# *In vitro* selenium bioaccessibility combined with *in vivo* bioavailability and bioactivity in Se-enriched microalga (*Chlorella sorokiniana*) to be used as functional food

Verónica Gómez-Jacinto[a,b,c], Francisco Navarro Roldán[b;e], Inés Garbayo-Nores[b,d], Carlos Vílchez-Lobato[b,d], Ana Arias Borrego*[a,b,c], Tamara García-Barrera*[a,b,c]

[a]*Environmental and Bioanalytical Chemistry Group. Departament of Chemistry "Profesor José Carlos Vílchez Martín". Faculty of Experimental Sciences. University of Huelva. Campus El Carmen. 21007-Huelva. Spain;*
[b]*Research Center of Natural Resources, Health and the Environment (RENSMA). University of Huelva. Spain.*
[c]*International Campus of Excellence on Agrofood (ceiA3). University of Huelva. Spain*
[d] *Algal Biotechnology Group. CIDERTA. University of Huelva. Parque Huelva Empresarial. 21007-Huelva. Spain.*
[e]*Department of Integrated Sciences, Cell Biology, Faculty of Experimental Sciences, University of Huelva, Spain*

***Corresponding Authors***

*E-mail: tamara@dqcm.uhu.es, Department of Chemistry "Professor José Carlos Vílchez Martín". Faculty of Experimental Sciences. University of Huelva. Campus El Carmen. 21007-Huelva. Spain. Telephone number: (+34) 959219962. Fax number: (+34) 959219942;*

*E-mail: ana.arias@dqcm.uhu.es, Department of Chemistry "Professor José Carlos Vílchez Martín". Faculty of Experimental Sciences. University of Huelva. Campus El Carmen. 21007-Huelva. Spain. Telephone number: (+34) 959219033. Fax number: (+34) 959219942;*

**Abstract:**

Speciation of seleno-metabolites and selenoproteins in Se-enriched *Chlorella sorokiniana* microalgae, *in vitro* and *in vivo* extracts has been performed by HPLC-ICP-MS. Selenium bioaccesibility in Se-enriched *Chlorela sorokiniana* evaluated by *in vitro* gastrointestinal digestion of the selenized microalga was 81 % (79% as SeMet). Mice supplemeted with Se-enriched *C. sorokiniana* presented increased Se concentration in kidney, reflecting a potencial mechanism of excretion by urine. In addition, selenium bioavailavility, which was in the range of 3-15% depending on the diet was 1.13-fold higher in mice fed with Se-enriched microalgae against the basal diet, but decreased with higher amounts of selenium. Regarding bioactivity (∑Se as selenoproteins in serum/total Se in serum = 76-85% depending on the diet), selenium and selenoproteins (SeP) increased in serum from animals fed high supplemental Se levels. These results showed that Se-enriched algae can be considered as an alternative selenized food for humans, due to their high Se bioavailability.

***Keywords***: Se-enriched microalgae, speciation, bioaccesibility, bioavailability, *Chlorella sorokiniana, Mus musculus*

## 1. Introduction

Selenium is a well known essential trace element for humans and animals, being the active center of numerous selenoproteins (Rayman, 2000). The deficiency of this element causes oxidative stress and in turn health disorders, such as Keshan disease, cardiovascular disease and myocardial infarction (Rayman, 2000, 2012). The relationship between selenium intake and status is not linear, but approximates more closely to a U-shape where adverse effects (e.g. on mortality and prostate cancer) are found at low and high selenium intake/status (Rayman, 2012). Adequate intakes (AIs) range from 15 µg/day for children aged one to three years to 70 µg/day for adolescents aged 15–17 years and adults ("Scientific Opinion on Dietary Reference Values for iron," 2015). However, the content of selenium in foodstuff varies with the regions and in some cases is not enough to provide the suitable intake of this element so there is a great interest in the production of selenium fortified foods and selenium-based nutritional supplements (functional foods) as well as animal feed supplements and nutraceuticals.

However, due to the narrow concentration range of selenium essenciality, special care should be taken for the preparation of functional foods. Moreover, selenium bioavailability and bioaccessibility in food should be also considered to guarantee the required assimilation, since frequently only a fraction is absorbed and transformed into a biologically available form. The selenium bioaccessible fraction can be defined as selenium that is soluble in the intestine, usually calculated by *in vitro* simulated gastrointestinal digestion. The selenium bioavailable fraction can be considered as the quantity of selenium absorbed through the intestinal barrier that finally reaches the systemic circulation, and the bioactive fraction as the quantity of Se transformed into active selenomolecules (Thiry, Ruttens, De Temmerman, Schneider, & Pussemier, 2012).

On the other hand, the chemical species of selenium present in food strongly determines the esenciallity/toxicity as well as bioavailability. In this sense, it has been reported that the selenoamino acid selenomethionine (SeMet), which is the major nutritional source of selenium for animals, is highly bioavailable (Schrauzer, 2003). For this reason, but also due to its high retention capacity in the organisms for a longer period of time after supplementation, this selenometabolite has been claimed as most suitable form of selenim for supplementation (Rayman, 2000).

*Chlorella,* is an unicellular green algae which is consumed in Europe, Japan, China and USA. Several health benefits have been described for humans and animals after the consumption of this chlorophyll-containing organism (Becker, 2007), mainly due to the presence of minerals, dietary fiber and a wide range of antioxidants substances (Pulz & Gross, 2004; Spolaore, Joannis-Cassan, Duran, & Isambert, 2006; Stengel, Connan, & Popper, 2011). In addition, microalgae are able to efficiently metabolize selenium into reduced selenometablites such as selenoamino acids, but also selenoproteins and volatile compounds (Gojkovic et al., 2013, 2014; Neumann, De Souza, Pickering, & Terry, 2003; Pelah & Cohen, 2005; Umysova et al., 2009). Selenium speciation studies previously perfomed in *C. sorokiniana*, demonstrated that there are three major amino acids after protein hydrolysation, namely: selenocysteic acid, Se-methyl selenocysteine (SeMeSec) and Se-methy lselenocysteine selenoxide) and three free aminoacids: selenocysteine (SeCys), selenocystine (SeCys2) and SeMet (Stadtman, 2003). Moreover, when *Chlorella* grows under low levels of sulphur in the presence of selenite, the main metabolite is SeMet (Veronica Gómez-Jacinto, García-Barrera, Garbayo, Vílchez, & Gómez-Ariza, 2012). Even more importantly, it has been reported that algal cells substitute sulphur by selenium after been exposed to sub-lethal, but higher than trace concentrations of Se. Thus, although selenium is metabolically reduced to selenide through specific enzymes which

drive its assimilatory reduction, it uses the sulphur enzymatic system under overloading conditions (Stadtman, 2003).

Usually, selenium speciation in food is performed on water-soluble extracts. However, the determination of selenium and especially, their chemical species in the gastrointestinal absorbable fraction are scarce. To obtain the bioaccesible fraction, stomach and small intestine digestion have been simulated by treating food with pepsin at pH 2 (gastric digestion) followed by the adition of amylase, pancreatin and bile salts at neutral pH (intestinal digestion) (Thiry et al., 2012). *In vitro* experiments can be used as a reference of biological processes and represent a valuable alternative to *in vivo* experiments, because they are faster, cheaper, simpler (Waisberg, Black, Waisberg, & Hale, 2004) and reduce the use of animals for experimentation. However, the use of model organisms, especially mammals are mandatory to validate the results.

The main objective of this paper is to evaluate the bioaccessibility of selenium in selenized *Chlorella sorokiniana* by *in vitro* digestion simulating main stomach and intestinal physiology events. This allows assessing the feasibility of Se-enriched *Chlorella sorokiniana* biomass as antioxidant functional food for animals and humans. In adition, the bioavaility of selenium (*in vivo* serum and organs determinations) from this functional food has been studied using the mice model (*Mus musculus*) fed selenized microalgae at three different concentrations (0.15, 0.5 and 1 µg Se/g feed). Selenium speciation was carried out in *C. sorokiniana* cells harvested from liquid cultures, where they are produced and in the *in vitro* digests, using reversed phase (RP) coupled to inductively coupled plasma mass spectrometry (ICP-MS). Moreover, selenoproteins and selenometabolites were quantified in mice serum and organs, using a metallomic analytical approach based on a two dimensional column switching system combining size exclusion and affinity chromatography coupled to ICP-MS, using species unspecific

isotopic dilution analisus (2D-SEC-AF-SUID-ICP-MS). The determination of selenoproteins allows also evaluating the bioactivity of selenium in selenized *C. microalgae*. Under our knowledge, this paper is the first one combining the *in vitro* selenium bioaccessibility with *in vivo* bioavailability and bioactivity in Se-enriched microalgae.

## 2. Materials and methods

### *2.1. Instrumentation*

Total selenium determination and speciation was performed in an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan) equipped with an octapole collision/reaction cell. Chromatographic separations of chemical species was performed by means of a Phenomenex Luna C18 column (250×4.6×5µm) mounted into an HPLC pump model 1100 as delivery system using an UV detector (Agilent, Wilmington, DE, USA) on line coupled to the ICP-MS.

Separation of selenoproteins was carried out by two 5 ml HiTrap ® Desalting Columns (GE Healthcare, Uppsala, Sweden) connected to two affinity columns, with stationary phases of heparin-sepharose (HEP-HP) and blue-sepharose (BLU-HP), both purchased from GE Healthcare, Uppsala, Sweden. The outlet of the HPLC column was directly connected via PEEK capillary tubing to the Micromist nebulizer of the ICP-MS. ICP-MS experimental parameters for $H_2$ reaction cell mode were optimized by using a 2% (*v/v*) $HNO_3$ aqueous solution containing $^{59}Co$, $^{7}Li$, $^{89}Y$, and $^{205}Tl$ (100 µg $L^{-1}$). Polyatomic interferences were overcame using $H_2$ at a flow-rate of 4 ml $min^{-1}$ in the plasma (such as $^{80}Ar_2$, $^{32}S^{16}O$, $^{10}Ca_2$, and $^{40}Ar^{40}Ca$). Instrumental settings are collected in Table 1.

Selenum species were extracted using an ultrasonic probe (HD 2200, Bandelin electronic, GmbH & Co. Kg, Berlín, Germany) with a MS 72 tip and the phase separation process was accelerated with a centrifuge model Sigma Laborzentrifugen 4-10 (Osterode, Germany). The mineralization of samples for total selenium determination was carried out into a microwave accelerated reaction system model MARS (CEM Corporation, Mattews, Carolina del Norte, USA) using MARSXpress vessels.

The determination of total chlorophyll and carotenoids in the extracts obtained from microalgal cells was carried out using an UV/vis spectrometer (Ultrospec 3100 pro, Biochromm Ltd). The photosynthetic activity was determined with a Clark electrode (Hansatech, UK). A Neubauer chamber and an optical microscope Olympus model CX41 were used to determine the number of cells. Cells were grown in a borosilicate glass Pyrex® graduated bottle (5 L) containing 4.5 L of culture.

### *2.2. Reagents and standard solutions*

Ultrapure water (18 MΩ cm) was obtained from a Milli-Q water purification system and was used throughout (Millipore, UK). All reagents used were of analytical grade and the standard solutions were stored at 4º C in darkness until the analysis. The following reagents were purchased from Sigma-Aldrich (Steinheim, Germany): EDTA, $MgSO_4 \cdot 7H_2O$, $CaCl_2$ x $2H_2O$, $KH_2PO_4$, $Na_2HPO_4 \cdot 2H_2O$, $NaHCO_3$, $Na_2EDTA \cdot 2H_2O$, $H_3BO_3$, $KNO_3$, $ZnSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, selenocystine ($SeCys_2$), D,L-selenomethionine (D,L-SeMet), Se-methyl selenocysteine (SeMetSec), $Na_2SeO_3$, bile salts, enzymes (e.g. pepsin, pancreatin and amylase) and Protease XIV. Sodium selenite ($Na_2SeO_4$) and tetraethylammonium chloride (TEAC) were obtained from Fluka (Buchs, Switzerland). Ammonium acetate, sodium hydroxide, nitric and hydrochloric acids were purchased from Merck (Darmstadt, Germany).

For the preparation of standards, $SeCys_2$, D,L-SeMet and SeMetSeCys were dissolved with ultrapure water adding 3% HCl (v/v) for the complete dissolution of $SeCys_2$ and SeMetSeCys. Inorganic selenium solutions were prepared by dissolving sodium selenate in 2% (v/v) $HNO_3$ solution and sodium selenite in 2% (v/v) HCl solution. Stock solutions of 1000 mg $L^{-1}$ were stored at 4º C in darkness, whereas working standard solutions were prepared daily by the appropriate dilution. Enriched $^{74}Se$ powder was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) and dissolved in the minimum volume of nitric acid (Suprapur grade), finally diluted to volume with ultrapure water.

The mobile phase used for RP-HPLC-ICP-MS was 0.075% TEAC, adjusted at pH 4.5 with HCl solution. The tuning solution containing $^{59}Co$, $^{7}Li$, $^{89}Y$, and $^{205}Tl$ (100 mg $L^{-1}$ each) was purchased from Agilent Technologies (USA). The reference material SELM-1 (selenium-enriched yeast, 1381.43 ± 63.21 mg $kg^{-1}$ of SeMet) was obtained from the National Research Council of Canada. Human serum certified reference material (CRM) BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium).

### 2.3. Procedures

#### *2.3.1. Preparation of control and Se-supplemented cultures of C. sorokiniana*

The preparation of the cultures was optimized previously and published elsewhere (Moreno et al., 2014). Briefly, microalgae *C. sorokiniana* (CCAP 211/8K) was obtained from the UTEX culture collection. The culture was maintained in Erlenmeyer flasks with modified M-8 medium for exponential grown phase and using the following incubation conditions: 30º C, pH 6.7 and a continuous illumination using a photon flux density of 160 µmol $m^{-2}$ $s^{-1}$. The basal culture medium used contained the following components (mol $L^{-1}$): $KH_2PO_4$ ($5.4x10^{-3}$), $Na_2HPO_4 \cdot 2H_2O$ ($1.5x10^{-3}$), $MgSO_4 \cdot 7H_2O$ ($1.6x10^{-3}$),

$CaCl_2 \cdot 2H_2O$ ($0.9x10^{-4}$), $KNO_3$ ($30x10^{-3}$), EDTA ($0.3x10^{-3}$), $Na_2EDTA \cdot 2H_2O$ ($0.1x10^{-3}$), $H_3BO_3$ ($1.0x10^{-6}$), $MnCl_2 \cdot 4\ H_2O$ ($0.7x10^{-4}$), $ZnSO_4 \cdot 7H_2O$ ($0.1x10^{-4}$), $CuSO_4 \cdot 5H_2O$ ($0.7x10^{-5}$), $NaHCO_3$ ($5x10^{-3}$). Medium sterilization was performed for 20 minutes at 120 ºC and 1 atm. In adition, cultures were bubbled with 5% (v/v) carbon dioxide-enriched air as carbon source. Aeration also kept cultures homogeneous.

Cell viability and the occurrence of bacterial contaminations were followed under optical microscope observations. Total biomass was determined by weighting dried cells (80ºC until constant weight) and measuring optical density. The number of cells were counted combining the optical microscope and the Neubauer chamber. The photosynthetic activity was measured by means of a Clark electrode. Finally, the total content of chlorophyll and carotenoids was determined following the Arnon´s method (Lichtenthaler, 1987) and the H. Lichtenthaler equations (Lichtenthaler, 1987).

On the other hand, prior to preparing Se-supplemented cultures of *C. sorokiniana*, the sub-lethal concentration of sodium selenate was determined. First, cultures of *C. sorokiniana* were grown in the presence of different sodium selenate concentrations in the range from 5 to 200 mg $L^{-1}$. Cultures were exposed to selenium during the exponential growth, resulting that 50 mg $L^{-1}$ was the sub-lethal concentration for the algae. This concentration of sodium selenate was used for further experiments. The stability of sodium selenate in the culture medium was studied by adding concentrations of sodium selenate from 5-200 mg $L^{-1}$ to different culture media, but without the algae. Under these conditions, the interconversion of sodium selenate in other chemical species of selenium was not observed.

#### *2.3.2. Preparation of control and Se-enriched algae for mice supplementation diets*

The experimental diets used in this work were based on conventional pellets for rodents (basal diet) and selenium-deficient pellets, both from Harlan Laboratories, Inc. (Indianapolis, IN, USA), which were grounded into powder using a jaw crusher (Retsch GmbH BB200) and a vibratory disc mill until the grain size was B100 mm. Figure 1 shows the different diets used in the study. The five diets were prepared as follows: (i) Diet I, control: composed of standard diet powder only; (ii) Diet II: 500 mg of microalgae powder without selenium (microalgae control) were mixed with standard diet powder for a final weit of 756 g. The final selenium content was 0.15 µg Se $g^{-1}$ feed in the control food (Diets I and II); (iii) Diet III: Se enriched algae feed pellets were prepared by supplementing the Se-deficient basal diet for rodents with Se-enriched algae to give a final Se content of 0.15 µg Se $g^{-1}$ in the diet (94.5 mg of microalgae powder grown in the presence of 50 mg $L^{-1}$ of Se plus Se-deficient diet powder for a final weight of 756 g); (iv) Diet IV: Se-enriched algae feed pellets were prepared by supplementing the basal diet rodent formulation with Se-enriched algae to give a final Se content of 0.5 µg Se $g^{-1}$ (the same as Diet III, but with 425 mg of microalga grown in the presence of 50 mg/L of Se); and (v) Diet V: the same as Diet IV, but with 1 µg Se/g (717 mg of microalga grown in the presence of 50 mg $L^{-1}$ of Se).

In all cases, the powders were mixed homogeneously, reconstituted with distilled water in a kneader (Fimar AM1, Rimini, Italy), and then made into pellets again with an extruder (Fimar MPF4), taking care that there was no cross contamination of selenium. The five reconstituted pellet diets were dried at low temperature in an oven with fan-assisted circulation to obtain the same degree of humidity as the original standard rodent diet. Dried pellets were stored at 4 ºC under vacuum until used.

All the diets contained the same amount of algae that was adjusted in each case by adding control algae without selenium when necessary. The composition of the formulated

control and mixed diet were analyzed for Se and other metals before to be administrated to mice.

### *2.3.3. In vivo experiments*

*Mus musculus* (inbred BALB/c strain) mice were purchased from Charles River Laboratory (Spain) (n = 30, initial weight 21.67 ± 1.95 g,). Male mice of 7 weeks of age were fed *ad libitum* with conventional pellets and with free access to water. The animals were allowed to acclimate during 5 days to adjust the selenium status and to adapt to the new environment before the experiment period. Mice were randomly divided into 5 experimental groups and housed in cages. Animals were fed with the diets described in the section 2.3.2 for 5 consecutive weeks. Body weights of the animals were recorded at the beginning of the experiment and repeated weekly in order to monitor their growth. Food intake and behaviour of mice were also monitored throughout the experiment.

After experiments, the animals were fasted for 8 h and individually anesthetized with inhaled isoflurane prior to sacrifice by exsanguination by cardiac puncture, dissected using a ceramic scalpel and finally organs transferred rapidly to dry ice. Individual organs were excised, weighed in Eppendorf vials, cleaned with 0.9% NaCl solution, frozen in liquid nitrogen and stored at -80º C until use. Samples for serum were collected in 1.8 mL tubes with the Advanced BDVacutainer SST II gel separator and suction system. Blood samples were first cooled in a refrigerator and protected from light for 60 min to allow clot retraction, and serum obtained after centrifugation at 1500 *g* during 15 min at 4º C. All animals received humane care in compliance with the guidelines for animal care proposed by the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

### *2.3.4. Determination of total selenium concentrations in C. sorokiniana, diets, mice organs and serum*

Total selenium concentrations were determined in *C. sorokiniana*. To this end, the cultures were centrifuged, the pellet isolated and freeze dried. Then, an amount of 100.0000 mg was weighted and mineralized using 800 mg of a 1:3 (v/v) dilution of $H_2O_2$ in $HNO_3$ at 400 W. Samples were mineralized in the microwave oven from room temperature ramped to 160ºC in 15 minutes and maintained 40 minutes at this last temperature.

Total selenium was also determined in all the organs and serum. For this purpose, individual organs were disrupted by cryogenic homogenization. For total metal determination, three samples of serum and pulverized organs of mice from each group were exactly weighted (100 mg) in 5-ml microwave vessels and 500 mg of a mixture containing nitric acid and hydrogen peroxidase (4:1 v/v) was added. The samples were mineralized using the above described conditions for the microwave oven.

For the determination of total selenium in the different mice diets, an amount of 100 mg of feed pellets was accurately weighed and submitted to acid digestion with 200 µL of $H_2O_2$ and 600 µL of $HNO_3$. The samples were mineralized using the above described conditions for the microwave oven.

Then, the solutions were made up to 2 g with ultrapure water and selenium analyzed by ICP-MS. All the solutions were filtered using 0.45 µm PVDF filters before ICP-MS meassurements (Operational parameters collected in Table 1).

### *2.3.5. In vitro simulated gastrointestinal digestion and speciation by RP-HPLC-ICP-MS (Bioaccessibility)*

Cells were disrupted by freezing the algae pellet with liquid nitrogen and selenium species extracted from 40 mg of the lyophilized algae by 2 min of sonication at 25% power after the addition of 5 mL of deionized water and 20 mg of Protease XIV. The extract was centrifuged at 6000 rpm for 5 min collecting the supernatant. The procedure was repeated with 5 mL of ultrapure water. Cell extracts were ultrafiltered using AMICON cut-off filters of 3 kDa (Millipore, Darmstadt, Germany). A volume of 500 µL of extract was transferred to the filters and centrifuged at 4000 *g* during 60 min at 4ºC. The extracts, wich contain molecules with molecular mass lower than 3 kDa, were taken to dryness under a $N_2$ gentle stream and finally reconstituted with 100 µL of mobile phase. Before the injection into the RP-HPLC-ICP-MS, the samples were filtered using a microfilter of 0.45 µ of PVDF (low organic adsorption). Chromatographic conditions are listed in Table 1.

The *in vitro* gastric/intestinal digestion method used was based on that described by Bathia *et al* (*Bathia et al., 2013).* About 0.2 g of dried algae were incubated with 4 mL of gastric acid, which consisted of 1% (w/v) pepsin in 0.15 M NaCl at pH 2 adjusted with 37% (v/v) HCl. After 1 min of shaking for initial degassing, the digestion was performed in a thermostatic bath at 37ºC for 4 hours under gentle shaking. After gastric digestion, the pH was adjusted to 6.8 with a saturated sodium bicarbonate solution. Then, 5 mL of intestinal juice containing 3% (w/v) pancreatin, 1.5% (w/v) amylase, 1% bile salts in 0.15 M NaCl were added. The mixture was first vigorously shaken for 1 min and further incubated uring 4 h in a thermostatic bath at 37º C under gentle shaking. Once the digestion was completed, samples were centrifuged at 7500 g and 4º C for 20 min, the supernatant was filtered through a 0.22 µ Millipore filter and solutions stored at -80º C until analysis by RP-HPLC-ICP-MS. Procedural blanks were run to check the presence

of selenium in the reagents. The *in vitro* enzymolysis procedure was carried out in triplicate and the results were expressed as the mean value ± standard deviation.

### *2.3.6. Selenium speciation in mice organs and serum by 2D-SEC-AF-SUID-ICP-MS (Bioavailability)*

Pools of organs from mice of each groups above described were treated following a procedure described elsewhere (García-Sevillano et al., 2012) and later analyzed by in series two-dimensional size exclusion and affinity chromatographic separations using a high performance liquid chromatograp coupled to ICP-MS (2D/SEC-AF-ICP-ORS-MS) (García-Sevillano, García-Barrera, & Gómez-Ariza, 2013; García-Sevillano et al., 2012). The absolute quantification of selenium containing proteins and selenometabolites in the different chromatographic peaks was carried out by post-column species-unspecific isotopic dilution (SUID) analysis as described by C. Sariego-Muñiz et al. (Sariego Muñiz, Marchante Gayón, García Alonso, & Sanz-Medel, 2001).

### *2.3.7. Measurement of the clinical parameters in blood from mice fed with Se-enriched algae*

Serum enzyme activities of alanine transferase, alkaline phosphatase, amilase, lipase and aspartate transferase and concentrations of bilirubin, albumin, ferritin, LDL, HDL, triglycerides and creatinine were determined on a Cobas 8000 modular analyzer (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Standard controls were run before each determination, and the values obtained for the different biochemical parameters were always within the expected ranges. The intra-assay variability of biochemical tests was relative to 5 repeated determinations of the control serum in the same analytical session, whereas inter-assay variability for each parameter was calculated on the mean values of control sera measured during 6 analytical sessions.

### *2.3.8 Statistical analysis*

All experimental results are given as the mean ± standard error of the means (S.E.M) of *n=6* observations, representing number of animals used in the experiment. The complete statistical analysis was conducted with Minitab 16 software (Minitab Inc., State College, PA, USA, 2007). Normality of data was evaluated by the Anderson-Darling test and probability values less than 0.05 ($p$ <0.05) were considered as statically significant different and able to be submitted to post hoc test. The statistically significant different among groups was calculated by means of a One-way ANOVA followed by the Tukey test when considered appropriate.

## 3. Results and discussion

### *3.1. Quality control of the analytical methods*

Selenium recovery experiments were performed spiking algae pellets with different concentrations of selenium applying the Total Selenium Determination approach described above. Resulting recoveries were always higher than 90% for selenium with relative standard deviation values (calculated for three separate aliquots) ranging from 3 to 15 %, which were considered acceptable. On the other hand, recovery experiments for individual species were performed. For SeMet, the reference certified material SELM-1 (selenium enriched yeast) was used. The recoveries obtained were always higher than 95% with % RSD (relative standard deviation) in the range 8 to 11% for all analytes. Interconversion of species was not observed. The limits of detection of $SeCys_2$, SeMetSec, SeMet and SeVI were 2.3 ng $g^{-1}$, 2.5 ng $g^{-1}$, 2.6 ng $g^{-1}$, and 2.9 ng $g^{-1}$, respectively.

### *3.2. Effects of selenium on growth rate of Chlorella sorokiniana*

Production of Se-enriched *C. sorokiniana* biomass requires cultivation to be performed under sub-lethal Se concentrations in the culture medium. Toxic effect of selenate greatly depends on microalgae genus and species. Consequently, prior to production of Se-enriched biomass, specific toxicity analysis is required in order to select sub-lethal Se concentrations. The microalgae culture status was monitored considering several physiological parameters, namely dry weight of biomass, pigment content and oxygen evolution measurement

As described in the experimental section, *C. sorokiniana* cells were grown under different concentrations of sodium selenate to determine the sub-lethal concentration of this chemical specie in the alga. Biomass concentration for control and selenized cultures are presented in Figure 2. Data show that biomass concentration decreased for about 50% (50 mg Se $L^{-1}$ culture) compared to control, but the culture remains growing, which means the cells remain viable.

Oxygen evolution measurement is used as an economic and sensitive method for rapid detection of toxic effects on algal cultures. Oxygen evolution rates decreases from 0.26 $\mu molO_2 \cdot mg^{-1}Chl \cdot h^{-1}$ in the control to 0.17 $\mu molO_2 \cdot mg^{-1}Chl \cdot h^{-1}$ in the culture exposed to 50 mg $L^{-1}$ of selenate (data not shown), which means that the algal cells remain viable. Then, in order to obtain biomass enriched in SeMet maintaining simultaneously cell viability, a sublethal selenate concentration of 50 mg $\cdot L^{-1}$ was used.

At the literature, selenium concentration range used in different exposure experiments varies significantly depending on the microalga species (Bennett, 1988; Fournier, Adam-Guillermin, Potin-Gautier, & Pannier, 2010; Ibrahim & Spacie, 1990; Morlon et al., 2005; Umysova et al., 2009). Related studies on *S. quadricauda* revealed that 50 mg $L^{-1}$ selenate in the medium was not lethal to microalga cultures as cells grew

and divided normally (Umysova et al., 2009), while *C. zofingiensis* was resistant to selenite concentrations up to 100 $mgL^{-1}$ (Pelah & Cohen, 2005).

The total selenium content was 355±8 µg $g^{-1}$ dry weight (DW) in *C. sorokiniana* exposed to sodium selenate (50 mg $L^{-1}$) for 120 hours, showing that *C. sorokiniana* is an efficient selenium accumulator. In previous studies, results showed a rapid selenium absorption in the first few hours at the cell surfaces (V. Gómez-Jacinto, García-Barrera, Garbayo-Nores, Vilchez-Lobato, & Gómez-Ariza, 2012; Veronica Gómez-Jacinto et al., 2012). The ability of other green algae to absorb inorganic selenium from the culture medium and accumulate this element has been also reported. The total selenium content in the microalgal biomass suggests that bioaccumulation of selenium increases almost proportionally to the selenium concentration in the microalgal cultures (Gojkovic et al., 2013, 2014; Neumann et al., 2003; Pelah & Cohen, 2005; Umysova et al., 2009). Toxicity of selenium to microalgal cells is closely related to its bioaccumulation in algal biomass and the biotransformation suffered by incorporated inorganic selenium.

### *3.3. Accumulation of SeMet in C. sorokiniana in presence of selenate*

Due to their reductive metabolism, *C. sorokiniana* carries out the enzyme-mediated selenite and selenate reduction to selenide which is incorporated preferentially into selenoproteins and Se-aminoacids (Neumann et al., 2003). Therefore, for selenium releasing from proteins, enzymatic digestion of the samples was employed and analysis of Se species was carried out.

Figure 3A shows the elution profile of standards (SeCys2, SeMetSeCys, SeMet and Se(VI)) obtained by RP-ICP-MS. Figure 3B shows the chromatogram corresponding to Se-species analysis from *Chlorella* samples grown in the presence of 50 mg $L^{-1}$ of selenate. This reveals the presence of $SeCys_2$, SeMetSeCys, SeMet and Se (VI). Selenate

was present 28.6±5 $\mu g \cdot g^{-1}$, but 95% of selenium content was transformed into organic species. SeMet is the main Se-specie accumulated in Se-enriched algae, accounting for 79% of total accumulated Se, which is consistent with previous results reported by other authors in green algae under similar experimental conditions. In this way, Umysová et al. (2009) reported that wild type of *Scenedesmus quadricauda* accumulated 300 $\mu g \cdot g^{-1}$ SeMet in the presence of 50 $mg \cdot L^{-1}$ of selenate (Umysova et al., 2009). In addition, it has been found that selenium is present in *Chlorella* as SeMet representing more than 70 % of selenium in the algae, also in good agreement with results obtained in selenized yeast (Umysova et al., 2009). Finally, Bottino et al. (1984), identified SeMetSeCys and SeCys amino acids in in *Chlorella* and *Dunaliella sp*. exposed to 10 $mg \cdot L^{-1}$ selenite (Bottino et al., 1984).

In our study *Chlorella sorokiniana* was exposed to 50 mg $L^{-1}$ accumulating 280.4±7 µg $g^{-1}$ of SeMet. This fact is of a great importance because SeMet is highly suitable for nutritional supplementation as it is more bioavailable, less toxic and can provide higher Se concentration in tissue than inorganic Se, as previously reported (Rayman, 2004; Schrauzer, 2003). SeMet is also one of the precursors of methyl selenol, a potent anticarcinogen which inhibits tumour invasion and angiogenesis (Kim, Oh, Park, & Chung, 2007). The concentration of $SeCys_2$ and SeMeSeCys was 12.3±2 and 38.6±5 µg $g^{-1}$, respectively. The intracellular concentration of SeMet in microalgae species exposed to Se species depends on a number of factors, among which Se concentration in the culture medium, culture age and sulfur concentration have a great impact (Gojkovic et al., 2013). Therefore, besides selecting sub-lethal Se concentrations, other experimental conditions suitable to address SeMet biosynthesis and accumulation should be considered in order to produce Se-enriched *Chlorella sorokiniana* biomass with high value as functional food.

### *3.4. Bioaccessibility of Se and its species in enriched Chlorella sorokiniana*

The determination of total selenium content and Se-species distribution in food is not enough to determine the bioaccessibility of this element. For this reason, it is necessary to determine the selenium and Se-species presence in gastrointestinal extracts. The *in vitro* digestion method used in this work comprises a simulation of both stomach and intestinal physiology. The analysis of total selenium and speciation studies in both digestions were used to predict the bioaccessibility of organic and inorganic selenium forms.

The soluble extract obtained after *in vitro* simulated gastrointestinal digestion of the selenized algae contained about 287.5±6 μg $g^{-1}$ of selenium. This means that 81% of the Se taken up by the algae was solubilized in conditions simulating human gastrointestinal digestion and thus was potentially bioavailable. The residual fraction from the *in vitro* method was acid-digested in triplicate and analyzed for total selenium, giving a concentration for total selenium of 73±8 μg $g^{-1}$. The fraction of selenium not solubilized after gastrointestinal digestion might be present in form of indigested Se-containing polysaccharides in its hemicellulotic cell wall structure. Previously reported data established that selenoaminoacids bioaccesibility is lower in Se-enriched *Chlorela vulgaris* (~49%), but higher as compared to Se-yeast (~21%) and a commercially available Se-enriched food supplement (~32%) (Vu et al., 2019). This discrepancy with data reported in the present work for selenium bioaccessibility in Se-enriched *Chlorella sorokiniana* may be related with the *Chlorella* species (*sorokiniana* vs *vulgaris*), but also with the chemical form of selenium being determined in the bioaccessibility study (selenoamino-acids-Vu et al., 2019/total selenium-this study) and the form of selenium used for the enrichment (selenate vs selenite) as well as the concentration (50 vs 2.25-4.5 $mg \cdot L^{-1}$).

For the characterization of selenium species liberated after *in vitro* enzymolysis, reverse phase HPLC-ICP-MS was used. In relation to this, the oxidation of SeMet after *in vitro* or enzymolysis procedures has been reported (Dumont, Vanhaecke, & Cornelis, 2004). In addition, Dumont *et al.* indicated that SeOMet is generated after gastrointestinal digestion as degradation product of SeMet even after keeping the extract lyophilized at -20º C under Ar atmosphere (Dumont et al., 2004). Other results obtained from selenized yeast showed that SeOMet is a degradation product after medium and long-term sample storage, while SeOMet was not detected when samples were immediately analysed (Gammelgaard, Cornett, Olsen, Bendahl, & Hansen, 2003). This chemical form of selenium could be just an analytical artefact as previously observed by Larsen, but not originated initially by the gastrointestinal digestion (Larsen, Sloth, Hansen, & Moesgaard, 2003).

Our results show that selenium speciation in the gastrointestinal extract was dominated by SeMet, which represented 79% (226±5 μg $g^{-1}$) of the total selenium bioaccessible. Oxidized SeMet was not detected. In relation to inorganic selenium species, selenate was found at 7.6 ± 2 μg $g^{-1}$ in gastrointestinal extract. By contrast, other authors determined that the bioaccesibilities of SeMet, SeMetSeCys and selenocysteine (SeCys) in Se-enriched *Chlorella vulgaris*, are very similar (Vu et al., 2019). However, the distribution of the chemical species of selenium is different in this microalga and for this reason, SeCys2 is not determined in *C. vulgaris*, an abundant chemical form of selenium present in *C. sorokiniana* after SeMet and SeMetSeCys. In adition, the *Chlorella* specie (vulgaris *vs* sorokiniana) could also influence the bioaccessibility results.

Since it is considered that selenium species found in the gastrointestinal juice constitute the bioaccessible fraction as a result of both gastric and intestinal digestion

(Cabañero, Madrid, & Cámara, 2004), the results described above suggest that almost all selenium content in algae is potentially bioaccessible, although many factors could affect it (Welch & Graham, 2005; Yoshida, Abe, Fukunaga, & Kikuchi, 2002).

Several dietary factors such as type and degree of food processing, diet composition, presence of other metals, concomitant ingestion of certain drugs, and physiology factors such as nutritional state, growth, and pregnancy, together with the presence or absence of dietary micronutrient inhibitors or enhancers substances, can reduce or promote the selenium bioaccessibility (Welch & Graham, 2005; Yoshida et al., 2002).

### *3.5. Characterization of mice diets*

Prior the administration of the different diets to animals, total selenium concentration and speciation was carried out. Selenium was not detected in microalgal cells from control cultures nor in the medium. Selenium concentration in Se-enriched alga was 355±8 $\mu g \cdot g^{-1}$ dry weight (DW), as described previously. The different diets used to feed animals provide selenium concentration values that are shown in Table 2, being SeMet the major Se-species found in Se-enriched algae, accounting for 79% of total selenium present, which denotes the suitability of this alga for SeMet intake. Other toxic elements are present at very low concentrations.

To ensure the suitability of different diets, the presence of toxic elements in them was evaluated (Table 2). The results show the absence of toxic metals in the algae.

### *3.6. Mice body weight evolution under Se supplementation*

Mice body weight changes in the different Se-supplemented groups along the time are shown in Figure 4. The means (S.E.M) correspond to $n=6$ observations, representing

number of animals used in the experiment. Tukey test has been performed and statistical differences among all the treatments are indicated using letter codes (mean values that do not share any letter are significantly different). There were no significant differences from the initial body weight among four groups. However, after day 10, the average body weight of mice fed supplemental algae with Diet III was significantly higher than those of other groups ($p<0.05$). The microalga found in diet III is lower than in other diets, and for this reason the final weight (which is the same for all diets) is complemented by commercial food, which in turn implies more commercial food present in diet III. As all mice received the same intake, those fed with diet III obtain more commercial food. On the other hand, commercial food is rich in protein, animal fats and carbohydrates (manufacturer information), while microalgae are rich in dietary fiber and low in lipids (Navarro et al., 2016), which means that there are lower calories in diet III than in others. In all cases, the weight suits the average psychological parameters as regards age and sex of mice.

During whole period of the study, no obvious symptoms of mice health impairement, or hair, nail or skin damage, were observed in all groups of animals.

### *3.7. Biochemical parameters in blood of mice fed with Se-enriched algae*

Blood sampling was performed by the same skilled technician for all samples, and all manipulations performed before and after blood collection were accurately settled, so that variability caused by blood sampling was negligible. In the present study, the level of haemolysis in all serum samples was scored by direct observation. The results obtained in the last day of the experiment are shown in Table 3.

Data obtained revealed that administration of Se at high doses (Diet V) increases the levels of alanine transaminase (ALT) and aspartate transaminase (AST). AST is

widely used to assess the liver function. ALT is a cytoplasmic enzyme while AST is found in both cytoplasm and mitochondria. ALT increased in a cirrhotic liver and AST in myocardial infarction, liver diseases, liver cancer and liver cirrhosis. Therefore, increase of ALT and AST is denoting a potential injury of excesive intake of selenium in Diet V, mainly in liver that is a primary defense organ that detoxifies drugs and xenobiotics.

Results collected in table 3 reveal that under the same experimental conditions, supplemental diets with 0.15 or 0.5 µg Se $g^{-1}$ did not change significantly ($p>0.05$) the serum levels of any of the biochemical parameters measured compared to control group, with the exception of tryglicerides which showed decreasing levels under diets supplemented with selenium.

### *3.8. Bioavailability and bioactivity of selenium from enriched Chlorella sorokiniana: in vivo total selenium distribution and speciation in mice M. musculus after selenized-algae feeding*

The presence of Se in the organs and serum of *M. musculus* mice fed with different Se diets was determined by ICP-MS after acid digestion, and the results are shown in Figure 5. Results are expressed as mean ± standard error of the means (S.E.M) of $n=6$ observations, representing number of animals used in the experiment. Tukey test has been performed and statistical differences among all the treatments are indicated using letter codes (mean values that do not share any letter are significantly different).

As expected, results show that selenium concentration in organs is always higher in the groups supplemented with higher amounts of selenium in the diet. Se concentration levels are similar in liver and serum ranging from 300 to 600 ng of Se $g^{-1}$ aproximately, but the selenium concentration in kidney increases drastically, especially for high Se-dose

diets (Diet IV and diet V), reflecting a potencial mechanism of excretion of Se by urine. These results also provide evidence about the preferential accumulation of selenium in specific organs. On the other hand, bioavailaility of selenium increases when this element is supplemented by the algae instead of the basal diet and decreases at higher concentrations (Table 4). Since the bioavailable fraction can be considered as the quantity of Se absorbed through the intestinal barrier and reaching the systemic circulation (Thiry, Ruttens, De Temmerman, Schneider, & Pussemier, 2012), the percentage of bioavailable Se can be calculated as Se determined in mice serum against Se intake. To this end, if we consider that the mean adult mice weight is 25 g approximately, and that each mice had approximately 1.2 g of blood and 300 mg of serum (Lagares Martinez, 2001), then the total amount of selenium in the body (systemic circulation) can be calculated (Table 4). Moreover, taking into account the selenium content in each diet and the daily-consumed feed (~5 g), the % of Se bioavailavility of the diets can also be calculated as the total Se (ng) in ~300 mg of serum/mice against total Se (ng) intake per day (~5g/day). As we can see, total selenium in serum is 1.13-fold higher in mice fed Diet III (Se is supplemented by the algae) than Diet II (Se is supplemented by the basal diet), that in turn indicates higher bioavailavility. However, the bioavailavility decreases in the diets with higher amounts of selenium (IV and V), which may suggest that the body capacity of Se assimilation is overloaded at concentrations higher than 0.15 μg Se $g^{-1}$ feed. Nevertheless, although the bioavailavility of selenium is lower, selenium increases in serum and in organs as above discussed. Moreover, at higher supplemental levels, the bioactivity is maintained as discussed in the following paragraphs. On the other hand, as commented before (section 3.4.), 81% of the Se taken up by the algae is bioaccesible (solubilized in conditions simulating human gastrointestinal digestion: selenium that is soluble in the intestine) and thus was potentially bioavailable, but only 3-15% has been determined to

be bioavailable (absorbed through the intestinal barrier and reaching the systemic circulation) depending on the diet. This discrepancy may be related with the fact that probably most of selenium is excreted and does not finally reach target organs. Thus, the complementary study of bioaccesible and bioavailable fractions is mandatory.

On the other hand, absolute quantification of Se containing proteins (selenoprotein P (SeP), extracellular glutathione peroxidase (eGPx) and selenoalbumin (SeAlb) as well as low molecular weight Se-metabolites has been performed in mice plasma using the speciation approach described in the materials and method section (Table 4). The elution profiles of selenoproteins in human serum certified reference material (CRM) BCR-637 (Institute for Reference Materials and Measurements, IRMM, Geel, Belgium)(García-Sevillano et al., 2013; García-Sevillano, García-Barrera, & Gómez-Ariza, 2014b) and mice plasma (García-Sevillano, García-Barrera, & Gómez-Ariza, 2014a; García-Sevillano et al., 2014b) have been previously obtained using the same analytical method for the separation and mass spectrometric identification after tryptic digestion.

In mammalian bloodstream, selenium is mainly associated to the three selenoproteins SeP, eGPx, SeAlb and Se-metabolites. SeP is the major Se-specie in mice serum, especially after feeding a diet supplemented with selenium-enriched algae Table 4.

The majority of selenium was associated to SeP followed by GSH-Px and SeAlb. The results show that selenium species were higher in serum from animals fed with high content Se diet. SeAlb concentration is the most affected by thet type of Se-enriched diet although GSH-Px presence in serum is also enhanced by selenium intake. However, the concentration of SeP increases gently with increasing selenium in the diet.

Butler *et al.* (1990), reported a significant increase in the percentage of Se in the albumin fraction of plasma in monkeys supplemented with Se as selenomethionine compared with supplementation of Se as selenite (Butler, Whanger, Kaneps, & Patton, 1990). Similarly, the distribution of Se in plasma fractions was investigated in pigs fed various levels of dietary SeMet, where there was a redistribution of Se between varios fractions in the blood. On a percentage distribution basis, the Se in SeP decreased, and that in the albumin fraction increased with increased dietary intakes of Se as SeMet (Gu, Xia, Ha, Butler, & Whanger, 1998) humans, increases in the ratios of SeAlb in either the plasma or the albumin fraction also occurred with increases of Se intake of these subjects (Gu et al., 1998).

Since the bioactive fraction can be described as the quantity of Se transformed into active selenomolecules (Thiry et al., 2012), this fraction can be calculated as selenium content in specific selenoproteins in serum against total selenium in serum (Table 4). It is notheworthy that, selenoproteins concentrations reported in Table 4 are given as ng of selenium per gram of sample. Since SeP and GPx present 10 and 4 atoms of Se in its structure, respectively, the absolute concentration of each selenoprotein can be calculated from this data. This conversion is not possible in the case of SeAlb, which is a Se-containing protein (not a selenoprotein, wich is genetically encoded as SeP and GPx) formed in human serum/plasma by random replacement of methionine by selenomethionine (SeMet)(Rayman, 2008).

Thus, we can conclude that the %Se bioactivity ranged from 76-85 being almost contant among the different supplemental Se doses and withouth correlation with Se-enriched *C. Sorokiniana* or basal diet.

It is notworthy that SeAlb, SeMet and total selenium concentrations in mice serum are significantly different after feeding with any diet (Table 4) in comparison with control

diet. Moreover, Se-metabolites present the highest F value and p-value < 0.01 (ANOVA one way) in mice serum after feeding with microalga containing diets (Diets III, IV and V) when comparing with control. On the other hand, the F value increases with the amount of microalga contained in the diet. Finally, all the selenoproteins, selenometabolites and total selenium are significantly different in mice serum fed diet V (higest content of microalga and selenium) against control.

## 4. Conclusions

The evaluation of Se-enriched algae as a possible dietary source of this element requires the determination of total concentrations of selenium, but also the distribution of different chemical species as well as their bioaccessibility/bioavailavility and bioactivity.

This study investigated *in vitro* Se bioaccessibility in *C. sorokiniana*, a green algae that accumulated substantial Se amounts when cultivated on Se-enriched cultures. About 81% of Se in the algae was extracted after *in vitro* simulated gastrointestinal digestion mainly as SeMet.

Microalgal biomass, having fast growth and high ability to metabolize Se into low toxic reduced Se-metabolites, particularly SeMet, can be used as a biological carrier of bioactive Se-amino acids appropriate for supplementation in human and animals. Accordingly, Se-enriched *C. sorokiniana* can be used as functional food, as ingredient in the formulation of nutraceuticals or as Se dietary supplement. Depending on the intended use, the production process can be adapted in order to achieve optimal Se concentrations in the algae.

Selenomethionine is the main form of selenium in *C. sorokiniana* and the results showed a redistribution of Se between various fractions of serum. On a percentage distribution basis, when Se increases using the algae as a vehicle, Se as selenoprotein P decreased while increased in the albumin and GSH-Px fractions.

When it is compared with control group, Se content in serum and organs increased significantly *($p<0.05$)* in selenium supplemented groups. In addition, increases in the ratios of selenium to albumin in either the serum or albumin fractions occurred when selenium intake increases in these animals. These results showed that Se-enriched algae can be considered as an alternative selenized food for humans, due to their high Se bioavailability.

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## Figures

**Figure 1**. Different mice diets used in the study.

**Figure 2**. Biomass concentration for control and selenized cultures.

**Figure 3A.** Elution profiles of the standards ($SeCys_2$, SeMetSeCys, SeMet and Se(VI)) by RP-

ICP-MS.

**Figure 3B.** Selenium species present in *Chlorella sorokiniana* grown on selenite-added culture medium. Selenium species following the elution order: $SeCys_2$, SeMetSeCys, SeMet and Se (VI).

**Figure 4.** Body weight changes of mice during the experiment. From day 1 to 5 mice were fed with the standard diet, from day 5 mice were fed with Diets I to V. Letter codes indicate significat differences among all the treatments, at the end of the experiment (mean values that do not share any letter are significantly different).

**Figure 5.** Concentration of Se in serum, liver and kidneys. Letter codes indicate significat differences among all the treatments (mean values that do not share any letter are significantly different).

# Tables

| ICP-MS conditions | |
|---|---|
| Forward power | 1,500 W |
| Plasma gas flow rate | 15 L $min^{-1}$ |
| Auxiliary gas flow rate | 1 L $min^{-1}$ |
| Carrier gas flow rate | 0.15 L $min^{-1}$ |
| Sampling depth | 7 mm |
| Sampling and skimmer cones | Ni |
| $H_2$ flow | 4 mL $min^{1}$ |
| Nebulizer | MicroMist (Glass Expansion) |
| Torch | Shield (with long life platinum shield plate) |
| $Q_{oct}$ | −18 V |
| $Q_p$ | −16 V |
| Points per peak | 1 |
| Integration time | 0.3 per isotope |
| Replicates | 1 |
| **Chromatographic conditions of 2D/SE-AF-HPLC-SUID-ICP-ORS-M** | |
| Sample loop | 100 µL |
| Flow rate | 1.3 mL $min^{-1}$ |
| Mobile phase A | 0.05 M ammonium acetate pH 7.4 |
| Mobile phase B | 1.5 M ammonium acetate pH 7.4 |
| Gradient | 0-7 min 100% A<br>6-18 min 100% B<br>18-20 min 100% A |
| Valve position | 1-10 min inject<br>10-17 min load<br>17-20 min inject |
| **Chromatographic conditions of RP-HPLC-ICP-ORS-MS** | |
| Column | Phenomenex Luna C18 (250x4.6x5µm) |
| Mobile phase | TEAC, pH 4.5 |
| Flow rate | 1 mL $min^{-1}$ |
| Sample loop | 20 µL |

**Table 1.** Operating conditions for SEC-ICP-MS, 2D/SE-AF-HPLC-SUID-ICP-ORS-MS and RP-HPLC-ICP-ORS-MS

| Elements | Recommended limits | Diet I | Diet II | Diet III | Diet IV | Diet V |
|---|---|---|---|---|---|---|
| Se | <0,6 $\mu g \cdot g^{-1}$ | 0,15 | 0,16 | 0,16 | 0,67 | 1,13 |
| Cu | 10-35 | 33,92 | 36 | 36 | 36 | 36 |
| Pb | 1,5 | <LOD | <LOD | <LOD | <LOD | <LOD |
| Hg | <0,1 | <LOD | <LOD | <LOD | <LOD | <LOD |
| As | <1 | 0,06 | 0,07 | 0,06 | 0,07 | 0,07 |
| Cd | <0,250 | <LOD | <LOD | <LOD | <LOD | <LOD |

**Table 2.** Concentrations of Se and toxic metals in differents diets

| Clinical parameters (mean ± SD) | Bilirubin mg/dL | Glucose | Albumin gr/dL | LDL mg/dL | HDL mg/dL | Alanine transpherase UI/L |
|---|---|---|---|---|---|---|
| DIET I | 0.04 ± 0.01 | 149.1 | 2.9 ± 0.5 | 77 ± 5 | 104.9 ± 8 | 92 ± 8 |
| DIET II | 0.05 ± 0.02 | 130.4 | 2.8 ± 0.7 | 58 ± 6 | 111.7 ± 11 | 107.3 ± 11 |
| DIET III | 0.04 ± 0.01 | 136 | 2.7 ± 0.4 | 64 ± 6 | 115 ± 9 | 94 ± 9 |
| DIET IV | 0.06 ± 0.01 | 136.2 | 2.8 ± 0.5 | 62 ± 4 | 110.2 ± 10 | 106.6 ± 9 |
| DIET V | 0.05 ± 0.02 | 122.8 | 2.9 ± 0.6 | 60 ± 7 | 99 ± 11 | 166.6 ± 15 |
| **Clinical parameters (mean ± SD)** | **Alkaline phosphatase UI/L** | **Amilase UI/L** | **Triglycerides mg/dL** | **Lipase UI/L** | **Creatinine mg/dL** | **Aspartate transpherase UI/L** |
| DIET I | 109 ± 11 | 2829.7 ± 251 | 266 ± 14 | 37.6 | 0.27 | 393.2 |
| DIET II | 104 ± 13 | 2581.8 ± 303 | 255 ± 12 | 34.4 | 0.25 | 446.1 |
| DIET III | 104 ± 15 | 2237 ± 189 | 273 ± 19 | 32.5 | 0.25 | 694 |
| DIET IV | 103 ± 12 | 2670.9 ± 225 | 123.9 ± 18 | 28.3 | 0.2 | 747 |
| DIET V | 111 ± 11 | 3455 ± 275 | 169.7 ± 16 | 27.7 | 0.24 | 1172.4 |

**Table 3.** Clinical parameters in blood from *Mus musculus* mice under differents doses of Se in diets

| Group | DIET I (basal diet 0.15 µg Se $g^{-1}$) | DIET II (basal diet + control algae 0.15 µg Se $g^{-1}$) | | | DIET III (Se deficient diet + Se algae 0.15 µg Se $g^{-1}$) | | | DIET IV (Se deficient diet + Se algae 0.5 µg Se $g^{-1}$) | | | DIET V (Se deficient diet + Se algae 1 µg Se $g^{-1}$) | | | Limits of detection (LOD) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean± S.E.M | Mean± S.E.M | ANOVA One way | | Mean± S.E.M | ANOVA One way | | Mean± S.E.M | ANOVA One way | | Mean± S.E.M | ANOVA One way | | Limits of detection |
| GPx (ng Se $g^{-1}$ serum) | 8.8 ± 1.9 | 8.8 ± 0.9 | p=1.000 | F=0.00 | 7.4± 0.8 | p=0.114 | F=2.99 | 9.4 ± 1.1 | p=0.503 | F=0.48 | 50.5 ± 10.4 | p=0.000* | F=92.99 | 0.2 |
| SeP (ng Se $g^{-1}$ serum) | 268.4 ± 12.7 | 242.3 ± 18.3 | p=0.017 * | F=8.26 | 259.0 ± 15.4 | p=0.262 | F=1.41 | 324.9 ± 12.3 | p=0.000* | F=6.30 | 328.9 ± 15.6 | p=0.000* | F=53.94 | 0.7 |
| SeAlb (ng Se $g^{-1}$ serum) | 22.7 ± 4.1 | 20.0 ± 3.1 | p=0.220 | F=1.71 | 29.6 ± 3.7 | p=0.013* | F=9.21 | 61.2 ± 10.5 | p=0.000* | F=73.54 | 134.7 ± 8.14 | p=0.000* | F=905.30 | 1 |
| Se-metabolites (ng Se $g^{-1}$serum) | 48.8 ± 2.1 | 51.5 ± 2.8 | p=0.092 | F=3.48 | 74.0 ± 5 | p=0.000* | F=147.21 | 75.4 ± 2 | p=0.000* | F=504.74 | 81.9 ± 2.89 | p=0.000* | F=515.24 | 0.4 |
| Total selenium (ng $g^{-1}$ serum) | **353.64 ± 18.51** | **342.57 ± 34.7** | p=0.506 | F=0.48 | **388.69 ± 23.5** | p=0.017* | F=0.017 | **481.06 ± 34.6** | p=0.000* | F=63.20 | **614.68 ± 25.5** | p=0.000* | F=408.62 | **0.04** |
| Total Se (ng) in ~300 mg of serum/mice | **107.3±5.2** | **102.6±11.6** | | | **115.7±7.5** | | | **145.3±11.3** | | | **184.7±8.5** | | | |
| Total selenium (ng $g^{-1}$ feed) | 150 | 160 | | | 160 | | | 670 | | | 1130 | | | |
| Total Se (ng) intake per day (~5g/day) | **750** | **800** | | | **800** | | | **3350** | | | **5650** | | | |
| %Se bioavailability | **14** | **13** | | | **15** | | | **4** | | | **3** | | | |
| %Se bioactivity (∑Se as selenoproteins in serum/total Se in serum) | 85 | 79 | | | 76 | | | 82 | | | 84 | | | |

**Table 4**. Quantification of selenium species in mice serum (*Mus musculus*) under differents diets. Asterik denote significative values, when compared to the control group -DIET I (one-way ANOVA, Tukey test).