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Biofortification of selenium in black soldier fly (*Hermetia illucens*) prepupae reared on seaweed or selenium enriched substrates

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RESEARCH ARTICLE

Abstract

Selenium (Se) is an essential trace element for livestock. The element can be supplemented to feed in inorganic or organic Se forms, where the chemical form in the diet affects the accumulation of Se in animal tissues. Insects are known to be natural bioaccumulators of different nutrients, but no studies have so far looked up on the capacity of insects to be biofortified with Se, as a potential future source for Se in feed. In this study, black soldier fly (BSF, *Hermetia illucens*) larvae were reared on three different substrates: (1) a control substrate (CTR diet) of plant-ingredients (Gainesville diet); (2) *Ascophyllum nodosum* diet (AN30%), with 30% substitution of the alfalfa meal with the brown algae; and (3) a Se diet, where the CTR diet was fortified with 0.3 mg/kg of Se. All experiments were carried out under dark condition, at 25 °C with 70% relative humidity for two weeks, and the final BSF prepupae were analysed for Se, metals and minerals, and Se species. The mean total Se content in the prepupae reared on Se dietary group was over five times higher compared to the CTR group, whereas lower Se levels were detected in AN30% dietary group. Se speciation analyses showed that organic selenomethionine (SeMet) was the major Se species present, both in CTR and AN30% group. For the Se group, SeMet was accounting for 54% of the total Se. The levels of SeMet were higher in the Se group compared to the CTR group, indicating that BSF prepupae are able to convert inorganic Se to organic Se. The overall results show that the substrates hereby studied affect the total Se and Se species, as well as the levels of other elements, in the BSF prepupae.

Keywords: speciation analysis, feed additive, selenite, selenomethionine, HPLC-ICP-MS

1. Introduction

Recently the European Union (EU) approved dried yellow mealworm (*Tenebrio molitor*) as a safe food. It was the first approved insect food in EU, and currently, 11 other insect foods are waiting for safety evaluations from EU. The inclusion of insects has been investigated for diet of carnivorous (Achionye-Nzeh and Ngwudo, 2003; Fasakin *et al.*, 2003; Sealey *et al.*, 2011) and omnivorous fish (Belghit *et al.*, 2019; Lock *et al.*, 2016; Renna *et al.*, 2017; St-Hilaire *et al.*, 2007), as well as for the diet of livestock and crustaceans (Moula and Detilleux, 2019; Yoo *et al.*, 2019). The most widespread insect studied in terms of feed resource is the black soldier fly (BSF; *Hermetia illucens*) (Pinotti *et al.*, 2019). In 2017 the BSF was approved as a feed material for farmed fish (Commission Regulation (EU) 2017/893) and from 17 August 2021 with the Commission Regulation (EU) 2021/1372, the use of insect-derived proteins is also allowed in poultry and pig feeds. Depending on the feeding medium, BSF larvae (BSFL) can be a good source of proteins, lipids, vitamins, and essential minerals (Barroso *et al.*, 2014; Henry *et al.*, 2015; Kouřimská and Adámková, 2016; Makkar *et al.*, 2014; Van Huis, 2013).

Selenium (Se) is an essential element, both for animals and humans (Adadi *et al.*, 2019), as it is involved in the functioning of several enzyme systems (Constantinescu-Aruxandei *et al.*, 2018). It is a micronutrient necessary

for optimal health, and is involved in various biochemical reactions, in particular in the processes of protecting cell from the damages of free radicals (D'Amato *et al.*, 2020) along with vitamin E. Selenium becomes part of selenoproteins and performs its action as scavenger in the form of glutathione peroxidase (Schiavon *et al.*, 2017) and is also a component of iodothyronine deiodinase, the enzyme that catalyses thyroxine deiodation to triiodothyronine (Kieliszek *et al.*, 2015). At doses too high compared to metabolic demand, Se becomes toxic to the body, similar to several other minerals, i.e. copper (Cu), iron and zinc (Zn) (Kobayashi *et al.*, 2018; Mocchegiani *et al.*, 2013; Scheiber *et al.*, 2013). An excess of Se in the diet can cause disease states such as oxidative stress (Berntssen *et al.*, 2018).

It is generally recognised that the mobility, toxicity, and bioavailability of trace elements, including Se, depends on their chemical forms, or chemical species (Ochsenkühn-Petropoulou *et al.*, 2016). Chemical species is defined as the specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure. It is known that organic Se forms, e.g. Se amino acids such as selenomethinone (SeMet), are more bioavailable than inorganic forms, such as selenite and selenate (Fontagné-Dicharry *et al.*, 2015; Lorentzen *et al.*, 1994). Fish and other animals given feed supplemented with organic Se forms accumulates Se more readily in tissue compared to animals given inorganic Se forms (Fontagné-Dicharry *et al.*, 2015; Le and Fotedar, 2014; Zhan *et al.*, 2011). Several scientific opinions by the European Food Safety Authority (EFSA) on the safety of organic Se-sources have concluded that the supplementation level of organic Se should be limited to ensure consumer safety (EFSA, 2011a, 2012). Subsequently, EU has regulated the use of several organic Se feed additives, at a supplementation level of maximum 0.2 mg Se/kg feed (EFSA, 2011b, 2013). The legislative differentiation between total Se and supplemented organic Se forms, as well as the known differences in bioavailability and toxicity between Se species, emphasise the need for suitable Se speciation techniques to determine the amount of organic Se.

The BSFs are scavenger animals (Makkar *et al.*, 2014), so it is possible to feed them with waste materials such as manure or fruit/vegetables waste (Liland *et al.*, 2017; Pinotti and Ottoboni, 2021; Sheppard *et al.*, 1994). Studies have shown that insects have the potential to recycle nutrients from waste by incorporating, e.g. amino acids and fatty acids (Pinotti *et al.*, 2019; Sealey *et al.*, 2011) which makes them attractive not only for the feed industry, but also in terms of bio-circular economy (Gasco *et al.*, 2020). In EU, there are restrictions on the feed that may be given to farmed animals, including insects. Insects can therefore only be fed materials of vegetal origin, with some exceptions, e.g. milk, eggs and blood products from non-ruminant animals. Insect producers in Europe have recently shown great interest in using other media for rearing insect larvae, and among resources that could be used as rearing substrates for farming insects are marine macroalgae or seaweed. Seaweeds are listed as authorised feed materials for food-producing animals and could therefore be used as substrate for insects. Rearing BSFL on substrates of marine origin, such as seaweed, increases the levels of marine nutrients in the larvae, e.g. omega-3 polyunsaturated fatty acids, iodine and vitamin E (Liland *et al.*, 2017). However, also other metals and minerals, i.e. arsenic (As), cadmium (Cd), mercury (Hg) and lead (Pb) have been seen to accumulate in insects reared on seaweed (Biancarosa *et al.*, 2018a).

The possibility of biofortifying insects with different nutrients can be exploited to improve the nutritional characteristics of feed/food (Ottoboni *et al.*, 2018; Spranghers *et al.*, 2017). In the case of Se biofortification, it is important to consider the chemical form of Se, since this can affect the bioavailability of the element. The organic form, selenoamino acids, is considered more bioavailable and also less toxic compared to inorganic forms. Most biofortification studies with Se have mainly used yeasts and algae (Adadi *et al.*, 2019; Kieliszek and Błażejak, 2013), and despite several scientific works concerning Se-biofortification strategies, the production of Se-enriched foods suitable for animal and human consumption is still challenging (D'Amato *et al.*, 2020). To get a better scientific basis for the use of different Se feed supplements, studies need to address the species-related transfer and accumulation of Se (Zhao *et al.*, 2019). Selenium species have previously been determined in animal tissues of chicken and lamb, and in fish and seafood using high pressure liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) after enzymatic digestion (Bierla *et al.*, 2008; Bryszewska and Mage, 2015; Kristan *et al.*, 2013). In a recent study, Se speciation analysis was performed on fish tissue and fish feed using enzymatic digestion and determination of organic Se using HPLC-ICP-MS (Sele *et al.*, 2018). Inorganic selenite and selenate were in the same study determined by an alkaline extraction prior to HPLC-ICP-MS analysis. Since BSF typically contain high amounts of protein and essential amino acids, their profile has been compared to fish meal (Barroso *et al.*, 2014; Makkar *et al.*, 2014), and a similar strategy for Se speciation analysis could be applied. The protective chitin layer can, however, challenge the extraction efficiency of Se. To our knowledge, no studies have so far looked up on the ability of insect to accumulate Se, and the Se species in the insect when reared on different Se sources.

The aim of this study was to expose BSF prepupae to different substrates containing Se to evaluate if the BSF prepupae can be biofortified with Se, and to study the effect of the different substrates. A further aim was to study the Se species in the biofortified BSF prepupae, and by this

contribute to a better understanding of the species related transfer of Se in insects. Methods for the determination of Se species in insects were established to reach this goal, with a focus on optimising the extraction procedure.

2. Materials and methods

Chemical and reagents

All chemicals used were analytical grade quality or better. For the sample preparation and analysis, ultrapure water (18.2 $M\Omega^{-cm}$) was produced in-house using a Milli-Q water purification system (Merck Millipore, Burlington, MA, USA). Seleno-DL-methionine (SeMet, ≥99% purity), sodium selenate (Se(VI), ≥98% purity), sodium selenite (Se(IV), 99% purity), protease type XIV from *Streptomyces griseus*, chitinase from *S. griseus*, ammonium phosphate dibasic, ammonium acetate, tris(hydroxymethyl)aminomethane, phosphoric acid, sodium hydroxide, methanol (HPLC grade), pyridine, hydrochloric acid, ammonia solution, hydrogen peroxide (H_2O_2, Emsure ACS, ISO, 32% w/w) were all obtained from Merck (Darmstadt, Germany). Nitric acid was further purified using a sub-boiling distillation unit (Savillex, Eden Prairie, Mn, USA). A multi element standard, Hg, gold, germanium (Ge), rhodium (Rh) and thulium (Tm) standards were obtained from Spectrascan (Teknolab, Ski, Norway). A tuning solution of lithium, yttrium, cesium and thallium (ICP-MS stock solution, tuning solution B) was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Certified reference materials of selenium enriched yeast (SELM-1) and TORT-3 (lobster hepatopancreas) were obtained from the National Research Council Canada (NRC, Ottawa, ON, Canada). The certified reference materials SRM 1566b (oyster tissue) was obtained from National Institute of Standards and Technology (Gaithersburg, MD, USA) and ERM-BC210a (wheat flower) from LGC (Middlesex, UK).

Samples

Rearing and harvesting

First instar BSF (*H. illucens*) larvae were taken from a stock colony of the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna 'Bruno Ubertini' (Brescia, Italy). Three different substrates were tested for their effects on the growth performance and composition of BSF larvae: (1) Gainesville diet (Hogsette, 1992) consisting of alfalfa, wheat bran and wheat flour with the ratios represented in Table 1 used as control substrate (CTR); (2) *Ascophyllum nodosum* diet (AN30%) with 30% substitution of the alfalfa meal with the brown algae; and (3) Selenium diet (Se) with Gainesville diet enriched with 0.3 mg/kg of Se in the form of sodium selenite (Table 1).

Nutrient composition

All diets were analysed for moisture, crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF), and ash as described by the Association of Official Analytical Chemists (AOAC) (2005). The moisture content of samples was determined by an oven-drying method (130 °C for 2 h) (Commission Regulation No. 152/2009). The CP content was measured according to the Kjeldahl method (proc. 2001.11; AOAC 2005). The NDF and ADF analyses were performed according to procedures of the AOAC (2005) (methods 2002.04 and 973.18, respectively), using an Ankom 220 fibre analyser (ANKOM™ Technology, Fairport, NY, USA). Ash was measured by using a muffle furnace at 550 °C (proc. 942.05; AOAC 2005). Moreover,

Table 1. Composition and selected nutritional factors of three test diets used to rear *Hermetia illucens* larvae.[1]

Constituent	Units	Amount (% on as is basis)		
		CTR	AN30%	Se
Alfalfa meal	%	30	-	30
Ascophyllum nodosum meal	%	-	30	-
Wheat bran	%	50	50	50
Corn meal	%	20	20	20
Total selenium	mg/kg	1.33	1.01	1.63
Relative to control	%	100	76	122
Crude protein	%	15.6	9.6	15.6
NDF	%	44.7	41.7	44.7
ADF	%	15.7	21.2	15.7
Ash	%	5.8	14.7	5.8

[1] ADF = acid detergent fibre; AN30% = *Ascophyllum nodosum* diet; CTR = control diet; NDF = neutral detergent fibre; Se = selenium diet.

the selenium concentration was determined by atomic emission spectrometry (Ng *et al.*, 1974) inductively coupled to argon plasma with optical detector.

Each treatment was tested in three experimental units (crates of size 40×30×15 cm) which were added their respective feeding media and 300 BSF larvae (eggs were placed aching on experimental feeding media). The larvae were subjected to a feeding regime of 190 mg fresh material per larvae every 2nd day, until they reached the 4th instar. At this stage, uneaten feeding media was removed and replaced as described above until larvae reached the 6th instar. The larvae were kept in complete darkness at 25 °C with 70% relative humidity during the experiment; only while feed was added was light present. Every 3rd day, 10 larvae were randomly counted from each container and weighed with an analytical balance (ME104E/M, Mettler-Toledo S.p.A. Milan, Italy). Every 4th day, the weight gained was calculated by comparing the obtained average larval weight with the previous mean larval weight. When insects reached the 6th instar, the larvae were separated manually from the substrate material.

The collected prepupae were washed with tap water and stored frozen at -20 °C. Samples of fresh substrate were also collected and stored at -20 °C pending chemical analysis. Total prepupal biomass was recorded per single crate. Prior to analysis, the larvae were grounded in liquid nitrogen using a mortar and pestle; since substrate material was already grounded this preliminary step was skipped. Both grounded prepupal biomass and substrate materials were freeze-dried until constant weight. While freeze-drying may result in less complete moisture removal compared to oven drying, this difference is minimal, and freeze-drying guarantees a better preservation of nutrients. Freeze dried material were stored at 4 °C until further analysis. For element determination and for Se speciation, biological replicates of n=3 were analysed.

Metal and mineral composition

Determination of elements

The freeze-dried prepupae and extracts from the Se speciation procedure were digested using a microwave-acid decomposition and analysis of multi-elements by ICP-MS based on the method described by Julshamn *et al.* (2007). In brief, approximately 0.2 g of freeze-dried samples and 1 ml of extracts from the Se speciation procedure were weighed in Teflon vessels and added 2 ml of concentrated HNO_3. The tubes were capped and placed in the ultrawave system (Milestone UltraWAVE, Sorisole, Italy) in a container with 130 ml of Milli-Q water and 5 ml of H_2O_2. The digests were diluted to a final volume of 25 ml (freeze-dried sample) or 10 ml (extract from Se speciation) with Milli-Q water.

The sample digests were analysed with an iCAP-Q ICP-MS using collision cell and FAST SC-4Q DX autosampler (both Thermo Fisher Scientific Inc.). An external calibration curve was prepared from freshly prepared multi-element solution, including Se (Spectrascan, Teknolab). The instrument was set in He KED (kinetic energy discrimination) mode for interference removal. A solution of internal standard comprising of Ge, Rh and Tm (Spectrascan) was used for correction of instrumental drift during the analysis. The instrument was tuned prior to analysis using a tuning solution (1 µg/kg tuning solution, Thermo Fisher, in 2% HNO_3 and 0.5% HCl). Isotope ^{78}Se was monitored.

The accuracy of the analysis was assessed using the certified reference material lobster hepatopancreas (TORT-3) and oyster tissue (SMR 1566b). The results obtained for total Se (mean ± SD) were 9.9±0.3 (n=6) and 2.0±0.1 mg/kg (n=6) for TORT-3 and Oyster Tissue, respectively, and agreed well with the certified reference values of 10.9±1.0 and 2.1±0.2 mg/kg for TORT-3 and oyster tissue, respectively. The results obtained for the other elements determined agreed well with the certified values (Table S1).

Determination of selenium species

In the present study, inorganic and organic Se species were determined using high performance liquid chromatography coupled to inductively coupled plasma mass spectrometry (HPLC-ICP-MS, 1260 Infinity HPLC and 7900 ICP-MS, both Agilent Technology, Santa Clara, CA, USA) based on the methods described in Sele *et al.* (2018). The ICP-MS was tuned prior to analysis using a tuning solution. A reaction gas of hydrogen was applied in the ICP-MS at a flow rate 2.5 ml/min, to prevent interferences, and Se isotopes ^{77}Se, ^{78}Se and ^{82}Se were monitored with an integration time of 0.1 sec.

Inorganic selenium species

In brief, inorganic Se species were determined by accurately weighing 0.2 g freeze-dried prepupae in 13 ml polypropylene tubes, adding 5 ml 0.1 M NaOH, mixing carefully for 1-2 min until all sample was dissolved, and then mixed on a rotator (model LD79, Labinco, Breda, the Netherlands) at 25 rpm/min for 1 h. The samples were left in a sonication bath for 15 min and centrifuged (3,500×*g*, 10 min; Eppendorf Centrifuge 5702, Hamburg, Germany), filtrated using 0.45 µm, 25 mm membrane filters with syringe (Merck Millipore).

The Se species selenite and selenate were separated using a strong anion-exchange column (Hamilton PRPX100, 150 × 4.6 mm, 5 µm, VWR, Radnor, PA, USA) with a gradient elution using a mobile phase of (A) 0.5 mM and (B) 200 mM ammonium acetate, both with pH 5.2 and 3% methanol. The sample were injected (50 µl) on the

column and separate using the following gradient: 0-2 min 90% (A) and 10% (B); 2.1-8 min from 10-100% (B); 8.1-16 min with 100% (B); 16.1-22 min 90% (A) and 10% (B) with a flow rate of 1 ml/min. There is no commercially available CRMs for selenite or selenate, but instead, a fish feed supplemented with selenite was used as an in-house control for the analysis.

Organic selenium species

The organic Se species were determined by applying a sequential enzymatic digestion procedure with protease and then chitinase to evaluate the extraction efficiencies of these enzymes on the organic SeMet in the insect samples. An overview of the analysis performed in this study is shown in Figure 1.

For the organic Se speciation, the samples were determined by accurately weighing 0.5 g freeze-dried prepupae in 13 ml polypropylene tubes, added 5 ml of 10 mM Tris-HCl, pH 8, containing 40 mg Protease type XIV (28 U/ml). The samples were mixed carefully for 1-2 min. The samples were left in a water bath (OLS200 Grant Instruments, Cambridge, UK), in the dark and at 37 °C, shaking at 60 rpm/min for 18 h. The samples were then cooled to room temperature and left in a sonication bath for 15 min before centrifugation (3,000×*g* for 10 min), the sample extracts were then moved into a new 13 ml polypropylene tube with Pasteur pipette.

The non-soluble fractions of the prepupae were further digested with a new freshly prepared batch of 0.7 mg chitinase dissolved in 5 ml Tris-HCl (10 mM, pH 8, 28 U/ml). The samples were again mixed carefully for 1-2 min. The samples were left in a water bath (OLS200 Grant Instruments), in the dark and at 37 °C, shaking at 60 rpm/min for 18 h. The samples were cooled to room temperature, left in a sonication bath for 15 min and centrifuged (3,000×*g* for 10 min), and soluble extract moved into new 13 ml polypropylene tubes with Pasteur pipette. All soluble extracts from both enzymatic digestions were filtrated using a 0.45 µm, 25 mm membrane filters with syringe (Merck Millipore), and further by centrifugal filters (Amicon Ultra-0.5 ml 10 kDa, Merck Life Science AS, Oslo, Norway) to separate the low molecular weight Se species from the high molecular weight compounds by centrifugation (14,000×*g* for 20 min, Eppendorf). The CRMs SELM-1 and ERM-BC210a, as well as the phosphate buffer (blanks) was treated in the same way as the samples.

The SeMet was separated from other organic and inorganic Se species in the extracts using a reversed-phase column (Gemini C6-phenyl 110 A (4.6 × 105 mm, 5 µm; Phenomenex, Castel Maggiore, Italy) with 25 µl injection volume, an isocratic elution using a mobile phase of 20 mM ammonium formate, pH 9 and with 3% methanol, and a flow rate of 1 ml/min.

The accuracy of the analysis was assessed using the CRMs SELM-1 (selenised yeast) and ERM-BC210a (wheat flower), both with certified values for SeMet. The results obtained for SeMet (mean ± SD) were 1,003.8±112.1 mg Se/kg (n=2) and 13.0±3.6 mg Se/kg (n=2) for SELM-1 and ERM-BC210a, respectively, and was within 2×SD of the certified reference values for SeMet of 1,284.4±104.7 mg Se/kg and 11.03±1.0 mg Se/kg for SELM-1 and ERM-BC210a, respectively.

Statistical analysis

Data were analysed using the GLM procedure of SPSS (SPSS Statistics, IBM Corp., Armonk, NY, USA), at significance level of 0.05, followed by Tukey's multiple comparison analysis used to determine the effect on total selenium and other elements of dietary treatments. The statistical analysis was conducted using R (version 3.6.3; R Core Team, 2019).

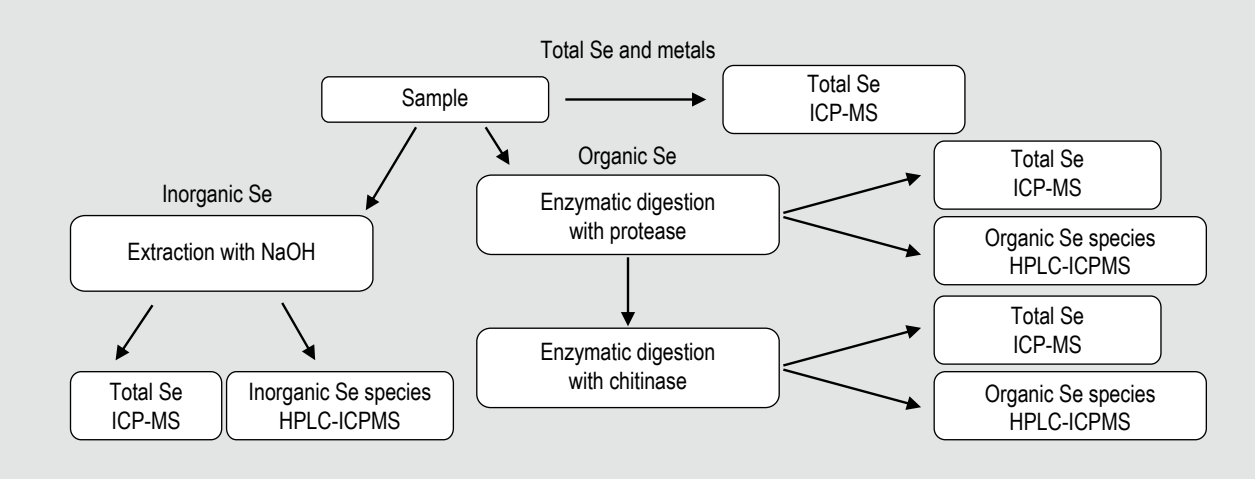

Figure 1. An overview of the analysis performed for determining total selenium and selenium species in the insect meals.

3. Results

Total selenium and other metal and minerals in the BSF prepupae

Total biomass produced, starting from 300 young larvae under different substrates were 20.9, 23.4, 20.7 g pupae for CTR, AN30% and Se dietary groups, respectively. The mean protein content was 536 g/kg DM in CTR, 446 g/kg DM in AN30%, and 351 g/kg DM in Se group. Although the focus of the study was Se, other selected minerals have been also analysed in the BSF prepupae. The concentrations (mean ± SD, n=3) of Se, As, Cd, Hg and Pb and the minerals Cu and Zn in the BSF prepupae are shown in Table 2. Different superscripts illustrate significant differences (P<0.05) among dietary groups (Table 2). Total Se levels were higher in the group reared on Se diet (1.17 mg/kg w/w) compared to the CTR group (0.41 mg/kg) and the insects group reared on algae (AN30% group, 0.21 mg/kg). For the metals As, Hg and Cd, higher concentrations were seen in the AN30% group, with concentrations of 6.40 mg/kg, 1.5 mg/kg, and 0.020 mg/kg of As, Hg, and Cd, respectively. In comparison, the CTR group contained concentrations of 0.28 mg/kg, 0.45 mg/kg, and 0.007 mg/kg of As, Cd, and Hg, respectively. For the minerals Cu and Zn, highest concentrations were seen in the Se group, with Cu and Zn concentrations of 18.7 mg/kg and 277 mg/kg, respectively. In comparison the CTR and AN30% group contained 13.1 mg/kg and 9.1 mg/kg of Cu, respectively, and 210 mg/kg and 170 mg/kg of Zn, respectively.

Selenium species in the BSF prepupae

For the Se speciation analysis, different extraction procedures were carried out (Figure 1), with determination of inorganic Se by an alkaline extraction procedure and determination of organic Se by enzymatic digestion using both protease and chitinase. All extracts were determined for total Se by acid digestion and analysis by ICP-MS, and an overview of the total Se in the extracts (mg/kg, mean with bars for standard deviation, n=3) are shown in Table 3. The sum of the Se concentrations in the extracts are higher than the total Se in the insect meals (Table 2). The mean concentrations of Se in the alkaline extractions were highest in the Se group, with 0.69±0.02 mg/kg, whereas the AN30% group contained lower selenium concentrations (0.07±0.01 mg/kg) than the CTR group (0.25±0.02 mg/kg). Se in the protease extracts were higher in the Se group (0.59±0.03 mg/kg) compared to the CTR group (0.29±0.05 mg/kg) and the AN30% group (0.12±0.01 mg/kg). In the soluble chitinase extracts the Se group contained the highest concentrations (0.19±0.02 mg/kg) compared to the CTR and AN30% group, with 0.08±0.01 mg/kg and 0.02±0.002 mg/kg, respectively. The concentrations in the extracts are presented in the Table 3.

Table 2. Total selenium (Se), arsenic (As), cadmium (Cd), mercury (Hg), lead (Pb), copper (Cu) and zinc (Zn) concentrations (mg/kg w/w, mean ± SD, n=3) in the black soldier fly prepupae reared on the experimental feeding medias. Significant differences among dietary groups (P<0.05) are illustrated by different superscripts.[1]

Samples	Se (mg/kg)	Relative to CTR (%)	As (mg/kg)	Cd (mg/kg)	Hg (mg/kg)	Pb (mg/kg)	Cu (mg/kg)	Zn (mg/kg)
CTR diet	0.41±0.04^{a}	100	0.28±0.05^{a}	0.45±0.05^{a}	0.007±0.001^{a}	0.33±0.04^{a}	13.1±1.7^{a}	210.0±0.1^{a}
AN30% diet	0.21±0.01^{b}	51	6.40±0.01^{b}	1.5±0.1^{b}	0.020±0.001^{b}	0.35±0.02^{ab}	9.1±0.6^{b}	170±10^{b}
Se diet	1.17±0.06^{c}	285	0.25±0.03^{a}	0.38±0.01^{a}	0.006±0.001^{a}	0.40±0.01^{b}	18.7±0.6^{c}	277±12^{c}

[1] AN30% = *Ascophyllum nodosum* diet; CTR = control diet; Se diet = selenium diet.

Table 3. Total selenium (Se) in the soluble extracts (mg/kg ww, mean ± SD, n=3) determined by ICP-MS, and the calculated recovery (%) of Se in the extracts compared to total Se (sum of the extracts) for the dietary groups.[1]

Samples	Soluble alkaline extract (mg/kg)	Soluble alkaline extract (%)	Soluble protease extract (mg/kg)	Soluble protease extract (%)	Soluble chitinase extract (mg/kg)	Soluble chitinase extract (%)
CTR diet	0.25±0.02	40	0.29±0.05	47	0.08±0.01	13
AN30% diet	0.07±0.01	44	0.12±0.01	43	0.02±0.002	13
Se diet	0.69±0.02	47	0.59±0.03	40	0.08±0.02	13

[1] AN30% = *Ascophyllum nodosum* diet; CTR = control diet; Se diet = selenium diet.

The Se speciation analysis using HPLC-ICP-MS made it possible to determine selenite and selenate, in the alkaline extracts, and SeMet in the soluble protease and chitinase extracts. Table 4 shows the concentrations of selenite, selenate, and SeMet (mean with standard deviation, mg/kg, n=3). For all samples the levels of selenate were low (<0.004 mg/kg). The concentration of selenite was low in the AN30% group, 0.074 mg/kg, compared to the CTR group, 0.13 mg/kg. The Se group contained a concentration of 0.53 mg/kg selenite. Similarly, the organic Se in the protease extracts was lower in SeMet concentration in the AN30% group (0.10 mg/kg) compared to the CTR group (0.27 mg/kg), whereas the Se group was higher in SeMet (0.50 mg/kg) compared to the CTR group. SeMet was also detected in the soluble chitinase extracts, however, in lower concentrations than in the soluble protease extracts. Similarly, the Se group had higher levels of SeMet compared to the CTR group and the AN30% group for the chitinase extracts. The chromatographic separation of selenite, selenate and SeMet in the insect groups are shown in Figure 2 (A and B).

4. Discussion

The purpose of biofortification is to increase the micronutrients in the edible parts of the plants or animals, achieved by either mineral fertilisation or feed supplementation, respectively (D'amato *et al.*, 2020). Selenium is an essential element both for humans and animals, and the biofortification of this element to insects would be valuable in terms of exploring the alternatives to e.g. addition of Se in feed.

Total selenium content and other elements in BSF prepupae

The amount of Se in BSF prepupae was affected by the Se concentration in the three diets administered to the insects. Using Se levels in the CTR diet as reference, Table 1 shows that the AN30% diet and Se-enriched diet possess 76 and 122% of Se, respectively. The prepupae fed the AN30% diet, fed with 24% less Se compared to the CTR diet, had a 51% drop in Se content compared to prepupae of CTR group. On the other hand, adding sodium selenite to the diet (with a 22% increase in Se compared to the CTR diet), caused a clear increase in Se in the insects. The prepupae fed the Se diet had 285 times higher Se levels than the CTR group, showing that it is possible to biofortify insects with Se. This type of biofortification of Se is well known and studied in yeast, producing Se-enriched yeasts (Adadi *et al.*, 2019; Kieliszek and Błażejak, 2013), but to our knowledge, no studies have so far looked on the ability of insects to accumulate Se. Yeast is able to accumulate minerals and has a fast metabolism that allows rapid proliferation and greater interaction of cells with the external environment (Kieliszek *et al.*, 2015). The BSFL has in similar manner been shown to grow fast, and with the ability of feeding on e.g. waste, it is considered a bicircular feed and food resource (Bortolini *et al.*, 2020). The results in this study show that when adding Se to the diet, the BSF prepupae increase its concentration in Se. For comparison, other protein sources, such as plant protein and fishmeal intended for fish feed production in Norway contained Se levels ranging from below 0.009 to 1.1 mg/kg (n=9) and from 1.6 to 3.2 mg/kg (n=9), respectively (Sele *et al.*, 2021). Se levels in commercially produced insect meal intended for fish feed ranged in Se levels from 0.15 to 0.30 mg/kg (n=4). The levels in the insect meal fortified with selenite is hence comparable, or higher in Se levels, than processed plant meals.

Brown algae can be high in some metals, i.e. As and Cd (Biancarosa *et al.*, 2018b). The insects reared on the AN30% diet contained significantly higher levels of the undesirable metals As, Cd, and Hg when compared to the CTR and Se group (Table 2). Feed ingredients used in animal feed in the EU must meet the requirements of Directive 2002/32/EC and amendments, which sets maximum levels (MLs) for undesirable substances such as heavy metals and As in feed and feed materials (based on 88% dry matter). The concentrations of Pb and Hg in the prepupae did not

Table 4. Total selenium (Se), selenite and selenate, and selenomethionine (SeMet; mean ± std, mg/kg w/w, n=3) in the protease and in the chitinase extracts of the insect prepupae, results obtained from the HPLC-ICPMS analysis.[1]

		Inorganic Se			Organic Se		
Samples	Total Se (mg/kg)	Selenite (mg/kg)	Selenate (mg/kg)	Inorganic Se of total Se (%)	SeMet in soluble protease (mg/kg)	SeMet in soluble chitinase (mg/kg)	SeMet of total Se (%)
CTR diet	0.41±0.04	0.13±0.03	0.006±0.007	27±7	0.27±0.07	0.053±0.008	78±16
AN30% diet	0.21±0.01	0.074±0.008	0.004±0.002	38±4	0.10±0.01	0.029±0.001	65±4
Se diet	1.17±0.06	0.53±0.02	0.007±0.004	48±3	0.50±0.03	0.13±0.01	54±4

[1] AN30% = *Ascophyllum nodosum* diet; CTR = control diet; Se diet = selenium diet.

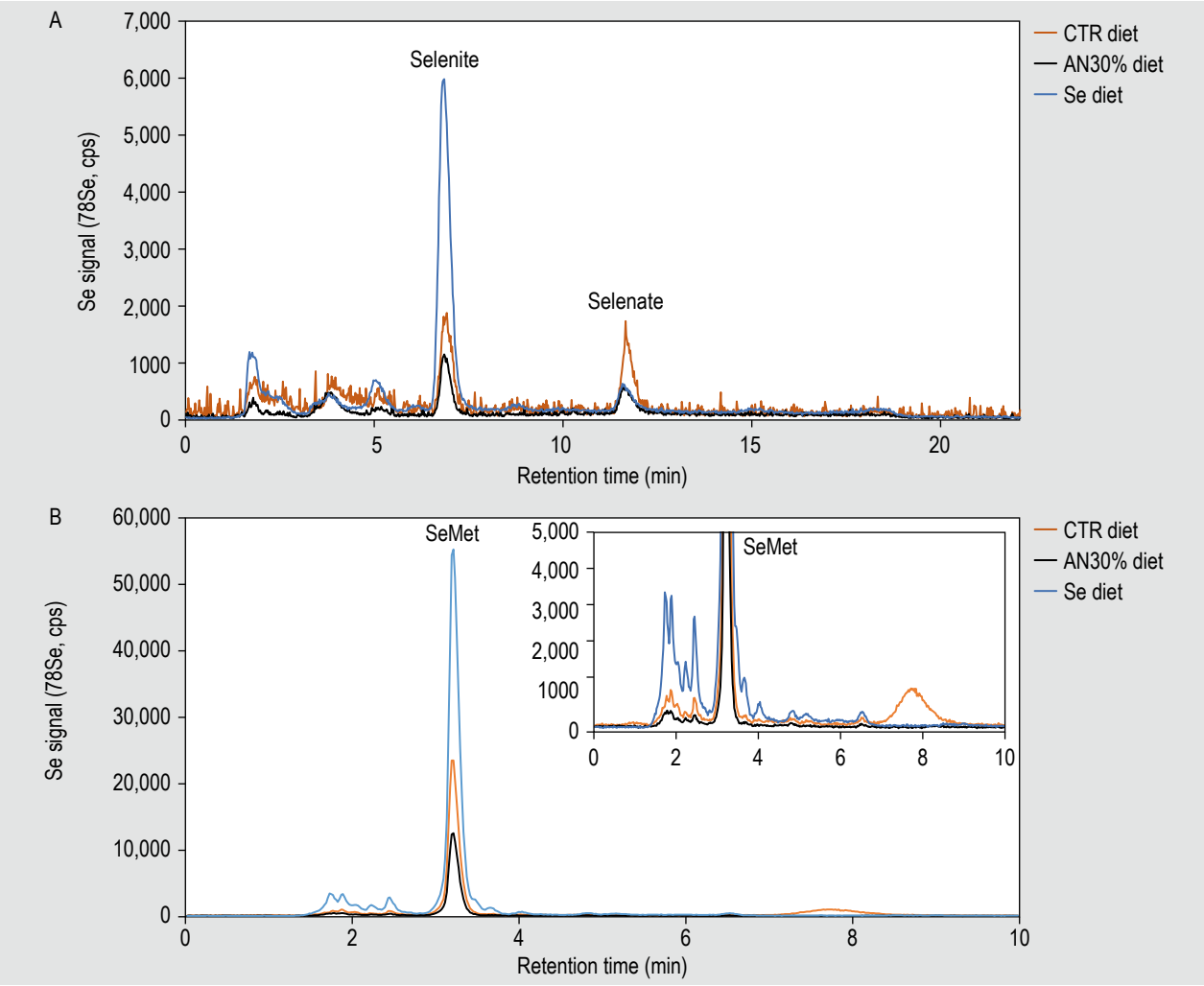

Figure 2. Chromatograms from the speciation analysis using HPLC-ICP MS for determining inorganic selenite and selenate (A) and organic selenomethionine (SeMet) (B) in the insect prepupae reared on the CTR diet (orange), the AN30% diet (black) and the Se diet (blue).

exceed the current MLs for these metals in feed materials or complete feed. However, using 30% algae inclusion in the diet of the BSF prepupae, As levels in the insect meal exceeded the ML for As in feed materials, set at 2 mg/kg. Higher ML is established for As in feed materials composed of macroalgae or feed materials manufactured from macroalgae, at 40 mg/kg. There are currently no specific MLs established for insect as a feed material in EU, which could be relevant since insects are now approved as a feed ingredient for animal feed. The results from this study show that including algae/seaweed in the insect feeding media can limit the use of BSFL as a feed, which has also been seen in previous studies where seaweed was included in the feeding media of BSFL (Biancarosa *et al.*, 2018a).

Lead (Pb), and the minerals Cu and Zn were significantly different for the AN30% group and for the Se group when compared with the CTR group (Table 2). Lowered levels of Cu and Zn were seen in the AN30% group compared to the CTR group, whereas higher levels were seen for the same minerals in the Se group. Previous studies have shown antagonist interaction between Se and Cu in salmon tissue and in rainbow trout (Berntssen *et al.*, 2000; Knox *et al.*, 1982). Diener *et al.* (2015) studied the bioaccumulation of heavy metals in BSFL, and where the larvae and prepupae showed a higher Cd level than present in the substrate, while Pb and Zn were not accumulated. Similar results were also obtained by Purschke *et al.* (2017) with a bioaccumulation of Cd and Pb in the larvae, with bioaccumulation factors of 9.1 and 2.3 for Cd and Pb, respectively.

Selenium species in BSF prepupae

In Se speciation, a complete extraction of the element is often challenging, mainly because of the binding of Se to proteins. Proteolytic enzymes are extensively used for the extraction of selenoamino acids from tissues (Bierla *et al.*, 2008; Bryszewska and Mage, 2015; Jagtap *et al.*,

2016) and proteolytic enzymes were therefore applied for the extraction of Se. Insects have an outer protective exoskeleton layer of chitin, a long-chain polymer of N-acetylglucosamine, an amide derivative of glucose (Chen *et al.*, 2018). This polysaccharide is likely non-digestible by a protease enzyme. To have a complete extraction of the insects, a sequential extraction was therefore applied, digesting the sample first with protease prior to chitinase for digesting the non-soluble residue. The enzymatic digest using protease contained 47% of the total Se in the CTR group, whereas 43 and 40% for the AN30% and the Se group, respectively. Only minor concentrations of Se were detected in the chitinase digests of all dietary groups, accounting for 13% of total Se, and with the highest concentration seen for the Se group. These results suggest that only a minor fraction of Se bind to the exoskeleton layer but that a higher accumulation occurs when the larvae is reared on a Se biofortified substrate.

The samples were furthermore digested for inorganic Se using an alkaline solution, previously shown preferable for the determination of inorganic selenite and selenate (Sele *et al.*, 2018). Se accounted for approximately 40-50% of the total Se in all dietary groups for the alkaline extracts (Table 3). The Se speciation analysis of the alkaline extracts showed that selenite and selenate were present in all insect groups, but that selenite was the major Se species. As expected, the Se group contained the highest level of selenite. Inorganic Se (the sum of selenite and selenate) ranged from 27% in the CTR group to 48% in the Se group (Table 4). The AN30% group contained 38% of the total Se as inorganic Se species. Algae absorb Se from water mainly in the form of inorganic selenite and selenate, then convert it into organic selenium replacing sulphur in the synthesis of selenoamino acids (Zhong and Cheng, 2017). The same mechanism for Se uptake as seen for algae is also true for terrestrial plants, including alfalfa (*Medicago sativa*). For plants, the roots absorb Se in the soil, being inorganic selenite and selenate, and convert it into selenoproteins (Wang *et al.*, 2020). For insects fed the Se diet, approximately 50% of the Se content was in the inorganic Se form, reflecting that the insects absorb a large amount of added selenite without further modification.

Selenomethionine was detected as the major Se species in the protease extracts of insects from all dietary groups analysed (Figure 2), with lowest level in the AN30% group (0.10 mg/kg), and highest level in the Se group (0.50 mg/kg). In addition to SeMet, also other Se-containing peaks were seen in the chromatograms, before and after the SeMet eluting peak. These results suggest the presence of also other minor organic Se compounds, particularly for the Se group. The identity of these Se peaks is currently not known, and the identification and quantification of unknown Se-containing compounds can be challenging in speciation analysis due to the lack of analytical standards. For the identification of unknown elemental species, complementary analytical techniques such as high-resolution mass spectrometry (HR-MS) would be required (Yamashita, 2010). In animals, selenite is metabolised to hydrogen selenide, and further to selenocysteine (SeCys), which is specifically incorporated into selenoproteins (Ogra and Anan, 2009). Analysis of SeCys is, however, challenging due to the highly reactive free selenol group, and requires stabilisation through derivatisation prior to analysis (Bierla *et al.*, 2008). SeCys has been detects as minors Se species in fish and animal tissues (Bryszewska and Mage, 2015; Jagtap *et al.*, 2016). In plants, SeCys is often transformed into non-protein amino acids, such as Se-methyl-selenocysteine, selenocystathionine and γglutamyl-MeSeCys (Brown and Shrift 1981). Previous studies have shown the presence of SeMet as major Se species in commercially produced insect meal, but also the presence of minor unknown Se species (Vaksdal, 2021). To our knowledge, this is the first study of Se species in insect meal after exposing BSFL to different substrates.

The amount of organic Se in the prepupae depend on several factors, including the concentration of different species in the diet and then the ability of the insect to absorb and accumulate those species (Andrahennadi *et al.*, 2007). The synthesis of selenoamino acids, and therefore selenoproteins, by organisms can be explained by the need to continuously decrease the toxicity of Se entering the food chain. Selenium can enter the food chain almost exclusively in inorganic form, absorbed by plants and algae in the form of selenite and selenate (Ponton *et al.*, 2020; Schiavon *et al.*, 2017; Wang *et al.*, 2020). Plants and algae reduce in part the toxicity of absorbed Se by synthesis of SeCys and then SeMet (Ponton *et al.*, 2020). Considering the toxicity of Se, its storage in the form of non-specifically protein bound SeMet is considered to be harmless. SeMet is one of the major Se species found in Se-enriched cereals (Cheajesadagul *et al.*, 2014; Cubadda *et al.*, 2010; Stadlober *et al.*, 2001), whereas SeCys has a more toxic effect through its non-selective incorporation into proteins (Dimkovikj *et al.*, 2015). Therefore, the transformation of SeCys into non-protein amino acids is a key mechanism in Se tolerance for plants (Brown and Shrift, 1981). The same thing can happen at the next level of the food chain: insects that feed on plants in turn convert part of the selenite present in plants into selenoproteins. In this study, the insects in the CTR group and the AN30% group had higher concentrations of organic SeMet compared to the Se group. For the Se group, around 50% of the total Se was present in organic Se forms, and higher concentrations of SeMet was detected in the Se group compared to the CTR group. These results could indicate that the prepupae are able to absorb selenite added in the diet and subsequently convert it into organic SeMet through their metabolism. Se speciation of the diets would however be needed to confirm this. Within insect production, the selection of an appropriate and tailored

substrate could lead to the production of a premium feed specialty (e.g. insect Se fortified meals), thus providing new opportunities for raw materials and diet formulations (Pinotti *et al.*, 2019; Schiavone *et al.*, 2017)

5. Conclusions

Since the bioavailability and toxicity of Se is dependent on the Se species present, a better understanding of the major Se species in BSF larvae when exposed to different types of substrates is important in terms of feed and food nutrient profile and safety. This study shows when rearing BSF prepupae on brown algae, the Se levels did not increase when compared with the CTR group, whereas the levels of other metals (As, Cd, Hg, Pb) and minerals (Cu and Zn) were significantly affected. The diet enriched with inorganic selenite caused an increased Se level in the BSF prepupae. Selenium speciation analysis showed the presence of selenite, selenate, SeMet as well as other unknown Se species in the BSF prepupae. Furthermore, the insect reared on inorganic selenite was having higher levels of selenite, but also higher levels of SeMet compared to the CTR group. Minor levels of SeMet were found in the chitinase extracts, suggesting that Se can be bound to the exoskeleton layer of insects. Overall, the study shows that BSF prepupae can be fortified with inorganic selenite and become a source of inorganic and organic Se in feed and in food.

Supplementary material

Supplementary material can be found online at https://doi.org/10.3920/JIFF2021.0153.

Table S1. Results obtained for total Se, As, Cd, Hg, Pb, Cu and Zn (mg/kg, mean ± SD, n=6) in the certified reference materials oyster tissue and TORT-3, and the accuracy (%) of the analysis based on the mean results and the certified values.

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Conflict of interest

The authors declare no conflict of interest.

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