. (2012). Current knowledge in species-related bioavailability of selenium in food. *Food Chemistry, 130*(4), 767-784.

Torres, S., Martínez, L. D., & Pacheco, P. H. (2018). Determination of arsenic species distribution in extra virgin olive oils from arsenic-endemic areas by dimensional

chromatography and atomic spectroscopy. *Journal of Food Composition and Analysis*, *66*, 121-126.

Uhrovčík, J. (2014). Strategy for determination of LOD and LOQ values–Some basic aspects. *Talanta*, *119*, 178-180.

Ullah, H., Liu, G., Yousaf, B., Ali, M. U., Irshad, S., Abbas, Q., & Ahmad, R. (2019). A comprehensive review on environmental transformation of selenium: recent advances and research perspectives. *Environmental geochemistry and health*, *41*(2), 1003-1035.

Vacchina, V., Bierla, K., Szpunar, J., & Lobinski, R. (2018). Quantification of SeMet and SeCys in biological fluids and tissues by liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP MS). In *Selenoproteins*, (pp. 153-162): Springer.

Yang, R., Liu, Y., & Zhou, Z. (2017). Selenium and Selenoproteins, from Structure, Function to Food Resource and Nutrition. Food Science and Technology Research, 23(3), 363-373.

560 **Table 1.** Comparison of the penalty points of EcoScale of extraction procedures for seleno-

561 amino acids from milk.

Parameters	**Experimental conditions**	**Penalty points**
NADES EXTRACTION		
Reagents (quantity, hazard)	LGH (3.09 mL)	1 (<10 mL, 0 hazard)
Energy	Ultrasonic bath (34 min, 160w)	0 (<0.1kW h^{-1})
Occupational hazard		0
Waste	□4 mL (0.94 g sample, 3.09 mL LGH)	1 (1-10 mL)
TOTAL PENALTY POINTS		**2**
REPORTED MILK TREATMENT		
Milk deffating		
Reagents (quantity, hazard)	Ciclohexane (20 mL)	1 x 8 (<10 mL, 8 hazard)
Energy	Centrifuge + heater	2
Occupational hazard	Emission of vapors and gases to the air	3
Waste	4 g sample, 20 mL ciclohexane	5 (>10 mL)
Penalty Points		18
Protein precipitation		
Reagents (quantity, hazard)	Acetone	1 x 4 (<10 mL, 4 hazard)
Energy	-	0
Occupational hazard	Emission of vapors and gases to the air	3
Waste		1 (<1 mL)
Penalty Points		8
TOTAL PENALTY POINTS		**26**

567 **Table 2.** Recovery study of free seleno-amino acids from sheep biofortified milk.

568

Se Amino Acids	Lyophylized Se-fortified sheep milk				Cow milk powder			
	Base Concentration (µg L^{-1})	Added Concentration (µg L^{-1})	Determined Concentration (µg L^{-1})	RR (%)	Base Concentration (µg L^{-1})	Added Concentration (µg L^{-1})	Determined Concentration (µg L^{-1})	RR (%)
Se Cysteine (SeCys)	76.2	25	111±10.3	109	29.8	25	51.7±5	94.5
	76.2	50	138±12.7	109	29.8	50	75.9±8.1	95.1
	76.2	100	190±21.4	108	29.8	100	125±17.2	96.6
	76.2	200	277±21.6	100	29.8	200	226±19.1	98.3
Se-Methyl-Se-Cysteine (Se-Met-Se-Cys)	99.4	25	119±20.2	95.6	38.0	25	61.1±6.2	92.5
	99.4	50	155±14.9	104	38.0	50	79.6±8.5	90.4
	99.4	100	205±16.6	103	38.0	100	132±14.7	95.6
	99.4	200	325±29.1	109	38.0	200	236±17.5	99.2
Seleno-Methionine (Se-Met)	69.7	25	97.2±11.1	103	33.6	25	54.5±5.5	93.1
	69.7	50	128±12.5	107	33.6	50	80.1±11.4	93
	69.7	100	182±15.3	107	33.6	100	115±22.3	85.9
	69.7	200	279±33.8	103	33.6	200	216±17.6	92.7

569 **Table 3**. Free seleno-amino acids concentration in milk powder samples.

Sample	SeCys (µg kg^{-1})	Se-Met-SeCys (µg kg^{-1})	SeMet (µg kg^{-1})	Total Selenium (mg kg^{-1})
Commercial / cow #1	29.8±3.1	38.0±3.6	33.6±2.1	1.15 ± 0.06
Commercial / cow #2	45.4±3.9	52.9±6.3	77.6±9.4	1.24 ± 0.06
Commercial / cow #3	39.5±4.7	44.6±4.9	58.2±5.2	1.07 ± 0.05
Freeze dried sheep's Milk (control)	31.8± 4.1	35.1±4.5	44.1±5.7	0.96 ± 0.05
Freeze dried sheep's Milk (enriched)	76.2±6.1	99.4± 8.9	69.7±8.07	2.19 ± 0.19
Certified Reference Material ERM®-BD150	ND	ND	17.8±2.30	0.2 ± 0.01

*ND: not detectable. LoD SeCys: 7.37 µg kg^{-1}, LoD Se-Met-SeCys: 9.64 µg kg^{-1}
n=10

570

571 **Figure Captions.**

572 **Figure 1.** Comparison of selenium relative recovery from cow powder milk and
573 lyophilized biofortified sheep milk between different NADES. LGH, lactic acid: glucose
574 5:1; CGH, citric acid: glucose 1:1; FCH, fructose: citric acid 1:1. Dilution: 18% (v v^{-1}).

575 **Figure 2.** Variations of signal to noise ratio (S/N) of Se^{82}, Se^{77} and Se^{78} according to radio
576 frequency (RF) power and nebulization gas flow rate (NGFR).

577 **Figure 3.** Response surfaces obtained using central composite design. (A) Response
578 surface of LGH volume vs. Sample quantity; (B) response surface of LGH % vs. LGH
579 volume (C); response surface of US time vs. LGH volume. LGH, lactic acid: glucose 5:1;
580 US, ultrasound.

581