Optimization of Analytical Method for Selenium Speciation in Fish Feed and Feed Ingredients Using a Chemometric Approach

MASTER THESIS

by

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ABSTRACT

Selenium (Se) is an essential element for humans, as well as for fish, including Atlantic salmon (*Salmo salar*). One of the main sources of Se and other elements for farmed salmon is the diet. Due to the replacement of marine-based ingredients with plant-based ingredients in fish feed, supplementation of Se has been considered necessary to maintain normal functions in fish. There are different chemical forms of Se, often characterized as Se species. Se species can be either inorganic or organic. Organic Se has higher a bioavailability than inorganic Se. However, inorganic Se is considered more toxic than organic Se. Other elements, such as the essential elements cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn), and non-essential elements such as cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As), may also be present in salmon. Some of these elements are known to interact with Se, but few studies have investigated the effects of the Se species on the interactions between Se and other elements. In fish feed, Se can be supplemented, both in forms of inorganic Se (e.g. selenite) and as organic Se (e.g. selenomethionine (SeMet) or SeMet produced by Se-enriched yeast. Maximum limits have been established for Se as a feed additive for animals by the European Commission, set to a total of 0.5 mg Se/kg. For SeMet and SeMet produced by the Se-enriched yeast (*Saccharomyces cerevisiae*), the limit is set to 0.2 mg Se/kg. Due to this difference in legislation for the supplementation of Se species, there is a need for analytical methods that can discriminate between the different Se species.

In this thesis, the overall aim was to apply chemometrics and other statistical approaches for handling data obtained from salmon a feeding trial with Atlantic salmon, and for the method optimization for determining Se species in fish feed and feed ingredients. This study evaluates the correlations between Se and other elements in salmon fed with feed supplemented with inorganic or organic Se species. Furthermore, the extraction procedure for an analytical method determining SeMet in fish feed and feed ingredients using was optimized using chemometric tools.

The correlations of Se to other elements were assessed in whole-bodies and fillets of salmon. The samples were from a previous feeding trial (Berntssen *et al.*, 2018b), and were analyzed by ICP-MS for determination of total Se, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb and Zn. To assess correlations between Se and other elements in salmon, two statistical methods, Pearson's and Spearman's correlation coefficients. The correlation study showed interactions between Se and Hg in whole-body and fillet samples, and Se and Cu in whole-body samples. It was also seen interactions between inorganic Se and As, and between inorganic Se and Fe in whole-body samples.

Method development was further performed to optimize a Se speciation method for the determination of organic Se in fish feed and feed ingredients analyzed by HPLC-ICP-MS (Sele *et al.*, 2018a). Different factors

were tested using experimental designs: i) extraction solutions (ammonium phosphate or a mixed buffer solution, both with pH 7), ii) an enzymatic pre-extraction step (with or without papain) and iii) enzymes for main extraction (protease type XIV, protamex, α-amylase and cellulase). Principal component analysis (PCA) was performed on the SeMet recovery as a response for the certified reference materials ERM BC210a (wheat flour) and SELM-1 (selenized yeast). From the experiments, the combination of protease and cellulase (1:1 ratio) for enzymatic digestion, with ammonium phosphate, and without a pre-extraction step was seen to be the most optimal method for extraction of organic Se. The optimized method was furthermore applied to experimental diets as well as commercial fish feed and feed ingredients (i.e. fish meal, plant meal and insect meal). The method was evaluated to be more accurate for the determination of SeMet in higher concentrations in feed but did not show sufficient recovery for Se in feed and feed ingredients at lower levels. Other Se peaks were observed in the chromatograms for some fish feed and fish meal, with unknown chemical structures.

From this thesis, the use of statistical and chemometric approaches was considered beneficial for assessing correlations between elements and the method development. From the correlation study, a large dataset was obtained, with many outliers. For this, visualization of data through boxplots was a useful tool for excluding the outliers. Shapiro-Wilk normality testing was also useful for determining data distributions for further assessment of Pearson's and/or Spearman's correlation coefficients. For method development, experimental design was useful for limiting the number of experiments, while facilitating for finding the best conditions for increasing SeMet recovery. Possible interactions between factors were also provided by using experimental design and PCA. Although more work is needed for an optimal Se speciation method, the results from this thesis show that experimental design and PCA are useful approaches also for future method development.

SAMMENDRAG

Selen (Se) er et essensielt grunnstoff for mennesker, dyr og fisk, inkludert atlantisk laks (*Salmo salar*). En av de viktigste kildene til Se og andre grunnstoff for oppdrettslaks er fiskefôr. Siden plante-baserte ingredienser har erstattet marine ingredienser i fiskefôr, er tilsetning av Se i fôr blitt ansett som nødvendig for å opprettholde normale kroppsfunksjoner i fisk. Det finnes ulike kjemiske former for Se, ofte kjent som Se-spesier. Se-spesier kan være uorganiske eller organiske, der organisk Se har høyere biotilgjengelighet enn uorganisk Se. Uorganisk Se regnes også som mer giftig enn organisk Se. Andre grunnstoff som kan være til stede i laks, er de essensielle grunnstoffene kobolt (Co), krom (Cr), kobber (Cu), jern (Fe), mangan (Mn) og sink (Zn), og ikke-essensielle grunnstoff som kadmium (Cd), kvikksølv (Hg), bly (Pb) og arsen (As). Noen av disse elementene er kjent for å interagere med Se, men få studier har undersøkt interaksjoner mellom Se og elementkonsentrasjoner knyttet til Se-spesier. I fiskefôr kan Se tilsettes som både uorganisk Se (f.eks. selenitt) og som organisk Se (f.eks. selenometionin (SeMet) og SeMet produsert av Se-beriket gjær). Maksimumsgrenser har blitt etablert for Se som tilsetningsstoff i dyrefôr av Europakommisjonen, som er i dag satt til 0,5 mg Se/kg. For SeMet og SeMet produsert av Se-beriket gjær (*Saccharomyces cerevisiae*) er grensen satt til 0,2 mg Se/kg. Ettersom egne grenser har for noen Se-spesier, er det et behov for analytiske metoder som kan skille mellom de ulike Se-spesiene.

Det overordnede målet i denne masteroppgaven var å anvende kjemometri og andre statistiske tilnærminger for å håndtere data innhentet fra Atlantisk laks fra et fôringsforsøk, og for å optimalisere en metode for bestemmelse av Se-spesier i fiskefôr og fôringredienser. Dette innebar blant annet å vurdere korrelasjoner mellom Se og andre grunnstoff i laks som ble fôret med uorganisk selenitt eller organisk SeMet. Videre ble ekstrasjonsprosedyren i en analysemetode optimalisert for bestemmelse av SeMet i fiskefôr og fôringredienser ved bruk av kjemometriske verktøy.

Korrelasjoner mellom Se og andre grunnstoff ble vurdert i helfisk og filet fra atlantisk laks fra et tidligere fôringsforsøk (Berntssen *et al.*, 2018b). Prøvene ble analysert ved hjelp av ICP-MS for å bestemme totalkonsentrasjoner av Se, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb og Zn. Både Pearson- og Spearman-korrelasjonskoeffisienter ble benyttet for å finne korrelasjonskoeffisienter mellom Se og andre elementer. Korrelasjonskoeffisientene viste interaksjoner mellom Se og Hg i både helfisk og filet, og mellom Se og Cu i helfisk. Interaksjoner mellom uorganisk Se og As, og mellom uorganisk Se og Fe i helfisk ble også sett.

En metodeutvikling ble videre utført for å optimalisere en Se-spesieringsmetode for bestemmelse av organisk Se i fiskefôr og fôringredienser ved bruk av HPLC-ICP-MS (Sele *et al.*, 2018a). Ulike faktorer ble testet ved hjelp av eksperimentell design: i) ekstraksjonsløsninger (ammoniumfosfat eller en blandet løsning, pH7 for begge), ii) enzymatisk pre-ekstraksjonstrinn (med eller uten papain) og iii) enzymer for

hoved-ekstraksjonstrinnet (protease type XIV, protamex, α-amylase og cellulase). Prinsipial komponent analyse (PCA) ble utført på SeMet-gjenfinning som respons for de sertifiserte referansematerialene ERM BC210a (hvetemel) og SELM-1 (Se-holdig gjær). Fra eksperimentene, var den mest optimale metoden å kombinere protease og cellulase (1:1-forhold) for enzymatisk fordøyelse med ammoniumfosfat, uten et pre-ekstrasjonstrinn. Den optimaliserte metoden ble anvendt på eksperimentelle fiskefôr, kommersielle fiskefôr og fôringredienser (dvs. fiskemel, plantemel og insektmel). Metoden ble vurdert som mer nøyaktig for bestemmelse av SeMet i høyere konsentrasjoner i fôr, men viste ikke tilstrekkelig gjenfinning for Se i fôr og fôringredienser ved lavere nivåer. Andre Se-topper med ukjent kjemiske struktur ble observert i kromatogrammer for noen fôr- og fiskemelsprøver.

I denne studien ble statistiske og kjemometriske metoder ansett som gunstig for å finne korrelasjoner mellom grunnstoff og for metodeutviklingen. Fra korrelasjonsstudien ble det innhentet et stort datasett med mange uteliggere. For dette var visualisering av data gjennom boxplott et nyttig verktøy for å ekskludere uteliggere. Shapiro-Wilks normalitetstesting var også nyttig for å bestemme datadistribusjoner for videre bestemmelse av Pearson- og/eller Spearman-korrelasjonskoeffisienter. For metodeutviklingen var eksperimentell design nyttig for å begrense antall eksperimenter og samtidig legge til rette for å de mest gunstige betingelsene for å øke SeMet-gjenfinning. Mulige interaksjoner mellom faktorer ble også tilgjengelig ved bruk av eksperimentell design og PCA. Selv om det kreves mer arbeid for å optimalisere metoden for Se-spesiering, viser resultatene fra denne masteroppgaven at eksperimentell design og PCA kan bli brukt som nyttige verktøy for videre metodeutvikling.

CONTENTS

ABBREVIATIONS

AU	Anson unit
cps	Counts per second
CRM	Certified Reference Material
DOE	Design of Experiments
dw	Dry weight
ESI	Electrospray ionization
FAA	Free amino acid
FF	Fish feed
FM	Fish meal
GC	Gas chromatography
GGMSC	γ-Glutamyl-selenomethylselenocysteine
GPX	Glutathione peroxidase
GSH	Glutathione
GS-Se-SG	Selenodiglutathione
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry
ICP-MS	Inductive coupled plasma mass spectrometry
IM	Insect meal
kDa	Kilodalton
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
ML	Maximum limit
mRNA	Messenger-RNA
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
n	Sample size
PC	Principal component
PCA	Principal Component Analysis
PEEK	Polyether ether ketone
PM	Plant meal
PP	Polypropylene

p-value	Probability value
QA	Quality assurance
QC	Quality control
r	Correlation coefficient
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RP	Reversed-phase
rpm	Revolutions per minute
rps	Revolutions per second
RSD	Relative standard deviation
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEC	Size-exclusion
SeCys	Selenocysteine
$SeCys_2$	Selenocystine
SeMet	Selenomethionine
SeMetSeCys	Selenomethylselenocysteine
SeOMet	Selenomethionine-Se-oxide
TOF	Time-of-flight
U	Unit
VIP	Variable importance plot
ww	Wet weight

1 INTRODUCTION

1.1 Background

In recent years, plant-based ingredients have been increasingly used in feed for farmed Atlantic salmon (*Salmo salar*) in Norway. Salmon feed has traditionally contained mainly marine-based feed ingredients such as fish meal and fish oil, with a 90% inclusion in the 1990s (Ytrestøyl *et al.*, 2015). However, there has been a decrease in the use of marine-based feed ingredients, to around 25% inclusion in 2016, with plant-based ingredients as the main substitute (Aas *et al.*, 2019). The decreased biomass availability and hence the resulting price increase has contributed to the increased use of plant-based ingredients as a replace of marine ingredients (Aas *et al.*, 2019). In commercial fish feed produced in Norway, several types of feed ingredients are being used, including fish meal, fish oil and plant-based ingredients such as soy protein, wheat gluten and carbohydrates from wheat, pea and tapioca (Aas *et al.*, 2019; Ytrestøyl *et al.*, 2015). Insect meal was approved in 2017 as a feed ingredient in aquaculture feeds by the European Commission (EC, 2017b).

Selenium (Se) is one of the elements naturally present in feed ingredients. Se has a major function as an antioxidant, which prevents cell damage, and is considered an essential mineral for fish (Janz, 2012; Zoidis *et al.*, 2018). In salmon, other essential elements may also be present, such as cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), and zinc (Zn), and also non-essential elements such as the heavy metals cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As). Some of these elements are known to interact with Se, whereas Se has shown to be effective in the prevention of heavy metal poisoning from Hg, Cd and Pb (Ralston *et al.*, 2007; Sørmo *et al.*, 2011; Zoidis *et al.*, 2018). On the other hand, Se is considered nutritional at specific levels, with a narrow range between the level of deficiency and intoxication, both of which may lead to various diseases and mortality (Suzuki, 2005; Thiry *et al.*, 2012). Fish meal is known to naturally a contain higher concentration of Se compared to plant-based ingredients (Ørnsrud *et al.*, 2020; Sanden *et al.*, 2017). Recently, there has also been an increase in research on alternative feed ingredients in salmonid feed (Silva *et al.*, 2020; Biancarosa *et al.*, 2019).

Due to reduced availability of Se from the replacement of fish meal to plant-based ingredients, supplementation of Se in fish feed has been considered necessary to maintain normal physiological processes in fish (Antony Jesu Prabhu *et al.*, 2020; Antony Jesu Prabhu *et al.*, 2014; Antony Jesu Prabhu *et al.*, 2019).

In fish feed, Se can be supplemented in different chemical forms, such as selenite (inorganic Se) and selenomethionine (organic Se) (Pedrero and Madrid, 2009). The different forms of Se are often referred to

as *Se species* (Ochsenkühn-Petropoulou *et al.*, 2016; Templeton *et al.*, 2000). For animal feeds, Se is regulated as a feed additive in the European feed legislation. The maximum limit (ML) established for total Se in animal feeds, including fish feed, is 0.5 mg Se/kg feed (Council Directive 70/524/EC and amendments). The organic species, selenomethionine and selenomethionine produced by the Se-enriched yeast (*Saccharomyces cerevisiae*), are regulated, being limited to supplementation of 0.2 mg/kg feed to ensure consumer safety (EC, 2003; EC, 2017b; EFSA, 2011a; EFSA, 2011b). Due to the legislations that also specify limits for organic Se, there is a need for analytical methods to identify inorganic and organic Se species in fish feed (Berntssen *et al.*, 2018a).

Two analytical methods for the determination of Se species - Se speciation methods, were recently published for the application on fish feed and salmon muscle tissue (Sele *et al.*, 2018a). These methods consist of an enzymatic extraction procedure for the organic Se species followed by a separation and detection using HPLC-ICP-MS. From the Se speciation it was shown that both inorganic Se forms, such as selenate and selenite, and organic forms, such as selenomethionine and selenocysteine, can be found in muscle tissue of farmed Atlantic Salmon and salmon feed (Sele *et al.*, 2018a). It was, however, specified that there were challenges in the extraction recoveries for Se in fish feed (Sele *et al.*, 2018a).

For Se speciation, extractions of organic Se species are usually performed through enzymatic digestions using enzymes or different combinations of enzymes and different extraction solutions, depending on the sample type. Combining two or more enzymes for extraction of Se has frequently been seen in other studies of Se speciation (Zhang and Yang, 2014; Cuderman *et al.*, 2010; Mounicou *et al.*, 2009; Wang *et al.*, 2013; Gao *et al.*, 2018; Oliveira *et al.*, 2016), as well more than one extraction step (Zhang and Yang, 2014; Sele *et al.*, 2018a; Mounicou *et al.*, 2009). Different extraction solutions (buffers) with multiple chemicals have also been evaluated for increased Se extraction efficiencies (Oliveira *et al.*, 2016).

For method development, a normal approach has been to use a one-factor-at-a-time strategy for evaluating the effects of the experiments. However, another strategy could involve the use of experimental design. Experimental design is considered more efficient for evaluating possible interactions between different factors (Silva *et al.*, 2019b; Miller and Miller, 2018; Montgomery, 2017). Experimental design is not often seen in Se speciation, but it has been successfully used for finding optimal conditions in a couple of Se speciation studies (Zhang and Yang, 2014; Gong *et al.*, 2018). Taking into consideration results from previous work performed in Se speciation, different enzymes, combinations of enzymes and extractions solutions can be tested for the development of analytical methods for speciation of organic Se, by using experimental design.

1.2 Aims, objectives and hypotheses of the thesis

The overall aim of this thesis was to use chemometrics and other statistical approaches for handling a large dataset, and for the method optimization for determining Se species in fish feed and feed ingredients. This study includes two parts:

i) To evaluate the correlations between Se and other elements in Atlantic salmon fed with feed supplemented with inorganic or organic Se species, using statistics and chemometrics for a large dataset.

ii) To optimize the extraction procedure for an analytical method determining the organic Se species, selenomethionine (SeMet), in fish feed and feed ingredients using chemometric tools.

Hypotheses

1. Statistical and chemometrics approaches can be applied to assess correlations between Se and other elements in salmon samples.
2. Chemometric approaches can be applied to optimize the extraction recovery for the organic Se species SeMet in fish feed and feed ingredients by testing the following experimental conditions:
   a. using different enzymes, or a combination of enzymes.
   b. using a different type of extraction solution.
3. Quality assurance by evaluating method parameters will provide valuable information about the quality and reliability of the optimized method.

Objectives

The objectives of this study were to:

I) Find potential correlations between Se and other elements in salmon samples by using Pearson's and Spearman's correlation coefficients.

II) Improve the extraction method for Se speciation by optimization using chemometrics, including experimental designs and principal component analysis (PCA). This objective was divided into the following tasks:
   a. To screen for relevant factors using different extraction solutions, enzymes and combinations of enzymes.
   b. To perform an optimization of the method by combining significant factors from the screening process.

III) Determine the selectivity, precision and trueness of the measurements to evaluate the quality of the optimized method.

IV) Apply the optimized method for determining organic Se species in fish feed and feed ingredients.

This master thesis is a part of the project "Method development Se speciation", funded by the Institute of Marine Research.

2 THEORY

2.1 Selenium (Se)

Selenium (Se) is a chemical element with an atomic number of 34 and an atomic mass of 78.961 u (Meija *et al.*, 2016), and is known as a metalloid in group 16 and period 4 in the periodic table. The element was discovered in 1817 by J.J. Berzelius while analyzing an impurity in the production of sulfuric acid. By the resemblance to tellurium, an element named after the Latin word *Tellus* (Earth), Se was named as a reference to the Greek god of the moon, *Selene* (Sonet *et al.*, 2016). Se is known to have similar properties as the neighboring elements in the same period, sulfur and tellurium, and is isomorphous with sulfur with the same oxidation states (-2, 0, +2, +4 and +6). The element is abundant as five stable isotopes: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se and ^{80}Se, and the weakly unstable isotope ^{82}Se (Sonet *et al.*, 2016). The most abundant isotopes are ^{80}Se (49.61%) and ^{78}Se (23.77%) (Pröfrock, 2016).

Se is distributed in the environment through processes such as weathering of rocks and soils, volcanic activity, wildfires and volatilization from water bodies and plants. Due to this, Se also occurs naturally in sea- and freshwater, with a natural background concentration from 0.01 to 0.1 µg/L (Janz, 2012). The background concentration of Se can vary greatly among geographical regions and can be as high as 5-50 µg/L in exposed water environments (Janz, 2012). The large variation of Se concentrations worldwide affects the levels of Se in aquatic organisms, such as fish (Janz, 2012).

2.2 The biological role of Se

Since the 1950s, Se has been recognized as essential to humans and animals (Sonet *et al.*, 2016). The concentration range of Se that is essential to humans and animals is considered very narrow, with a recommended intake of Se at 55 µg/day (Zoidis *et al.*, 2018; Hariharan and Dharmaraj, 2020) and an upper level intake of 400 µg/day for humans (Yusà and Pardo, 2015). Se deficiency symptoms can be apparent at intakes less than 12 µg/day, whereas uptake of more than 500 µg/day can be highly toxic (Alonso *et al.*, 2015).

Se at nutritional levels is preventative against various cancer diseases and has been linked to reduced effects of HIV in infected patients, as well as preventing neurodegenerative disorders, such as Alzheimer (Sonet *et al.*, 2016). It may also reduce the toxic effect of mercury and other heavy metals, which has been shown in rats, chicken and fish (Ralston *et al.*, 2007; Sørmo *et al.*, 2011; Zoidis *et al.*, 2018). On the other hand, excessive doses of Se has been linked to increased risks of cancer and heart diseases in humans (Poljšak and Fink, 2014).

Deficiency problems are associated with muscular, thyroid, immune, cardiovascular and neurological disorders in humans and animals (Sonet *et al.*, 2016; Hosnedlova *et al.*, 2017). In calves, lambs, foals and children, Se deficiency can be apparent as white muscular disease or nutritional muscular dystrophy, and as yellow fat disease in foals (Hosnedlova *et al.*, 2017).

2.2.1 Biological function of Se in Atlantic salmon

Fish tissues contain a high level of polyunsaturated fatty acids, which are essential in cell membranes (Martínez-Álvarez *et al.*, 2005). Due to this, fatty fish such as Atlantic salmon (*Salmo salar*) and other salmonids are prone to oxidative stress. A relatively high level of antioxidants from dietary Se is therefore necessary for salmon to prevent deficiency problems (Antony Jesu Prabhu *et al.*, 2020). This applies especially to farmed fish that are fed with diets of high plant content (Aas *et al.*, 2019).

The chemical form of Se must be considered when supplementing, since there are differences in the bioavailability of organic and inorganic Se species. It has been shown that organic Se sources (SeMet) have higher bioavailability than inorganic Se sources (selenite) (Ørnsrud and Lorentzen, 2002; Wang and Lovell, 1997). Also, inorganic Se species (e.g. selenate and selenite) are considered more toxic for salmon than the organic Se species (e.g. SeMet and SeCys) (Berntssen *et al.*, 2017; Thiry *et al.*, 2012). It has been shown that toxic levels of dietary Se from the selenite has led to mortality in salmon at nominal Se concentrations of 25 and 30 mg/kg diet, whereas no mortality occurred in salmon fed with similar levels of the SeMet. This shows that salmon has a higher tolerance to the organic Se species than inorganic Se species (Berntssen *et al.*, 2018b). On the other hand, liver pathology and kidney dysfunction were seen in salmon fed SeMet supplemented diets at ≥21 mg Se/kg diet, and increased liver oxidative stress and liver damage were observed in salmon fed with diets supplemented with selenite at 5.4-11 mg Se/kg diet (Berntssen *et al.*, 2018b). Muscular dystrophy has also been observed in fish due to vitamin E and Se deficiency (Combs and Combs, 1986).

At nutritional levels, dietary Se is required to maintain a stable internal environment of the body (body Se homeostasis) and improved health status of Atlantic salmon (Antony Jesu Prabhu *et al.*, 2020). In recent studies, it has been found that the minimal level of dietary Se for post-smolt salmon is 0.27 mg/kg, with a required level to maintain body Se homeostasis at 0.65 kg/mg diet, which is above the existing legal limit of 0.5 mg Se/kg (Antony Jesu Prabhu *et al.*, 2020).

2.2.2 Essential and non-essential elements in Atlantic salmon

In addition to Se, fish contain other elements, such as cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), manganese (Mn) and zinc (Zn), also considered essential elements. Similar as for Se, too low or too high intake of i.e. Cr, Cu, Fe and Zn can cause deficiency or intoxication, respectively. Fish can contain high levels of heavy metals such as cadmium (Cd), mercury (Hg), lead (Pb) and arsenic (As) (Marcovecchio *et al.*, 2015). These elements are considered to be non-essential elements and are associated with severe negative effects (Marcovecchio *et al.*, 2015).

The levels of elements can vary between different organs from the fish bodies. For assessing the element contents in the edible tissue, fillet can be extracted from the fish for analysis. For determining the health and quality of the fish, whole-bodies and organs such as the liver and gill can be analyzed to assess the element contents (Marcovecchio *et al.*, 2015). According to the online database for nutrients and contaminants in seafood from "Seafood data" from Institute of Marine Research (2020c) (data from 2006 to 2009), it is seen that the element concentrations in the fillet of farmed Atlantic salmon are ranging from 0.55 to 1.6 mg/kg (N = 14) for As, from 2.3 to 3.2 mg /kg (N = 14) for Fe, from 3.4 to 4.2 mg/kg (N = 14) for Zn, from 0.14 to 0.33 mg/kg (N = 14) for Hg, whereas the concentrations for Cr (N = 4), Cd (N = 14) and Pb (N = 14) are below LOQ.

In previous studies, metal interactions have been evaluated for any beneficial or harmful effects (Antony Jesu Prabhu *et al.*, 2019; Berntssen *et al.*, 2000; Fontagné-Dicharry *et al.*, 2015; Hilton, 1989; Lorentzen *et al.*, 1998; Silva *et al.*, 2019a). Studies on Se and Hg in mammals, and fish consumed by humans, have shown that there is a protective effect of Se on the toxic Hg species methylmercury (Burger *et al.*, 2013). It has also been proposed that Hg has a protective role against the toxic effects of Se (Vukšić *et al.*, 2018; Burger *et al.*, 2013; Sørmo *et al.*, 2011). In a recent study, it was shown that the interactions between Zn, Se and Mn additive sources significantly affect the availability of these elements in Atlantic salmon diets (Silva *et al.*, 2019a).

2.3 Se species

Se naturally occurs in the marine and terrestrial environment. It is rarely found in its pure elemental form (Se^0) in nature. The element can be present in different chemical forms, *Se species. Chemical species* are defined by the International Union of Pure and Applied Chemistry (IUPAC) as: "Chemical elements: specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecular structure" (Templeton *et al.*, 2000).

In natural samples several species of Se occur, but mainly salts, such as sodium selenite (SeO_3^{2-}) and sodium selenate (SeO_4^{2-}) (inorganic Se), and organic Se species, such as selenomethionine (SeMet) and selenocysteine (SeCys) are observed (Sonet *et al.*, 2016). There are also other organic Se species, selenocystine ($SeCys_2$), γ-glutamyl-selenomethylselenocysteine (GGMSC) and selenomethylselenocysteine (SeMetSeCys), present in plants (Sentkowska, 2019) and selenoneine present in fish (Alonso *et al.*, 2015). Figure 1 shows an overview of the most common Se species in living organisms.

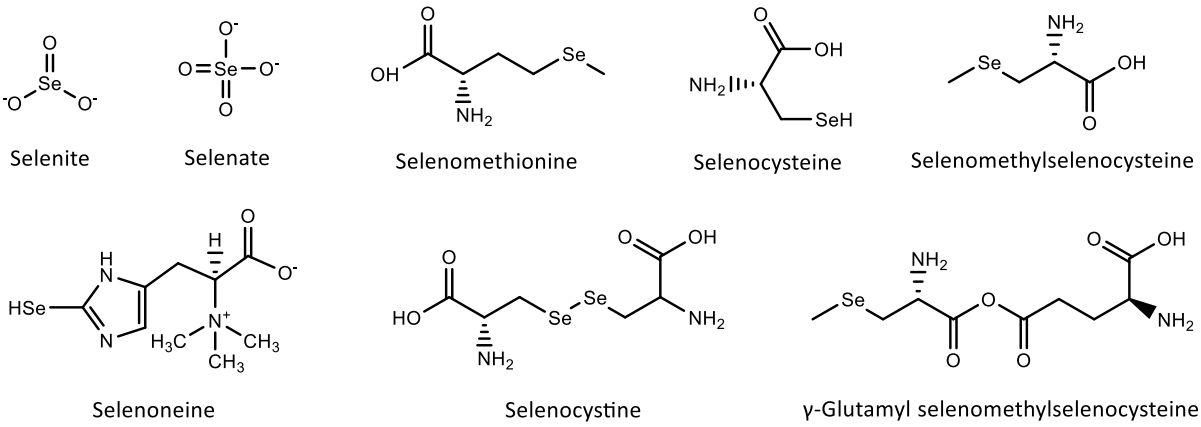


Figure 1: Selected Se species reported in living organisms.

The bioavailability and toxicity of the element are affected by the chemical forms present (Thiry *et al.*, 2012). Inorganic Se is considered as the most toxic Se species (Alonso *et al.*, 2015). Compared to inorganic Se species, organic Se species, such as SeMet, are often considered more apparent in terms of bioavailability (Ruiz-de-Cenzano *et al.*, 2015; Thiry *et al.*, 2012). The most common organic Se in nutritional sources are the amino acids SeCys and SeMet (Suzuki, 2005). Se and is known to be an important element in a number of enzymes known as selenoproteins, which are crucial for biological functions (Álvarez-Pérez *et al.*, 2018; Sonet *et al.*, 2016; Thiry *et al.*, 2012). The organic Se species, SeMet and SeCys, are known to form Se-containing proteins, where SeCys forms selenoproteins which are crucial for biological functions (Suzuki, 2005; Álvarez-Pérez *et al.*, 2018). SeMet, on the other hand, forms unspecific-Se-containing proteins that are regarded not as selenoproteins (Kurokawa and Berry, 2013; Godin *et al.*, 2015).

2.3.1 The protective role of Se

Selenoproteins are known to have antioxidant effects against reactive oxygen species (ROS), which includes free radicals and compounds that can generate free radicals, such as hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH) (Gilbert and Colton, 2002; Amit and Priyadarsini, 2011). In animals, ROS are

produced during normal processes in the body (Phaniendra *et al.*, 2014; Gilbert and Colton, 2002). Excess production of ROS is often associated with oxidative stress, which causes damage to DNA, proteins and lipids (Zoidis *et al.*, 2018; Arteel and Sies, 2001). Other species that are similar to ROS are reactive nitrogen species (RNS), which includes compounds like nitride oxide radical (NO or NO•) and nitrite (NO_2^-) (Krumova and Cosa, 2016; Phaniendra *et al.*, 2014). Antioxidants inhibit oxidation from the free radicals and can therefore be used in specific doses to prevent the negative effects, by balancing the levels of ROS/RNS produced (Phaniendra *et al.*, 2014). Selenoproteins, such as glutathione peroxidases (GPX), iodothyronine deiodinases, thioredoxin reductases and selenoprotein P, are characterized as enzymatic antioxidants that prevent the formation of free radicals by scavenging ROS (Zoidis *et al.*, 2018; Arteel and Sies, 2001). The impact of increased production of selenoproteins on the balance between the ROS/RNS levels and antioxidants from supplementation of Se is illustrated in Figure 2.

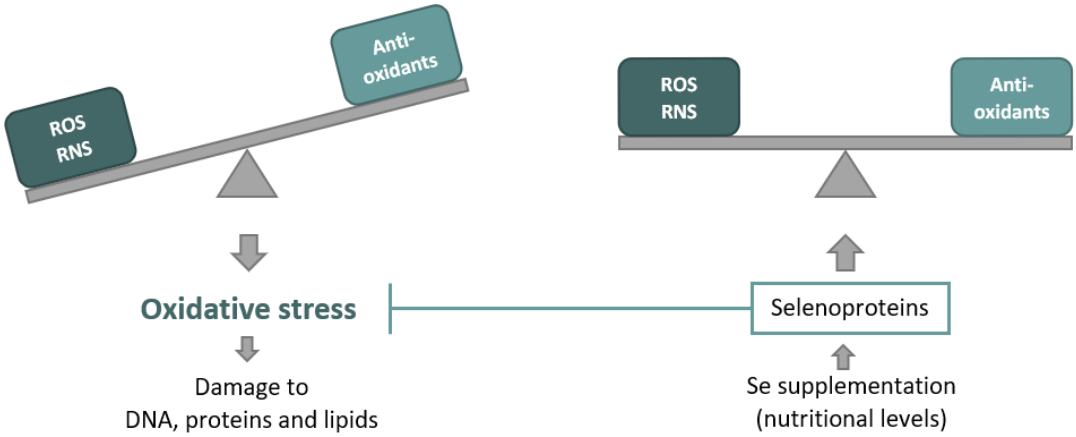


Figure 2: The protective role of selenoproteins against oxidative stress when levels of ROS/RNS produced in the body and level of antioxidants are balanced, modified from Kang *et al.* (2020).

2.3.2 Se metabolism

The metabolism of Se in humans and animals include processes of absorption, transportation, transformation and excretion (Kang *et al.*, 2020). The metabolism of Se in fish is not well established (Janz, 2012; Pacitti *et al.*, 2016) but is assumed to be similar to animals at high Se levels (Mechlaoui *et al.*, 2019). Selenide (H_2Se) plays a major role in the production of selenoproteins (Ogra and Anan, 2009). There are different metabolic pathways to selenoproteins in animals, presented briefly in Figure 3.

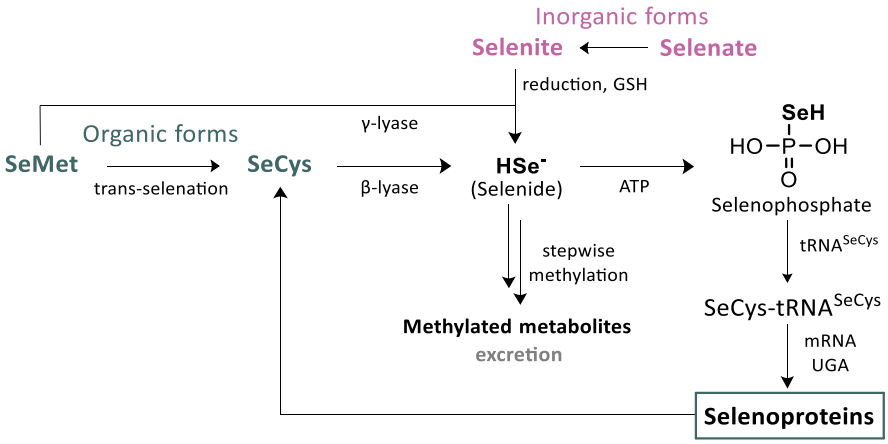


Figure 3: Metabolic pathway for Se, adapted from Suzuki (2005).

In the pathway to H_2Se, SeMet can be transformed to SeCys by trans-selenation and will further be transformed to H_2Se by cleavage of C-Se bonds through β-lyase or γ-lyase. Some organic Se species will be oxidized to selenite or selenate, which can also be reduced to H_2Se by glutathione (GSH) (Suzuki, 2005; Ogra and Anan, 2009). The H_2Se is then activated as selenophosphate through ATP, which carries methylated SeCys to the messenger-RNA (mRNA). From there on, the methylated SeCys is incorporated into a protein by a stop codon (UGA) as SeCys residue, producing a selenoprotein (Ogra and Anan, 2009). The excess of H_2Se will be methylated stepwise into the metabolites, which are excreted through urine and exhalation (Suzuki, 2005).

2.4 Se levels and Se species in food

For most human populations, bread, cereals, seafood and meat are the major food sources of Se (Tinggi, 2008; Zand *et al.*, 2015; Hariharan and Dharmaraj, 2020). The Se concentrations in bread, cereals (e.g. barley, rice, rye and wheat flour) and Se enriched cereals can range from 0.009 mg/kg to 0.1 mg/kg (Stadlober *et al.*, 2001; Cubadda *et al.*, 2010). In meat, including beef, pork, lamb and chicken, Se concentrations can range from 0.038 to 0.656 mg/kg (Barclay *et al.*, 1995; Reykdal *et al.*, 2011; Lombardi-Boccia *et al.*, 2005; Holland *et al.*, 1991; Murphy and Cashman, 2001).

For seafood, generally high mean concentrations of Se are seen: 0.32-1.57 mg/kg in pelagic fish from Central North Pacific, 0.293-0.881mg/kg in seafood from Thailand and 0.173-0.678 mg/kg in fish from Italy (Kaneko and Ralston, 2007; Schaeffer *et al.*, 2005; Sirichakwal *et al.*, 2005; Alegría-Torán *et al.*, 2015). In Norway, relatively high concentrations are also reported in seafood (Table 1). In wild marine organisms, Se concentrations have been seen to range from 0.37 to 0.80 mg/kg in molluscs, including blue mussels (*Mytilus edulis*) and great scallop (*Pecten maximus*) and from 0.23 to 1.2 mg/kg in crustaceans, e.g. brown

crab (*Cancer pagurus*) and Norway lobster (*Nephrops norvegicus*). In farmed molluscs, the Se concentrations can vary from 0.25 to 0.67 mg/kg. These data show that the Se levels in farmed marine organisms are lower than in wild organisms (Table 1). This also applies for fish, with Se concentrations that range from 0.23 to 0.62 mg/kg in wild fish (fillet), and from 0.12 to 0.44 mg/kg in farmed fish (fillet). For Atlantic Salmon, Se concentration at 0.43 mg/kg has been reported in wild salmon, while Se concentrations from 0.12 to 0.25 mg/kg have been reported in farmed salmon.

Table 1: Se concentrations (mg/kg ww) in seafood, retrieved from the online database for nutrients and contaminants in seafood (data from 2006 to 2019, "Seafood data", (Institute of Marine Research, 2020c), accessed 14.02.2021).

Category	Type (wild)	Se (mg/kg)	Type (farmed)	Se (mg/kg)
Fish (fillet)	Atlantic salmon	0.43 (N=1)	Atlantic salmon	0.12-0.25 (N=14)
	Atlantic cod	0.23-0.29 (N=14)	Atlantic cod	0.14-0.26 (N=10)
	Atlantic halibut	0.39-0.51 (N=9)	Atlantic halibut	0.16-0.44 (N=7)
	Turbot	0.47-0.62 (N=2)	Turbot	0.20-0.30 (N=3)
	Atlantic mackerel	0.41-0.59 (N=12)	Rainbow trout	0.12-0.26 (N=13)
	Atlantic herring	0.43-0.62 (N=7)	Arctic char	0.15-0.32 (N=6)
	Haddock	0.30-0.36 (N=3)		
Molluscs (edible parts)	Blue mussel	0.37-0.80 (N=11)	Blue mussel	0.45-0.76 (N=14)
	Great scallop, with roe	0.32-0.55 (N=14)	Great scallop, with roe	0.25-0.67 (N=11)
Crustaceans (white edible parts)	Brown crab	0.93-1.2 (N=8)		
	Red king crab	0.23-0.33 (N=2)		
	Snow crab	0.70-0.95 (N=2)		
	Lobster	0.54-0.63 (N=2)		
	Norway lobster	0.83-1.1 (N=2)		
	Shrimp (unpeeled)	0.39-0.54 (N=12)		

2.4.1 Se supplementation in fish feed

Some Se species are used as supplements in commercial multivitamins, dietary foods and animal feeds (Quintaes and Diez-Garcia, 2015). Supplementation of Se has been necessary in foods and feeds, particularly to plant-based ingredients, due to insufficient levels of Se in several regions of the world, caused by acid rain and excessive fertilization (Zand *et al.*, 2015). For farmed fish, fish meal has been an important source of Se in commercial feeds (Sørensen, 2011). Today, commercial fish feed is mainly plant-based (Aas *et al.*, 2019) and may be supplemented with Se to maintain normal growth. The Se concentrations in commercial fish feed produced in Norway in recent years are ranging from 0.3 to 2.3 mg/kg (Table 2).

Table 2: Se mean concentration (mg/kg) and the Se concentration range (min-max, mg/kg) in commercial fish feed produced in Norway reported through the Norwegian surveillance program for fish feed.

Year	Se concentration, mean (mg/kg)	Se concentration, min-max (mg/kg)	Reference
2019	0.6 (n=93)	0.3-1.5 (n=93)	(Ørnsrud *et al.*, 2020)
2018	0.7 (n=76)	0.24-2.3 (n=76)	(Sele *et al.*, 2019)
2017	0.8 (n=40)	0.3-1.8 (n=40)	(Sele *et al.*, 2018b)

To protect the consumer, farmed animal and the environment, the European Commission have established maximum limits (MLs) for undesirables (i.e. Hg, As, Pb and Cd), and maximum content for feed additives in animal feeds (EC, 2020). For Se supplementation, the current limit for maximum content is set to 0.5 mg Se/kg for animals. The approved feed additives of Se are the organic Se sources, hydroxy-analogue of SeMet, L-SeMet, DL-SeMet and SeMet produced from Se-enriched yeast (*Saccharomyces cerevisiae)* and the inorganic Se source, sodium selenite (EC, 2003; EC, 2015; EC, 2017a). For SeMet and SeMet produced from Se-enriched yeast (*Saccharomyces cerevisiae*), a maximum content has been set to 0.2 mg Se/kg for animals (EC, 2003; EC, 2017b; EFSA, 2011a; EFSA, 2011b). The limit for Se-enriched yeast was set from evaluations made by the European Food Safety Authority (EFSA), an agency that provides independent scientific advice that forms policies and legislations made by the European Commission. The advice they give is based on risk assessment on food and feed safety, nutrition, animal health and welfare, plant protection and plant health. For regulation, the analytical methods for determination of Se are well-established, but since the legislations specify limits for organic Se in animal feed, there is also a need for analytical methods for determination of organic Se and inorganic Se species in fish feed (Berntssen *et al.*, 2018a; Sele *et al.*, 2018a).

2.5 Analytical procedures for Se speciation

2.5.1 Se speciation

Through Se speciation, information about the species of inorganic or organic Se can be provided (Fairweather-Tait *et al.*, 2010). The term *speciation analysis* has been defined by the International Union of Pure and Applied Chemistry (IUPAC) as *analytical activities of identifying and/or measuring the quantities or one of more individual chemical species in a sample* (Templeton et al., 2000). Three steps are generally involved in speciation analysis: i) extraction of species, ii) separation of species and iii) detection and quantification (Wrobel and Wrobel, 2015). The procedures for speciation can be based on different separation types; i) non-chromatographic, ii) liquid chromatography or electrophoresis and iii) gas chromatography. Various analytical methods are established for speciation analysis for Se, but the most used separation method is liquid chromatography (LC) (Pyrzynska and Sentkowska, 2019).

2.5.1.1 Extraction of procedures

For speciation analysis, the extraction procedure is an important step, with an aim to achieve quantitative recovery of elemental species without changing the chemical identities (Ochsenkühn-Petropoulou *et al.*, 2016). The extraction of species has been performed using various chemical solvents (e.g. nitric acid (HNO_3), HNO_3/methanol, methanol/water, water, trifluoracetic acid, tetramethylammonium hydroxide and methanesulfonic acid) and enzymes (e.g. pronase E, pepsin, pancreatin and trypsin) (Alonso *et al.*, 2015). The solvents and enzymes have also been assisted with microwave, ultrasound or accelerated solvent extraction (Alonso *et al.*, 2015; Wrobel and Wrobel, 2015). For speciation of selenoproteins or Se-containing proteins, enzymatic digestion is considered an appropriate method since they can break specific bonds and thereby selectively release analytes, such as peptides and amino acids, from the sample matrix (Alonso *et al.*, 2015). The choice of enzyme must be carefully evaluated to match the sample type and analytes of interest. Some enzymes can also be combined to achieve certain cleavages in the proteins (Alonso *et al.*, 2015).

The enzymes that have been frequently applied for Se speciation in recent years include protease (Cubadda *et al.*, 2010; Cuderman *et al.*, 2010; Duncan *et al.*, 2017; Gao *et al.*, 2018; Hsieh and Jiang, 2013; Mellano *et al.*, 2013; Oliveira *et al.*, 2016; Sele *et al.*, 2018a; Siwek *et al.*, 2005; Vu *et al.*, 2018), pepsin (Wang *et al.*, 2013), pancreatin (Oliveira *et al.*, 2016; Wang *et al.*, 2013) and trypsin (Siwek *et al.*, 2005; Zhang and Yang, 2014). Other types of enzymes used for determining organic Se in marine and plant-based samples are papain and flavourzyme (Zhang and Yang, 2014), and lipase (Cuderman *et al.*, 2010; Gao *et al.*, 2018; Mellano *et al.*, 2013; Sele *et al.*, 2018a). The proteolytic protease XIV has also been used in combinations with other enzymes in many of these studies (Cubadda *et al.*, 2010; Cuderman *et al.*, 2010; Gao *et al.*, 2018; Mellano *et al.*, 2013; Oliveira *et al.*, 2016; Sele *et al.*, 2018a).

2.5.2 Chromatography

Chromatography is an analytical technique used to separate compounds in mixtures and is usually applied for quantitative analysis (Miller, 2005). Chromatographic separation is performed by a partition of the compounds between two phases, where one phase (the mobile phase) moves relative to the other (the stationary phase) (Figure 4). Separation takes place inside the chromatographic column, which contains both the stationary and the mobile phase. The compound mixture is injected into the mobile phase at time t_0 (Figure 4a), and the compounds in the mixture will be distributed between the two phases with distribution ratios depending on the properties of the analyte molecules and the properties of the two phases. The molecules are in dynamic equilibrium, which means there is a continuous exchange of molecules between the phases. In such a system, the molecules will move with a velocity that is equal to

the fraction of the analyte in the mobile phase multiplied by the mobile phase velocity (Harris, 2010). In Figure 4 it is schematically shown how some analytes move faster (solute A, Figure 4.b) than the other analytes (solute B). This is explained by a larger fraction of the solute A being in the mobile phase, which means they have lower retention by the stationary phase. In analytical column chromatography, there is a detector at the end of the column that (ideally) gives a signal that is proportional to the amounts (mass or concentration) of the analytes. The time the analytes elutes from the column is called the retention time, t_R. The chromatogram is a plot of the detector signal versus the time from the injection (t_0). The signals from each analyte are typically referred to as chromatographic peaks (Harris, 2010).

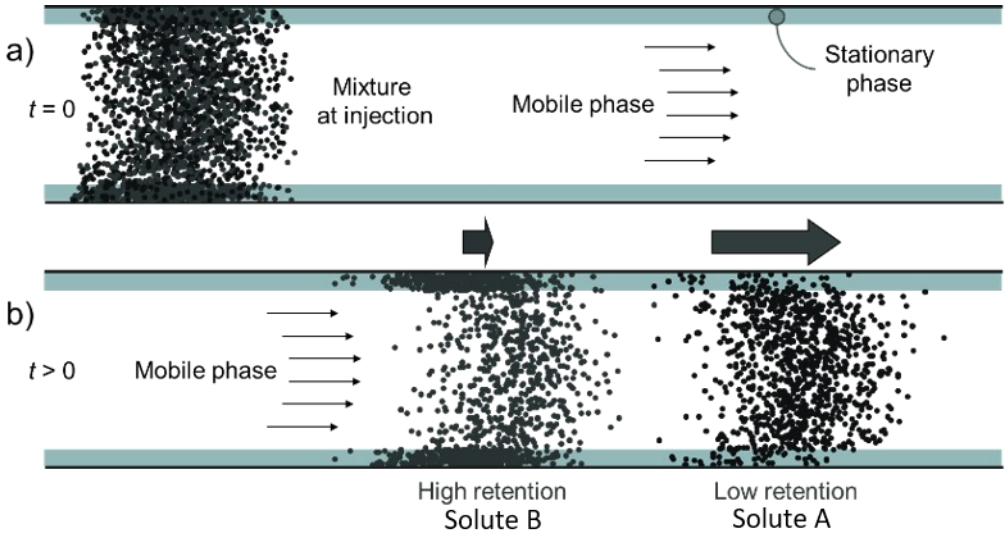


Figure 4: Elution of two solutes of different retentions. Solute A and B are a) not separated at t = 0, and are b) separated at t > 0. Adapted from lecture notes by Svein A. Mjøs, University of Bergen.

The chromatographic retention of a compound is measured by the retention factor, *k* (equation (1)):

$$k = \frac{n_s}{n_m} = \frac{t_R - t_m}{t_m} \tag{1}$$

where n_s and n_m are the number of molecules (at equilibrium) in the stationary and mobile phases respectively, and t_m is the time the mobile phase uses through the column.

The difference in retention factors between two analytes are critical for their separation and is referred to as chromatographic selectivity. This is expressed by the separation factor, α (equation (2)):

$$\alpha = \frac{\mathrm{k}_B}{\mathrm{k}_A} \tag{2}$$

where k_A and k_B are the retention factors for the first and last eluting peaks, respectively.

The chromatographic techniques are roughly divided into gas chromatography (GC) and liquid chromatography (LC). Gas is used as the mobile phase in GC, while a liquid solution is the mobile phase in LC (Miller, 2005). In LC, the chromatographic selectivity is typically achieved from differences in polarity (normal phase and reversed-phase LC), ionization (ion-exchange chromatography), size (size exclusion chromatography) or by macromolecular interactions dependent on shape as well as properties of functional groups (affinity chromatography). There are also other modes of separation, and many are a mixture of several types of interactions (Miller, 2005).

2.5.3 Chromatographic separation of Se species

Generally, separation of Se species can be performed with LC methods including reversed-phase (RP), RP ion-pairing, anion-exchange, cation-exchange or by size-exclusion (SEC) coupled to Inductive Coupled Plasma Mass Spectrometry (ICP-MS) (Alonso *et al.*, 2015). The SEC with Tris-HCl buffer as mobile phase is mainly used for separation of high-molecular-mass proteins with selenoamino acids (Pyrzynska and Sentkowska, 2019). Some of the separation methods can also be combined to perform sequential separation for a mapping of fractions or selenopeptides, e.g. by using capillary or nano-High Performance Liquid Chromatography (HPLC) coupled to ICP-MS or by combining SEC with anion-exchange or RP chromatography (Alonso *et al.*, 2015).

In RP-LC, separation is performed by polarity, where the mobile phase will contain polar solvents with the stationary phase being non-polar. This leads to polar compounds eluting first, while the less polar compounds will be more retained and the be the last to elute. In normal phase chromatography, the mobile phase will instead contain non-polar solvents and the stationary phase will be polar, where the more polar compounds will be more retained. For analytes with acidic or basic functional groups, the pH of water-based mobile phases in RP-LC will have a high influence on the retention factors. It is therefore often critical that the mobile phases are properly buffered. In this work, the Se species have been separated by polarity, by an RP-HPLC system. Mobile phases commonly used in RP-LC are mixtures of water with methanol or acetonitrile, where hydrophilic selenoamino acids will not be retained (Pyrzynska and Sentkowska, 2019). In other Se speciation studies with RP-HPLC, columns of C8 or C18 hydrophobic alkyl chains have been used as the stationary phase (Goenaga Infante *et al.*, 2009; Bierla *et al.*, 2018).

2.5.4 Mass spectrometry (MS)

Mass spectrometers (MS) are often used as detectors coupled with LC and GC, providing quantitative and qualitative data. Mass spectrometry is a type of analyzer that provide information about the elemental composition or the structure of an analyte (Becker, 2009). There are numerous types of mass

spectrometers, all with the principle that the velocity and direction of ions can be controlled by electric or magnetic fields (Becker, 2009). When coupled to chromatographic instrumentation, the separated analytes are introduced to the mass spectrometer, where the analytes are ionized, separated by mass-to-charge ratio and detected (Figure 5).

Figure 5: A basic set-up for mass spectrometry with an ion source for ionizing materials, an ion separator that separates the ions by their mass-to-charge (m/z) ratios and an ion detector that detects the ions (Becker, 2009).

A mass spectrometer is equipped with an ion source that is often operated at low pressure or near vacuum. Examples of ion sources are electron impact source (EI), electrospray ionization (ESI), laser ion source (LIMS), secondary ion source (SIMS) and inductively coupled plasma ion source (ICP). Materials introduced to the ion source will be ionized to mostly positively charged ions, but negative ionization can also be applied (Wilschefski and Baxter, 2019). The ions will then be separated in a mass analyzer, such as a quadrupole mass filter (Becker, 2009).

The quadrupole consists of metal electrode rods placed in a square array (Wilschefski and Baxter, 2019). The rods are charged with voltages resulting in an electric field. When ions are introduced to the quadrupole, they are accelerated in an oscillating motion between the rods (Becker, 2009). Ions with unstable oscillation will collide with the rods, which means that only ions with a certain mass-to-charge (m/z) ratios will reach the detector (de Hoffmann et al., 2007). Signals from the detector will then be picked up by a computer connected to the system, which also is used to operate the chromatograph and mass spectrometer.

2.5.4.1 Inductive Coupled Plasma Mass Spectrometry (ICP-MS)

Inductive coupled plasma mass spectrometry (ICP-MS) is a type of mass spectrometer that is used for the measurement of elements at trace levels (trace elements). It is primarily designed to analyze liquids. When analyzing solid materials, the sample needs to be dissolved by chemical digestion before introduced to the ICP-MS (Wilschefski and Baxter, 2019).

The ICP-MS includes many parts, including a nebulizer, spray chamber, a torch for ionization by plasma from argon gas, a mass analyzer and a detector. The nebulizer introduces the sample to the ICP as a liquid aerosol. Typically, the sample is delivered to the nebulizer by an autosampler and a peristaltic pump. When introduced, the aerosol enters a spray chamber, where the larger droplets will be removed and the finer

mist of aerosols will be ionized to mainly positive charged ions (Wilschefski and Baxter, 2019). The plasma is formed either by argon or helium gas, and the temperature of the argon plasma is 6000-7000 °C (Alonso *et al.*, 2015). The ions will be transported to the mass analyzer, which is most commonly a quadrupole. Other common types of mass analyzers in ICP-MS includes triple-quadrupoles (tandem mass spectrometry), time-of-flight (TOF) and electric/magnetic sectors (Wilschefski and Baxter, 2019). Electron multiplier is the most common detector in ICP-MS (Wilschefski and Baxter, 2019).

There are many advantages in using ICP-MS, which includes a low detection limit at 0.001-0.01 µmol/L (Wilschefski and Baxter, 2019), low sample volume and simple sample preparation. Nevertheless, interferences and instrumental drift can often occur in ICP-MS and need to be corrected (Wilschefski and Baxter, 2019). To prevent interferences from affecting the accuracy of the analysis, different techniques are involved in the removal or correction of interferences and instrumental drift.

To exclude interferences to analytes, collision or reaction gases can be used based on the type of analyte (Wilschefski and Baxter, 2019). For Se speciation, a gas of hydrogen (H_2) as a reaction gas is used, to avoid interferences on the major isotopes of Se (^{80}Se, ^{78}Se and ^{76}Se) from Ar_2^+ (Alonso *et al.*, 2015). In quantification of Se using ICP-MS, the most prominent polyatomic interferences are from $^{40}Ar^{40}Ar$, $^{40}Ar^{40}Ca$ and $^{79}Br^{1}H$ for the isotope ^{80}Se, and $^{40}Ar^{38}Ar$, $^{40}Ar^{37}ClH$ and $^{38}Ar\ ^{40}Ca$ for the isotope ^{78}Se (Pröfrock, 2016).

For the determination of total element concentrations by ICP-MS, standards of relevant analytes containing known concentrations are analyzed to establish a calibration curve. These types of standards are called multi-element calibration standards in ICP-MS (Becker, 2009). To correct changes in instrument operating conditions and sample-specific matrix effects that may affect analyte signals, internal standards are also commonly used (Wilschefski and Baxter, 2019). The internal standards are selected based on the analyte and sample matrix. One or more elements with similar properties to the analyte, and not present in the sample matrix, are chosen for internal standard. The same concentrations of internal standard is then added to each sample, standard and blank, either online or offline. The measurements are then calculated based on the analyte to standard signal ratio (Becker, 2009; Wilschefski and Baxter, 2019).

The ICP-MS can be coupled with chromatographic instrumentation, such as HPLC or GC. To an HPLC, the end of the HPLC column is connected with a capillary tube to the nebulizer in the ICP-MS (Wilschefski and Baxter, 2019). The HPLC-ICP-MS is the most common set-up used for speciation analysis where the species are detected by the ICP-MS after the chromatographic separation (Pyrzynska and Sentkowska, 2019; Alonso *et al.*, 2015). Figure 6 shows a schematic diagram of the HPLC-ICP-MS system.

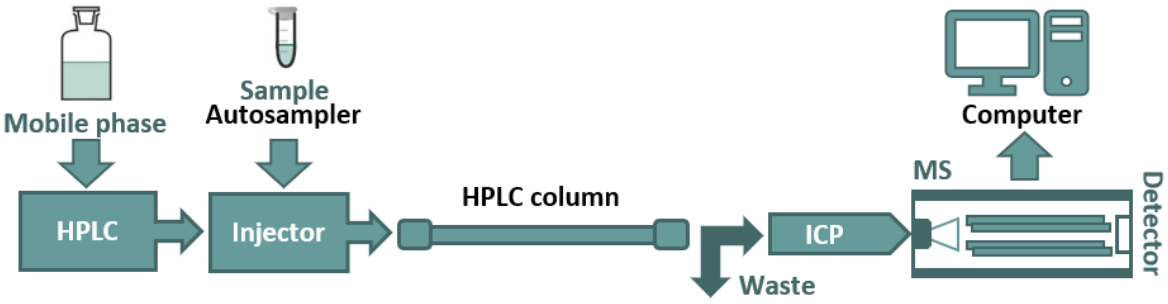


Figure 6: Schematic of HPLC-ICP-MS instrumental setup.

In Se speciation, the identification and quantification of unknown Se-containing compounds can be challenging due to the lack of analytical standards. To overcome this challenge, complementary techniques such as high-resolution mass spectrometry (HR-MS) can be used for identifying the molecular structure of unknown Se compounds (Casiot *et al.*, 1999; Mounicou *et al.*, 2009). Other set-ups for identification of unknown compounds include ESI coupled to tandem mass spectrometer (MS/MS) (Hsieh and Jiang, 2013; Tie *et al.*, 2015), ESI Time-of-Flight (TOF) MS and Orbitrap MS (Shao *et al.*, 2014), and can be coupled to HPLC or to HPLC parallel with ICP-MS (Dernovics and Lobinski, 2008; Goenaga Infante *et al.*, 2009). For the determination of SeCys, a derivatization step is required to stabilize the SeCys prior to analysis (Pedrero and Madrid, 2009; Godin *et al.*, 2015).

2.6 Statistics and chemometrics

2.6.1 Multivariate experimental design

Experimental design and optimization is a concept used for systematic evaluation of problems related to research, development and production, by the execution of informative experiments (Eriksson *et al.*, 1998; The National Academies of Sciences - Engineering - Medicine, 1995; Thelin *et al.*, 1996). To solve relevant problems, a design strategy using multivariate experimental design can be made to find the most optimal conditions for observed variables called *responses*. The responses are dependent on the changes in multiple independent variables, also called factors (x). The observed response can be described as a function, *y = f(x)* (Thelin *et al.*, 1996).

With two variables (x_1 and x_2) and three variables (x_1, x_2 and x_3), the response (y) can be described respectively as the general models, equation (3) and (4):

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_{12} \quad (3)$$

and

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{12}x_{12} + b_{13}x_{13} + b_{23}x_{23} + b_{123}x_{123}. \quad (4)$$

In multivariate experimental design, the goal is to determine the regression coefficients, *b*, by using the relation between the observed responses *y* and the variables *X* (x_1, x_2, …, x_n) from general models such as equation (4). The amplitude of the regression coefficients is then used to evaluate the importance of each individual factor (x) or factor interaction to the response (y). With eight regression coefficients, at least eight different experiments are involved with a minimum of two levels of each factor in an experimental design. The simplest experimental design fulfilling these requirements is called a *factorial design*.

A full factorial design studies the responses of every combination of factors and factor levels in an attempt to find the best combination. A full factorial design with two levels and *k* factors can be written as 2^k. The simplest case for a full factorial design with two levels will be with two factors (2^2), which will have four experiments ($2^2 = 4$). A 2^3 full factorial design will thus have eight experiments. The levels of the factors are often coded as "-" for low level and "+" for high level (Lundstedt *et al.*, 1998). Figure 7 shows examples of a full factorial design of 2^3 for three factors and 2^2 for two factors with two levels in "standard order". The 2^3 full factorial design is visualized with a cube diagram representing the three factors. The "standard order" is a non-randomized run order, starting with experiment 1 at "low" level for all three factors (NIST/SEMATECH, 2012).

Experiment	Factors		
	x_1	x_2	x_3
1	-	-	-
2	+	-	-
3	-	+	-
4	+	+	-
5	-	-	+
6	+	-	+
7	-	+	+
8	+	+	+

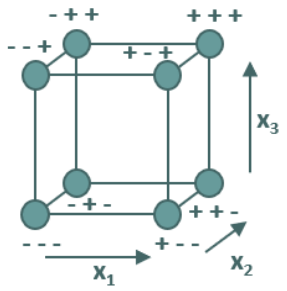


Figure 7: Examples of 2^3 full factorial design and 2^2 full factorial design with four experiments in standard order and (shaded cells), and a cube diagram representing the eight experiments in 2^3 full factorial design modified from Lundstedt *et al.* (1998). The levels of the factors are coded as "-" for low level and "+" for high level.

A full factorial design is a balanced design, which means that all factors have the same number of high and low levels and that the sum of each factor column is zero (in coded values). This property minimizes confounding effects, which means that regression coefficients are proportional to the effects of the different factors. Full experimental designs can be applied to look at the main effects of the factors and factor interactions, and the product of the factors in every combination can be evaluated. The experiments

of the factor interactions will have level signs calculated by multiplication of the relevant factors (Lundstedt et al., 1998).

Another example of an experimental design is the reduced factorial design. This type of design has an advantage by covering as much as possible of a full factorial design without performing all experiments. This can be done when third or higher-order interactions are negligible and can be excluded from the multivariate model (Lundstedt *et al.*, 1998; Jamshidnezhad, 2015). An example is a reduced factorial design with three factors and two levels, a 2^{3-1} reduced factorial design with four experiments. The 2^{3-1} reduced factorial design will include factors x_1 and x_2 of the 2^2 full factorial design (Figure 7) as the first and second factor, and a third factor will be equivalent to x_1x_2 interaction, the product of factor x_1 and x_2 (Lundstedt *et al.*, 1998).

As part of an experimental procedure, full or reduced factorial designs can be applied for *screening* procedures, performed to evaluate the influence of the factors and possible factor interactions on the responses. The most relevant factors are then selected for further studies for optimization (Lundstedt *et al.*, 1998).

Other types of experimental designs can give three or more levels for each factor, such as composite designs and Doehlert designs. These designs are response surface designs and can be used to achieve more complex models, with squared terms of the main effects, to determine the exact optimum for a response. To estimate the reliability of the multivariate models, residuals (unexplained variance), can be calculated from additional replicated experiments in the experimental design, or replicates of experimental points called center points with factors set at their mid values (Lundstedt *et al.*, 1998). The variables that are involved in an experimental design are described in Table 3.

Table 3: Variables involved in an experimental design.

Type of viable	Description
Continuous (quantitative)	Continuous numeric factors involving any value within a range (e.g. temperature).
Discrete (quantitative)	Discrete numeric factors involving certain values within a range (e.g. the floors of a building or the sample number in an analytical sequence).
Categorical (qualitative)	Non-numerical property that cannot be described by a scale with no natural order between the categories (e.g. type of enzyme).
Binary	Factor with only two values. Usually used to describe a presence or absence of factors.

When including factors of the categorical or binary type in experimental designs, having more than two levels and center points is typically not an option. Replicates of experiments in the original design must

therefore be used for estimation of residuals. Multivariable data obtained from experiments performed using experimental design can be visualized through principal component analysis (PCA).

2.6.2 Principal Component Analysis (PCA)

Principal component analysis (PCA) is a multivariable statistical method that uses orthogonal transformations to reveal structures in the data in a complex dataset (Shlens, 2014). The data are compressed into latent variables called principal components (PC) by linear combinations of data with maximum variance. The items in the linear combinations are expressed as vectors called *scores* and *loadings* (Isaksson and Næs, 1996). The PCs will provide an easier way of interpreting the data while minimizing information loss (Jolliffe and Cadima, 2016). In PCA, projections onto latent variables make a two-dimensional plot when working with a dataset of two factors (x, y). When working with three factors, a three-dimensional PCA plot can be made. Figure 8 shows an illustrated PCA transformation of six objects with two factors, by plotted objects on the x-y plane onto two principal components, making a *score plot*.

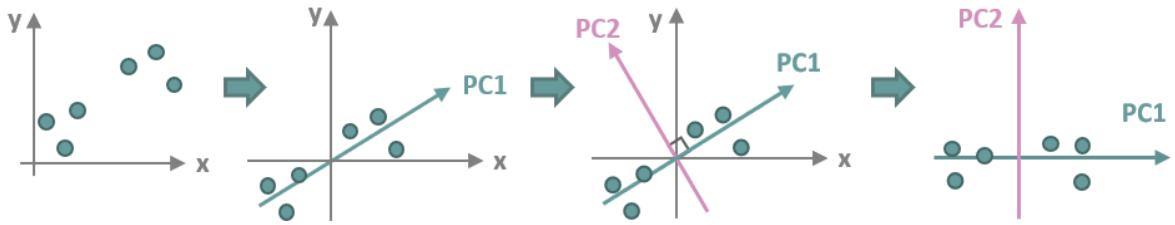


Figure 8: Transformation of a dataset with six objects from the x-y plane to the PC1-PC2 plane, modified from Eriksson (2020).

The objects are first centered by the average measurement of each factor in a PCA. A line will then be fitted to the centered objects by minimizing the distances of the objects to the line (minimize the sum of squared errors). This also means that the line will be fitted where the distance between the projection points are maximized (maximizing variance) according to Pythagoras theorem (see Figure 9). The projected points are placed on the line orthogonally to the line from the objects. The fitted line will be the first principal component (PC1), while the second principal component (PC2) will be orthogonal to PC1.

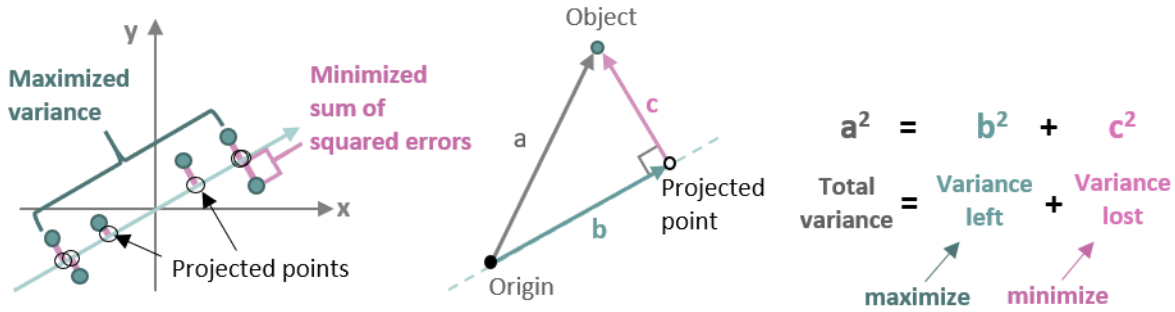


Figure 9: Latent variable projection based on Pythagoras theorem, modified from Williams (2016).

In addition to score vectors, factors can be interpreted by loading vectors in a *loading plot*. Score vectors describe the relationship between the PCs and objects in the data matrix, and loadings vectors describe the relations between the PCs and the original factors. Score plots and loading plots can be interpreted to find relations, similarities, differences and groupings between objects, factors and responses, and can also be combined in a biplot (Isaksson and Næs, 1996). In biplots, correlations can found by studying the angles between the vectors of the factors and objects (Martens, 2001).

2.7 Quality assurance (QA) and quality control (QC)

To minimize uncertainties and experimental errors in the results from analytical analyses, quality assurance (QA) is important. The QA is a part of the quality management system and it is required for accredited laboratories. This involves correct training of laboratory analysts, the use of validated and well documented analytical methods, record keeping, appropriate environment and storage for equipment and chemicals, procedures for maintenance and routines for calibration of equipment and instruments (Prichard and Barwick, 2007).

Quality control (QC) describes the measures made for ensuring the quality of the results, i.e. measurements of blanks, QC samples, repeated sampled, blind samples, chemical standards and spike samples. For monitoring a method performance over time, the measurements of QC samples can be plotted in control charts. A QC sample consists of a material that is stable and homogenous and can be characterized in-house or by a third-party as a certified reference material (CRM) with well-established target values called certified values. Measurements of this type of sample can be used to ensure the variations in the results for the applied analytical method (Prichard and Barwick, 2007).

Experimental errors can be classified by systematic or random errors. Random error is inevitable and the result of natural variation by chance, causing the results to vary in an unpredictable way. Systematic error is the result of a factor consistently affecting the results, which can give higher or lower results than expected. The systematic type of error can be corrected by data analysis, while random errors cannot be corrected for a set of measurements. However, random errors can be minimized by making repeated measurements, since the effect of random error on the mean will decrease with increasing number of measurements.

Method validation is a process performed before implementing a new or existing analytical method. The term is defined in the international quality standard (ISO/IEC 17025; general requirements for the competence of testing and calibration laboratories), as *the confirmation by examination and provision of*

objective evidence that the particular requirements for a specific intended use are fulfilled. A validation process is required for each method to ensure that the laboratory can demonstrate the requirements specified by customers and the quality system (Prichard and Barwick, 2007). Method validation can be performed by conducting an interlaboratory comparison approach or by a single-laboratory approach. An interlaboratory comparison approach is used when a method is developed for a wide-ranging use, while a single-laboratory approach is used for developing a method that will be used in only one laboratory (Eurachem, 2014). Parameters such as uncertainty and metrological traceability are a part of method validation. Other parameters that are included in method validation are selectivity, precision, bias/trueness (accuracy), measurement range, the limit of detection (LOD) and the limit of quantification (LOQ), and ruggedness (Prichard and Barwick, 2007).

2.7.1.1 Selectivity

Selectivity refers to the ability an analytical method has to measure particular analytes in sample matrixes without interferences from other components with similar properties (Prichard and Barwick, 2007). The term can also be referred to as specificity, and can be determined by evaluating blank samples for analyte signals (Rao, 2018).

2.7.1.2 Precision, repeatability and reproducibility

Precision is the degree of agreement between independent repeated measurements. The parameter does not relate to the true value but is dependent on the distribution of random error. Precision by repeated measurements using the same method and the same sample can be expressed as *repeatability* and *reproducibility* through relative standard deviation (RSD, %). Reproducibility is calculated from measurements under different conditions such as different analysts, instruments and laboratories with the same method and the same sample (Prichard and Barwick, 2007). The reproducibility in a single laboratory is often described with the term *intermediate precision* (Eurachem, 2014; Prichard and Barwick, 2007). To improve precision, a minimum of seven replicates are measured to reduce random errors (Prichard and Barwick, 2007). The RSD (%) can be evaluated by using the Horwitz ratio for expected and acceptable RSD (%) based on the Horwitz function (NMKL 5, 2003; Eurachem, 2014; Rao, 2018).

2.7.1.3 Trueness

Trueness is the difference between the mean value of test results and a reference value for a sample material. Trueness is usually determined by comparing the mean of measurements with a certified value of a CRM (Prichard and Barwick, 2007). Another approach for determining trueness can be to use spiked samples with a known concentration of an analyte (Prichard and Barwick, 2007). This approach can be applied for evaluating possible interferences like matrix effects (Prichard and Barwick, 2007).

3 MATERIALS AND METHODS

All laboratory equipment, instruments, chemicals and reagents, samples and certified reference materials (CRMs) used in this project were provided by the Institute of Marine Research. The laboratory work was performed at the inorganic chemistry laboratory of the institute in Bergen, Norway, accredited to the quality standard NS-ISO 17025:2017. A general overview of the experimental steps conducted in this study, i) the correlation analysis and ii) the method development, are shown in Figure 10.

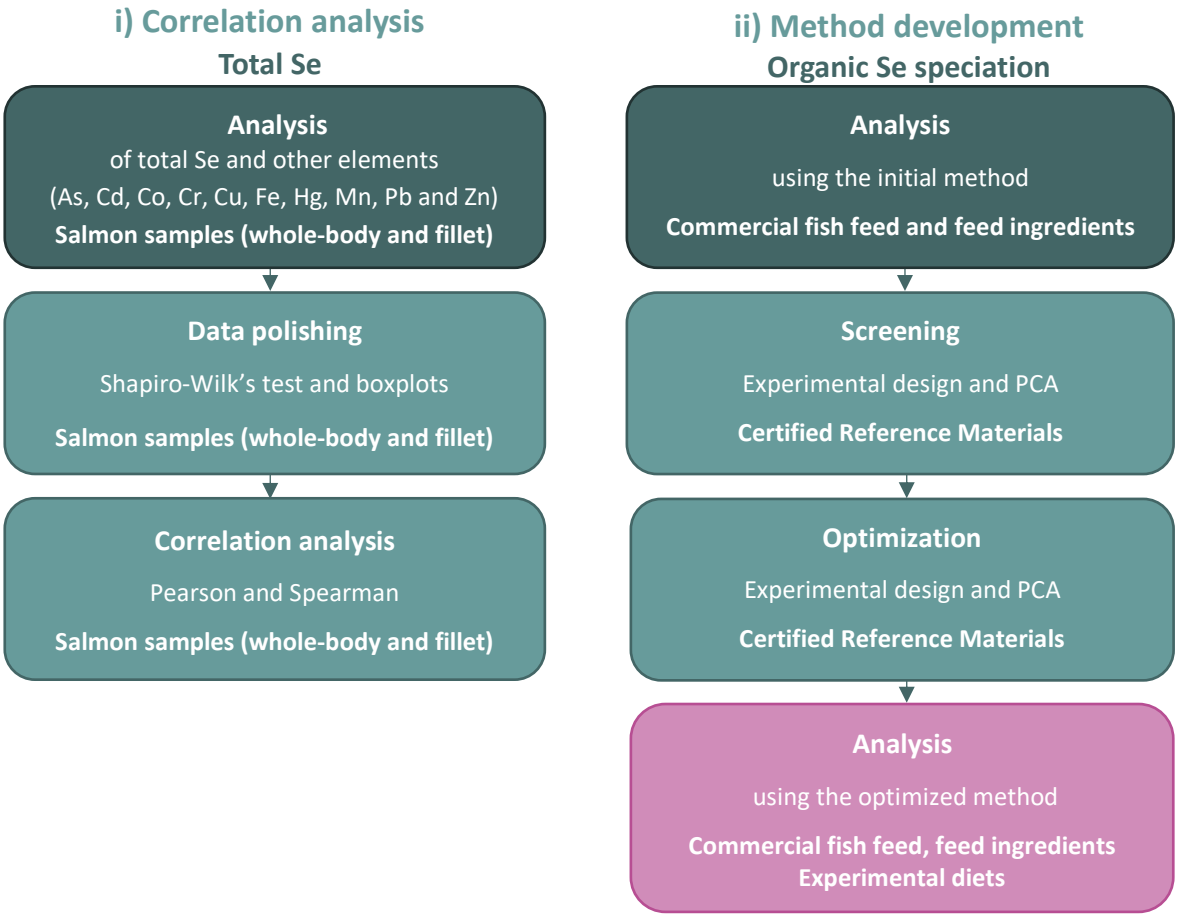


Figure 10: An overview of the experimental steps in this work; i) correlation analysis and ii) method development.

In brief, for the correlation analysis, the salmon samples were determined for total Se, as well as other elements, i.e. arsenic (As), cadmium (Cd), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), mercury (Hg), manganese (Mn), lead (Pb) and zinc (Zn). Both whole-body and fillet samples of farmed Atlantic Salmon (*Salmo salar*) that had been fed with Se supplemented diets were analyzed. Correlations between the concentrations of Se and other elements were determined using statistical methods, including Shapiro-Wilk's normality test and visualization using boxplots for data polishing, Pearson's correlations and Spearman's Rank-Order correlation.

For the method development for Se speciation, the extraction procedure was optimized using two certified reference materials (CRMs). The significant factors affecting the sample extraction were screened for and optimized using chemometric approaches, such as experimental designs and PCA. The factors were evaluated using the results from total Se determination and Se speciation of sample extracts. The optimized method was quality controlled by evaluation of performance characteristics related to method development and method validation, and the method was applied to samples, including fish feed and feed ingredients of fish meal, plant meal and insect meal.

3.1 Materials

3.1.1 Chemicals and reagents

Deionized and filtrated water was used for all steps requiring H_2O in the sample preparation steps and analyses, and was available from a Milli-Q Reference Water Purification System (18.2 MΩ cm, EMD Millipore Corporation, MA, USA). All chemicals and reagents that were used in this project were of analytical grade, given in Table 4.

Table 4: Chemicals and reagents used for sample preparation and analysis.

Product	Supplier
Multi element standard (Prod.no. SS 6083S)	Spectrascan, Teknolab (Ski, Norway)
Mercury (Hg) 1000 µg/mL (Prod.no. SS 1532)	Spectrascan, Teknolab (Ski, Norway)
Gold (Au) 1000 mg/L Certipur® ICP standard (170321 Supelco)	Merck KGaA (Darmstadt, Germany)
Rhodium (Rh) 1000 µg/mL (Prod.no. SS 1550)	Spectrascan, Teknolab (Ski, Norway)
Germanium (Ge) 1000 µg/mL (Prod.no. SS 1130)	Spectrascan, Teknolab (Ski, Norway)
Thulium (Tm) 1000 mg/L Certipur® ICP standard (170361 Supelco)	Merck KGaA (Darmstadt, Germany)
Stock tuning solution, Ba, Bi, Ce, Co, In, Li and U (Prod.no SS 6088SS)	Spectrascan, Teknolab (Ski, Norway)
Hydrogen peroxide (H_2O_2) 30% solution, Perhydrol (CAS 7722-84-1)	Merck KGaA (Darmstadt, Germany)
Nitric acid (HNO_3) ≥ 69% Suprapur® (CAS 7697-37-2)	Merck KGaA (Darmstadt, Germany)
ICP-MS stock tuning solution (Part #5188-6564)	Agilent (Waldbronn, Germany)
Ammonium phosphate dibasic ($(NH_4)_2HPO_4$) ≥99.0% (CAS 7783-28-0)	Merck KGaA (Darmstadt, Germany)
Ortho-Phosphoric acid (H_3PO_4), 85% solution (CAS 7664-38-2)	Merck KGaA (Darmstadt, Germany)
Ammonia (NH_3), 25% solution (CAS 1336-21-6)	Merck KGaA (Darmstadt, Germany)
Formic acid (HCOOH) 98-100% (CAS 64-18-6)	Merck KGaA (Darmstadt, Germany)
Sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) for analysis (CAS 10049-21-5)	Merck KGaA (Darmstadt, Germany)
Sodium citrate tribasic dihydrate ($HOC(COONa)(CH_2COONa)_2 \cdot 2H_2O$) (CAS 6132-04-3)	Sigma-Aldrich (Oslo, Norway)

Calcium chloride ($CaCl_2$) Reag.Ph Eur (CAS 10043-52-4)	Merck KGaA (Darmstadt, Germany)
Sodium dodecyl sulphate (SDS) ACS reagent ≥99.0% (CAS 151-21-3)	Sigma-Aldrich (Oslo, Norway)
Sodium hydroxide (NaOH), 50% solution (CAS 1310-73-2)	Merck KGaA (Darmstadt, Germany)
Seleno-DL-methionine (SeMet) ≥99% purity (CAS 1464-42-2)	Sigma-Aldrich (Oslo, Norway)
Ammonium formate (HCO_2NH4) ≥97% (CAS 540-69-2)	VWR Chemicals (Bergen, Norway)
Methanol (CH_3OH) >99.9%, hypergrade for LC-MS (CAS 67-56-1)	Sigma-Aldrich (Oslo, Norway)
Gemini® 5 µm C6-Phenyl 110 Å, LC Column 150x4.6 mm, Ea (00F-4444-E0)	Phenomenex (California, USA)

3.1.2 Enzymes

All enzymes used in this work were obtained from Sigma-Aldrich (Oslo, Norway). The enzymes and the enzymatic strength of the products are shown in Table 5.

Table 5: Enzymes used for extractions of organic Se in this project.

Product	Unit activity	Application step
Papain from *Carica papaya* (76220)	≥3 units/mg powder	Pre-extraction
Protease type XIV from *Streptomyces griseus* (P5147)	≥3.5 units/mg powder	Main extraction
Protamex®, protease from *Bacillus* sp. (P0029)	>1.5 AU-N/g powder*	Main extraction
α-Amylase from porcine pancreas (A3176)	≥5 units/mg powder	Main extraction
Cellulase from *Aspergillus niger* (C1184)	≥0.3 units/mg powder	Main extraction

*Corresponds to approximately ≥ 0.555 units/mg. One Anson unit (AU) = 370 units, according to Sigma-Aldrich (2020).

3.1.3 Equipment and instruments

A list of equipment and instruments used during laboratory work is given in Table 6. Water bath and analytical balances, as well as other equipment, e.g. pipettes, refrigerators, freezers and incubators (not listed in the table), were routinely controlled by technicians.

Glassware, such as volumetric flasks and glass bottles (not listed in the table) were also used for the laboratory work. To minimize metal contamination, all glassware and Milestone digestion vials were washed using an acid steam cleaning system (Milestone TraceCLEAN). Larger glassware, such as volumetric flasks and glass bottles, were washed with Thernard's solution (500 mL Milli-Q water, 100 mL H_2O_2 (30%) and 200 mL HCl (37%)). All acid-washed glassware was rinsed thoroughly with Milli-Q water before and after acid wash.

Table 6: Laboratory equipment and instruments used in this project.

Product	Supplier
Explorer® Analytical balance	Ohaus (Nänikon, Switzerland)
Balance XPR204	Mettler Toledo (Greifensee, Switzerland)
Precision balance MS6002TS	Mettler Toledo (Greifensee, Switzerland)
Cryogenic miller 6875D Freezer/Mill	SPEX SamplePrep (New Jersey, USA)
Knife Mill GM 200	Retsch (Haan, Germany)
Ultra centrifugal mill ZM 100	Retsch (Haan, Germany)
Labconco FreeZone 18 Liter Console Freeze Dry System	Labconco (Kansas, USA)
Milestone digestion vials (TFM or quarts) with TFM caps	Milestone Srl (Sorisole, Italy)
Falcon™ 15 mL conical centrifuge tubes	Thermo Fischer (Waltham, USA)
Falcon™ 50 mL conical centrifuge tubes	Thermo Fischer (Waltham, USA)
Sterile 13 ml centrifuge tubes (PP)	Sarstedt (Nümbrecht, Germany)
HPLC vials (PP), snap top, 1 mL (Part #5182-0567)	Agilent (Waldbronn, Germany)
HPLC vials (PP), snap top with glass insert, 250 µL (Part #9301-0977)	Agilent (Waldbronn, Germany)
HPLC vial snap caps (PTFE) with silicone septa for (Part #5182-0550)	Agilent (Waldbronn, Germany)
Amicon Ultra-0.5 mL centrifugal filters 10 kDa with 2 mL micro-centrifuge tubes	Merck KGaA (Darmstadt, Germany)
Micro-centrifuge tubes 1.5 mL	Eppendorf (Hamburg, Germany)
Grant OLS200 water bath	Grant Technologies (Cambridge, UK)
Centrifuge 5702	Eppendorf (Hamburg, Germany)
Centrifuge 5424 R	Eppendorf (Hamburg, Germany)
WTW SenTix 81 pH electrode with WTW InoLab pH meter	Xylem (New York, USA
KNF Laboport vacuum pump N816.3 KT18, 50Hz, 100W	KNF Neuberger GmbH (Freiburg im Breisgau, Germany)
Filter membrane, RC 47 mm, pore size 0.45 µm (Part #5191-4337)	Agilent (Waldbronn, Germany)
Vortex mixer MS1 Minishaker	IKA (Staufen, Germany)
Milli-Q Reference Water Purification System	EMD Millipore Corporation (MA, USA)
TraceCLEAN Acid steam cleaning system*	Milestone Srl (Sorisole, Italy)
UltraWAVE Microwave Acid Digestion System	Milestone Srl (Sorisole, Italy)
Thermo Scientific iCAP Q ICP-MS	Thermo Fischer (Waltham, USA)
Agilent HPLC 1260 Infinity II Bio-Inert LC System	Agilent (Waldbronn, Germany)
Agilent ICP-MS 7900	Agilent (Waldbronn, Germany)

*Concentrated nitric acid (HNO_3) was used for the acid steam cleaning system.

3.1.4 Samples

3.1.4.1 Salmon samples

Samples of farmed Atlantic salmon for the correlation analysis were obtained from a previous study (Berntssen *et al.*, 2018b). The fish were fed a control diet (no supplementation; 0.45 mg/kg), and diets supplemented with inorganic Se (selenite) at concentrations defined as low (approximately 5 mg/kg) or medium levels (11 mg/kg) and organic Se (L-selenomethionine) at concentration defined as low (6.2 mg/kg), low-medium (16.2 mg/kg) and medium (21 mg/kg). The study by Berntssen *et al.* (2018b) aimed to assess the toxic levels of Se in Atlantic salmon. The samples included three biological replicates of pooled samples (n = 5) of salmon from three different fish tanks fed with the same diet. The samples were analyzed in total Se for the previous study (Berntssen *et al.*, 2018b). For the correlation analysis in this project, pooled samples were analyzed in technical replicates (n = 3) for the elements As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb, Se and Zn.

3.1.4.2 Fish feed for Atlantic salmon and feed ingredients

Commercial fish feed and feed ingredients analyzed in this project were obtained from the National Surveillance program for fish feed for 2019 (Ørnsrud *et al.*, 2020). An overview is shown in Table 7, with total Se concentrations in each sample (Ørnsrud *et al.*, 2020). Fish feed (FF; n = 6), fish meal (FM; n = 3), plant meal (PM; n = 2) and insect meal (IM; n = 2) were analyzed for the application of the optimized method and compared to the initial method.

Table 7: Samples of commercial fish feed and feed ingredients for Se speciation and the total Se concentrations ± SD (mg/kg ww, n =3) obtained through the surveillance program for fish feed for 2019 (Ørnsrud et al., 2020).

Sample name	Samples
FF1 - FF6	Fish feed
FM1 - FM3	Fish meal
PM1 - PM2	Plant meal
IM1 - IM2	Insect meal

* n = 1-2.

Experimental diets from two previous studies (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b) were selected for evaluation of the optimized method. The experimental diets had different supplement sources, different supplementation concentrations as well as different diet composition. An overview of the experimental diets is shown in Table 8, with supplemented Se species, nominal Se concentrations supplemented to diets and diet composition (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b). Diet A and B were supplemented with L-selenomethionine close to the legal limit of 0.5 mg/kg feed. Diet D-F were experimental diets supplemented at higher Se levels (5 and 15 mg Se/kg diet), while diet C was a

basal diet related to diet D-F. The details of the diet compositions from Antony Jesu Prabhu *et al.* (2020) and Berntssen *et al.* (2018b) are given in Appendix A.

Table 8: Experimental diets used for application of method, Se species supplemented to the diets, concentrations of Se (mg Se/kg ww) in diets and diet compositions.

Sample	Supplemented Se species	Supplement conc. (mg Se/kg diet)[(a)]	Diet composition (ingredients)
Diet A [(1)]	SeMet	0.15	Marine: 11.5%, Plant: 84% (Wheat: 30%), Other[(b)]: 4.5%
Diet B [(1)]	SeMet	0.4	
Diet C [(2)]	No supplement (basal)	0	Marine: 22.2%, Plant: 70% (Wheat: 30%), Other[(b)]: 8.1%
Diet D [(2)]	SeMet	5	
Diet E [(2)]	SeMet	15	
Diet F [(2)]	Selenite	5	

1: Diet described in Antony Jesu Prabhu *et al.* (2020). 2: Diet described in Berntssen *et al.* (2018b).
a: Nominal concentrations (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b). b: See Appendix A for details.

3.1.4.3 Certified reference materials (CRMs)

The CRMs utilized in this project are given in Table 9.

Table 9: Certified reference materials (CRMs) applied in this project.

Product	Supplier	Application
TORT-3 (lobster hepatopancreas)	National Research Council Canada (Ontario, Canada)	Total Se and element determination
SRM 1566b (oyster tissue)	National Institute of Standards and Technology (Gaithersburg, USA)	Total Se and element determination
ERM BB422 (fish muscle)	Institute for Reference Materials and Measurements (Geel, Belgium)	Total Se and element determination
ERM BC210a (wheat flour)	LGC (Teddington, UK)	Se speciation
SELM-1 (selenized yeast)	National Research Council Canada (Ontario, Canada)	Se speciation

The certified values for total Se and SeMet in ERM BC210a and SELM-1 are given in Table 10, while the certified values for ERM BB422, TORT-3 and SRM 1566b are given in later sections.

Table 10: Certified values (mean ± SD) for total Se (mg Se/kg) and SeMet (mg SeMet/kg) obtained from certificates for CRMs ERM BC210a (wheat flour) and SELM-1 (selenized yeast), recalculated SeMet certified values (mg Se/kg).

CRM	Total Se (mg Se/kg)	SeMet (mg SeMet/kg)	SeMet (mg Se/kg)*
ERM BC210a	17.23 ± 0.91	27.4 ± 2.6	11.03 ± 1.05
SELM-1	2013 ± 70	3190 ± 260	1284 ± 105

* Converted into concentration of SeMet in unit mg Se/kg, using equation (8) (see section 3.9.1).

3.2 Homogenization of samples

3.2.1 Salmon samples

The whole-body and fillet samples of Atlantic salmon were lyophilized prior to analysis, as elemental analyses are commonly conducted on dry materials (Alonso *et al.*, 2015). Fresh samples were stored at -20 °C prior to lyophilization. The samples were then thawed and weighed, prior to lyophilization using the Labconco Freeze dry system. After lyophilization, the samples were grounded, homogenized, and weighed again for moisture calculations of water content. The lyophilized samples were stored at room temperature until further analyses.

3.2.2 Commercial fish feed and feed ingredients

Samples of commercial fish feed and feed ingredients were homogenized and grinded using a mill from Retsch. All grinded samples of commercial fish feed and feed ingredients were stored at 2-8°C prior to analysis.

3.2.3 Experimental diets

A cryogenic mill (6875D Freezer/Mill) was used for milling and homogenization of pellets of the experimental diets (Table 8). The mill pre-cools multiple samples with liquid nitrogen and grinds the samples by an impact rod in each vial. A polycarbonate vial set was used for the samples. The settings used for pellets (approximately 3 g) are shown in Table 11. The ground samples of experimental diets were stored at 2-8°C prior to analysis.

Table 11: Settings used for grinding experimental diets (3 g) using cryogenic mill.

6875 Freezer/Mill settings	
Pre-cool	15 min
Run time	2:00 min
Cool time	1
Cycles	3
Rate	12 CPS

3.3 Overview of analytical work

An overview of the analytical work conducted in this project is shown in Figure 12. Salmon samples and samples of fish feed and feed ingredients were determined for total Se using ICP-MS. Other elements (As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb and Zn) were also determined in salmon samples. For the method development, the organic Se species SeMet was determined in the soluble fractions extracted in fish feed

and feed ingredients after enzymatic digestions (pre-extraction and/or main extraction) using HPLC-ICP-MS. Both non-soluble and soluble fractions after the enzymatic digestion were determined for total Se using ICP-MS.

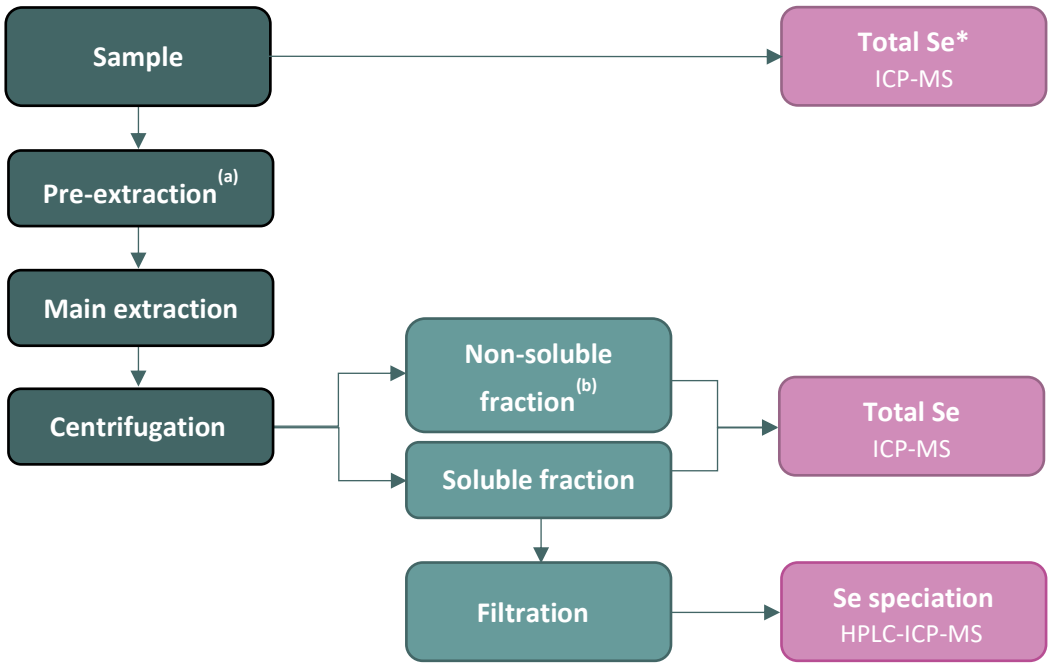


Figure 11: Flowchart of the analytical work in this study.

*For the correlation study, the following elements are also included: As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb and Zn.
a: Pre-extraction was only performed for some of the experiments in the method development.
b: The non-soluble fraction was only analyzed for total Se for the experimental diets. Whereas the soluble fraction was only analyzed for total Se the method development steps.

3.4 Determination of total Se and other elements using ICP-MS

3.4.1 Digestion of samples and sample extracts

For the determination of total Se, micro-wave assisted acid digestion was performed on the sample materials using an UltraWAVE digestion system (Milestone). Samples and CRMs were approximately weighed to 0.2 g in digestion vials containing 0.5 mL Milli-Q water. Two mL concentrated HNO_3 was added to each vial, including blank samples. The vials were then capped and placed in a container of 130 mL Milli-Q water and 5 mL H_2O_2 in the UltraWAVE system, digested for 2 h under a pressure of 40 bar. After digestion and cooling, the samples were diluted to 25 mL with Milli-Q water and stored in 50 mL polypropylene tubes prior to analysis by ICP-MS.

The same digestion procedure was conducted for the soluble and non-soluble fractions. The soluble fractions were weighed, approximately 1.0 g, digested in the UltraWAVE system, and diluted to 10 mL with Milli-Q water. The non-soluble fractions were dried for 24 h at 90 °C in an incubator prior to digestion. The dried material was weighed in the digestion vial (approximately 0.04 g), digested in the UltraWAVE system, and diluted to 10 mL with Milli-Q water. All sample extracts were stored at room temperature in 15 mL polypropylene tubes prior to analysis by ICP-MS.

3.4.2 Analysis by ICP-MS

A multi-element analysis was performed to determine the concentration of total Se in the acid digests using ICP-MS. The method was also used to determine the total concentrations of As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb and Zn for the salmon samples. The analysis was performed using a Thermo Scientific iCAP Q ICP-MS with a collision cell. The ICP-MS was equipped with an SC-4 DX autosampler with a FAST valve from Elemental Scientific (Omaha, USA), and was operated with Qtegra iCap Q software (Thermo Scientific) on a computer connected to the system. The method is accredited for the elements Cu, Zn, As, Se, Cd, Hg and Pb (Appendix A) (Institute of Marine Research, 2020a).

For analysis, a multi-element solution and a Hg single-element solution were freshly diluted to appropriate concentrations with 5% HNO_3 (v/v) to establish a calibration curve for the analysis. Internal standards of germanium (Ge), rhodium (Rh) and thulium (Tm) were added to the samples, calibration standards and blank samples, for correction of possible matrix effects or changes in operating conditions in the analyses. A gold single-element solution was also freshly prepared and was added to the calibration curve solution and internal standard to stabilize Hg ions. A tuning solution (1 ppb) was freshly prepared and used for tuning at start-up for monitoring possible interferences from oxide species and double charged ions. The instrumental settings for ICP-MS are presented in Table 12.

Table 12: The instrumental settings for the ICP-MS when determining total Se and other elements.

	ICP-MS (iCAP Q)
Plasma power (Ar, 99.999% purity)	1550 W
Nebulizer gas flow (Microflow PFTA-ST)	1.05 L/min
Auxiliary gas flow	0.8 L/min
CCT1 gas flow (He, 99.999% purity)	4.6 mL/min
Integration time	0.1 s
Isotopes monitored	^{78}Se, ^{75}As, ^{111}Cd, ^{59}Co,^{52}Cr, ^{63}Cu, ^{56}Fe, ^{202}Hg, ^{55}Mn,^{208}Pb, ^{66}Zn

3.5 Determination of organic Se species using HPLC-ICP-MS

3.5.1 Extraction procedures

The extraction method for organic Se in fish feeds was based on a method for Se speciation (Sele et al. 2018), and this method was the basis for the method optimization. For the extraction procedure, approximately 0.2 g of sample material was weighed and suspended in 2.5 mL or 5 mL buffer containing an enzyme (≥28 U/mL). Each sample was mixed thoroughly using a vortex mixer before placing the sample tubes in a water bath at 37 ^{o}C for 20 h, shaking at 100 rpm/min, for a main extraction. All extractions were inactivated by placing the sample tubes in a water bath at 100 ^{o}C for 15 min. After inactivation of the enzymes, all samples were centrifuged at 3500 rpm for 10 min. To separate the lower molecular weight Se species from higher molecular weight species, 0.5 mL of the soluble fraction of each sample was transferred to a microcentrifuge tube with an Amicon Ultra-0.5 mL 10 kDa filter. The samples were filtered by centrifugation at 14 000 g for 20 min and the filtered samples was transferred to 2 mL centrifuge tubes and stored at -20 ^{o}C prior to analysis on HPLC-ICP-MS. Unfiltered extracts and non-soluble fractions were also stored at -20 ^{o}C prior to microwave assistant digestions and analysis by ICP-MS. The samples were thawed to room temperature prior to further analysis.

An additional pre-extraction step was included for selected samples in the screening and optimization step. For the pre-extraction, 2.5 mL buffer containing papain enzyme (≥28 U/mL) was added to the sample. Similar to the procedure for the main extraction, the sample was mixed thoroughly and extracted in a water bath at 37 ^{o}C for 20 h, shaking at 100 rpm/min. After cooling the sample to room temperature, the enzymes were inactivated by a water bath at 100 ^{o}C for 15 min. The main extraction procedure was then conducted by addition of 2.5 mL enzyme solution containing one of the following enzymes: protease, protamex, α-amylase or cellulase. The samples were once again mixed thoroughly and were extracted in a water bath at 37 ^{o}C for 20 h, shaking at 100 rpm/min, before inactivation of enzymes.

3.5.2 Se speciation analysis with HPLC-ICP-MS

For the determination of Se species, the chromatographic separation was performed using an HPLC (1260 Infinity II Bio-Inert LC System, Agilent) with autosampler, and the separated Se species were detected using an ICP-MS (7900, Agilent). The ICP-MS was equipped with a MiraMist nebulizer (Agilent Technologies) and the HPLC-ICP-MS was operated using the computer software MassHunter (Agilent Technologies). The organic Se species were separated on an RP HPLC column with a stationary phase of C6 linked phenyl with TMS end-capping, dimensions of 4.6 mm internal diameter, 150 mm length, 5 µm particle size and 110 Å pore size (Part no. 00F-4444-E0), Phenomenex, California, USA).

For the analysis, an external calibration curve of SeMet was applied, with concentrations of 0, 0.5, 1, 5, 10 25, 50 µg Se/L made from a standard solution of Seleno-DL-methionine. All sample extracts were diluted to achieve concentrations within the range of the calibration curve. The following dilution factors were applied: blank samples, commercial fish feed and feed ingredients were diluted 4 times, ERM BC210a (wheat flour) was diluted 10 times, SELM-1 was diluted 2000 times, while experimental diets were diluted 4 or 8 times prior to analysis.

The mobile phase was prepared by dissolving an appropriate amount of ammonium formate to reach the desired ionic strength (20 mM) in an aqueous 1% (v/v) MeOH solution, followed by adjustment of pH to 9 with NH_3 and HCOOH. The mobile phase was filtrated prior to analysis using a 0.45 µg filter and a filtration system containing a funnel, flask, suction tubes and a vacuum pump (Table 6).

Prior to analysis, the ICP-MS was tuned using a solution of lithium (Li), yttrium (Y) and thallium (Tl) for performance check on their respective counts per second (^{7}Li: > 3000 counts, ^{89}Y: > 15 000 counts and ^{205}Tl: > 9000 counts, with RSD% < 5), at pump velocity of 0.12 rps (Institute of Marine Research, 2020b). A solution of ^{78}Se was monitored for the tuning, with an in-house limit of ^{78}Se: > 1500 counts per second. The HPLC column was prepared by purging and flushing it with Milli-Q water prior to the analysis.

For analysis, the sample eluent from the HPLC column was connected to the nebulizer in the ICP-MS via a PEEK-tubing. An octopole reaction gas of H_2 was applied in the ICP-MS at a flow rate of 2.5 mL/min, to prevent interferences from polyatomic argon to the monitored isotope, ^{78}Se. In addition to the isotope ^{78}Se, ^{76}Se was also monitored with an integration time at 0.1 s. Due to high signal interferences from ^{40}Ar^{40}Ar, one of the most abundant Se isotopes, ^{80}Se, was not monitored. The instrumental settings applied are shown in Table 13.

Table 13: Instrumental settings for HPLC and ICP-MS.

Reversed-phase HPLC (Agilent 1260 system)	
Column	Phenomenex Gemini C6-phenyl 110 A (105 X 4.6 mm,
Injection volume	25 µL
Operating pressure	< 200 bar
Mobile phase	20 mM ammonium formate + 1% MeOH (pH 9)
Mobile phase flow rate	1.0 mL/min
Elution program	Isocratic
ICP-MS (Agilent 7900)	
Carrier gas flow (Ar, 99.999% purity)	1.15 – 1.25 L/min
Forward/reflected power	1550 W
Plasma gas flow	15.0 L/min
Makeup gas flow	0.12 L/min
H_2 gas flow (collision-reaction cell)	2.5 mL/min
Spray chamber temperature	2 °C
Integration time	0.1 sec
Isotopes monitored*	^{78}Se, ^{76}Se

3.6 Method development for the determination of organic Se species

3.6.1 Application of initial method for organic Se speciation

Samples of commercial fish feed and feed ingredients were analyzed for organic Se species using the initial method based on Sele *et al.* (2018a). In brief, enzymatic digestion was performed on approximately 0.2 g samples, with ≥ 28 U/mL protease XIV in 2.5 mL of 1 mM ammonium phosphate as buffer, analyzed using anion-exchange column (Sele *et al.*, 2018a). For this project, the analyses were instead performed using an RP column. The enzymatic digestion was also performed on approximately 0.2 g samples with ≥ 28 U/mL protease XIV in this project, but in 2.5 mL buffer mixture, referred to as "Mix". The buffer was composed of 20 mM sodium phosphate, 50 mM sodium citrate, 5 mM calcium chloride and 1 mM sodium dodecyl sulphate (SDS) in Milli-Q water, and pH adjusted to pH 7 with H_3PO_4 or NaOH. The buffer solution is previously described in Oliveira *et al.* (2016). The extraction procedure was performed at 37 °C for 24 h and analyzed with the same HPLC-ICP-MS set-up as the other samples.

3.6.2 Enzymatic digestion procedures for the experimental designs

For the method development, the buffer "Mix" (see section 3.6.1) and the buffer "AmPh" (1 mM ammonium phosphate in MilliQ-water, pH 7, adjusted with H_3PO_4 and NH_3) were tested in combinations with different enzymes. The enzymes tested were protease XIV, protamex, α-amylase and cellulase for the main extraction, and papain for the pre-extraction step.

To assess the extraction procedures, factors and possible interactions between factors, a screening step using experimental design was applied. After screening for the selected factors, an optimization step was performed using a second experimental design.

An overview of the mass of enzyme powders, the final concentrations of the enzyme buffer solutions, containing papain (≥28 U/mL), protease (≥28 U/mL), protamex (≥28 U/mL), α-amylase (≥28 U/mL), cellulase (≥28) and a combination of protease and cellulase (≥56 U/mL) used for the experimental designs are shown in Table 14.

Table 14: Enzymes used, with mass of enzyme powder (mg), volume of buffer solution (mL), concentration of enzyme (mg/L) and enzyme activity (U/mL) when using 0.2 g sample. The final volume and activity are the volume and activities after addition to the sample tubes for the relevant extraction steps.

Screening and optimization

		Added to sample				Final	
	Enzyme	**Mass (mg)**	**Volume (mL)**	**Concentration (mg/mL)**	**Activity (U/mL)**	**Volume (mL)**	**Activity (U/mL)**
1	Papain	23.3	2.5	9.33	≥28	2.5	≥28
2a	Protease	40.0	2.5	16.0	≥56	5	≥28
2b	Protamex	252	2.5	101	≥56	5	≥28
2c	α-Amylase	28.0	2.5	11.2	≥56	5	≥28
2d	Cellulase	467	2.5	187	≥56	5	≥28
2e	Protease	20.0	1.25	16.0	≥56	2.5	≥28
	Cellulase	233	1.25	187	≥56	2.5	≥28
	Combined (Sum)				≥112	5	≥56

1: In the pre-extraction step.
2: In the main extraction; *a*, *b*, *c* and *d* were applied for the screening step; *a* and *e* were applied for the optimization step.

3.6.2.1 Screening of factors influencing organic Se speciation analysis

The screening was conducted on the CRMs ERM BC210a (wheat flour) and SELM-1 (selenized yeast). The factors screened for were i) the buffer solution, ii) pre-extraction and iii) enzymes (Table 15). For evaluating the extraction recovery with multiple enzymes and two different extraction solutions, a 2^3 full experimental design (Figure 7) was applied with two different settings, i) experiment A and ii) experiment B, with a total of 32 samples. The experimental conditions for experiment A and experiment B are shown in Table 15.

Table 15: The experimental conditions used for 2^3 full factorial design for experiment A and experiment B, applied to the CRMs ERM BC210a (wheat flour) and SELM-1 in the screening.

Conditions for experiment A

Factor	Name	Low level (-)	High level (+)
X1	Buffer[a]	AmPh[i]	Mix[ii]
X2	Pre-extraction[b]	No	Yes
X3	Enzyme[c]	Protease	Protamex

Conditions for experiment B

Factor	Name	Low level (-)	High level (+)
X1	Buffer[a]	AmPh[i]	Mix[ii]
X2	Pre-extraction[b]	No	Yes
X3	Enzyme[c]	α-Amylase	Cellulase

a: The extraction buffer, being either i) 1 mM NH_3PO_4 or ii) mixed buffer of 20 mM $NaPO_4$, 50 mM sodium citrate, 5 mM CaCl, 1 mM SDS.
b: A pre-extraction using papain from *Carica papaya,* prior to the main extraction.
c: The enzyme used during main extraction.

The extraction buffers tested were the buffers "Mix" (+) and "AmPh" (-). The factor "pre-extraction" indicates an enzymatic extraction performed with papain before the main extraction, "yes" (+), or no pre-extraction "no" (-). The following enzymes: protease, protamex, α-amylase and cellulase were screened, and these four enzymes were set as levels for the factor "enzyme" (X3), with "protease" (-) and "protamex" (+) in experiment A, and "α-amylase" (-) and "cellulase" (+) in experiment B. The factors "buffer" (X1) and "pre-extraction" (X2) were similar for experiment A and B.

The details for the experimental design, experiment A and experiment B, are explained in later in section 4.2.2. To study the variation, experiment 1 was conducted using the "low" level for all three factors for experiment A and B. The "low" levels were "AmPh" (-) for "buffer" (X1), "no" (-) for "pre-extraction" (X2) and "protease" (-) for "enzyme" (X3). Since all factors are categorial without the possibility for a center level, triplicates of experiment 1 were included as a replacement for the center points (0).

3.6.2.2 Optimization of method for organic Se speciation on fish feed and feed ingredients

For the optimization step, experimental designs of two factors and two levels, 2^2 full factorial design (experiment C) were used (Figure 7). The experimental settings used for experiment C are shown in Table 16 and were used for the optimization step. The factors, "pre-extraction" (X2) and "enzyme" (X3) tested in experiment A and experiment B, were considered relevant and were included in experiment C as the two factors. The levels for "enzyme" (X3) were different in experiment C compared to experiment A and B, by using the enzyme protease alone, "protease" (-), or combined with cellulase in a 1:1 ratio, "combined" (+).

Table 16: The experimental conditions used for 2^2 full factorial design (experiment C), applied to ERM BC210a (wheat flour) and SELM-1 for optimization.

Conditions for experiment C			
Factor*	**Name**	**Low level (-)**	**High level (+)**
X2	Pre-extraction[a]	No	Yes
X3	Enzyme[b]	Protease	Combined[c]

a: A pre-extraction using papain from Carica papaya prior to the main extraction.
b: Enzyme used during main extraction.
c: Combination of protease XIV from Streptomyces griseus and cellulase from Aspergillus niger (1:1 ratio).

The four experiments in the 2^2 full factorial design for each CRM given in Table 16, were analyzed in triplicates. The buffer solutions "AmPh" was applied during the optimization. Other conditions that were kept constant in the optimization step were the 5 mL volume of the buffer solution, 1 mM concentration of buffer solution, 0.2 g of sample, 20 hours extraction time, 37 °C extraction temperature and the instrumental settings for HPLC and ICP-MS. The details for the experimental design, experiment C, are explained in later in section 4.2.3.

3.6.3 Analysis using the optimized method

The optimized factors were set for the method, and this method was further applied for the determination of organic Se species in the experimental diets, commercial fish feed and feed ingredients. The optimized method composed of 5 mL buffer solution containing protease and cellulase (1:1; ≥56 U/mL) in the ammonium phosphate buffer ("AmPh"), whereas the initial method composed of 2.5 mL buffer solution containing protease type XIV (≥28 U/mL) in the "Mix" buffer. An overview of the differences between initial and optimized method is shown in Table 17.

Table 17: Enzymes used for the extractions, with mass of enzyme powder (mg), volume buffer solution (mL), concentration of enzyme (mg/L) and enzyme activity (U/mL) for the extraction methods, using 0.2 g sample.

			Application			
Extraction method		**Enzyme**	**Mass (mg)**	**Volume (mL)**	**Concentration (mg/mL)**	**Activity (U/mL)**
a	**Initial**	Protease	20	2.5	8.0	≥28
b	**Optimized**	Protease	20.0	2.5	8.0	≥28
		Cellulase	233	2.5	93.3	≥28

a: The mixed buffer was used as buffer solution.
b: Ammonium phosphate was used as buffer solution.

3.7 Quality assurance

To assure that reliable measurements were made for the analyses in this study, several measures were involved, including the use of standards, quality control samples and blank samples. For all analyses, CRMs were included as control samples. The control samples were included both at the beginning and the end of each sequence for the analyses and were evaluated in terms of certified values and controls charts, when available. The control samples for the determination of total Se and other elements were ERM BB422 (fish muscle), TORT-3 (lobster hepatopancreas) and SRM 1566b (oyster tissue). For Se speciation, the CRMs ERM BC210a and SELM-1 were used as control samples. ERM BC210a (wheat flour) and SELM-1 (selenized yeast) were also applied for the optimization of the method using experimental design.

Calibration curves for each element analyzed by ICP-MS and for SeMet when performing Se speciation by HPLC-ICP-MS, were evaluated based on correlation coefficients required to be above 0.995. For the multielement determination by ICP-MS, internal standards were also added for correction of instrumental drift in the analyses (see section 3.4.2). Furthermore, blank samples (reagents without sample) were treated the same way as the samples in the extraction procedures and analysis, and used for correction of results for Se speciation.

For the method development for Se speciation, the evaluation of performance characteristics was also performed, based on definitions related to method development and validation studies (Prichard and Barwick, 2007; Eurachem, 2014; Eurachem and CITAC, 2016)". For the optimized method, intermediate precision as reproducibility, selectivity, and trueness from the measurements of ERM BC210a and SELM-1 were evaluated.

3.7.1 Intermediate precision as reproducibility

The reproducibility (R) for the speciation analysis and determination of SeMet was assessed from five subsequent days of analysis for determination of SeMet (mg Se/kg) in the CRMs ERM BC210a (wheat flour) and SELM-1 (selenized yeast). The reproducibility was calculated as RSD (%) (see section 3.9.1). The intermediate precision was further evaluated by comparing obtained RSD (%) from the reproducibility for each CRM with expected RSD (%) and acceptable RSD (%) for the analyte concentrations based on Horwitz ratio (NMKL 5, 2003).

3.7.2 Selectivity

To determine the selectivity, chromatograms of the ERM BC210a and SELM and samples of experimental diets, commercial fish feed and feed ingredients were evaluated. The chromatograms were checked for any Se signals at the retention time of the analyte, SeMet. This can provide information about any matrix

interferences in different sample types and different analyte concentrations. The selectivity was also determined by evaluation of any signals in the retention time area of SeMet in blank samples.

3.7.3 Trueness

For determining the trueness of the method, SeMet measurements of CRMs were evaluated with certified values (see section 3.1.4.3, Table 10. It was also estimated trueness based on the SeMet concentrations in experimental diets supplemented with SeMet at different concentrations, with specified supplementation levels given in Table 8 (section 3.1.4.2; (Antony Jesu Prabhu et al., 2020; Berntssen et al., 2018b)). The trueness for CRMs and experimental diets was calculated by determining the analytical recoveries (%), described in section 3.9.1.

The recovery of spiked samples was applied for evaluating the trueness of the method for commercial feed and feed ingredients. Fish feed (n = 1), fish meal (n = 1), plant meal (n = 1) and insect meal (n = 1) were spiked with 5 µg Se/L of SeMet standard after the enzymatic digestion, prior to HPLC-ICP-MS analysis. The spiked samples were analyzed together with unspiked samples, and the measured concentrations were used for calculating the trueness as spike recovery (%), described in a later section (3.9.1).

3.8 Statistics and data analysis for correlation study

3.8.1 Deviations (%) for technical replicates

Determination of Se and other elements such as As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb and Zn in whole-body and fillet samples of Atlantic salmon was performed by ICP-MS. The element concentrations were measured in the technical replicates (n = 3) of biological replicates (n = 3) of the samples fed with the same type of diet.

For each element, deviations (%) were calculated from the technical replicates (n = 3) using equation (5). The acceptable deviations (%) for the whole-body and fillet samples were set to 25% and 10%, respectively. For the method development, acceptable deviations (%) were also set to 10% for replicates for SeMet and total Se analysis in CRMs, fish feed and feed ingredients.

$$\%Deviation = \frac{x_{min} - x_{max}}{\bar{x}} \cdot 100\% \,, \qquad (5)$$

where:

- x_{min} is the minimum concentration of the replicates (mg/kg),
- x_{max} is the maximum measurement of the replicates (mg/kg),
- $\bar{x}$ is mean measurement of replicates (mg/kg).

3.8.2 Data polishing of datasets for salmon samples for statistical analysis

A data polishing was performed as a pre-treatment on datasets containing element measurements in whole-body and fillet samples (Appendix C, Table C1 and Table C2), by exclusions of extreme values (outliers). For this procedure, an additional replicate (n = 1) measured in 2017 (Berntssen *et al.*, 2018b) was added for each whole-body and fillet sample to the recent measurements (n = 3). To make all measurements within each subset comparable for the exclusion of outliers, all replicates were normalized by their respective mean prior to the outlier evaluation. The normalization was done using equation (6).

$$x_{normalized} = \frac{x}{\bar{x}}, \tag{6}$$

where:

- $x_{normalized}$ is normalized value,
- x is measured value (mg/kg),
- $\bar{x}$ is mean measurement of replicates (mg/kg).

The normalized datasets were imported to the statistical software R (Version 4.0.3) and the integrated development environment RStudio. The normalized subset for each element was visualized through normality plots, histograms and boxplots. Data points outside of the whiskers of the boxplots were identified as outliers and were excluded from further statistics. Shapiro-Wilk's test for normality (95% confidence interval) was performed for all normalized subsets, both before and after the exclusion of outliers. The exclusion was, however, not performed on subsets containing element concentrations below LOQ for the relevant element. The LOQ values for the determination of elements using ICP-MS are shown in Appendix B.

After excluding the outliers, the original non-normalized datasets were used to find mean concentrations for the technical replicates in the salmon samples (Appendix D). Further calculations were made on the biological replicates (n = 3) to find mean concentrations of the elements found in salmon fed with the same diet, assuming normality for the pooled samples (McMahan *et al.*, 2013; Zar, 1998).

3.8.3 Assessment of correlation coefficients for Se interactions in salmon samples

Polished datasets containing concentrations of elements (mg/kg ww) in the salmon samples were organized into four datasets (Datasets i, ii, iii and iv) (Appendix C, Table C6) for the determination of correlation coefficients. The datasets were organized based on the type of sample (whole-body or fillet) and the Se species supplemented to the salmon diets (inorganic or organic Se). The datasets are described

in Table 18 and were imported to R for the calculation of correlation coefficients and probability values (p-values).

Table 18: Datasets applied for the determination of correlation coefficients. All datasets contained subsets of total concentrations (mg/kg ww) of the elements Se, As, Cu, Fe, Hg, Mn and Zn.

Dataset i	Whole-body from fish fed with inorganic Se supplemented feed
Dataset ii	Whole-body from fish fed with organic Se supplemented feed
Dataset iii	Fillet from fish fed with inorganic Se supplemented feed

Pearson's correlations (parametric statistics) were applied for the normally distributed datasets of whole-body samples. For the datasets for fillet samples including non-normal distributed subsets, Pearson's correlations and Spearman's rank-order correlations (robust statistics) were applied. The two methods were applied for a comparison of the correlation coefficients for fillet samples. Pearson's correlations and Spearman's rank-order correlations were applied using the 'psych' package in R on polished datasets. Probability values (p-values) of each correlation coefficient were investigated to evaluate their significance with a 99% confidence interval. In correlation matrices, significance levels are shown as stars associated to the p-values (p-values (0, 0.001, 0.01, 0.05, 0.1, 1) <=> symbols ("***", "**", "*", ".", " ", " ")) (Revelle, 2020). The degree of correlation between Se and other elements was interpreted from the absolute magnitude of each correlation factor (r) (Rumsey, 2011; Calkins, 2005);

- $|1| < r < |0.9|$: very strongly correlated,
- $|0.9| < r < |0.7|$: strongly correlated,
- $|0.7| < r < |0.5|$: moderately correlated,
- $|0.5| < r < |0.3|$: weakly correlated,
- $|0.3| < r < 0$: not correlated.

3.9 Statistics and data analysis for Se speciation

3.9.1 Evaluation of recovery of Se and Se species

The analytical recoveries (%) for total Se and SeMet in CRMs and experimental diets were calculated using equation (7).

$$Analytical\ recovery\ (\%) = \frac{c_{measured}}{c_{target}} \cdot 100\ , \qquad (7)$$

where:

- $c_{measured}$ is measured concentration of Se (mg Se/kg) or SeMet (mg SeMet/kg),
- c_{target} is target concentration for Se (mg Se/kg) or SeMet (mg SeMet/kg).

Certified values for total Se and SeMet for the CRMs were set as target concentrations. For experimental diets, total Se concentrations in the samples determined using ICP-MS were set as target concentrations for total Se, while supplemented concentrations of SeMet were used as target concentrations for SeMet in the extracts.

The original measurements of SeMet were reported in µg Se/L, and it was necessary to convert the certified values for SeMet given in mg SeMet/kg to mg Se/kg. The concentrations of SeMet to mg Se/kg were converted using equation (8).

$$c_{Se} = c_{SeMet} \cdot \frac{M_{Se}}{M_{SeMet}}, \tag{8}$$

where:

- c_{Se} is the concentration of Se in SeMet (mg Se/kg),
- c_{SeMet} is concentration of SeMet (mg SeMet/kg),
- M_{Se} is the atomic mass of Se (g/mol),
- M_{SeMet} is the molecular mass of SeMet (g/mol),
- c_{SeMet} is concentration of SeMet (mg SeMet/kg).

For the experimental diets, concentrations of SeMet were available from the Se inclusion data and were used as target values for SeMet. The target values for the experimental diets are shown in Table 19.

Table 19: Target concentrations for SeMet (mg Se/kg) based on inclusion of SeMet in experimental diets and target concentrations for total Se (mg Se/kg) from measured concentrations in sample (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b).

Sample	Total Se concentration (mg Se/kg)*	SeMet, target concentration (mg Se/kg)
Diet A	0.38	0.15
Diet B	0.63	0.4
Diet C	0.45	0
Diet D	8.1	5
Diet E	19.4	15
Diet F	5.9	0

*Obtained from Antony Jesu Prabhu *et al.* (2020) and Berntssen *et al.* (2018b).

To calculate extraction recovery (%) for organic Se fraction of total Se in a sample, equation (9) was used.

$$Extraction\ recovery\ (\%) = \frac{c_{Organic\ Se}}{c_{Total\ Se}} \cdot 100\ , \tag{9}$$

where:

- $c_{Organic\ Se}$ is the concentration of Se in SeMet (mg Se/kg),
- $c_{Total\ Se}$ is the concentration of total Se (mg Se/kg).

Spike recovery (%) was calculated from SeMet concentrations of spiked samples (commercial fish feed and feed ingredients, the SeMet concentrations in unspiked samples and the added concentrations of SeMet. Spike recovery (%) was calculated using equation (10), where *C* is the concentration (Harris, 2010).

$$Spike\ recovery\ (\%) = \frac{C_{spiked\ sample} - C_{unspiked\ sample}}{C_{added}} \cdot 100. \quad (10)$$

For quality assurance, precision for the determination of SeMet was calculated as the relative standard deviation (RSD, %) for reproducibility by equation (11), from the mean value and standard deviation (SD).

$$\%RSD = \frac{SD}{mean} \cdot 100\%. \quad (11)$$

3.9.1.1 Se measurements in extracts

For the soluble extracts analyzed for total Se using ICP-MS (iCAP Q), the concentrations retrieved from the measurements in µg/L were converted into concentrations with unit mg/kg, which could be compared to the total Se concentrations in the original sample. Dilution factors were calculated using the mass of weighed samples (m), added volume of extraction solution to the samples (V = 5 mL) and number of dilutions (D) after digestion (D≠1 when diluted more after digestion), with the following equation:

$$Dilution\ factor = \frac{m \cdot D}{1000 \cdot V}. \quad (12)$$

Further, the dilution factors calculated using equation (12) were used for calculating the concentration in the sample with equation (13).

$$c_{sample} = \frac{c_{diluted\ sample}}{Dilution\ factor}, \quad (13)$$

where:

- c_{sample} is concentration in sample (mg/kg),
- $c_{diluted\ sample}$ is concentration measured in the diluted sample (mg/kg).

3.9.2 Experimental designs

The experimental designs used in this project were generated using the R Commander package (version 2.6-2) with an experimental design plug-in (RcmdrPlugin.DoE, version 0.12-3) for R (version 4.0.3). The order of the experiments in the experimental designs was also randomized by the same package prior to performing the experiments. Blind samples for the extractions were treated the same way as other samples for each experiment and were included in the runs. Significant effects and interactions from the SeMet and total Se results were studied by data analysis using principal component analysis (PCA), for choosing further conditions in a screening step and optimal conditions in an optimization step.

3.9.3 Principal Component Analysis

The data obtained from the screening and optimization steps were analyzed using a multivariate analysis software from Pattern Recognition Systems, Sirius 10.0, for exploratory analysis using principal component analysis (PCA), and variable analysis.

The PCA responses that were studied included i) %recovery of SeMet in ERM BC210a (wheat flour), ii) recovery (%) of total Se in ERM BC210a (wheat flour), iii) recovery (%) of SeMet in SELM-1 and iv) recovery (%) of Tot Se in SELM-1, shortened to i) “SeMet Wheat”, ii) “Tot Se Wheat”, iii) “SeMet SELM-1” and iv) “Tot Se SELM-1” for PCA.

To find potential correlations between experiments, factors and responses in the experimental designs, an exploratory analysis was performed on all responses and individual responses. Standardization was used for weighting of the responses. The correlations were interpreted based on the angles between the vectors of the factors and objects in biplots, i.e. the angle between the vectors of factor x and factor y;

- $0° < \theta < 90°$: x and y are positively correlated,
- $90° < \theta < 180°$: x and y are negatively correlated,
- $\theta = 90°$: x and y are not correlated,
- $\theta = 0°$: x and y have strong positive correlation,
- $\theta = 180°$: x and y have strong negative correlation.

Variable analysis was performed by evaluating bar graphs of regression coefficients along with the respective variable importance plots (VIP) for regression coefficients. The weightings for this analysis was set to “w=1” to get a better estimate of the factors’ significance. A potential increase or decrease of each factor and factor levels were interpreted based on the regression coefficient sign, and their significance was evaluated using VIP plots. In VIP plots factors above the line (Comp.1 = 1) were considered significant for the response. For the experiments performed in replicates, mean recoveries (%) were used for the

responses, while median recoveries (%) were used in the optimization step to evaluate any differences from mean recoveries (%) in variance analysis.

3.9.4 Sign test for commercial fish feed and feed ingredients

To evaluate the optimized method, SeMet concentrations from the optimized method and SeMet concentration obtained from analysis using the initial method were compared. The comparison between the two methods was performed by testing for significant differences in the mean concentration from polished datasets. Pre-treatment for polishing the data for commercial fish feed and feed ingredients was performed by outlier exclusion using the same procedure used for data for salmon samples, described under section 3.8.2. A two-sample sign test for non-parametric data with a 95% confidence interval was performed using the 'BSDA' package in R.

4 RESULTS AND DISCUSSION

4.1 Total Se and other elements in samples of Atlantic salmon

The correlation coefficients between Se and other elements (As, Cu, Fe, Hg, Mn and Zn) were determined for the salmon fed inorganic Se and the salmon fed organic Se, to evaluate for any differences in the interactions to Se. Many studies have studied minerals interactions to Se or interactions between other minerals than Se in salmon and salmon feed (Antony Jesu Prabhu *et al.*, 2019; Berntssen *et al.*, 2000; Fontagné-Dicharry *et al.*, 2015; Hilton, 1989; Lorentzen *et al.*, 1998; Silva *et al.*, 2019a), but less focus has been given to mineral interactions with salmon fed different Se species. The aim was to assess the correlation between Se and other elements in salmon by statistical and chemometric approaches. Further discussions regarding the biological and toxicological aspects were not made for the data in this study.

4.1.1 Deviations in the element measurements

The measurements of replicated samples showed that total element concentrations in whole-body samples ranged from 0.13 to 12 mg/kg for Se, 0.47 to 0.74 mg/kg for As, 1.1 to 2.9 mg/kg for Cu, 9.5 to 14 mg/kg for Fe, below LOQ to 0.01 mg/kg for Hg, 0.62 to 1.8 mg/kg for Mn and 13 to 52 mg/kg for Zn (Appendix C, Table C1). In fillet samples, measurements ranged from 0.11 to 12 mg/kg for Se, 0.51 to 0.89 mg/kg for As, 0.30 to 0.46 mg/kg for Cu, 1.8 to 3.8 mg/kg for Fe, 0.09 to 0.29 mg/kg for Mn, below LOQ to 0.01 mg/kg for Hg and 3.7 to 4.9 mg/kg for Zn (Appendix C, Table C2). All measurements or most of the measurements in the elements Cd, Co, Cr and Pb had concentrations below LOQ. Due to this, these elements were excluded from the dataset and were not used for further analysis.

Deviations (%) in the element measurements

The measurements for total Se and other elements in the whole-body and fillet samples of Atlantic salmon showed large deviations between technical replicates for several samples of the elements: Cd, Cr, Cu, Mn, Pb and Zn in whole-body samples and fillet samples (Appendix C, Table C3).

From the calculated deviations (%) between technical replicates (equation (5)), it was seen that the whole-body samples had replicates with deviations above the acceptable limit for whole-body samples (25%) for n = 3 (Appendix C, Table C3). The whole-body samples had deviations (%) from 0.6% up to 58%. Similar errors were seen for the fillet samples, but in this case, more samples were above the acceptable error limit (10%) with errors from 0.2% up to 85%. Some Fillet samples were chosen for reanalysis to evaluate if the high deviations were related to the extraction procedure. The calculated deviations of the reanalyzed samples (Appendix C, Table C4) showed multiple samples with deviations above 10%. The deviations (%)

for the reanalyzed fillet samples ranged from 0.2% to 59% (Table C4 in Appendix C), which resembled what was seen in the first analysis on the fillet samples (0.2-85%). This can suggest that the large deviations are likely related to the sample composition, and/or the homogenization of samples.

To cope with samples with large deviations, normalized datasets of the element measurements (Appendix C, Table C5) were used to identify extreme values in boxplots (Appendix C, Figure C1 and Figure C2). A dataset with measurements performed in fillet samples (n = 4 - 7) was made. This included the concentrations from the analysis, reanalysis and an earlier measurement from 2017 (Berntssen *et al.*, 2018b). This was done with an assumption that some of the errors between all replicates would increase when the additional replicates were added due to the separate analysis from 2017. However, the most extreme replicates were evaluated as outliers and were excluded from the datasets, while keeping the more accurate ones for the correlation analysis.

4.1.2 Data polishing prior to correlation analysis

Since normality of datasets is required for reliable evaluation of significance using Pearson's correlations, data polishing of non-normal distributed data was performed as an attempt to achieve normality, by anomaly detection (Rousseeuw and Hubert, 2017). From Shapiro-Wilk's normality tests, the distributions of the normalized subsets of the element measurements are summarized in Table 20 for the whole-body samples and the fillet samples, before and after outlier exclusions.

Table 20: Distribution of the element measurements in whole-body and fillet samples before and after outlier exclusions from Shapiro-Wilk's normality test.

Element	Whole-body samples		Fillet samples	
	Before outlier exclusions	After outlier exclusions	Before outlier exclusions	After outlier exclusions
Se	Non-normal	Normal	Non-normal	Normal
As	Non-normal	Normal	Non-normal	Normal
Cu	Non-normal	Normal	Non-normal	Normal
Fe	Non-normal	Normal	Non-normal	Normal
Hg	Normal	Normal	Non-normal	Non-normal
Mn	Normal	Normal	Non-normal	Non-normal
Zn	Non-normal	Normal	Normal	Non-normal

The results of Shapiro-Wilk's normality tests for the whole-body samples shows that normality was rejected ($p < 0.05$) for subsets of Se, As, Cu, Fe and Zn prior to outlier exclusions (Table 20). However, these elements became normally distributed (Shapiro-Wilk's normality test, $p > 0.05$) after data polishing by outlier exclusions. Thus, Pearson's correlations could be used to find correlation coefficients. For the fillet samples, normality (Shapiro-Wilk's normality test, $p > 0.05$) was rejected for all elements besides Zn prior to outlier exclusions and was also rejected for multiple elements (Hg, Mn and Zn) after the exclusion. The

p-values for the Shapiro-Wilk's normality tests before and after outlier exclusion for whole-body samples (Figure C3) and fillet samples (Figure C4) are available in Appendix C.

4.1.3 Concentrations of total Se and other elements in salmon samples

Concentrations of the elements Se, As, Cu, Fe, Hg, Mn and Zn in whole-body and fillet (Table 21) of Atlantic salmon, given as mean with standard deviation (mg/kg ww, n = 3; biological replicates). The mean concentrations were calculated from the polished datasets of the technical replicates (n = 4 to 7).

Table 21: Total concentration of elements Se, As, Cu, Fe, Hg, Mn and Zn in samples of Atlantic samples fed the different diets (mean ± SD, mg/kg ww, n = 3; biological replicates) determined by ICP-MS.

Whole-body samples							
Diets	**Se**	**As**	**Cu**	**Fe**	**Hg**	**Mn**	**Zn**
Basal (0.45)	0.15 ± 0.01	0.63 ± 0.04	1.5 ± 0.2	11.5 ± 0.6	0.0091 ± 0.0005	1.2 ± 0.1	23.1 ± 6.7
Selenite (5.4)	0.74 ± 0.03*	0.50 ± 0.03	1.4 ± 0.1	10.7 ± 0.4	0.004 ± 0.001**	1.0 ± 0.1	23.0 ± 4.0
Selenite (11)	1.4 ± 0.1*	0.64 ± 0.05	2.0 ± 0.3	10.4 ± 0.5	0.005 ± 0.0002	1.2 ± 0.1	30.3 ± 4.4
SeMet (6.2)	2.0 ± 0.1	0.64 ± 0.02	1.5 ± 0.2	11.3 ± 0.6	0.0083 ± 0.0003	1.3 ± 0.2	37.8 ± 7.3
SeMet (16)	5.8 ± 0.1	0.61 ± 0.02	1.5 ± 0.2	11.6 ± 0.5	0.0069 ± 0.0002	1.2 ± 0.3	30.7 ± 6.7
SeMet (21)	6.8 ± 0.1	0.65 ± 0.03	1.6 ± 0.4	12.6 ± 1.1	0.0074 ± 0.0005	1.2 ± 0.2	27.8 ± 7.0
SeMet (39)	9.71 ± 0.04*	0.65 ± 0.02	2.1 ± 0.7	12.8 ± 1.2	0.007 ± 0.001	1.2 ± 0.2	35.5 ± 9.4
Fillet samples							
Diets	**Se**	**As**	**Cu**	**Fe**	**Hg**	**Mn**	**Zn**
Basal (0.45)	0.121 ± 0.001	0.72 ± 0.03	0.34 ± 0.02	2.4 ± 0.2	0.009 ± 0.001	0.14 ± 0.02	4.1 ± 0.1
Selenite (5.4)	0.27 ± 0.02	0.53 ± 0.01	0.36 ± 0.02	2.3 ± 0.3	0.005 ± 0.001	0.12 ± 0.01	4.2 ± 0.1
Selenite (11)	0.33 ± 0.01	0.84 ± 0.05	0.34 ± 0.02	2.1 ± 0.2	0.0053 ± 0.0002	0.14 ± 0.02	4.3 ± 0.2
SeMet (6.2)	2.0 ± 0.1	0.68 ± 0.05	0.34 ± 0.01	2.3 ± 0.2	0.008 ± 0.002	0.13 ± 0.01	4.3 ± 0.1
SeMet (16)	6.1 ± 0.1	0.68 ± 0.04	0.37 ± 0.05	2.7 ± 0.5	0.007 ± 0.001	0.13 ± 0.02	4.3 ± 0.1
SeMet (21)	7.8 ± 0.3	0.70 ± 0.04	0.375 ± 0.002	2.4 ± 0.1	0.006 ± 0.001	0.12 ± 0.01	4.3 ± 0.2
SeMet (39)	11.5 ± 0.4*	0.79 ± 0.05	0.36 ± 0.06	2.33±0.03*	0.007 ± 0.001	0.14 ± 0.01	4.2 ± 0.2

* n = 2. ** Measurement below LOQ.

The results show that the total Se concentrations increased with increasing Se supplementation in the diet for both whole-body (0.15-9.71 mg Se/kg) and fillet samples (0.121-11.5 mg Se/kg) (Table 21). These results are consistent with what was seen in a previous study using similar samples (Berntssen *et al.*, 2018b). The Se concentrations in salmon fed with the basal diet (0.45 mg Se/kg) were 0.15 mg/kg and 0.121 mg Se/kg in the whole-body and fillet, respectively. The Se concentrations found in the fillet of farmed Atlantic salmon ranged from 0.12 to 0.25 mg/kg (n = 14) (data from 2006 to 2009, "Seafood data" database, (Institute of Marine Research, 2020c). The Se concentrations determined in the fillet samples fed the basal diet can be compared to the Se concentrations found in the fillet of farmed salmon in Norway.

Some of the other elements, such as Mn in whole-body, and Cu, Fe, Mn and Zn in fillet samples, had relatively stable concentrations for all dietary groups. This was also expected since only Se was supplemented to the diets. When comparing the element concentrations seen for the whole-body samples and the fillet samples, a clear difference can be seen in the concentrations of Fe and Zn for the two sample types (Table 21). The concentrations of Fe ranged from 10.4 to 12.8 mg/kg in whole-body samples, and from 2.1 to 2.7 mg/kg for the fillet samples. Similarly, concentrations of Zn, ranged from 23.0 to 37.8 mg Zn/kg in whole-body, and from 4.1 to 4.3 mg Zn/kg in fillet samples, which are much lower than in whole-body samples. Similar observations can also be made from the total concentrations of Cu and Mn, with slightly lower differences. The concentrations of Cu ranged from 1.4 to 2.1 mg Cu/kg in whole-body samples and from 0.34 to 0.375 mg Cu/kg in fillet samples. The concentrations of Mn ranged from 1.0 to 1.3 mg Mn/kg in whole-body samples and from 0.12 to 0.14 mg Mn/kg in fillet samples. One possible explanation for the higher total concentrations of Fe, Zn, Cu and Mn in the whole-body samples in comparison to the fillet samples, could be that the whole-body samples contain organs, such as gills and liver, while the fillet samples do not. These organs generally contain higher levels of metals (Yan and Wang, 2002; Lobos *et al.*, 2019).

For whole-body samples, the levels found for Cu (1.4 to 2.1 mg/kg) and Fe (10.4 to 12.8 mg/kg) (Table 21) were coherent with concentrations found in whole-body of post-smolt Atlantic salmon in another feeding trial (Antony Jesu Prabhu *et al.*, 2019). In the work of Antony Jesu Prabhu *et al.* (2019), the levels of Cu and Fe were 1.05 mg/kg and 12 mg/kg, respectively. The salmon of the whole-body samples compared with were fed a control diet. The final weight for the salmon was 482 ± 17 g in the study of Antony Jesu Prabhu *et al.* (2019), similar to the weight of the salmon used in this study (352 to 469 g; (Berntssen *et al.*, 2018b)). Lower concentrations of Mn and slightly higher concentrations of Zn were found in the whole-body samples in this study, in comparison to the whole-body samples fed control diet in the work of Antony Jesu Prabhu *et al.* (2019).

The concentrations of As, Fe and Zn in the fillet samples correspond with the concentrations normally seen in farmed Atlantic Salmon (fillet) ("Seafood data", (Institute of Marine Research, 2020c). The levels of Cd, Cr and Pb were below LOQ, which also correspond to the "Seafood data" database. The only differences found were in the Hg concentrations. Lower concentrations of Hg were found in the fillet samples in this study, in comparison to the database, which could be because of the higher Se levels in the salmon in this study, since Se is known to have a protective effect on the toxic Hg (Burger *et al.*, 2013) There were no data available in the database for the elements Co, Cu and Mn in farmed Atlantic Salmon (fillet).

4.1.4 Correlations between Se and other elements in Atlantic salmon

To study the correlation between Se and other elements in salmon, correlation coefficients (r = |0-1|) between elements were found using Pearson's correlations (r_p) for the whole-body samples and fillet samples. For comparison to Pearson's correlation coefficients, Spearman's Rank-order correlations (r_s) were also included for the fillet samples due to non-parametric subsets (Artusi *et al.*, 2002), to obtain more accurate identification of possible Se interactions with other elements.

The Pearson's correlations for whole-body samples fed with a) inorganic Se supplemented diets and b) organic Se supplemented diets are shown in correlation matrices in Figure 12. The correlation coefficients from Pearson's and Spearman's correlations are shown in Figure 13 for fillet samples of salmon fed inorganic Se and in Figure 14 for fillet samples of salmon fed organic Se.

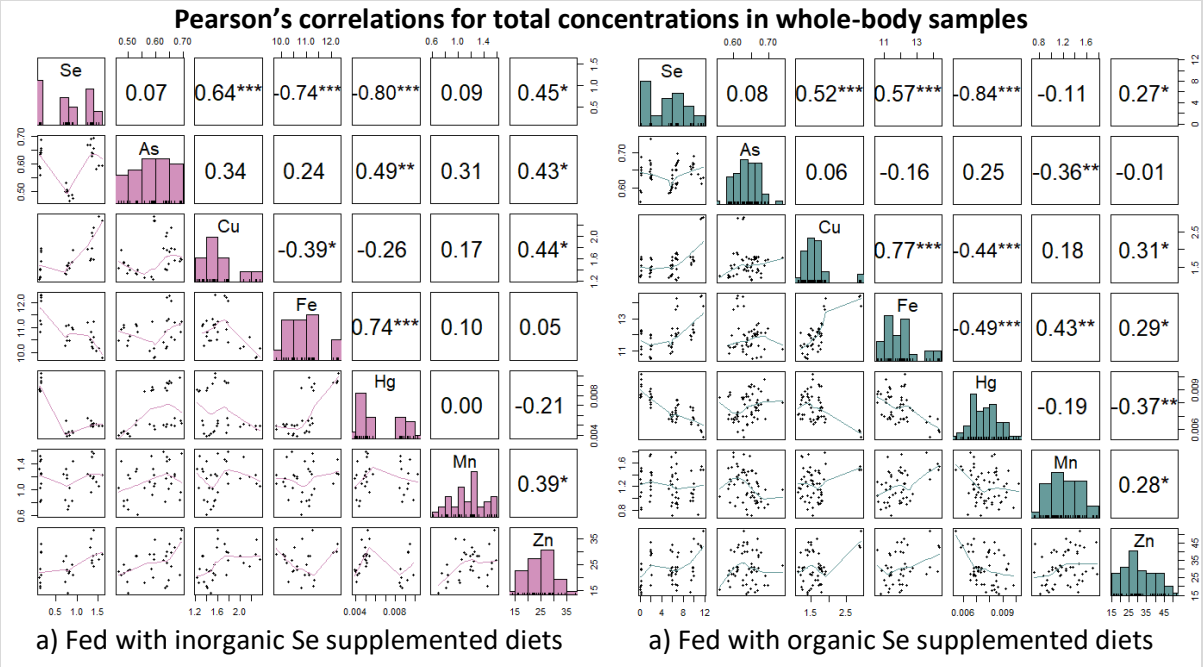


Figure 12: Correlation matrix of Pearson's correlation for the total concentrations of Se, As, Cu, Fe, Hg, Mn and Zn in whole-body of Atlantic salmon fed a) organic Se and b) inorganic Se, with correlation coefficients and significance level (p-values (0, 0.001, 0.01, 0.05, 0.1, 1) <=> symbols ("***", "**", "*", ".", " ", " "), distribution of the data and bivariate scatter plots with fitted lines.

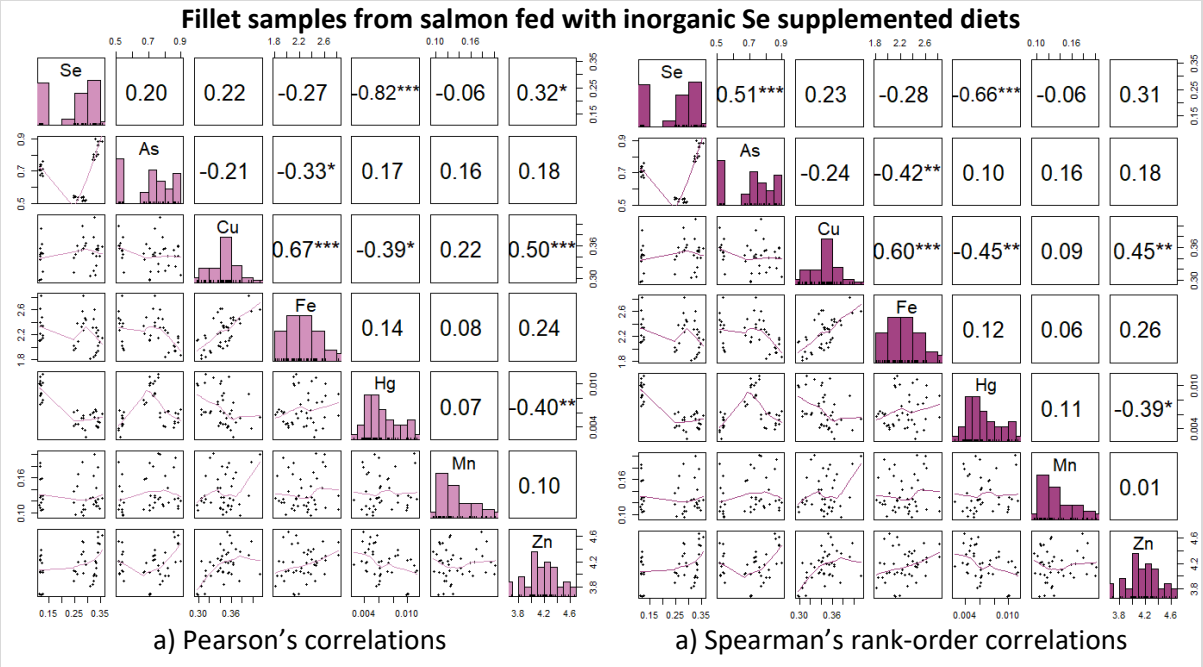


Figure 13: Correlation matrix for the total concentrations of Se, As, Cu, Fe, Hg, Mn and Zn in fillet from salmon fed with inorganic Se supplemented diets, with correlation coefficients a) Pearson's correlations and b) Spearman's rank-order correlations, significance level associated to p-values (p-values (0, 0.001, 0.01, 0.05, 0.1, 1) <=> symbols ("***", "**", "*", ".", " ", " "), distribution of the data and bivariate scatter plots with fitted lines.

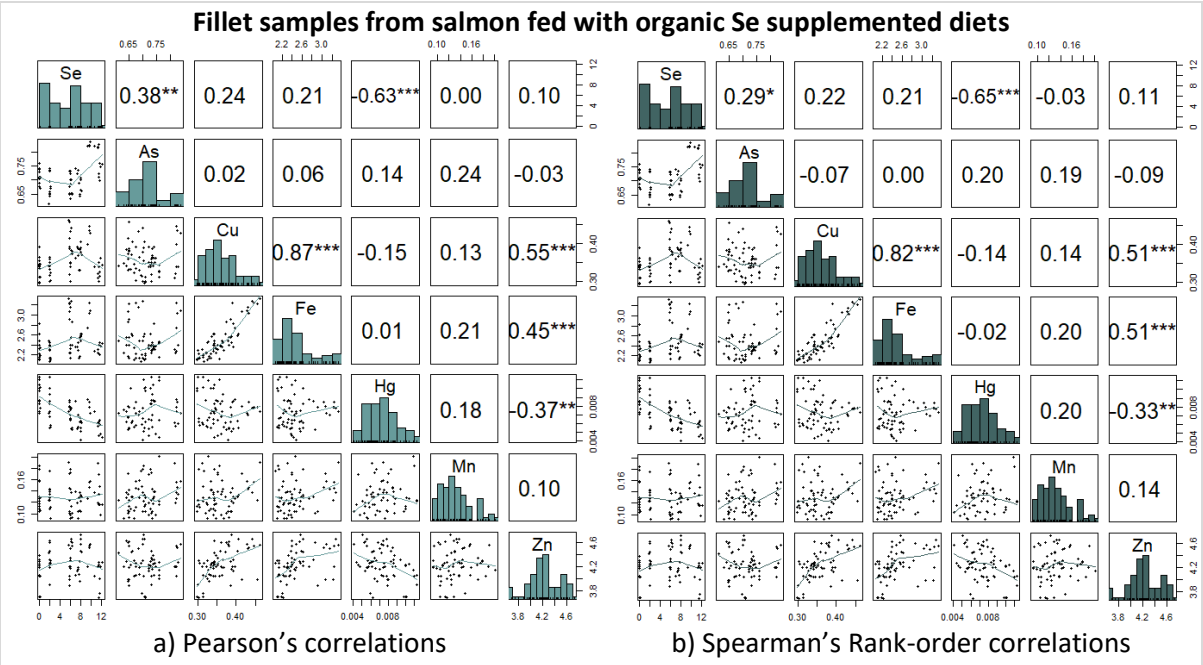


Figure 14: Correlation matrix for the total concentrations of Se, As, Cu, Fe, Hg, Mn and Zn in fillet from salmon fed with organic Se supplemented diets, with correlation coefficients a) Pearson's correlations and b) Spearman's rank-order correlations, significance level associated to p-values (p-values (0, 0.001, 0.01, 0.05, 0.1, 1) <=> symbols ("***", "**", "*", ".", " ", " "), distribution of the data and bivariate scatter plots with fitted lines.

Correlation coefficients in whole-body samples of Atlantic salmon

The correlation matrices for the whole-body samples (Figure 12) show few correlations between Se and other elements when fed with diets supplemented with inorganic Se (a) and organic Se (b). The main similarities seen between the two datasets are the strong negative correlations for Se and Hg with r_p=-0.80 (p = 0.00) in whole-body of salmon fed inorganic Se and r_p = -0.84 (p = 0.00) for whole-body of salmon fed organic Se. Another interaction was also seen between Se and Cu, with moderate positive correlations with r_p = 0.64 (p = 0.00) for whole-body of salmon fed inorganic Se and r_p = 0.52 (p = 0.00) for whole-body fed organic Se. These correlation coefficients were highly significant (***). This indicates that there are interactions between Se and Hg, and between Se and Cu. This means that there is an antagonist interaction between Se and Hg and a synergistic interaction between Se and Cu in the whole-body of Atlantic salmon. The interaction between Se and Cu that was seen in the results has also been observed in the liver of rainbow trout (*Oncorhynchus mykiss*) and in wild and farmed salmon in earlier studies (Hilton, 1989; Hilton and Hodson, 1983; Julshamm and Utne, 1985; Poppe *et al.*, 1986).

Furthermore, a strong negative correlation (r_p = -0.74, p = 0.00) can be seen for Se and Fe in whole-body of salmon fed inorganic Se. For the whole-body of salmon fed organic Se, the Se and Fe interaction had instead a moderate positive correlation (r_p = 0.57, p = 0.00). This could indicate that organic Se could interact differently with Fe in salmon than how inorganic Se would. The remaining elements, As and Mn, were considered not correlated to Se in whole-body of salmon fed with either inorganic or organic Se, with r_p = 0.07 (p = 1; Figure 12.a) and r_s = 0.08 (p = 1; Figure 12.b) for As, and r_p = 0.09 (p = 1; Figure 12.a) and r_s = -0.11 (p = 1; Figure 12.b) for Mn. A weak correlation was seen for Se and Zn in whole-body of salmon fed inorganic Se (r_p = 0.45, p = 0.22), while there was no correlation between Se and Zn in whole-body of salmon fed organic Se (r_p = 0.27, p = 0.47).

Correlation coefficients in fillet samples of Atlantic salmon

The correlation matrices for the fillet samples (Figure 13 and Figure 14) also showed moderate to strong negative correlation between Se and Hg for salmon fed inorganic Se (r_p = -0.82, p = 0.00 (Figure 13.a) and r_p = -0.63, p = 0.00 (Figure 14.a)). Similarly, a moderate negative correlation was seen for Se and Hg in the fillets of salmon fed organic Se (r_s = -0.66, p = 0.00 (Figure 13.b) and r_s = -0.55, p = 0.00 (Figure 14.b)), which also corresponds well with the whole-body samples. For the fillet samples, all correlation coefficients for Se and Hg were highly significant (***) as well. The results of Se and Hg interaction in these data is in coherence with results from other studies where Se have been shown to be against the toxic effects of Hg in fish (Zhang *et al.*, 2019; Burger *et al.*, 2013), and that Hg may also be protective against toxic effects of Se (Burger *et al.*, 2013; Sørmo *et al.*, 2011; Vukšić *et al.*, 2018).

For the fillet samples of salmon fed inorganic Se (Figure 13), no correlation (r_p = 0.20, p = 1) and moderate positive correlation (r_s = 0.51, p = 0.02) were seen between Se and As. For fillet of salmon fed organic Se (Figure 14), weak positive correlation (r_p = 0.38 (p = 0.03)) and no correlation (r_s = 0.29 (p = 0.33)) were seen between Se and As. Based on Spearman's Rank-order correlations, this would indicate that inorganic Se (selenite) supplementation could lead to a slight increase in As concentration in the salmon fillet. One explanation for the higher correlation coefficient of inorganic Se to As compared to organic Se to As. The interaction of Se with As has been proposed by another study, as they revealed that Se can decrease the toxicity of As, and that As can decrease the levels of Se (Sun *et al.*, 2014), that. Several studies have been made to understand the toxic effects of As and Se, but the topic needs to be further investigated (Ali *et al.*, 2020).

The other elements, Cu, Fe, Mn and Zn were considered not correlated to Se in the fillet samples with r =|0-0.3|for both approaches and diet types. The elements Fe and Cu were considered more correlated to Se in the whole-body in general, which might be explained by this sample type containing organs such as liver and kidneys, which typically contain higher levels of these elements (Yan and Wang, 2002; Lobos *et al.*, 2019).

Pearson's correlations vs Spearman's rank-order correlations

When comparing Pearson's correlations with Spearman`s rank-order correlations, some differences could be seen for the correlation coefficients. When using Spearman's rank-order correlations a change in the correlations for Se with some of the elements were seen, i.e., going from no correlation (r_p = 0.20, p = 1) to moderate positive correlation (r_s = 0.51, p = 0.02) for Se-As, and from strong negative (r_p = -0.82, p = 0.00) to moderate negative correlation (r_s = -0.66, p = 0.00) for Se-Hg in salmon fed inorganic Se (Figure 13). In fillet of salmon fed organic Se (Figure 14), weak positive correlation (r_p = 0.38, p = 0.03) changed to no correlation (r_s = 0.29, p = 0.33) for Se-As.

Figure 14 shows heavily tailed distributions for some of the elements (i.e. Cr, Hg and Mn) that were not normally distributed, while the distributions of all subsets for the included elements in whole-body were normal. Even though Pearson's correlations were applied to these datasets, it is often stated that Pearson's correlation requires normality (Hauke and Kossowski, 2011), and that non-normality may reduce the performance when using parametric statistics (Bishara and Hittner, 2012). This means that Pearson's is suitable for assessing correlation coefficients for elements in whole-body samples, but may not be the most suitable method for assessing correlations coefficients in fillet samples of salmon fed either inorganic or organic Se.

The other method applied to the fillet samples data, Spearman's Rank-order correlation, is typically used for non-parametric datasets (Artusi *et al.*, 2002). It is also known to be a more robust type for heavily tailed distributions (de Winter et al., 2016; Croux and Dehon, 2010). Based on this, Spearman's rank-order correlations would be the preferred method for assessing correlation coefficients in the fillet samples of salmon fed either inorganic or organic Se.

Since the same type of correlation method was chosen within the same type of sample, the element interactions found in salmon fed inorganic Se can be compared with the element interactions found in salmon fed organic Se. On the other hand, the correlation method more suitable for whole-body samples was different from the correlation method more suitable for fillet samples. Due to this, a comparison between the correlation coefficients in the whole-body samples and the fillet samples could be misleading without using the same method. A possibility for achieving comparable results could be to apply Spearman's rank-order correlation on the normally distributed datasets of the whole-body samples. However, this was not performed since there are no assumptions of normality for non-parametric methods (Artusi *et al.*, 2002). Performing a non-parametric method on normally distributed data is therefore assumed to give inaccurate results. For further studies of the biological aspects of the element interactions, the best option would be to compare the element interactions only within the same type of salmon sample, when using different approaches for determining correlation coefficients.

4.2 Method development for Se speciation

4.2.1 The selected factors for testing

With an aim to increase the extraction recovery for SeMet in fish feed and feed ingredients, the factors decided to be included were i) the solution of buffer (X1), ii) a pre-extraction step using papain (X2) and iii) the type of enzyme applied for the main enzymatic digestion (X3).

The buffers that were tested in this study was i) 1 mM ammonium phosphate, pH 7, and ii) a mixed buffer, composing of a mixed solution of $NaPO_4$, sodium citrate, CaCl and SDS. The mixed buffer has previously been included in an extraction method for Se speciation in cattle feeds and beef samples (Oliveira *et al.*, 2016). The extraction method in the work of Oliveira *et al.* (2016) was optimized for the organic Se species SeMet, $SeCys_2$ and selenomethionine-Se-oxide (SeOMet). Due to this, the mixed buffer was chosen for testing, along with ammonium phosphate, which has been used in the work of Sele *et al.* (2018a).

Previous studies have shown that papain can be applied for the enzymatic hydrolysis of allergenic proteins in wheat flour, gluten (Chen *et al.*, 2012) and gliadin (Li *et al.*, 2016). Papain is also one of the most common enzymes applied for modification of wheat or gluten protein in studies of free amino acid (FAA) and for reducing allergenic effects from the proteins (Yang and McCalla, 1968). Since wheat is commonly present in fish feed (Aas *et al.*, 2019; Ytrestøyl *et al.*, 2015), it was considered relevant to test papain for Se speciation in feed. Papain has also previously been used as a pre-extraction step for Se speciation tissues of bay scallops (*Argopecten irradians*), where it was selected due to the enzyme's broad specificity to break peptide bonds, especially into amino acids with side chains (Amri and Mamboya, 2012). The enzyme was used in a pre-extraction step prior to main enzymatic digestion, where a combination of flavourzyme, carboxypeptidase and trypsin was applied (Zhang and Yang, 2014). In the work of Zhang and Yang (2014), it was concluded that SeMet was completely released in the sample matrix with SeMet recoveries ranging from 92 to 103%, and it was therefore of interest to test papain as a pre-extraction step, prior to other enzymes in this study.

Based on a literature search, combining enzymes could provide higher extraction efficiency for Se speciation (Zhang and Yang, 2014; Cuderman et al., 2010; Mounicou et al., 2009; Wang et al., 2013; Gao et al., 2018; Oliveira et al., 2016). Enzymes that have been tested in combination with other enzymes for Se speciation include proteases such as protease XIV (Cuderman *et al.*, 2010; Mounicou *et al.*, 2009; Cubadda *et al.*, 2010; Gao *et al.*, 2018; Mellano *et al.*, 2013; Fang *et al.*, 2009; Oliveira *et al.*, 2016; Sele *et al.*, 2018a), α-amylase (Cuderman *et al.*, 2010; Wang *et al.*, 2013; Cubadda *et al.*, 2010; Fang *et al.*, 2009) and cellulase (Cuderman *et al.*, 2010; Mounicou *et al.*, 2009; Casiot *et al.*, 1999). Also, protamex (protease from *Bacillus* sp.) has been shown prominent in releasing amino acids in a study for nutritional and sensory qualities in

peanut meal (Su *et al.*, 2011). In the study of Su *et al.* (2011), protamex was used for reducing undesirable proteins in peanuts. Based on this literature search, protease XIV, protamex, α-amylase and cellulase were selected for the screening step of the method development. Sonication by an ultrasonic bath or a probe was also considered, as sonication has been frequently applied in other Se speciation studies (Cubadda *et al.*, 2010; Fang *et al.*, 2009; Mellano *et al.*, 2013; Mounicou *et al.*, 2009; Wang *et al.*, 2013; Xiao *et al.*, 2017; Xiao *et al.*, 2021; Zhang and Yang, 2014). This step was not tested in this project due to limited time.

The CRMs ERM BC210a (wheat flour) and SELM-1 (selenized yeast) are certified for Se and SeMet and were applied for the method development. There are no commercially available CRMs for Se and Se species in fish feed. ERM BC210a (wheat flour) and SELM-1 are, to our knowledge, the only CRMs commercially available with certified values for total Se and SeMet. Since fish feed generally contains plant ingredients, including wheat (Aas *et al.*, 2019; Ytrestøyl *et al.*, 2015) the CRM ERM BC210a is a relevant sample type for method development and quality assurance. SELM-1 is a CRM commonly used for quality control in Se speciation analysis (Bierla *et al.*, 2018; Oliveira *et al.*, 2016; Pedrero and Madrid, 2009; Sele *et al.*, 2018a; Vu *et al.*, 2018; Xiao *et al.*, 2017; Xiao *et al.*, 2021; Zhang and Yang, 2014; Zhou *et al.*, 2018)

4.2.2 Screening for relevant factors

The concentrations obtained for total Se (mg/kg ww) and SeMet (mg/kg ww) from the screening step for the CRMs, ERM BC210a (wheat flour) and SELM-1 (selenized yeast), are shown in Table 22 (experiment A) and Table 23 (experiment B) with the number of experiments in standard order. The responses are presented as total Se ("TotSe") recovery (%) and SeMet recovery (%) in ERM BC210a ("Wheat") and SELM-1, calculated from the measured values and certified values (n = 1). Experiment (exp) A1 and B1 are given as a mean ± SD (n = 3).

Table 22: Experiment A; 2^3 full factorial design for screening, number of experiments, factors and levels with codes, and concentrations of total Se (mg/kg ww) and SeMet (mg/kg ww), and the calculated recoveries (%) in ERM BC210a and SELM-1**.** Recoveries (%) were calculated using equation (7).

				Wheat flour		SELM-1	
Exp	**X1 (Buffer)**	**X2 (Pre-extraction)**	**X3 (Enzyme)**	**Total Se (mg/kg ww)**	**SeMet (mg/kg ww)**	**Total Se (mg/kg ww)**	**SeMet (mg/kg ww)**
A1 (n = 3)	- (AmPh)	- (no)	- (protease)	19.3 ± 0.7	6.6 ± 0.3	2140 ± 73	954 ± 65
A2	+ (Mix)	- (no)	- (protease)	15.9	6.8	2062	1001
A3	- (AmPh)	+ (yes)	- (protease)	18.5	5.4	2202	915
A4	+ (Mix)	+ (yes)	- (protease)	17.5	5.1	1947	941
A5	- (AmPh)	- (no)	+ (protamex)	17.3	0.4	2005	39
A6	+ (Mix)	- (no)	+ (protamex)	22.6	0.9	2041	56
A7	- (AmPh)	+ (yes)	+ (protamex)	20.5	3.3	2259	224
A8	+ (Mix)	+ (yes)	+ (protamex)	19.6	2.1	2147	219

				Responses as recovery (%)			
Exp	**X1 (Buffer)**	**X2 (Pre-extraction)**	**X3 (Enzyme)**	**TotSe Wheat A**	**SeMet Wheat A**	**TotSe SELM-1 A**	**SeMet SELM-1 A**
A1 (n = 3)	- (AmPh)	- (no)	- (protease)	112 ± 4	60 ± 3	105 ± 4	74 ± 5
A2	+ (Mix)	- (no)	- (protease)	92	61	102	78
A3	- (AmPh)	+ (yes)	- (protease)	107	49	108	71
A4	+ (Mix)	+ (yes)	- (protease)	101	47	96	73
A5	- (AmPh)	- (no)	+ (protamex)	100	4	99	3
A6	+ (Mix)	- (no)	+ (protamex)	131	9	100	4
A7	- (AmPh)	+ (yes)	+ (protamex)	119	30	111	17
A8	+ (Mix)	+ (yes)	+ (protamex)	114	19	106	17

Table 23: Experiment B; 2^3 full factorial design for screening, number of experiments, factors and levels with codes, and concentrations of total Se (mg/kg ww) and SeMet (mg/kg ww), and the calculated recoveries (%) in ERM BC210a and SELM-1**.** Recoveries (%) were calculated using equation (7).

				Wheat flour		SELM-1	
Exp	**X1 (buffer)**	**X2 (Pre-extraction)**	**X3 (enzyme)**	**Total Se (mg/kg ww)**	**SeMet (mg/kg ww)**	**Total Se (mg/kg ww)**	**SeMet (mg/kg ww)**
B1 (n = 3)	- (AmPh)	- (no)	- (α-amylase)	14 ± 7	3.3 ± 0.5	1703 ± 277	301 ± 79
B2	+ (Mix)	- (no)	- (α-amylase)	16	0.1	1709	201
B3	- (AmPh)	+ (yes)	- (α-amylase)	19	3.8	2014	396
B4	+ (Mix)	+ (yes)	- (α-amylase)	19	2.7	2094	353
B5	- (AmPh)	- (no)	+ (cellulase)	13	3.3	563	99
B6	+ (Mix)	- (no)	+ (cellulase)	19	1.8	483	30
B7	- (AmPh)	+ (yes)	+ (cellulase)	18	4.3	1805	568
B8	+ (Mix)	+ (yes)	+ (cellulase)	19	2.4	2019	415

				Responses as recovery (%)			
Exp	**X1 (buffer)**	**X2 (Pre-extraction)**	**X3 (enzyme)**	**TotSe Wheat**	**SeMet Wheat**	**TotSe SELM-1**	**SeMet SELM-1**
B1 (n = 3)	- (AmPh)	- (no)	- (α-amylase)	80 ± 41	30 ± 5	84 ± 14	23 ± 6
B2	+ (Mix)	- (no)	- (α-amylase)	94	0.9	84	16
B3	- (AmPh)	+ (yes)	- (α-amylase)	107	35	99	31
B4	+ (Mix)	+ (yes)	- (α-amylase)	108	25	103	27
B5	- (AmPh)	- (no)	+ (cellulase)	73	30	28	8
B6	+ (Mix)	- (no)	+ (cellulase)	111	16	24	2
B7	- (AmPh)	+ (yes)	+ (cellulase)	107	39	89	44
B8	+ (Mix)	+ (yes)	+ (cellulase)	111	22	99	32

The total Se concentrations in ERM BC210a were measured from 15.9 to 19.6 mg Se/kg in experiment A. The experiment with the highest recovery of total Se for ERM BC210a was A6 (ammonium phosphate as buffer, no pre-extraction and protease as enzyme). The highest total Se concentrations for SELM-1, with 2259 mg Se/kg, were obtained by using ammonium phosphate with pre-extraction, and protamex as enzyme (exp A7). In experiment A (Table 22) the SeMet concentrations measured in ERM BC210a ranged from 0.4 to 6.8 mg Se/kg. In SELM-1, the measured SeMet concentrations ranged from 39 to 1001 mg Se/kg. The highest concentrations of SeMet in ERM BC210a and SELM-1 were measured in exp A2 at 6.8 and 1001 mg Se/kg.

For the ERM BC210a in experiment B, total Se concentrations measured were from 13 to 19 mg Se/kg. The measured total Se concentrations ranged from 1947 to 2259 mg Se/kg in experiment A and from 483 to 2094 mg Se/kg in experiment B for SELM-1. For experiment B, the highest total Se concentrations were measured from exp B8 (mixed solution as buffer, with pre-extraction and cellulase as enzyme) for ERM BC210a (19 mg Se/kg) and SELM-1 (2019 mg Se/kg). SeMet concentrations measured in ERM BC210a ranged from 0.1 to 4.3 mg Se/kg in experiment B (Table 23). In SELM-1, the measured SeMet concentrations ranged from 30 to 568 mg Se/kg in experiment B. In experiment B, exp B7 (ammonium phosphate as buffer, with pre-extraction and cellulase as enzyme) gave the highest SeMet concentrations in ERM BC210a at 4.3 mg Se/kg and in SELM-1 at 568 mg Se/kg. This shows that the experiments that gave the highest total Se measurements did not give the highest measurements of SeMet.

Clear differences between using protease and protamex for the main extraction are seen in experiment A (Table 22). Experiment A shows that the lowest SeMet recoveries were obtained by using protamex (4-30%), while the highest SeMet recoveries were from using protease (42-61%), which suggest that protease is the most effective enzyme. In contrast, the experiments in experiment B (Table 23) gave lower SeMet recoveries in general at 25 ± 12% in ERM BC210a and 23 ± 14% in SELM-1, compared to 35 ± 23% in ERM BC210a and 42 ± 35% in SELM-1 in experiment A. The total Se recoveries were also lower for SELM-1 in experiment B (76 ± 32%) than in experiment A (103 ± 5%). This also suggests that protease is the most effective enzyme in general for increasing total Se and SeMet recoveries.

4.2.3 Optimization

Table 24 shows the measured concentrations of total Se and SeMet in CRMs, ERM BC210a and SELM-1 using 2^2 full factorial design for optimization with the factors pre-extraction (X2) and enzyme (X3). The concentrations of total Se in extracts and SeMet in extracts (mg/kg, mean ± SD, n = 3) and the recoveries (%) are shown in Table 24.

Table 24: Experiment C; 2^2 full factorial design for optimization, number of experiments, factors and levels with codes, and concentrations of total Se (mg/kg ww) and SeMet (mg/kg ww), and recoveries (%) in ERM BC210a and SELM-1 (mean ± SD, n = 3). Recoveries (%) were calculated using equation (7).

			Wheat flour		SELM-1	
Exp	**X2 (Pre-extraction)**	**X3 (Enzyme)**	**Total Se (mg/kg ww)**	**SeMet (mg/kg ww)**	**Total Se (mg/kg ww)**	**SeMet (mg/kg ww)**
1	- (no)	- (protease)	18.9 ± 0.7	4.2 ± 0.8	2174 ± 41	986 ± 15
2	+ (yes)	- (protease)	17.8 ± 0.4	5.8 ± 0.3	2230 ± 89	1008 ± 37
3	- (no)	+ (combined)	17.7 ± 0.2	7.3 ± 0.3	2163 ± 66	1010 ± 33
4	+ (yes)	+ (combined)	18.3 ± 0.4	5.9 ± 0.2	2147 ± 43	997 ± 13

			Responses as recoveries (%)			
Exp	**X2 (Pre-extraction)**	**X3 (Enzyme)**	**TotSe Wheat**	**SeMet Wheat**	**TotSe SELM-1**	**SeMet SELM-1**
1	- (no)	- (protease)	109 ± 4	38 ± 7	107 ± 2	77 ± 1
2	+ (yes)	- (protease)	103 ± 2	53 ± 3	110 ± 4	78 ± 3
3	- (no)	+ (combined)	103 ± 1	66 ± 3	106 ± 3	79 ± 3
4	+ (yes)	+ (combined)	106 ± 2	53 ± 2	106 ± 2	78 ± 1

The SeMet recoveries in ERM BC210a and SELM-1 ranged from 38 to 79 % recovery (Table 24) and the data analysis on these responses were applied for choosing the optimal conditions. The highest SeMet recoveries were in SELM-1, ranging from 77 to 79 %, while the SeMet recoveries in ERM BC210a ranged from 38 to 66 %. Even though the SeMet recoveries were lower in the ERM BC210a, both data analyses were considered individually, with a focus on ERM BC210a, due to the high composition of plant-based ingredients in diets the developed method would be applied for. Further optimization using response surface designs was not performed due to the qualitative (nominal) factor levels.

4.2.4 PCA for the screening

Analysis using PCA was performed separately for experiment A and experiment B, using the calculated recoveries for total Se and selenomethionine in the screening design as responses.

4.2.4.1 All responses for the screening

Biplots with all four responses, “TotSe Wheat”, “TotSe SELM-1”, “SeMet Wheat” and “SeMet SELM-1”, included in the analysis for screening the factors in experiment A and experiment B, are shown in Figure 15.

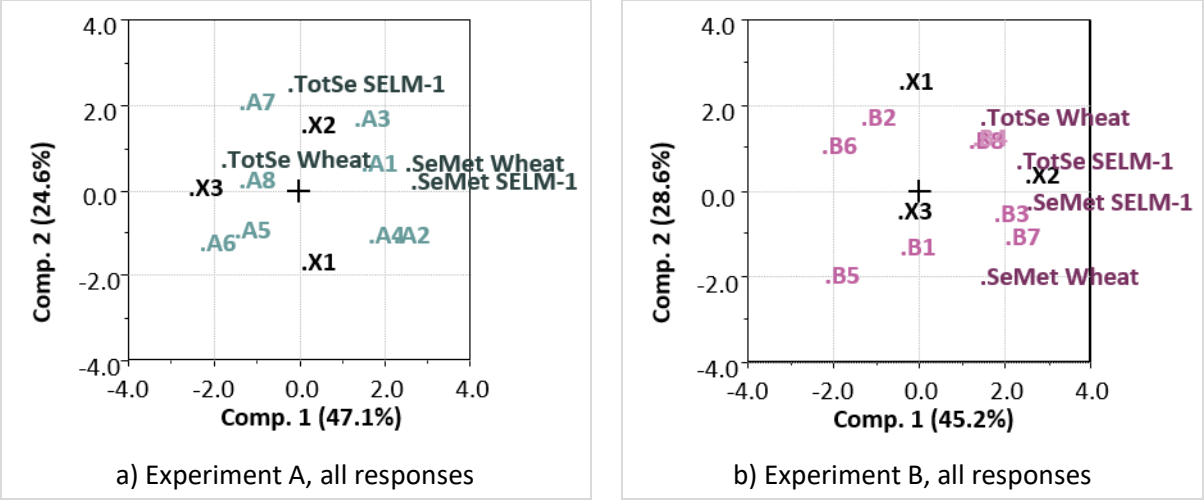


Figure 15: Biplots (Comp. 1 vs 2) showing the relations between experiments (1-8) and variables for factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3), and all responses "TotSe Wheat", "TotSe SELM-1", "SeMet Wheat" and "SeMet SELM-1" for screening.

Experiment A (All responses)

For experiment A (Figure 15.a), one can clearly see that the experiments are placed (exp A1-A8) in two groups separated by the first principal component (PC1). The experiments including protamex as enzyme (exp A5-A8) are on the left side while experiments with protease as enzyme are on the right side (exp A1-A4). The two responses for SeMet are basically explained by PC1, and the factor "enzyme" X3 has the most extreme value along this PC, with a negative sign. This indicates that choice of enzyme (X3) has a significant effect on recoveries in experiment A. A clear negative correlation between the choice of enzyme (X3) to the SeMet recoveries and positive correlation to total Se recovery in ERM BC210a can also be seen in experiment A. From this, protease (-) should be applied to increase SeMet recoveries in both CRMs. Furthermore, using protamex (+) as enzyme will increase total Se recovery in ERM BC210a.

It is also shown that the choice of enzyme (X3) is not correlated to total Se recovery in SELM-1, which means that this choice will not affect total Se recovery in SELM-1. Other than the choice of enzyme, the buffer and pre-extraction will not affect any SeMet recoveries in the CRMs or total Se recovery in ERM BC210a, based on the low correlation. For increasing total Se recovery in SELM-1 using ammonium phosphate as buffer and including pre-extraction in the extraction procedure is preferred, since these factors are negatively correlated ("buffer") and positively correlated ("pre-extraction") to the recovery of total Se in SELM-1.

Experiment B (All responses)

In experiment B (Figure 15.b), including a pre-extraction step had a clear effect on both total Se and SeMet recoveries in the CRMs. In experiment A, including a pre-extraction step did not affect the SeMet recoveries in general. The other factors, "buffer" (X1) and "enzyme" (X3) are less correlated to the total Se responses

and are negatively correlated to the SeMet responses in experiment B. This indicates that ammonium phosphate for buffer is preferred to increase SeMet recovery in ERM BC210a, even though ammonium phosphate could give a decrease of total Se recovery in ERM BC210a. This also means that "Mix" as buffer will increase total Se recovery in ERM BC210a and decrease the SeMet recovery in ERM BC210a by using the conditions used in experiment B. The choice of enzyme (X3) in experiment B, seems to be weakly positively correlated to the SeMet recovery in ERM BC210a. The choice of enzyme is not correlated with SeMet recovery in SELM-1 and is negatively correlated to the total Se responses in ERM BC210a and SELM-1. This means that having α-amylase (-) as enzyme is preferred to increase total Se recoveries, and cellulase (+) is preferred to increase SeMet recoveries.

Experiment A and B (all responses)

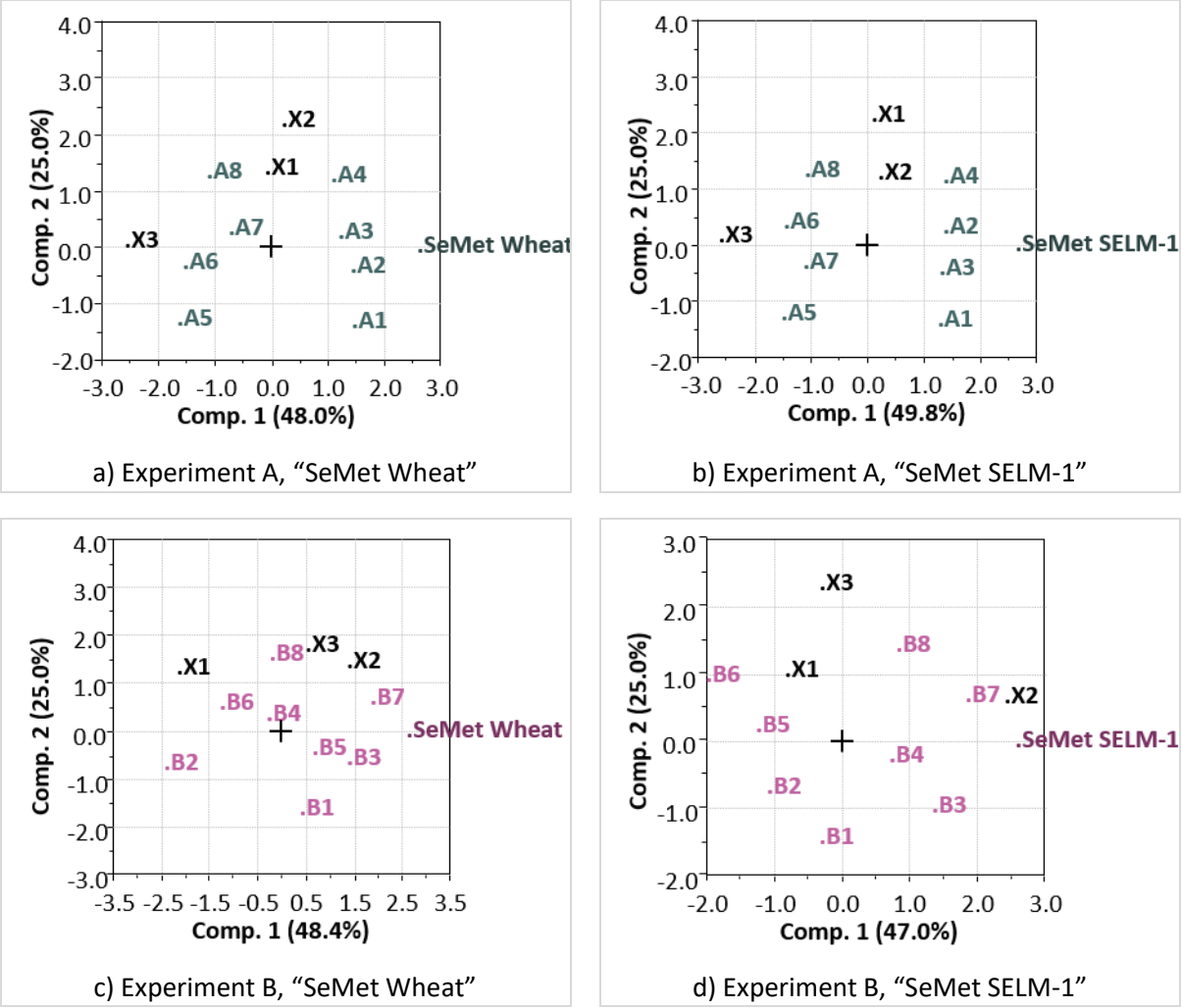
The PCA with all responses shows that different conditions have different effects on different responses. Conditions that may increase one response may decrease another. The analysis indicates that ammonium phosphate should be used as buffer, pre-extraction should be applied, and the enzymes protease and cellulase would be preferred for increasing SeMet recoveries.

4.2.4.2 Individual responses for the screening

For a better understanding of which factors that affect each response, interpretation of PCAs on individual responses of the total Se and SeMet recoveries were also performed. However, the main interpretation of the screening was performed through PCA with individual responses of the SeMet recoveries, since these were the desired recoveries to improve for method development.

The biplots and bar graphs of regression coefficients with variable importance plots (VIP) from PCA performed on total Se recoveries can be found in Appendix F. Similarly to the results from the PCA with all four responses, the PCAs with individual responses for total Se recoveries showed that other conditions were beneficial for increasing total Se recovery than what was seen for SeMet.

From the PCAs of SeMet as responses, the biplots with one response ("SeMet Wheat" or "SeMet SELM-1") for screening the factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3) with the eight experiments in experiment A and B are shown in Figure 16.



a) Experiment A, "SeMet Wheat"

b) Experiment A, "SeMet SELM-1"

c) Experiment B, "SeMet Wheat"

d) Experiment B, "SeMet SELM-1"

Figure 16: Biplots (Comp. 1 vs 2) showing the relations between experiments (1-8) and variables for factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3), and the individual responses for "SeMet Wheat" (SeMet recovery in ERM BC210a) and "SeMet SELM-1" (SeMet recovery in SELM-1) for screening.

Experiment A (SeMet recovery as responses)

Figure 16.a-b from experiment A shows a group of experiments to the left with protamex (+) as enzyme (exp A5-A8), and another group to the right using protease (-) as enzyme (exp A1-A4). This observation was also seen in the PCA using all responses, which means that using protease (-) will increase SeMet recoveries, since these experiments were closer to the SeMet responses in the biplot (Figure 16.a-b). For the factor for enzyme (X3), it is shown that protease (-) increases SeMet recovery in ERM BC210a and SELM-1 in experiment A (Figure 16.a-b). The experiments that were the most correlated to SeMet recoveries in the CRMs in experiment A (Figure 16.a-b), are experiment A3 and A2. These are experiments with protease as enzyme, but with different types of buffer, with or without pre-extraction.

Experiment B (SeMet recovery as responses)

In experiment B, groups can also be found (Figure 16.c-d), but these are grouped based on different conditions for ERM BC210a and are unclear for SELM-1. The experiments are separated by the type of buffer (X1) for SeMet recovery in ERM BC210a in experiment B (Figure 16.c). Exp B1, B3, B5 and B7 are closer to "SeMet Wheat". This shows that using ammonium phosphate as buffer will increase SeMet recovery in ERM BC210a. For experiment B (Figure 16.c-d) it is seen that cellulase (+) could be the best choice for increased SeMet recovery in ERM BC210a. Exp B7 and B3 are the ones most correlated to the SeMet recoveries in the CRMs and they are both experiments with ammonium phosphate including a pre-extraction step.

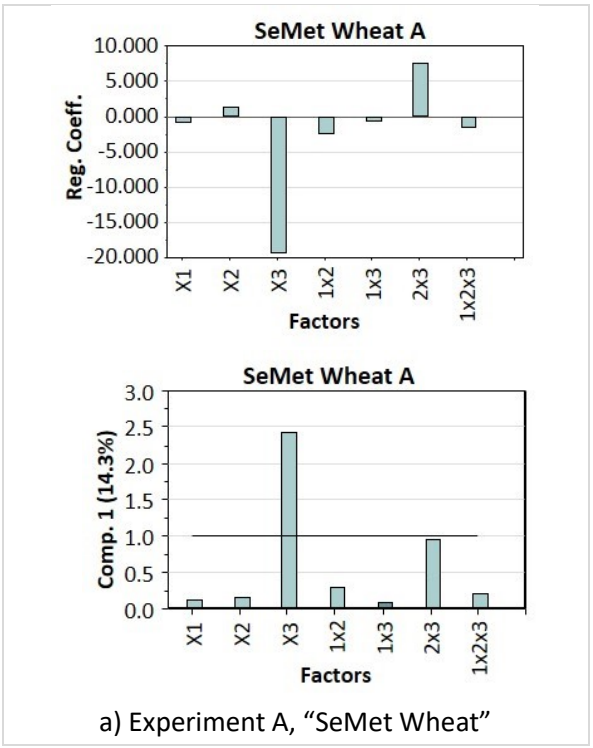
Experiment A and B (SeMet recovery as responses)

An observation that could be made from both experiment A and B, is that SeMet recoveries in the CRMS can be increased by including a pre-extraction step. The choice of buffer seem to be differently correlated to the responses in experiment A (Figure 16.a-b) with a slightly positive correlation, compared to experiment B (Figure 16.c-d) with a slight negative correlation. This means that using the mixed buffer could increase the SeMet recovery with the conditions in experiment A, and using ammonium phosphate could increase the SeMet recovery using the conditions in experiment B.

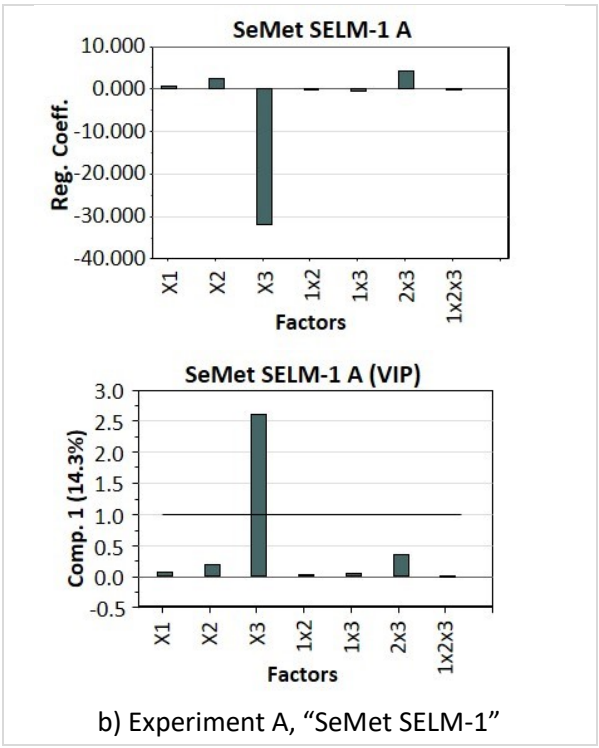
In summary, the biplots show that protease is the most effective enzyme for increased SeMet recoveries in both ERM BC210a and SELM-1, while ammonium phosphate and pre-extraction with cellulase as main enzyme also is beneficial. Nevertheless, these considerations are only based on the biplots and the significance of each factor must be established with statistical analysis for final conclusions.

Regression coefficients and VIP (SeMet recovery as responses)

Bar graphs of regression coefficients for the main factors and interactions for screening with one response of SeMet, "SeMet Wheat" and "SeMet SELM-1", are shown in Figure 17 for experiment A and in Figure 18 for experiment B. Figure 17 and Figure 18 also include variable importance plots (VIP) for the same factors and respective interactions.

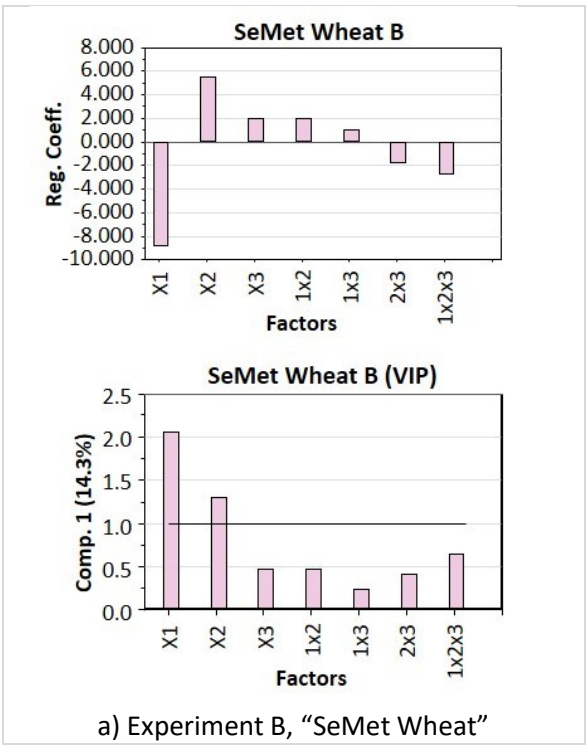


a) Experiment A, "SeMet Wheat"

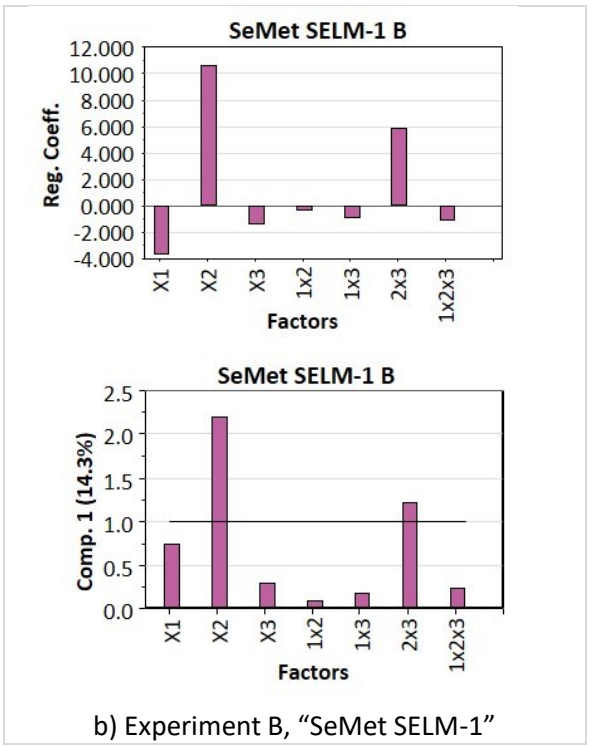


b) Experiment A, "SeMet SELM-1"

Figure 17: Bar graphs of regression coefficients and variable importance plots (VIP, comp. 1) of factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3), and factor interactions in screening with one response a) "SeMet Wheat" (SeMet recovery in ERM BC210a) and b) "SeMet SELM" (SeMet recovery in SELM-1).



a) Experiment B, "SeMet Wheat"



b) Experiment B, "SeMet SELM-1"

Figure 18: Bar graphs of regression coefficients and variable importance plots (VIP, comp. 1) of factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3), and factor interactions in screening with one response a) "SeMet Wheat" (SeMet recovery in ERM BC210a) and b) "SeMet SELM-1" (SeMet recovery in SELM-1).

The VIP plots (Figure 17) show that the only significant factor for the responses “SeMet Wheat” and “SeMet SELM-1” in experiment A is enzyme (X3). This factor has a negative regression coefficient for both SeMet responses (Figure 17.a-b). This means that using protease as enzyme will significantly increase the recovery of SeMet in ERM BC210a and SELM-1.

From the VIP plots in experiment B (Figure 18.a-b), the significant factors and interactions are “buffer” (X1) and “pre-extraction” (X2) for SeMet in ERM BC210a, and “pre-extraction” (X2) and the interaction between “pre-extraction” and “enzyme” (2x3) for SeMet in SELM-1. The bar graphs show that using ammonium phosphate as buffer (-) and including a pre-extraction step (+) are preferred to increase SeMet recovery in ERM BC210a, when using the conditions in experiment B. As interpreted from the magnitude of the regression coefficients obtained from experiment B, a pre-extraction step (+) with cellulase (+) as enzyme is preferred to increase SeMet recovery in SELM-1. The combination that could give a slightly higher increase of SeMet recovery in SELM-1 is to include a pre-extraction step and cellulase as enzyme.

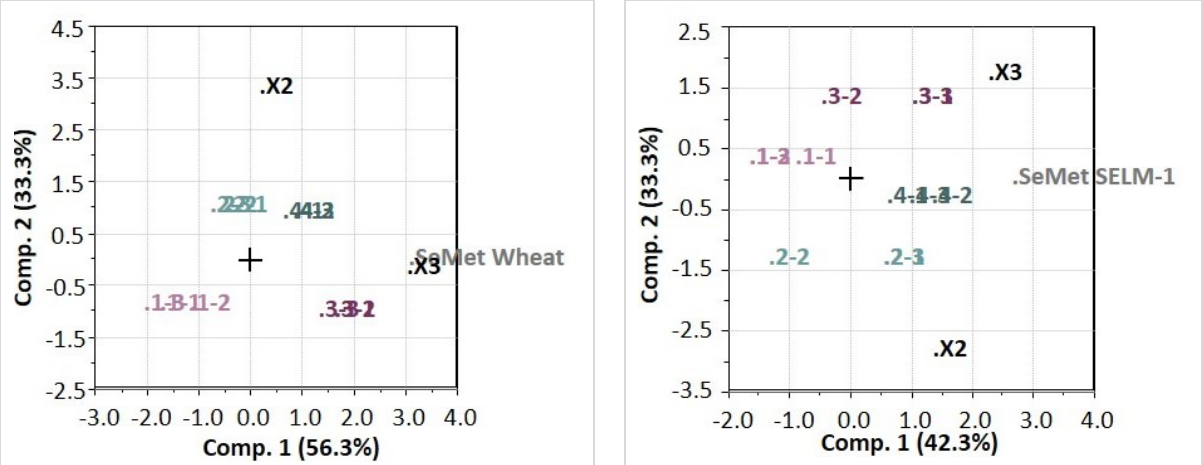
Based on the interpretation from experiment A and B, the buffer considered to be the best option for further application on fish feed was ammonium phosphate. The mixed buffer was only considered significant for increasing total Se recoveries and it was not included for further testing. The pre-extraction step was considered significant in experiment B and kept for further testing. For the factor “enzyme” (X3), protease from experiment A and cellulase from experiment B were chosen as the best options. Based on previous information, the enzymes protamex and α-amylase were not chosen for further testing. Furthermore, it was considered relevant to test a combination of the protease and cellulase, to evaluate if this could promote an increase in the hydrolysis of Se proteins more than only using a single enzyme.

4.2.5 PCA for the optimization

For the optimization, the main interpretation of the results was performed through PCA with individual responses of “SeMet Wheat” and “SeMet SELM-1”. This was done considering the factors that have the most effect on the SeMet recoveries. The biplots with one response, “SeMet Wheat” and “SeMet SELM-1”, included in the analysis for screening the factors “pre-extraction” (X2) and “enzyme” (X3) with triplicates of four experiments are shown in Figure 19. PCA using the medians of the triplicates (Appendix F) as responses was also checked to evaluate any differences from using mean values. However, the PCA using the mean values showed no significant differences, and using the mean values was considered to be acceptable for the main evaluation in PCA.

In addition, PCA with all four responses, “TotSe Wheat”, “TotSe SELM-1”, “SeMet Wheat” and “SeMet SELM-1”, and PCAs with individual responses, “TotSe Wheat” and “TotSe SELM-1” (total Se recoveries)

were performed. However, the total Se recoveries for the two CRMs presented very similar values (all above 100%) and for that reason these results were not considered relevant to be included in the main thesis but they can be seen in Appendix F.

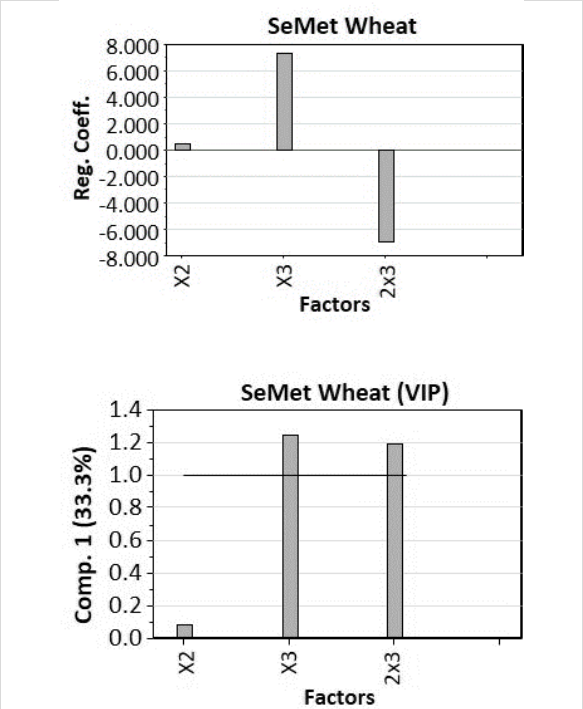


a) Experiment C, "SeMet Wheat"

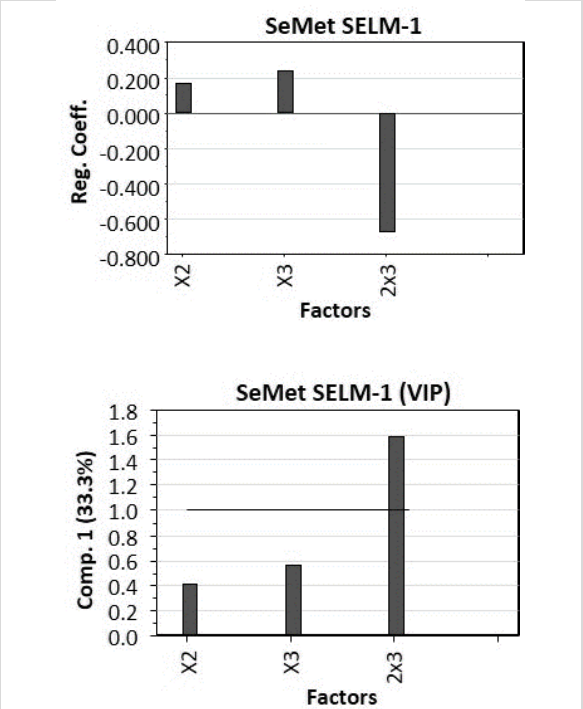
b) Experiment C, "SeMet SELM-1"

Figure 19: Biplots (Comp. 1 vs 2) showing the relations between experiments 1-4 and variables for factors "pre-extraction" (X2) and "enzyme" (X3), and individual responses a) "SeMet Wheat" (SeMet recovery in ERM BC210a) and b) "SeMet SELM-1" (SeMet recovery in SELM-1) for optimization.

The combined enzyme (+) is preferred to increase SeMet recoveries in both CRM. This can be seen from the loadings in the biplot (Figure 19.a), which shows that the choice of enzyme (X3) is strongly correlated to SeMet recovery in ERM BC210a (wheat flour). The choice of enzyme (X3) has also a positive correlation to SeMet recovery in SELM-1 (Figure 19.b). However, the factor pre-extraction (X2) does not seem to affect the SeMet recovery in ERM BC210a (Figure 19.a) and SELM-1 (Figure 19.b) due to no correlation. From both biplots (Figure 19), the experiments that have the best conditions for increased SeMet recoveries are experiment 3 and 4. These are experiments with the combined enzyme that matches well with the interpretation of the loadings. To evaluate the significance of the factors, bar graphs and variable importance plots (VIP) of regression coefficients for the main factors and interactions were analyzed (Figure 20).



a) Experiment C, "SeMet Wheat"



b) Experiment C, "SeMet SELM-1"

Figure 20: Bar graphs of regression coefficients and variable importance plot (VIP, comp. 1) of the factors "pre-extraction" (X2) and "enzyme" (X3) and factor interactions for optimization with one response a) "SeMet Wheat" (SeMet recovery in ERM BC210a) and b) "SeMet SELM-1" (SeMet recovery in SELM-1).

For the SeMet recovery in ERM BC210a (wheat flour) (Figure 20.a), the most significant factor is enzyme (X3), with a positive regression coefficient. This shows that choosing the combined enzyme (+) will significantly increase SeMet recovery in ERM BC210a, but not for SELM-1 (Figure 20.b). The interaction between "pre-extraction" (X2) and "enzyme" (X3) is also significant for SeMet recoveries in both CRMs (Figure 20.a-b). This means that excluding pre-extraction (-) while using the combined enzyme (+) gives the most significant increase for SeMet in ERM BC210a and SELM-1.

Based on the results obtained in this study, the optimal conditions for increasing the extraction of SeMet in fish feed are the use of a combination of protease and cellulase with ammonium phosphate as buffer without pre-extraction using papain.

4.2.6 The effects of enzymes for the digestion of SeMet in CRMs

In this study, various enzymes and combinations of enzymes were applied during the screening and the optimization. An overview of all enzyme combinations tested using ammonium phosphate as a buffer is shown in Figure 21. The results are shown for the recovery for SeMet (%, mean ± SD, n = 1 - 6) in ERM BC210a (wheat flour) and SELM-1 (selenized yeast). The recoveries were calculated using the determined

concentrations of SeMet when using HPLC-ICP-MS and the certified values for SeMet as target concentrations (equation (7)).

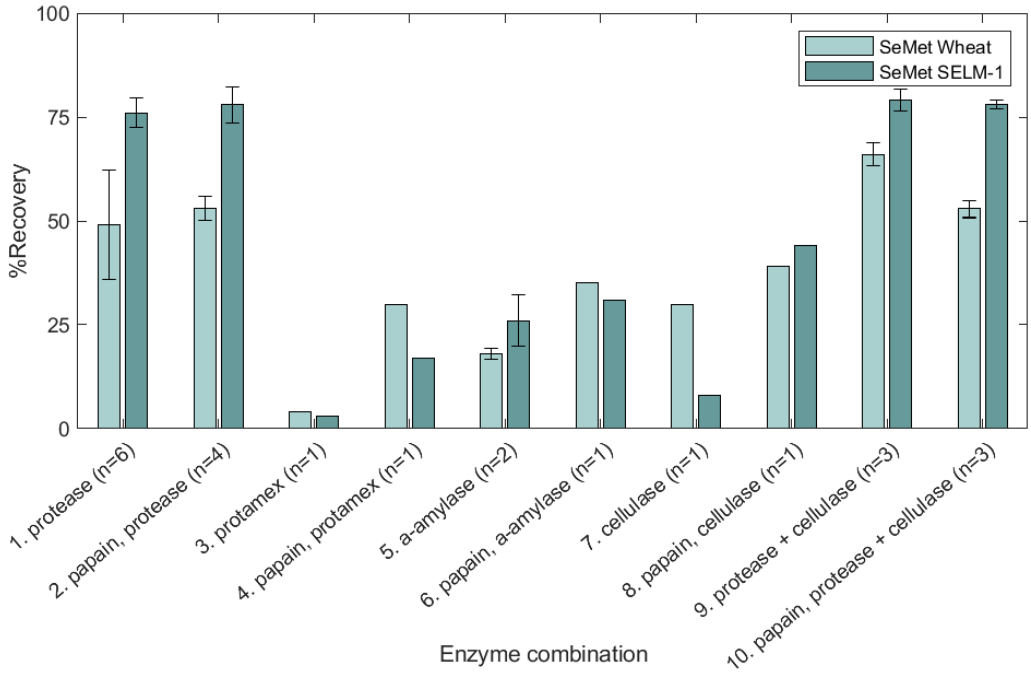


Figure 21: Bar graph of the recovery of SeMet (%) in the certified reference materials ERM BC210a (wheat flour) and SELM-1 (selenized yeast). Data was collected during the screening and optimization steps using different enzymes with ammonium phosphate as buffer, with or without papain for pre-extraction. The recoveries were calculated by comparing the concentration of SeMet determined by HPLC-ICP-MS and the certified values as described in equation (7).

In general, the highest SeMet recoveries seen were from combination 1, 2, 9 and 10 (Figure 21). All previous combinations included protease and had SeMet recoveries that ranged from 49 to 66% for ERM BC210a and from 76 to 79% for SELM-1. When testing only protease, the SeMet recoveries were 49% (ERM BC210a) and 76% (SELM-1). However, a minor increase in the recovery was seen when including other enzymes together with protease such as papain (combination 2) or cellulase (combination 9) (Figure 21). The enzyme combination giving the highest SeMet recovery for both ERM BC210a (66%) and SELM-1 (79%) was when applying protease and cellulase (1:1), without papain pre-extraction (combination 9, Figure 21).

Compared to combination 9, similar SeMet recovery in SELM-1 was seen in combination 10, where a pre-extraction was included for protease and cellulase. However, lower SeMet recovery was seen in ERM BC210a with combination 10. Since today's fish feed composition generally have a high inclusion of plant-materials, including wheat (Aas *et al.*, 2019; Ytrestøyl *et al.*, 2015), the pre-extraction step in combination 9 was seen as the most efficient combination of all the enzyme combinations tested. The conditions used in combination 9, were regarded as the most optimal method.

The effects of a pre-extraction enzymatic step

Although the optimal conditions (combination 9) did not include the pre-extraction step, the pre-extraction step showed an overall increased effect with other enzyme combinations. In general, the pre-extraction using papain (combination 2, 4, 6 and 8) increased the SeMet recovery for both CRMs compared to the equivalent extraction procedures without the pre-extraction step (combination 1, 3, 5, 7). Thus, the pre-extraction step using papain seems to facilitate the extraction of SeMet, when using protease, protamex, α-amylase or cellulase alone in the main enzymatic digestion.

As previously mentioned in section 4.2.1, papain was also used for pre-extraction in Se speciation in tissues of marine bay scallops (Zhang and Yang, 2014). In the work of Zhang and Yang (2014), the main enzymatic digestion consisted of a combination of flavourzyme, carboxypeptidase and trypsin. In the same study, SeMet recovery in SELM-1 was as high as 98.8% (Zhang and Yang (2014)). A recovery for SeMet in SELM-1 as high as described by Zhang and Yang (2014) was not achieved in this study when using papain (Figure 21). The lower recovery for SeMet in SELM-1 might have been caused by using other types of enzymes for main enzymatic digestion when comparing with what was used by Zhang and Yang (2014).

Enzymes and enzyme combinations

The enzymes protease, protamex, α-amylase and cellulase, applied for the main extraction, were tested individually with and without the pre-extraction step in the screening. Protease and cellulase were also tested in a combination, with and without the pre-extraction step.

Combining protease with cellulase (combination 9) increased the SeMet recovery for both CRMs, compared to using protease alone (combination 10) (Figure 21). A comparison of protease and cellulase in combination, to protease alone, has also been tested in another Se speciation study (Cuderman *et al.*, 2010). A combination of protease and cellulase gave significantly lower extraction recoveries (34% lower) than using protease alone in Se-enriched buckwheat (*Fagopyrum esculentum*) sprouts. Furthermore, a combination of protease and lipase was seen to give a 10% increase in Se recovery (%) compared to using protease alone (Cuderman *et al.*, 2010). Protease together with lipase in a sequential extraction was also tested for the extraction of Se species in fish feed, but showed no significant increase in the extraction recovery for SeMet compared to using protease alone (Sele *et al.*, 2018a).

α-Amylase was not seen to give significantly higher SeMet recovery for the CRMs alone and it was not further tested in a combination with protease. The combination of α-amylase and protease was applied in the work of Cuderman *et al.* (2010) with no significant difference in the recovery of SeMet compared to using protease alone.

From combination 3 and 4, protamex showed lower SeMet recoveries compared to other combinations (Figure 21). In comparison to protease, protamex gave a significantly lower effect. Since protamex is another type of protease (protease from *Bacillus* sp.), it was expected that this enzyme would have similar properties to protease XIV. However, the enzyme combinations with protamex gave relative low SeMet recovery when compared to the other recoveries.

Based on the different results obtained from the enzyme combinations in this project, the CRMs ERM BC210a and SELM-1 might need other types of enzymes or enzyme combinations for increased SeMet recovery for the Se speciation method. Recoveries for different types of samples might be highly dependent on the type of enzymes used for releasing Se species from sample matrix. Other types of enzymes or enzyme combinations might therefore also be needed to increase SeMet recovery in fish feed and feed ingredients.

4.3 Se speciation analysis

4.3.1 Se species in experimental diets

The concentrations of total Se (mg/kg, mean ± SD, n =3) in the experimental diets, in the soluble fractions, in the non-soluble fractions and the calculated extraction recovery of Se (%) in these fractions for the experimental diets are given in Table 25. The extraction recovery (%) was calculated using equation (9).

Table 25: Total Se (mg/kg ww, mean ± SD, n = 3) in experimental diets (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b), and total Se (mg/kg ww, mean ± SD, n = 3) and recovery (%) in the soluble and non-soluble fractions from the extractions (calculated using equation (9)), and the mass balance of Se recovery (%) from the fractions (the sum of Se in soluble and non-soluble fractions).

Sample	Total Se (mg/kg)	Total Se in soluble fraction (mg/kg)	Recovery of Se in soluble fraction (%)	Total Se in non-soluble fraction (mg/kg)	Recovery of Se in non-soluble fraction (%)	Mass balance of Se recovery from fractions (%)
Diet A	0.38 ± 0.01	0.43 ± 0.01*	113	0.061 ± 0.002*	16	130
Diet B	0.63 ± 0.02	0.71 ± 0.02*	113	0.083 ± 0.002*	13	126
Diet C	0.45 ± 0.04	0.42 ± 0.02	94	0.1532 ± 0.0005*	34	128
Diet D	6.2 ± 0.2	6.3 ± 0.1*	102	0.82 ± 0.02*	13	115
Diet E	16.2 ± 0.3	16.4 ± 0.2	101	1.8 ± 0.1*	11	113
Diet F	5.4 ± 0.09	3.6 ± 0.1	67	1.48 ± 0.01	27	94

* n = 2.

The concentrations of SeMet (mg Se/kg, mean ± SD, n = 3) was determined in the experimental diets using the optimized method and Se speciation by HPLC-ICP-MS (Table 26). The total Se in the diets, the supplemented levels of SeMet and recovery (%) for SeMet (calculated using equation (7)) are shown in Table 26.

For the experimental diets, a relatively large variation is seen in the recovery of total Se in the soluble fractions, ranging from 67 to 113% (Table 25). This shows that the extraction efficiency, or extraction yield, is variable when using the optimized extraction method on these diets. Besides diet F, all the experimental diets had a recovery for Se in the soluble fractions between 90 and 120%. For diet C, D and E the Se recoveries were within 95-105%, which shows that the optimized method digested and released organic Se in these experimental diets. Diets A, B, D and E are diets supplemented with SeMet at different concentration levels at 0.15, 0.4, 5, and 15 mg Se/kg, respectively.

The lowest recovery for Se was seen for diet F, with 67% Se recovery in the soluble fraction (Table 25). A lower recovery when using an enzymatic digestion procedure was expected, since diet F was supplemented with an inorganic source of Se (selenite). Previous studies have shown that an alkaline solution is the preferred extraction solution for selenite in fish feed (Sele *et al.*, 2018a).

Table 26: Total Se in diets (mg/kg ww, mean ± SD), supplemented level of SeMet (mg Se/kg) (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b), the SeMet concentration in soluble fraction (mg Se/kg ww, mean ± SD, n = 3) of the experimental diets determined by HPLC-ICP-MS and recoveries (%) for SeMet. Recoveries (%) were calculate using equation (7).

Sample	Total Se (mg/kg)[b]	Supplemented SeMet (mg Se/kg)	SeMet in soluble fraction (mg Se/kg)	SeMet recovery of supplemented SeMet (%)	SeMet recovery of total Se in sample (%)
Diet A	0.38 ± 0.01	0.15	0.08 ± 0.02*	50	20
Diet B	0.63 ± 0.02	0.4	0.25 ± 0.01	62	40
Diet C	0.45 ± 0.04	0	0.016 ± 0.004*	>100	4
Diet D	6.2 ± 0.2	5	3.36 ± 0.03	67	54
Diet E	16.2 ± 0.3	15	10.3 ± 0.4	69	64
Diet F[a]	5.4 ± 0.09	0	0.004 ± 0.002*	>100	0.1

a: Diet F was supplemented with selenite instead of SeMet.
b: Total Se concentrations in experimental diets (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b).
* n = 2.

The determined SeMet concentrations ranged from 0.004 to 10.3 mg/kg when using the optimized method (Table 26). This corresponded to a recovery for SeMet ranging from 0.1 to 64% of total Se in the samples. The lowest SeMet recoveries were detected in diet F, with concentration of 0.004 mg Se/kg. Since diet F was not supplemented with SeMet, the trace amounts of SeMet is most likely from the presence of natural bound SeMet in the marine feed ingredients. In the basal diet C (not supplemented), the SeMet concentration was 0.016 ± 0.004 mg Se/kg which accounted only for 4% of the total Se in the diet. These results indicate that the optimized method is not so efficient for extraction of naturally occurring SeMet in the feed ingredients, if assuming that SeMet is the main source of Se present in the feed ingredients (Bryszewska and Måge, 2015; Sele *et al.*, 2018a).

The determined concentrations of SeMet corresponded to recoveries for SeMet of 50 to above 100 % when compared with the supplemented SeMet levels (Table 26). Since the measured SeMet concentrations were higher than the zero amount of SeMet supplemented in diet C and F, the SeMet recovery were set to be above 100%. The lowest recovery of SeMet was seen for diet A (50%), which was supplemented with the lowest concentration of SeMet (0.15 mg Se/kg), followed by diet B (62%), diet D (67%) and diet E (69%), with increasing concentrations of SeMet supplementation. These results indicate a more efficient extraction of SeMet as the concentrations of SeMet increases in fish feed. Furthermore, these results shown that there is a challenge to get a complete extraction of SeMet at concentrations below 0.5 mg Se/kg, which is the legal limit for Se in animal feed (Council Directive 70/524/EC and amendments).

4.3.2 Se species in commercial fish feed and feed ingredients

The total Se concentrations (mg/kg, mean ± SD, n = 3) in the samples of commercial fish feed (FF) and feed ingredients (fish meal (FM), plant meal (PM) and insect meal (IM)) are presented in Table 7. These samples were obtained from the National surveillance program for fish feed for 2019 (Ørnsrud *et al.*, 2020). The concentrations of SeMet (mg/kg ww, n = 3) in the soluble fraction obtained when using the optimized extraction method and the initial extraction method for Se speciation (Sele *et al.*, 2018a), are compared in Table 27. The recoveries for SeMet (%) are calculated and presented (Table 27).

Table 27: Total Se (mg/kg ww, n = 3) in commercially produced fish feed, fish meal, plant meal and insect meal obtained from the National surveillance program for fish feed (Ørnsrud *et al.*, 2020). SeMet concentrations (mg/kg ww, n = 3) determined by HPLC-ICP-MS when applying the optimized extraction method and when applying the initial extraction method (based on Sele et al., 2018)), and the recovery (%) for SeMet of total Se for both methods. Recovery (%) was calculated using equation (7).

		Optimized method		Initial method	
Sample	**Total Se (mg/kg)**	**SeMet (mg Se/kg)**	**Recovery of SeMet (%)**	**SeMet (mg Se/kg)**	**Recovery of SeMet (%)**
FF1	0.96 ± 0.06	0.14 ± 0.01*	14	0.164 ± 0.004	17
FF2	1.42 ± 0.05*	0.38*	27	0.397 ± 0.009	28
FF3	0.38 ± 0.01	0.12*	31	0.147 ± 0.003	25
FF4	0.49 ± 0.01	0.108 ± 0.002*	22	0.10*	29
FF5	0.83 ± 0.01*	0.093 ± 0.004	11	0.14*	18
FF6	1.29*	0.448 ± 0.003	35	0.417 ± 0.006*	32
FM1	3.12*	0.31 ± 0.01	10	0.28*	8.9
FM2	2.79*	0.29 ± 0.01	10	0.29*	10
FM3	2.54*	0.43 ± 0.02	17	0.450 ± 0.002*	18
PM1	0.215 ± 0.003	0.088 ± 0.003	41	0.078*	36
PM2	0.81 ± 0.03*	0.41 ± 0.02*	50	0.432 ± 0.001*	54
IM1	0.153 ± 0.005	0.049 ± 0.0001*	32	0.039*	25
IM2	0.41*	0.085 ± 0.004	21	< LOQ	-

* n = 1-2.

The total Se concentrations in the fish feeds were ranged from 0.38 to 1.42 mg/kg. The feed ingredients ranged from 0.153 to 3.12 mg/kg, where the concentrations in the plant meals (0.215 to 0.81 mg/kg) and insect meals (0.153 to 0.41 mg/kg) were lower than the Se concentrations in the fish meals (2.54 to 3.12 mg/kg). These results were comparable to Se concentrations reported by the surveillance program for 2019 for fish feed (0.3 to 1.5 mg/kg, n = 93), fish meal (1.7 to 3.2 mg/kg, n = 10), plant meal (below 0.01 to 1.40, n = 10) and insect meal (0.1 to 0.4 mg/kg, n = 10) (Ørnsrud *et al.*, 2020).

The SeMet concentrations in all samples ranged from 0.049 to 0.43 mg Se/kg when using the optimized method for Se speciation (Table 27). The SeMet concentrations ranged from 0.093 to 0.448 mg Se/kg for fish feed, 0.29 to 0.43 mg Se/kg for fish meal, 0.088 to 0.41 mg Se/kg for plant meal and 0.049 to 0.085 mg Se/kg for insect meal. When applying the optimized method, the recovery for SeMet ranged from 10 to 50% when compared to total Se in the sample. The highest recovery was seen for plant meals with recoveries from 41 to 50%, whereas the lowest recoveries (10 - 17%) were seen for fish meals and for some of the feeds with high total Se concentrations (Table 27).

In general, the recovery for SeMet was lower for the commercial feeds and feed ingredients compared to what was found for the experimental diets (section 4.3.1). However, the total Se concentrations in the commercial feeds and feed ingredients were lower than most of the experimental diets. Hence, the low recovery for SeMet could be related to the insufficient extraction at lower total Se concentrations, similar to what was seen for the experimental diets. There are other factors that could also affect the recovery, e.g. other Se species present in the feeds and feed ingredients. SeMet is the most common Se species in marine samples (Bryszewska and Måge, 2015; Sele *et al.*, 2018a), and is therefore assumed to be present in these samples. There was no detailed information available about the Se content in the commercial fish feed and feed ingredients. Other species that could be present in fish feed and feed ingredients could be species such as SeCys. For determination of this species, a derivatization step would be required for extraction of SeCys prior to enzymatic digestion, which has been seen in other Se speciation studies (Godin *et al.*, 2015; Bierla *et al.*, 2018)

For the insect meal samples the recovery for SeMet was 32% (IM1) and 21% (IM2) and in a similar range as the fish feed. The highest recoveries were found in plant meal with 41% (PM1) and 50% (PM2). The total Se concentrations in the plant meal samples were 0.215 and 0.81 mg/kg. In general, the lowest recoveries were seen for fish meal and insect meal. These results could indicate that the optimized method is more efficient in extracting Se from plant-based ingredients than Se present in marine-based ingredients.

The results obtained with the optimized method were compared with the results obtained with the initial method for Se speciation (Table 27). The SeMet concentrations when using the initial method ranged from

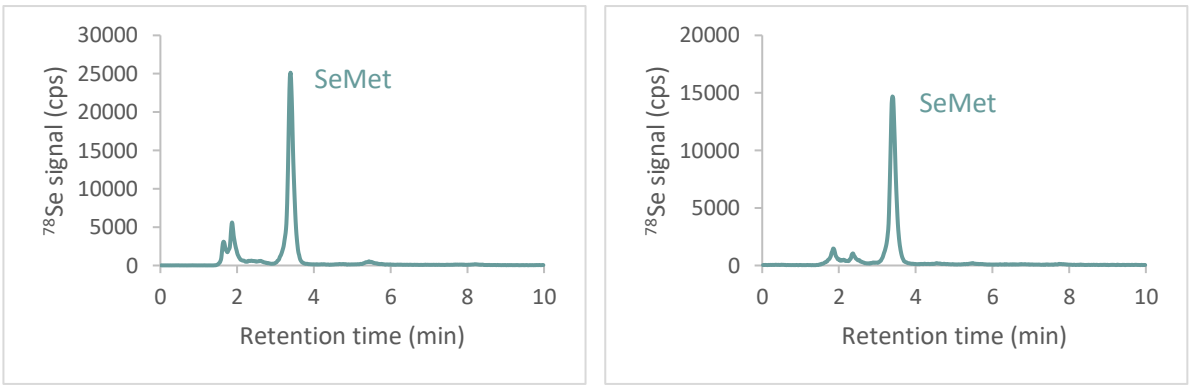
0.039 and 0.450 mg Se/kg, and recoveries for SeMet ranged from 0 to 54%. The SeMet concentrations when using the optimized method ranged from 0.049 and 0.448 mg Se/kg, and recoveries for SeMet ranged from 10 to 50%. Hence, quite similar concentrations and recoveries were seen when comparing the optimized and initial method. The most notable differences between the two methods are seen for the results for the plant meals and insect meals (Table 27). A minor increase in the recoveries was seen when using the optimized method in the plant-based ingredients and insect meals, with one of the insect meals (IM2) increasing recovery from 0 to 21%. These results may indicate that the method has been improved for these types of sample material, rather than marine-based samples. This can be related to the method development being performed with plant-based CRM ERM BC210a (wheat flour).

In general, the mean SeMet concentrations and standard deviations were similar for the optimized method and the initial method. A two-sample sign test was performed on the SeMet concentrations, and there were no significant differences ($p = 0.0218$) between the optimized method and the initial method**.**

Several of the feed samples had total Se concentrations above the upper limit at 0.5 mg/kg for animal feeds (Council Directive 70/524/EC and amendments), which has also been reported in the monitoring program (Ørnsrud *et al.*, 2020; Sele *et al.*, 2019). Two of the analyzed commercial fish feed had SeMet concentrations above the upper limit for SeMet or SeMet produced from Se-enriched yeast (*Saccharomyces cerevisiae*) in animal feeds, set to 0.2 mg Se/kg (EC, 2003; EC, 2017b; EFSA, 2011a; EFSA, 2011b). The samples with SeMet concentrations above 0.2 mg/kg were fish feed with total Se concentrations over 0.5 mg Se/kg. For the fish feed obtained from the surveillance program, detailed information on the ingredients is only available upon request, and the samples are analyzed without any specific information on their composition. The concentrations of total and Se species naturally occurring in the ingredients in the complete feed are not known. The analysis shows that SeMet are present in all feed ingredients analyzed in this study (Table 27). Since the chemical form of this Se species used as additives could be the same as naturally occurring Se species, it is difficult to say whether the SeMet is from the additive or from the feed ingredients. Some fish feed could contain larger fractions of feed ingredients with naturally higher Se concentrations than other feed, such as fish meal. Fish feed is therefore a challenging sample type to analyze, since different analytical methods are acquired for extracting different Se species. For selecting the most suitable method for Se speciation in fish feed, information about the type of Se species for supplementation or the information about the feed ingredients in the feeds sample would be recommended.

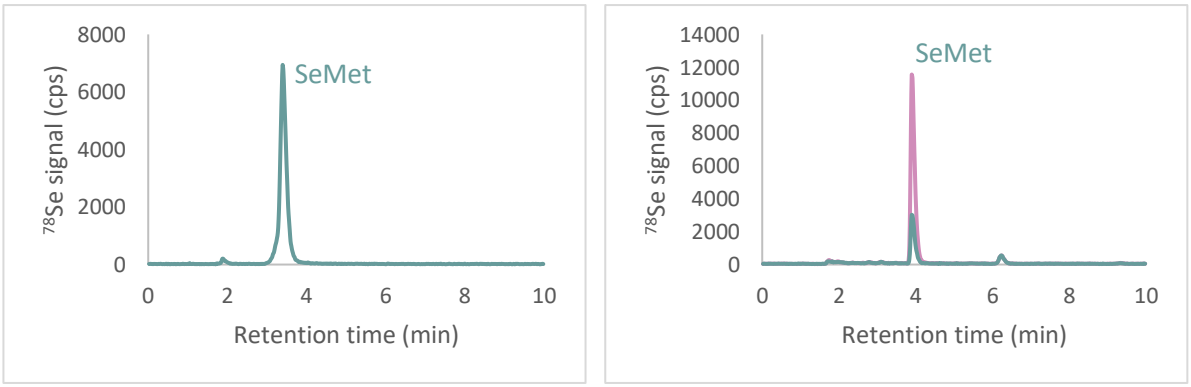
4.3.3 Chromatographic profiles for Se species in experimental diets, commercial fish feed and feed ingredients

The HPLC-ICP-MS chromatograms for the CRMs ERM BC210a and SELM-1 when analyzed with the optimized method for Se speciation are shown in Figure 22. For comparison, a chromatogram of a standard SeMet solution (10 ppb) and spiked sample of fish feed (FF1), can be seen in Figure 23.



a) ERM BC210a (10x dilution) b) SELM-1 (2000x dilution)

Figure 22: Chromatogram of a) ERM BC210a and b) SELM-1 (b) when analyzed by HPLC-ICP-MS, with SeMet eluting at retention time of 3.4 min.



a) SeMet standard solution (10 ppb) b) FF1 spiked with SeMet standard solution (5 µg/L)

Figure 23: Chromatogram of SeMet standard solution (10 ppb) with SeMet eluting at retention time of 3.4 min (a) and spiked sample of fish feed (FM1) eluting at retention time of 3.9 min (b), analyzed by HPLC-ICP-MS.

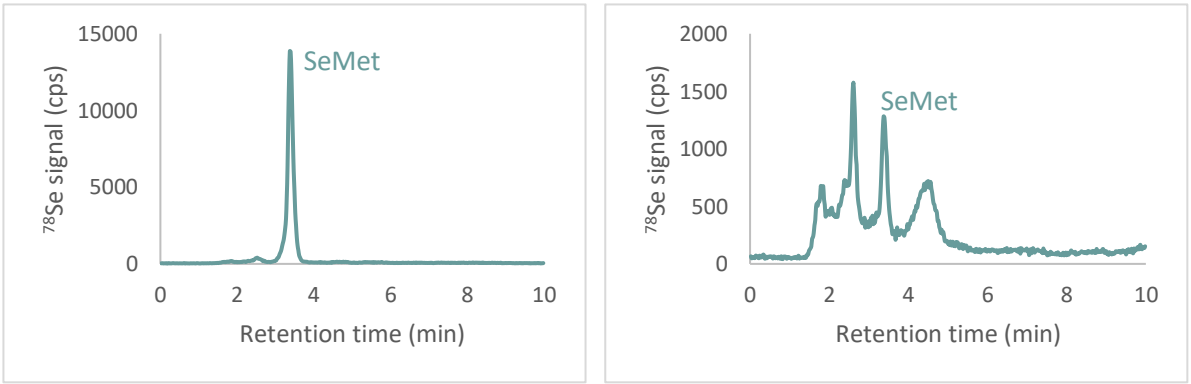
The chromatograms of ERM BC210a and SELM-1 show mainly two or three chromatographic peaks for Se (Figure 22). The first chromatographic peaks have retention times (r.t.) of 1-3 min, whereas the main peak with r.t. of 3.9 min corresponds to SeMet, shown by the standard solution (Figure 23.a). The identity of SeMet peak in the ICP-MS chromatograms, were also verified by spiking- experiments by SeMet standard for selected samples of fish feed and feed ingredients (Figure 23.b). For the spiked sample shown in Figure

23.b, the analyte retention time was having a minor shift compared to Figure 23.a, which can be explained by the necessity of replacing a well-used column with a newly purchased one.

The Se peaks in the start of the chromatograms for ERM BC210a and SELM-1 (Figure 22) indicates the presence of other Se species in the extracts. This is in coherence with what has been seen in previous Se species studies of SELM-1, where other Se species, such as selenite (Oliveira *et al.*, 2016; Xiao *et al.*, 2017), SeOMet and $SeCys_2$ (Oliveira *et al.*, 2016) have been identified. The CRM ERM BC210a have not been that thoroughly studied before, and the Se peaks are yet to be identified. However, another CRM of wheat flour (NIST 1567a) was used by Cubadda *et al.* (2010) and Hsieh and Jiang (2013), where both studies showed the presence of low Se signals corresponding to $SeCys_2$. In the work of Hsieh and Jiang (2013), the Se species selenate, selenite and SeMetSeCys were also identified in NIST 1567a and in the extract of NIST 1567a using ESI-MS/MS.

Experimental diets

Chromatograms of experimental diets supplemented with 5 mg Se/kg, diet D (SeMet) and diet F (selenite) are shown in Figure 24



a) Diet D (SeMet supplemented, 5 mg Se/kg) (8x dilution)

b) Diet F (selenite supplemented, 5 mg Se/kg) (4x dilution)

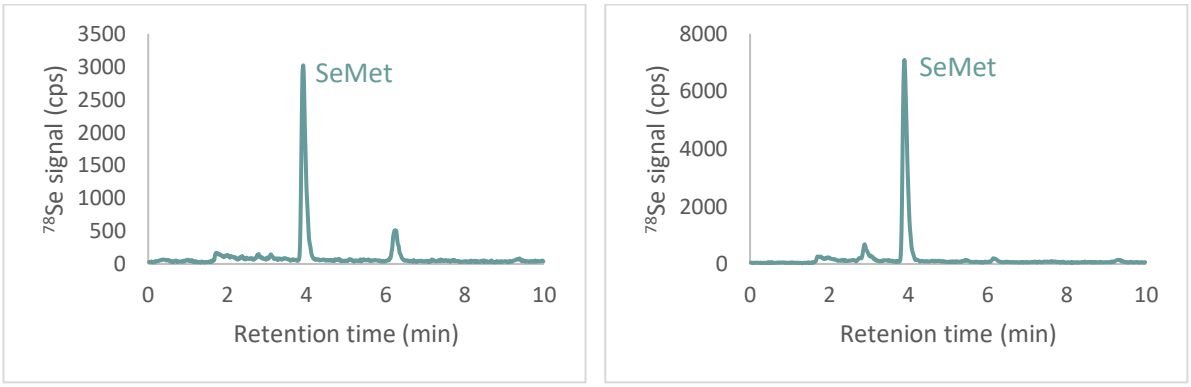
Figure 24: The HPLC-ICP-MS chromatogram of the experimental diets; diet D, supplemented with SeMet (a) and diet F, supplemented with inorganic selenite (b).

The chromatogram for diets supplemented with SeMet, such as diet D (Figure 24.b), show a clear signal for SeMet (r.t of. 3.4 min). Similar chromatographic profiles were seen for the other diets supplemented with SeMet and basal diet (diet C), with a clear SeMet signal (Appendix G, Figure G1). The chromatogram for diet F shows that the chromatographic peak for SeMet (r.t. of 3.4 min) is not baseline separated, and that the signal is interfered by another Se signal, making it hard to integrate the SeMet peak (Figure 24.a). The cause of the interferences is likely related to the high concentration of selenite (5 mg Se/kg) supplemented

to this diet, compared to the low amount of SeMet in the diet. Similar observations were seen by Sele *et al.* (2018a) when applying enzymatic digestion for feeds supplemented with selenite.

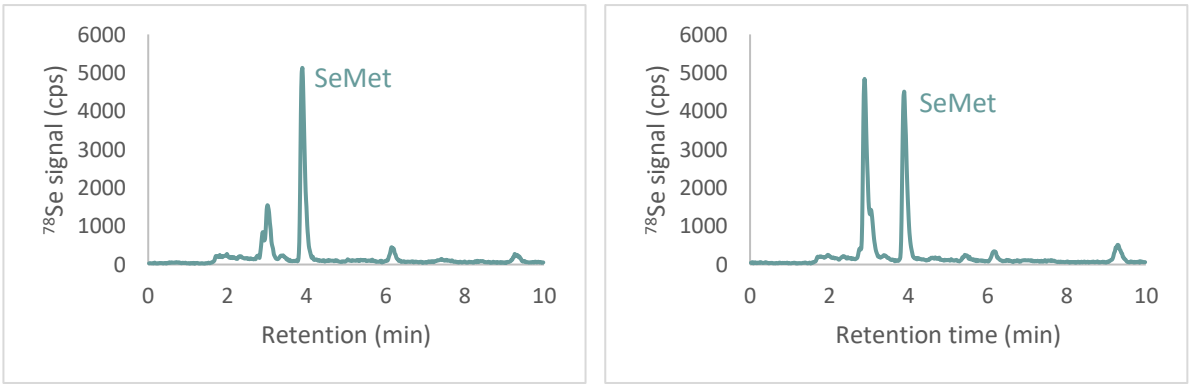
Commercial fish feed and feed ingredients

Chromatograms of selected commercial fish feed (FF1 and FF6) are shown in Figure 25. For selected feed ingredients, chromatograms are shown in Figure 26 for fish meal (FF1 and FF2), and in Figure 27 for insect meal (IM1) and plant meal (PM2).



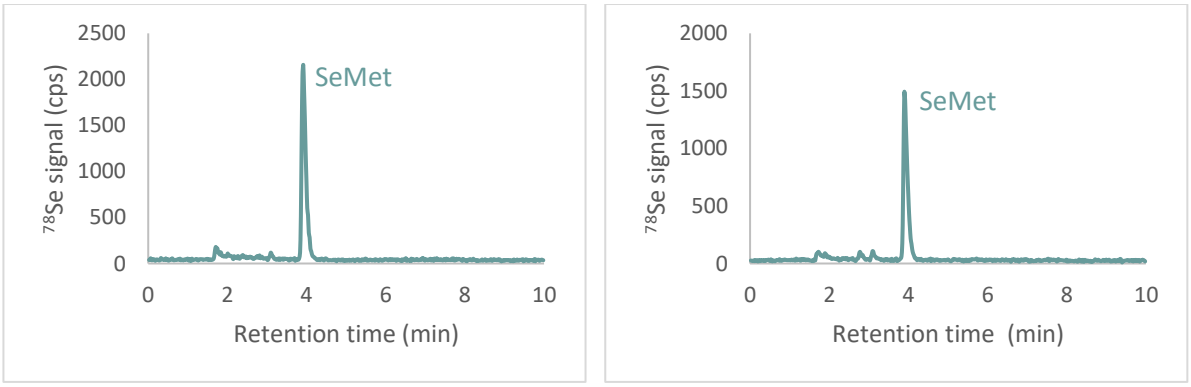
a) FF1 (4x dilution) b) FF6 (4x dilution)

Figure 25: Chromatograms of fish feed samples FF1 (a) and FF6 (b) when analyzed by HPLC-ICP-MS, with SeMet eluting at retention time of 3.9 min.



a) FM1 (4x dilution) b) FM2 (4x dilution)

Figure 26: Chromatograms of fish meal samples FM1 (a) and FM2 (b) when analyzed by HPLC-ICP-MS, with SeMet eluting at retention time of 3.9 min.



a) PM1 (4x dilution) b) IM1 (4x dilution)

Figure 27: Chromatograms of plant meal sample PM1 (a) and insect meal sample IM1 (b) when analyzed by HPLC-ICP-MS, with SeMet eluting at retention time of 3.9 min.

The analyte r.t. of 3.9 min achieved with the samples of commercial feed and feed ingredients (Figure 25, Figure 26 and Figure 27), differ from the r.t. of 3.4 min achieved with the experimental diets (Figure 24), which also can be explained by the replacement of the column.

In addition to SeMet, the chromatograms for some of the fish feed and fish meal samples showed the presence of other signals for Se, e.g. with r.t. of 2.9, 6 and 10 min. These peaks are likely other Se species, with chemical structure not known. The chromatographic peak with r.t. of 2.9 min was prominent in the three fish meal samples FM1 (Figure 26a), FM2 (Figure 26b) and FM3. Particularly, FM2 had higher signal intensity for this chromatographic peak (r.t. of 2.9 min) (Figure 26b). When integrated, this chromatographic peak had a similar peak area as SeMet in FM2. For FM1 (Figure 26a) and FM3 (Appendix G, Figure G3.a) this peak was minor Se peaks compared to SeMet. For FM1 and FM2, other peaks with lower signals than SeMet were observed at 6 min and 10 min (Figure 26), and at 10 min for FM3 (Appendix G, Figure G3.a). A similar peak of lower intensity with r.t. of 6 min was also observed in one of the fish feed, FF1 (Figure 25.a).

Other unknown Se signals with clearly defined peaks were not seen in fish feed, FF2-FF6 (Figure 25.b; Appendix G, Figure G2) and in plant meal and insect meal (Figure 27; Appendix G, Figure G3.b-c). Overall, these results show that other unknown Se compounds, especially in fish meal, are present, but the identity of these compounds are currently not known. Based on the chromatographic profiles for the CRMs and several of the feeds and feed ingredients, it is shown that other Se species are extracted with the applied extraction method. The unidentified Se peaks were not identified in this study. Identification of species is a challenge when using ICP-MS since the instrument only provides elemental information, and not any information about the molecular structures.

To identify unknown peaks in ICP-MS, the samples are commonly spiked with standards of the species that could be present. However, standards are not always available for every species. The unknown species that were observed in fish meal samples could be identified by spiking the samples with Se species that might be present, such as selenate, selenite, SeCys, $SeCys_2$, SeMetSeCys, GGMSC and SeOMet. Another approach would be to apply the same chromatographic settings in a HPLC-HR-MS/MS system, for identifying the unknown Se species in the samples. Other set-ups that could be used include ESI-MS/MS and ESI-TOF-MS, which have been applied in other Se speciation studies (Hsieh and Jiang, 2013; Tie *et al.*, 2015; Dernovics and Lobinski, 2008; Goenaga Infante *et al.*, 2009).

The low SeMet recovery for the samples can be caused by a possible presence of inorganic Se, such as selenate and/or selenite, or also SeCys and SeMetSeCys, which are species that have been identified in samples of wheat (Cubadda *et al.*, 2010; Duncan *et al.*, 2017; Li *et al.*, 2008; Tsai and Jiang, 2011). The species SeMetSeCys and GGMSC has also been observed in wheat cultivar grains (Duncan *et al.*, 2017). Since fish feed and plant ingredients can contain wheat, these Se species can be possible species in these samples. The unidentified Se signals were, on the other hand, very low compared to the SeMet signal seen in CRMs, experimental diets, plant meal samples and some of the fish feed.

The unidentified Se peaks had much lower signal than SeMet in all samples of feed and feed ingredients. With a recovery of SeMet far from 100% (10 to 50%) in commercial feed and feed ingredients (Table 27), there might be a possibility of more Se species in these samples, besides the detected SeMet and the unidentified peaks. More Se species might be extracted, but might be unretained by the chromatographic conditions used in this method. The unretained Se species could therefore be a part of the remaining fractions of the total Se, and the chromatographic conditions used for the method might have affected the results in this study.

4.4 Quality assurance

To assure the quality of the analysis, CRMs were included as control samples for all measurements for total element determination (ICP-MS) and for Se speciation analysis (HPLC-ICP-MS). For the optimized Se speciation method, an evaluation of performance characteristics was also performed, and based on established procedures and definitions related to method development and validation studies (Prichard and Barwick, 2007; Eurachem, 2014; Eurachem and CITAC, 2016).

4.4.1 Control samples

The measured element concentrations (mean ± SD) for the CRMs ERM BB422, TORT-3, and SRM 1566b used for the total element determination were all within 2xSD of the certified values (Table 28).

Table 28: The measured concentrations of Se (mean ± SD, mg/kg dw) and other elements in the CRMs ERM BB422, TORT-3 and SRM 1566b, and compared to the certified values.

CRM	Element	Measured concentration (mean ± SD, mg/kg dw)	Certified value ± SD (mg/kg dw)
ERM BB422 (n = 13)	Se	1.36 ± 0.05	1.33 ± 0.13
	As	12.8 ± 0.3	12.7 ± 0.7
	Cd	0.008 ± 0.002	0.0075 ± 0.0018
	Cu	1.71 ± 0.09	1.67 ± 0.16
	Fe	8.9 ± 0.4	9.4 ± 1.4
	Hg	0.56 ± 0.02	0.601 ± 0.030
	Mn	0.37 ± 0.02	0.368 ± 0.028
	Zn	16.7 ± 1.0	16.0 ± 1.1
TORT-3 (n = 8)	Se	10.6 ± 0.9	10.9 ± 1.0
SRM 1566b (n = 4)	Se	2.0 ± 0.1	2.06 ± 0.15

4.4.2 Evaluation of performance characteristics of the method

Method validation is usually considered to be closely tied to method development (Prichard and Barwick, 2007; Eurachem, 2014; Eurachem and CITAC, 2016). Many of the method performance characteristics such as selectivity, precision, trueness, LOD and LOQ, measurement range and ruggedness, are normally evaluated as part of a method development (Prichard and Barwick, 2007). In this project, full method validation was not performed. Instead, an assessment of selected method performance characteristics, including selectivity, intermediate precision and trueness were evaluated for the developed method.

4.4.2.1 Selectivity

The selectivity for SeMet measurements was determined by evaluating the HPLC-ICP-MS chromatograms of blank samples, of the certified reference materials (ERM BC210a and SELM-a), experimental diets and commercial fish feed and feed ingredients (section 4.3). Except for the experimental diet F, the

chromatographic peaks for SeMet show good resolution, with a baseline separation for the analyte and no interferences from other Se signals. The optimized extraction method did not cause additional interferences in the chromatographic analysis, and the selectivity for SeMet in the sample types and concentration range studied was overall good. On the other hand, the optimized method might not be selective when the fish feed contains unnaturally high concentrations of other species, such as the inorganic species selenite.

The blank samples analyzed in each sequence had SeMet concentrations that ranged from 0.02 to 0.05 mg Se/kg. These concentrations could be from the ammonium phosphate buffer and/or the combined enzyme of protease and cellulase. Since all samples and blank samples were supplied with the same extraction solution, there might have been analyte interferences from the extraction solution in all samples. The interferences could affect the concentrations determined in the samples. However, all sample measurements were corrected based on the blank sample measurements.

4.4.2.2 Intermediate precision

The determined concentrations of SeMet (mg Se/kg, mean ± SD, n = 3) in ERM BC210a and SELM-1 from five subsequent days of analysis and the intermediate precision as reproducibility for the method, given as the RSD (%) of SeMet (mg Se/kg, mean ± SD, n = 3), are shown in Table 29. RSD (%) was calculated using equation (11).

Table 29: Calculated RSD (%) based on the determinations of SeMet concentrations (mg Se/kg dw mean ± SD, n = 5 days of analysis) in ERM BC210a and SELM-1, the expected RSD(%) and the acceptable RSD (%) based on Horwitz ratio (NMKL 5, 2003). RSD (%) was calculated using equation (11).

Sample	Mean SeMet (mg/Se kg dw)	RSD (%)	Expected RSD	Acceptable RSD
ERM BC210a	7.85 ± 0.40	5.1	16	32
SELM-1	968.9 ± 57.4	5.9	5.6	11

The RSDs were 5.1% for ERM BC210a and 5.9% for SELM-1 (Table 29) and were within acceptable RSD levels at 32% and 11% based on the Horwitz ratio (NMKL 5, 2003). For ERM BC210a, the RSD (%) was within both the expected value (16%) and acceptable value (32%) for the relevant concentration. The RSD (%) for SELM-1 was higher than the expected value (5.6%) but was within the acceptable value (11%) for the concentration level. The RSDs are, however, based on only five days of measurements, having replicate samples of (n = 1 to 3) (Appendix G, Table G3). For a more accurate assessment of reproducibility, duplicated or triplicated measurements from a minimum of seven days should be applied to evaluate the spread over a longer period (Prichard and Barwick, 2007). Although repeatability was not assessed in this

study, the reproducibility is typically two or three times larger than the repeatability for a method (Prichard and Barwick, 2007), and could be assumed applicable also for this method.

Furthermore, for a more accurate assessment of the precision as reproducibility, relevant sample matrices, i.e. fish feed should be applied. This is currently not possible since CRMs of fish feed with certified values for SeMet are not commercially available. Assessment of precision can also be performed on reference materials that have been controlled in-house or by a third-party, which follows similar criteria as CRM (Prichard and Barwick, 2007).

4.4.2.3 Trueness

Trueness is the degree of agreement between the true content of an analyte and the result obtained (NMKL 5, 2003). The trueness evaluated from the results from five subsequent days of analysis of SeMet in ERM BC210a and SELM-1 is shown in Table 30. Trueness was also assessed by using the experimental diets, where the analytical recoveries (%) for SeMet in the diets (diet A, B, D and E) (Table 26) was calculated by comparing the results with the supplemented concentrations (Antony Jesu Prabhu *et al.* (2020); Berntssen *et al.* (2018b)).

Table 30: Trueness given as the analytical recoveries (%, mean ± SD, n = 3) for SeMet in ERM BC210a (certified value for SeMet: 11.03 ± 1.05 mg/kg) and SELM-1 (certified value for SeMet; 1284 ± 105 mg/kg) in five days of analysis. Trueness was calculated using equation (7).

Day	ERM BC210a SeMet (mg Se/kg)	ERM BC210a, trueness (analytical recovery, %)	SELM-1 (mg Se/kg)	SELM-1, trueness (analytical recovery, %)
1	7.29 ± 0.3	66 ± 3	1010 ± 33	79 ± 3
2	8.01*	73*	985*	77*
3	7.58 ± 0.13*	69 ± 1	919*	72*
4	8.19*	74*	900*	70*
5	8.19 ± 0.2	74 ± 2	1031 ± 51	80 ± 4
Trueness (n = 5)		71 ± 4		77 ± 5

* n = 1-2

The trueness for the SeMet measurements was 71 ± 4% for ERM BC210a and 77 ± 5% for SELM-1 (Table 30). With a 95% confidence interval, the calculated trueness for the optimized method is not acceptable, based on mean certified values for ERM BC210a (11.03 ± 1.05 mg Se/kg) and SELM-1 (1284 ± 105) (section 3.1.4.3, Table 10). Based on the lower level of the certified values, the trueness is 79±4% for ERM BC210a and 82±5% for SELM-1. These values for trueness were higher than the trueness from the mean certified levels for ERM BC210a (t-test, p = 0.014285), but was not significantly different from the trueness from the mean certified levels for SELM-1 (t-test, p = 0.05126).

The trueness based on the SeMet measurements and supplementation in the experiment diets were 62 ± 9 (Table 26). This is lower than the calculated trueness from CRMs. Using the supplemented levels as the target value is not of similar reliability as a certified value in CRMs, since the final concentration in the diets might be different than the intended. The trueness from measurements in experiment diets can therefore only be considered as estimates.

Trueness was also determined for commercial fish feed and feed ingredients by performing spike-experiments. Selected samples of fish feed, fish meal, plant meal and insect meal were spiked with a standard solution of SeMet (5 µg/L) to assure the chromatographic assignment of a peak to SeMet. The SeMet concentrations (µg Se/L) of each sample type, fish feed (n = 1), fish meal (n = 1), plant meal (n = 1) and insect meal (n = 1), SeMet concentrations (µg Se/L) of the samples spiked with 5 µg Se/L of SeMet standard after enzymatic digestion, and chromatographic (spike) recoveries (%) calculated using equation (10) are shown in Table 31.

Table 31: SeMet concentrations (µg Se/L) in samples of fish feed (n = 1), fish meal (n = 1), plant meal (n = 1) and insect meal (n = 1) and in spiked samples (5 µg Se/L SeMet), and the chromatographic recoveries from respective samples calculated using equation (10).

Sample	Sample concentration (µg Se/L)	Sample concentration after spiked (µg Se/L)	Difference (µg Se/L)	Recovery (%)*
FF1	1.51	6.69	5.18	104
FM2	3.02	8.36	5.34	107
PM1	0.88	5.22	4.34	87
IM2	0.81	6.16	5.36	107
Mean ± SD, chromatographic recovery (%)				101 ± 10

It is seen that the recoveries of the SeMet spiked samples were 87-107% (Table 31), which shows that the determination of SeMet is sufficient from the chromatography. The samples were spiked after the extraction step was performed. This means that the recoveries from spiked samples do not consider the extraction procedure itself, and therefore not the whole analytical procedure. The mean recovery (%) calculated for the spiked samples was 101 ± 10% for SeMet, which shows that all SeMet spiked to the samples are recovered. The trueness for SeMet in CRMs and the experimental diets, which were significantly lower than what was seen for spiked samples is therefore likely related to an insufficient extraction of SeMet in the samples.

The aim of the method development was to optimize the method by improving the extraction recovery for SeMet in fish feed and feed ingredients. However, the low analytical recoveries were seen for the CRMs and the experimental samples show that the extraction method is not still optimal for the extraction of

SeMet. Further work needs to be performed to improve the extraction efficiency of SeMet in both CRMs and fish feeds.

4.4.3 Source of errors – Se speciation

Method development

Some of the enzyme combinations (enzyme combination 5, 6, 7 and 8, Figure 21) gave low recoveries of SeMet in ERM BC210a and SELM-1, with a larger spread in the recoveries than other enzyme combinations. These combinations were evaluated in experiment B of the screening step, which included the enzymes α-amylase and cellulase. The SeMet concentrations and recoveries in experiment B (Table 23) showed large variations, as well as lower concentrations than in experiment A (Table 22). There were also some deviations between the replicates with SELM-1 in experiment B, which was seen in the PCA biplot (Figure 19.b).

One explanation for the large variation in the concentrations in experiment B could be that samples in experiment B were not shaken during the pre-extraction step, while they were shaken during the main extraction. Another reason could be linked to challenges that were experienced in the separation of the soluble fraction from the non-soluble fraction after enzymatic digestion in experiment B, and not in experiment A. The poor separation could be caused by the amount of enzyme powder in the extraction solution for the combined enzyme, as well as the two-step enzymatic process including a pre-extraction step with papain. The original amount of protease was kept, with addition of cellulase. The large amount of enzyme powder did affect the solubility of some of the samples, which could have affected SELM-1 more than ERM BC210a, since less spread is seen in the replicated measurements in ERM BC210a.

All samples in experiment B were treated the same way, even though they were not treated the same as in experiment A. Due to this, the low recoveries for total Se and SeMet in experiment B are not considered to affect the significance of the factors within experiment B.

Application of method

Two of the experimental diets, diet A and B, had somewhat higher amounts of Se in the soluble fraction than the total Se in the whole sample, resulting in over 100% recovery (Table 26). These results can be explained by the additional uncertainties in the total Se determinations of the extracts when performing acid digestion of extracts, or by the homogeneity of the samples. Fish feed is a challenging sample matrix to homogenize, with a high fat and protein content. The measurement results can therefore have a large spread in the element concentrations due to poor homogenization.

Another source of error in the experimental diets could be related to the different homogenization methods applied. For the experimental diet samples, homogenization was performed by impact with liquid nitrogen for a more pulverized sample for application of the optimized method, while another type of homogenization method was applied for the samples when total Se was determined (Antony Jesu Prabhu *et al.*, 2020; Berntssen *et al.*, 2018b). The deviations (%) for total Se concentrations in the soluble and non-soluble fractions were within 5% for the SeMet supplemented diets (diet A, B, D and E) (Appendix G, Table G1). For diet C and F, however, deviations of 8.4% and 5.7% were found for total Se in replicates of soluble fractions (Appendix G, Table G1). Ideally, the deviations of triplicate measurements should be below 5% with a level of confidence of 95% (Prichard and Barwick, 2007).

The deviations for most of the mean SeMet concentrations (n = 3) were above 5%, but below 10% for the commercial fish feed, feed ingredients and CRMs (Appendix G, Table G2). Large deviations (%) were found in some of the experimental diets for the mean SeMet concentrations: diet A (33%), diet C (34%) and diet F (55%) (Appendix G, Table G1). The high deviations in these samples might have affected the SeMet recoveries discussed in this study. Since only two of the samples, diet C and diet F, were not SeMet supplemented diets with very low SeMet concentrations from the analysis, these samples were not considered for evaluating SeMet recovery in SeMet supplemented feed. Diet A also contained lower SeMet concentration but was supplemented with SeMet. The large deviation means that the SeMet recovery in diet A should be considered as more uncertain. To increase the precision in the measurements of the samples with higher deviations, more replicates should have been analyzed to potentially decrease the uncertainties.

5 CONCLUSIONS

Correlation study in Atlantic Salmon

For the salmon samples, strong negative correlations were found between Se and Hg, which corresponds to well-established knowledge about the protective role of Se against toxic effects of Hg. In whole-body samples, moderate positive correlations were found between Se and Cu, which also corresponds to earlier studies in salmonids. A moderate positive correlation was also found between Se and As in fillet samples of salmon fed inorganic Se and a strong negative correlation between Se and Fe in whole-body of salmon fed inorganic Se. Weaker or no interaction was found between Se and As, and between Se and Fe, in salmon fed organic Se in the same sample types, indicating that inorganic Se supplemented in diets interact differently to As and Fe than organic Se.

The best approach for determining the correlation coefficients for the whole-body samples of Atlantic salmon fed with either inorganic or organic Se was Pearson's correlation. The subsets of the elements Hg, Mn and Zn in the dataset for fillet samples of salmon were not normally distributed, and Spearman's rank-order correlations meant for non-parametric data were applied in addition to Pearson's correlations. The Spearman's rank-order correlations gave different degrees of correlation between Se and As, and between Se and Hg compared to Pearson's correlations. Spearman's Rank-order correlation was therefore chosen as the best approach for the fillet samples.

Method development – Se speciation in fish feed and feed ingredients

Compared to the mixed buffer, ammonium phosphate was considered as the best buffer solution for increasing SeMet recoveries in both CRMs tested (i.e. ERM BC210a and SELM-1). Therefore, ammonium phosphate was applied as a buffer for further optimization. Among the enzymes tested, protease XIV and cellulase gave a noticeable increase in SeMet recovery. Compared to α-amylase, cellulase was considered the most effective. The recovery for SeMet in the CRMs increased when including a pre-extraction step with papain prior to the main enzymatic digestion step with cellulase.

From the optimization, the combination of protease and cellulase showed an increase in SeMet recovery in ERM BC210a compared to protease alone, while the interaction between the combined enzyme (protease and cellulase) and no pre-extraction step showed an increase in SeMet recovery for both ERM BC210a and SELM-1. Based on this, the most optimal conditions for increasing SeMet recoveries is to omit a pre-extraction step, and to use a combined enzyme of protease and cellulase for enzymatic digestions

with ammonium phosphate as a buffer. The optimized method was further applied to experimental and commercial feeds and feed ingredients.

The comparison between the optimized method and the initial method showed no significant difference ($p = 1$) in the SeMet recovery. The largest increase in SeMet recovery was seen for one of the plant meals and for the insect meal when using with the optimized method compared to the initial method. The optimized method was seen to be more suitable for plant-based materials than marine-based materials, since the highest extraction efficiency for SeMet was found in plant meal (41-50%) and the lowest in fish meal (10-17%).

For experimental diets supplemented with SeMet, 50 to 69% extraction efficiency for SeMet was achieved, while for commercial fish feed and feed ingredients the extraction efficiency for SeMet ranged from 10 to 50%. These results indicate that the optimized method is more accurate for determining SeMet in fish feed when SeMet is present in higher concentrations. Some quality parameters (i.e. selectivity, trueness and intermediate precision) for the optimized Se speciation method were assessed. A good selectivity was shown for SeMet in the CRMs (ERM BC210a and SELM-1), commercial fish feed, feed ingredients, and for experimental diets using the optimized method. It was also shown that this method might not be selective for fish feed that contains high concentrations of supplemented inorganic Se. The trueness evaluated from the measured concentrations of SeMet in CRMs, and experimental diets supplemented with SeMet, ranged from 50 to 69%. This is not considered optimal for an analytical method and shows that the method needs to be further improved to increase SeMet extraction efficiency for fish feed and feed ingredients. The intermediate precision was not fully assessed but it was considered acceptable based on the available measurements of CRMs ERM BC210a and SELM-1.

The HPLC-ICP-MS chromatograms shown clear chromatographic peaks for SeMet in all samples of fish feed and feed ingredients. This means that SeMet was detected in all samples of fish feed and feed ingredients. Two of the fish feed samples had higher SeMet concentrations than the established MLs for organic Se in animal feed. However, it was not possible to distinguish if the SeMet measured was supplemented or naturally occurring from the feed ingredients. In the chromatograms, unidentified Se peaks were detected in some of the fish meal and fish feed. These Se peaks were not identified with the current methodology, since there are challenges in the identification of species using ICP-MS.

Overall, the statistical and chemometric approaches showed to be valuable in this thesis. Exclusion of outliers through visualization of normalized data and normality tests were useful for handling a larger dataset with large deviations and for further finding correlation coefficients. For method development, experimental design and PCA were useful for finding optimal conditions for Se speciation of organic Se. By

using experimental designs, the number of experiments was limited, while facilitating for finding the best conditions for increasing SeMet recovery through PCA. From multivariate experimental design, information about not only the effect of every factor on the responses was provided, but also information about the interactions between factors. With numerous choices of enzymes and other steps that are usually involved in extraction methods for Se speciation, experimental design and PCA should be considered for the future development of Se speciation methods.

6 FUTURE PERSPERCTIVES

Correlation study in Atlantic Salmon

Further studies could be performed on the biological and toxicological aspects of Se from the results obtained using the polished dataset. In addition, further work on other types of tissues, such as liver and brain from the same fish trial would give valuable information on the interaction of Se with other elements. However, the sample homogenization method, data management and the statistical approaches used should be carefully considered.

Se speciation in fish feed and feed ingredients

In order to increase the SeMet recovery (%), it could be interesting to perform further testing on other types of enzymes seen in previous Se speciation studies (e.g. flavourzyme, carboxypeptidase, trypsin, proteinase k, pancreatin). It could also relevant to evaluate the combination of protease XIV and cellulase with different ratios, the effect of protease combined with other enzymes (e.g. α-amylase), addition of a sonication step, several extraction solutions, extractions times and temperatures. If using an extended screening, the optimization steps could be performed using other types of experimental design (e.g. response surface design). Preferably the method optimization and method validation should be performed using a fish feed reference material. Considering that this material is not commercially available, preparing an in-house reference material could be useful.

For further work on Se speciation, the unknown species that were observed in fish meal samples could be identified by spiking the samples with Se species that might be present, such as selenate, selenite, $SeCys_2$, SeMetSeCys, GGMSC and SeOMet. Another approach would be to apply the same chromatographic settings in a HPLC-HR-MS/MS system, for identifying the unknown Se species in the samples. Furthermore, a derivatization step could also be performed prior to enzymatic digestion for assessing SeCys in the fish feed and feed ingredients.

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APPENDICES

Appendix A – Compositions of experimental diets

The composition of basal diet of experimental diet A and B, previously studied by Antony Jesu Prabhu *et al.* (2020), are given in Table A1. For experimental diets, diet C-F, previously studied by Berntssen *et al.* (2018b), the composition is given in Table A2.

Table A1: Diet composition I; Composition of basal experimental diet for Atlantic salmon post-smolt, modified from (Antony Jesu Prabhu et al., 2020).

Diet composition I

Source	Ingredient	Percentage
Plant	Whole wheat	10
Plant	Corn gluten	10
Plant	Wheat gluten	20
Plant	Soy protein concentrate	30
Plant	Faba beans, whole	1
Marine	Fish meal	1
Marine	Fish oil	10.5
Plant	Rapeseed oil	13
Other	Micro-ingredients and Se-free premixes	4.4
Other	Yttrium premix	0.1

Source	Percentage
Marine	11.5
Plant	84
Other	4.5

Source	Percentage
Wheat	30

Table A2: Diet composition II; Composition of basal experimental diet for Atlantic Salmon smolt, modified from (Berntssen et al., 2018)

Diet composition II

Source	Ingredient	Percentage
Marine	Fish meal	10
Plant	Soya protein concentrate	10
Plant	Wheat gluten	17
Plant	Maize gluten	10
Plant	Pea protein 50	5
Plant	Pea protein >75	5
Plant	Wheat	10.5
Marine	Fish oil	12.2
Plant	Rape seed oil	12.2
Other	Micro-nutrient mixture	8.1

Source	Percentage
Marine	22.2
Plant	69.7
Other	8.1

Source	Percentage
Wheat	27.5

Appendix B – LOD and LOQ for determination of Se and other elements using ICP-MS

Limit of detection (LOD) and limit of quantification (LOQ) for determination of total concentration of the elements Se, As, Cd, Co, Cr, Cu, Fe, Hg Mn, Pb and Zn using ICP-MS are presented in Table B1, acquired from the established method (Institute of Marine Research, 2020a). LOD is the lowest amount of an analyte that can be quantified with a reasonable certainty (NMKL 4, 2005), determined as 3xSD from a minimum of 20 blind samples. The values of LOD are determined as 10xSD from a minimum of 20 blind samples, based on 0.2 g samples diluted to 25 mL for LOQ in mg/kg dw (Institute of Marine Research, 2020a).

Table B1: LOD and LOQ for determination of total concentrations of elements using ICP-MS (Institute of Marine Research, 2020a).

Element	LOD (µg/L)	LOQ (µg/L)	LOQ (mg/kg dw)
Se	0.02	0.08	0.01
As	0.02	0.08	0.01
Cd	0.01	0.04	0.005
Co*	0.05	0.2	0.02
Cr*	0.07	0.2	0.03
Cu	0.2	0.8	0.1
Fe*	0.2	0.8	0.1
Hg	0.01	0.04	0.005
Mn*	0.07	0.2	0.03
Pb	0.07	0.2	0.030
Zn	1.2	4	0.5

* Not accredited.

Appendix C – Data polishing for correlation study

The first number of the codes, i.e. "1-1-1", in the tables of Appendix C represents the samples and replicates of samples of both whole-body and fillet of Atlantic salmon. The first digit of the codes represents the diet fed to the salmon, the second digit is the number of a biological replicate, while the third digit represents the number of a technical replicate for a sample.

The salmon fed with basal diet starts with the code "1". The salmon fed with selenite supplemented diets with Se concentration 5.4 mg Se/kg and 11 mg Se/kg starts with the codes "2" and "3", respectively. The salmon fed with SeMet supplemented diets starts with the codes "6" for 6.2 mg Se/kg, "7" for 16 mg Se/kg, "8" for 21 mg Se/kg and "9" for 39 mg Se/kg.

Measurements of elements in salmon samples

The measured concentrations of Se, As, Cd, Co, Cr, Cu, Fe, Hg, Mn, Pb and Zn in whole-body samples and fillet samples are shown in Table C1 and Table C2, respectively.

Table C1: Measurements of relevant elements (mg/kg) in technical replicates whole-body samples fed with diets supplemented with Se. Extreme values identified as outliers are marked in strikethrough.

Diet - Bio.rep - T.rep	Se	As	Cd*	Co*	Cr*	Cu	Fe	Hg	Mn	Pb*	Zn
1-1-1	0.134	0.5597	0.0015	0.0038	0.0119	1.263	~~10.24~~	0.0084	1.034	0.0005	18.05
1-1-2	0.145	0.5859	0.0016	0.0040	0.0360	1.283	~~12.84~~	0.0085	1.520	0.0010	23.32
1-1-3	0.131	0.5904	0.0011	0.0038	0.0107	1.234	~~10.09~~	0.0086	1.320	0.0009	19.91
1-1-4	~~0.157~~	0.5893	0.0016	0.0041	0.0067	~~1.835~~	11.22	0.0088	0.9294	0.0003	~~36.04~~
7-1-1	5.959	0.6084	0.0010	0.0046	0.0109	1.309	11.42	0.0071	1.366	0.0004	30.99
7-1-2	5.784	0.6001	0.0010	0.0050	0.0198	1.252	11.20	0.0068	1.706	0.0005	30.62
7-1-3	5.640	0.5864	0.0013	0.0047	0.0116	1.267	10.83	0.0071	1.241	0.0007	29.15
7-1-4	5.298	0.5852	0.0009	0.0042	0.0058	1.211	11.34	0.0068	1.155	0.0001	26.85
9-1-1	11.35	0.6119	0.0013	0.0045	0.0092	2.874	14.40	0.0058	1.550	0.0011	42.31
9-1-2	11.32	0.6257	0.0017	0.0048	0.0140	2.860	14.34	0.0059	1.483	0.0027	43.29
9-1-3	11.79	0.6276	0.0016	0.0049	0.0100	2.902	13.77	0.0054	1.487	0.0039	51.78
9-1-4	10.79	0.6384	0.0015	0.0052	0.0081	~~1.937~~	~~12.44~~	0.0063	0.7243	0.0002	44.60
2-1-1	0.756	0.5312	0.0111	0.0053	0.0232	1.319	11.06	0.0054	1.326	0.0011	28.29
2-1-2	0.688	~~0.4710~~	0.0099	0.0047	0.0212	1.157	~~9.50~~	~~0.0045~~	1.245	0.0006	26.30
2-1-3	~~0.784~~	0.5291	0.0114	0.0058	0.0225	1.332	11.13	0.0050	1.287	0.0009	28.36
2-1-4	~~0.591~~	0.5345	0.0041	0.0048	0.0182	1.400	10.81	~~0.0061~~	0.6223	0.0004	~~13.29~~
8-1-1	7.168	0.6779	0.0018	0.0044	0.0155	1.211	12.15	0.0079	1.006	0.0012	39.55
8-1-2	6.729	0.6505	0.0016	0.0042	0.0094	1.141	11.63	0.0071	1.110	0.0004	28.74
8-1-3	6.632	0.6497	0.0021	0.0041	0.0069	1.161	~~11.35~~	0.0072	1.172	0.0003	31.15
8-1-4	7.017	0.6716	0.0017	0.0047	0.0068	~~1.625~~	~~13.34~~	0.0078	0.8410	0.0002	37.06
3-1-1	1.334	0.6926	0.0023	0.0044	0.0096	1.600	10.83	0.0054	0.967	0.0004	34.98
3-1-2	1.311	0.6700	0.0020	0.0043	0.0076	1.576	10.51	0.0049	1.210	0.0002	31.76
3-1-3	1.398	0.6940	0.0020	0.0051	0.0098	1.592	11.24	0.0052	1.557	0.0003	38.23
3-1-4	1.245	0.6698	0.0015	0.0041	0.0078	1.763	11.20	0.0057	1.242	0.0003	~~19.25~~
6-1-1	1.958	0.6384	0.0021	0.0055	0.0114	1.504	11.79	0.0076	1.315	0.0003	45.60
6-1-2	1.917	0.6281	0.0023	0.0055	0.0068	1.429	12.03	0.0084	1.755	0.0003	45.24
6-1-3	1.931	0.6312	0.0021	0.0053	0.0082	1.440	11.98	0.0079	1.436	0.0003	39.85
6-1-4	1.878	0.6461	0.0008	0.0044	0.0099	1.234	~~10.58~~	0.0082	1.716	0.0004	~~22.66~~
2-2-1	0.825	0.4646	0.0063	0.0047	0.0164	1.438	10.46	0.0040	1.031	0.0004	22.13

2-2-2	0.896	0.4855	0.0060	0.0048	0.0166	1.532	10.99	0.0044	0.8275	0.0003	24.80
2-2-3	0.811	0.4841	0.0058	0.0050	0.0208	1.521	11.10	0.0043	0.7550	0.0007	21.69
2-2-4	0.913	0.4746	0.0077	0.0052	0.0182	1.750	10.93	0.0042	1.446	0.0005	20.79
8-2-1	6.754	0.6087	0.0017	0.0054	0.0154	1.919	14.26	0.0068	1.590	0.0003	28.48
8-2-2	6.551	0.5998	0.0015	0.0051	0.0149	1.895	13.58	0.0065	1.777	0.0003	27.06
8-2-3	6.903	0.6128	0.0011	0.0056	0.0164	1.857	13.80	0.0070	1.321	0.0005	31.29
8-2-4	6.655	0.6122	0.0010	0.0046	0.0106	~~1.445~~	~~12.31~~	0.0073	1.013	0.0004	29.53
6-2-1	1.991	0.6528	0.0013	0.0044	0.0121	1.406	10.55	0.0087	1.046	0.0003	41.21
6-2-2	2.013	0.6708	0.0014	0.0046	0.0095	1.393	11.35	0.0091	1.199	0.0007	38.21
6-2-3	1.994	0.6663	0.0012	0.0047	0.0181	1.357	10.58	0.0080	1.272	0.0002	41.20
6-2-4	1.856	0.6778	0.0009	0.0047	0.0064	1.319	10.64	0.0089	0.9348	0.0002	~~16.91~~
9-2-1	9.776	0.6650	0.0013	0.0040	0.0101	1.802	12.58	0.0076	0.9520	0.0002	28.68
9-2-2	9.896	0.6710	0.0015	0.0041	0.0137	1.796	12.71	0.0074	1.075	0.0004	30.37
9-2-3	9.753	0.6574	0.0012	0.0040	0.0097	1.716	12.36	0.0069	1.014	0.0004	31.03
9-2-4	9.292	0.6608	0.0006	0.0042	0.0126	1.781	11.94	0.0077	0.9575	0.0003	16.82
3-2-1	1.598	0.5931	0.0023	0.0042	0.0070	2.277	9.792	0.0049	1.229	0.0004	28.87
3-2-2	~~1.707~~	0.5979	0.0026	0.0039	0.0077	2.282	9.808	0.0046	1.096	0.0003	34.45
3-2-3	1.518	0.5941	0.0020	0.0040	0.0075	2.349	10.16	0.0049	1.057	0.0003	26.91
3-2-4	1.459	0.5798	0.0046	0.0047	0.0076	~~1.780~~	10.07	0.0051	1.123	0.0002	28.86
7-2-1	5.903	0.6353	0.0011	0.0046	0.0104	1.789	11.53	0.0070	1.061	0.0004	21.13
7-2-2	5.907	0.6263	0.0011	0.0047	0.0106	1.696	10.97	0.0070	0.9222	0.0005	23.69
7-2-3	5.936	0.6502	0.0016	0.0047	0.0109	1.542	11.43	0.0073	0.7115	0.0005	28.12
7-2-4	5.820	0.6036	0.0014	0.0047	0.0063	~~1.242~~	11.30	0.0069	0.8623	0.0002	26.11
1-2-1	~~0.172~~	0.6490	0.0012	0.0041	0.0120	1.502	11.32	0.0092	1.013	0.0006	18.86
1-2-2	0.148	0.6364	0.0014	0.0047	0.0132	1.412	10.81	0.0087	0.8160	0.0005	16.00
1-2-3	0.143	0.6408	0.0012	0.0039	0.0122	1.407	11.26	0.0085	1.449	0.0004	16.59
1-2-4	0.153	0.6885	0.0015	0.0043	0.0083	1.556	11.12	0.0098	0.8413	0.0004	20.93
9-3-1	9.975	0.6896	0.0012	0.0041	0.0105	1.699	12.13	0.0068	1.151	0.0004	25.94
9-3-2	9.530	0.6386	0.0011	0.0038	0.0103	1.578	11.69	0.0064	1.083	0.0006	39.98
9-3-3	9.490	0.6538	0.0010	0.0036	0.0106	1.615	11.89	0.0065	1.469	0.0006	23.48
9-3-4	9.940	0.6685	0.0015	0.0040	0.0079	1.665	12.20	0.0068	1.250	0.0004	47.53
3-3-1	1.351	0.6429	0.0025	0.0091	0.0147	2.062	10.22	0.0051	1.512	0.0014	25.69
3-3-2	1.366	0.6396	0.0029	0.0048	0.0096	2.002	10.15	0.0048	1.181	0.0012	28.20
3-3-3	1.388	0.6571	0.0024	0.0045	0.0106	2.158	10.62	0.0050	1.203	0.0003	24.18
3-3-4	1.357	0.6285	0.0024	0.0043	0.0052	1.785	10.41	0.0050	1.587	0.0003	26.66
1-3-1	0.1529	0.6426	0.0021	0.0040	0.0095	1.751	12.19	0.0093	1.253	0.0008	29.90
1-3-2	0.1535	0.6575	0.0018	0.0042	0.0111	1.783	12.06	0.0093	1.100	0.0007	29.48
1-3-3	0.1593	0.6499	0.0020	0.0039	0.0099	1.721	12.25	0.0094	1.473	0.0008	32.93
1-3-4	0.1592	~~0.7030~~	0.0014	0.0042	0.0054	1.551	12.29	0.0102	1.245	0.0004	~~14.87~~
6-3-1	2.109	0.6244	0.0012	0.0049	0.0186	1.727	11.33	0.0084	1.440	0.0007	38.78
6-3-2	2.069	0.6369	0.0011	0.0049	0.0100	1.514	11.40	0.0085	1.391	0.0005	26.66
6-3-3	2.028	~~0.7384~~	0.0011	0.0047	0.0082	1.772	11.11	0.0080	1.151	0.0008	31.84
6-3-4	1.983	0.6258	0.0007	0.0044	0.0071	~~1.282~~	11.12	0.0083	1.270	0.0005	20.76
8-3-1	6.683	0.6634	0.0014	0.0041	0.0105	1.775	11.92	0.0076	0.9104	0.0017	21.29
8-3-2	6.973	0.6977	0.0013	0.0043	0.0125	1.724	12.38	0.0082	0.9020	0.0003	20.85
8-3-3	6.623	0.6639	0.0012	0.0040	0.0122	1.747	12.10	0.0074	1.433	0.0005	21.06
8-3-4	6.804	0.6680	0.0010	0.0045	0.0156	1.448	12.04	0.0082	0.9257	0.0007	17.68
2-3-1	0.7621	0.5004	0.0043	0.0048	0.0150	1.471	10.48	0.0041	1.226	0.0006	22.53
2-3-2	0.7187	0.4981	0.0042	0.0048	0.0141	1.346	10.28	0.0041	0.8394	0.0014	18.09
2-3-3	0.7719	0.5050	0.0050	0.0051	0.0169	1.404	10.64	0.0039	1.182	0.0008	22.94
2-3-4	0.7847	0.5010	0.0043	0.0045	0.0134	1.475	9.749	~~0.0048~~	0.7001	0.0010	14.05
7-3-1	5.928	0.6018	0.0030	0.0060	0.0753	1.566	~~14.31~~	0.0067	1.228	0.0010	39.88
7-3-2	6.057	0.6132	0.0023	0.0055	0.0389	1.487	12.40	0.0066	1.403	0.0012	43.81
7-3-3	5.742	0.5912	0.0023	0.0053	0.0205	1.553	~~11.70~~	~~0.0057~~	1.295	0.0011	40.99
7-3-4	5.802	0.5956	0.0014	0.0052	0.0149	1.471	11.99	0.0068	1.523	0.0010	27.01

* Measurements of Cd, Co, Cr and Pb were below LOQ.

Table C2: Measurements of relevant elements (mg/kg ww) in technical replicates fillet samples fed with diets supplemented with Se. Extreme values identified as outliers are marked in strikethrough.

Diet - Bio.rep - T.rep	Se	As	Cd*	Co*	Cr	Cu	Fe	Hg	Mn	Pb*	Zn
1-1-1	0.1282	0.6703	0.0001	0.0025	0.0627	0.3451	2.336	0.0078	0.1061	0.0014	4.038
1-1-2	0.1236	0.6887	0.0001	0.0024	0.0675	0.3636	2.482	0.0074	0.1586	0.0024	4.177
1-1-3	0.1180	0.6771	0.0001	0.0024	0.0603	0.3346	2.218	0.0072	0.1580	0.0082	4.046
1-1-4	0.1176	0.7053	0.0001	0.0019	0.0009	0.3559	2.111	0.0110	0.1850	0.0002	4.039
7-1-1	6.039	0.7028	0.0001	0.0025	0.0174	0.3014	2.131	0.0066	0.1783	0.0016	4.299
7-1-2	5.970	0.7240	0.0001	0.0029	0.0183	0.3402	2.321	0.0065	0.0976	0.0020	4.166
7-1-3	6.089	0.7335	0.0002	0.0028	0.0225	0.3515	2.398	0.0064	0.1719	0.0069	4.331
7-1-4	5.880	0.7333	-0.0001	0.0025	0.0065	0.3196	2.243	0.0087	0.1008	0.0002	4.045
9-1-1	11.323	0.8198	0.0002	0.0026	0.0307	0.3219	2.233	0.0057	0.1191	0.0056	4.188
9-1-2	11.587	0.8287	0.0002	0.0027	0.0356	0.3399	2.397	0.0060	0.1344	0.0059	4.266
9-1-3	11.315	0.8168	0.0001	0.0024	0.0284	0.3110	2.313	0.0061	0.1901	0.0008	4.197
9-1-4	10.769	0.8221	0.0001	0.0028	0.0017	0.3398	2.273	0.0074	0.1369	0.0002	4.038
2-1-1	0.2624	0.5370	0.0002	0.0036	0.0274	0.3500	2.089	0.0044	0.1658	0.0033	4.195
2-1-2	0.2474	0.5385	0.0002	0.0036	0.0241	0.3371	2.027	0.0042	0.1361	0.0006	4.057
2-1-3	0.2539	0.5430	0.0001	0.0034	0.0171	0.3447	1.973	0.0044	0.0968	0.0008	4.040
2-1-4	0.2483	0.5480	-0.0001	0.0034	0.0015	0.3105	1.956	0.0061	0.1173	-0.0001	3.896
8-1-1	7.373	0.7224	0.0001	0.0024	0.0115	0.3911	2.363	0.0054	0.1194	0.0007	4.119
8-1-2	7.616	0.7369	0.0001	0.0027	0.0139	0.3676	2.299	0.0056	0.0916	0.0004	4.176
8-1-3	7.521	0.7353	0.0001	0.0024	0.0270	0.3876	2.452	0.0070	0.1036	0.0008	4.158
8-1-4	7.035	0.7429	-0.0001	0.0028	0.0023	0.3628	2.361	0.0086	0.1384	-0.0001	4.047
3-1-1	~~0.3608~~	~~0.8239~~	0.0001	0.0024	0.0267	0.3692	2.423	0.0046	0.1693	0.0012	4.399
3-1-2	0.3315	0.7940	0.0000	0.0025	0.0359	0.3540	2.311	0.0042	0.1116	0.0007	4.178
3-1-3	0.3390	0.8041	0.0000	0.0026	0.0243	0.3544	2.272	0.0041	~~0.2280~~	0.0030	4.349
3-1-4	0.3217	0.7880	0.0000	0.0024	0.0255	0.3750	2.450	~~0.0075~~	0.1139	0.0002	3.893
3-1-5	0.3210	0.7727	0.0000	0.0024	0.0240	0.3463	2.318	0.0060	0.1269	0.0001	3.847
3-1-6	0.3263	0.7709	0.0000	0.0024	0.0188	0.4120	2.609	0.0054	0.2009	0.0001	4.000
3-1-7	0.3340	0.8189	-0.0001	0.0025	0.0018	0.3404	2.227	0.0062	0.1426	0.0003	4.104
6-1-1	2.160	0.6758	0.0000	0.0028	0.0210	0.3041	2.138	0.0058	0.1116	0.0011	4.255
6-1-2	2.157	0.6714	0.0000	0.0026	0.0207	0.3242	2.106	0.0056	0.0921	0.0008	4.288
6-1-3	2.084	0.6528	0.0001	0.0027	0.0243	0.3333	2.133	0.0055	0.1424	0.0004	4.176
6-1-4	2.029	0.6941	0.0000	0.0029	0.0007	0.3166	2.069	~~0.0092~~	0.1762	0.0001	4.245
2-2-1	0.2945	0.5253	0.0000	0.0036	0.0590	0.3638	2.442	0.0026	0.1222	0.0004	4.155
2-2-2	0.2902	0.5153	0.0002	0.0039	0.1017	0.3594	2.468	0.0036	0.1058	0.0052	4.214
2-2-3	0.2800	0.5177	0.0002	0.0038	0.0733	0.3962	2.580	0.0038	0.1865	0.0053	4.521
2-2-4	0.2828	0.5249	-0.0001	0.0035	0.0028	0.3548	2.264	~~0.0055~~	0.1136	0.0001	4.021
8-2-1	8.002	0.7078	0.0001	0.0032	0.0501	0.4050	2.627	0.0055	0.1337	0.0023	4.644
8-2-2	8.031	0.7030	0.0001	0.0033	0.0534	0.3884	2.574	0.0051	0.1223	0.0057	4.725
8-2-3	8.088	0.7003	0.0001	0.0032	0.0506	0.3786	2.473	0.0041	0.1036	0.0019	4.563
8-2-4	7.751	0.6897	-0.0001	0.0026	0.0006	0.3294	2.138	~~0.0076~~	0.1284	0.0000	4.068
6-2-1	2.078	0.7365	0.0000	0.0027	0.0378	0.3652	2.449	0.0094	0.1430	0.0007	4.303
6-2-2	2.040	0.7435	0.0000	0.0028	0.0294	0.3510	2.312	0.0086	0.1386	0.0007	4.246
6-2-3	2.054	0.7330	0.0000	0.0028	0.0393	0.3593	2.371	0.0083	~~0.2814~~	0.0004	4.456
6-2-4	2.010	0.7332	-0.0001	0.0025	0.0075	0.3005	2.282	0.0113	0.1244	0.0001	3.901
9-2-1	11.57	0.7244	0.0000	0.0027	0.0371	0.3512	2.403	0.0046	0.1703	0.0015	4.225
9-2-2	11.84	0.7471	0.0000	0.0028	0.0334	0.3616	2.379	0.0048	0.0980	0.0004	4.190
9-2-3	12.22	0.7591	0.0001	0.0029	0.0330	0.3434	2.427	0.0044	0.1162	0.0003	4.267
9-2-4	11.60	0.7121	0.0000	0.0027	0.0313	0.3019	2.225	0.0062	0.1168	0.0002	3.689
9-2-5	11.63	0.6989	0.0000	0.0027	0.0304	0.3143	2.213	0.0059	0.1483	0.0002	3.751
9-2-6	11.65	0.7609	-0.0001	0.0028	0.0027	0.3250	2.455	0.0064	0.1157	0.0003	3.990
3-2-1	0.3407	0.8567	0.0000	0.0027	0.0263	0.3521	2.016	0.0039	0.1787	0.0006	4.666
3-2-2	~~0.3554~~	0.8583	0.0009	0.0026	0.0302	0.3406	2.008	0.0045	~~0.2918~~	0.0011	~~4.892~~
3-2-3	0.3388	0.8618	0.0001	0.0028	0.0300	~~0.4226~~	1.941	0.0048	0.1233	0.0011	4.578
3-2-4	0.3335	0.8211	0.0001	0.0025	0.0283	0.3229	1.917	0.0062	0.1172	0.0000	3.984
3-2-5	0.3228	0.8044	0.0000	0.0023	0.0305	0.3141	1.848	0.0061	0.1636	0.0000	3.973
3-2-6	0.3099	~~0.7764~~	0.0000	0.0023	0.0236	0.3047	1.815	0.0057	0.1244	0.0001	3.813
3-2-7	~~0.3016~~	0.8639	-0.0001	0.0027	0.0043	0.3213	1.936	0.0060	0.1941	0.0003	4.201

7-2-1	6.317	0.6486	0.0001	0.0032	0.0483	0.3738	2.868	0.0053	0.1005	0.0018	4.601
7-2-2	6.248	0.6503	0.0001	0.0030	0.0478	0.3820	2.543	0.0054	0.1135	0.0006	4.605
7-2-3	6.307	0.6514	0.0000	0.0029	0.0329	0.3802	2.402	0.0055	0.0939	0.0004	4.543
7-2-4	6.009	0.6322	-0.0001	0.0025	0.0029	0.3474	2.234	0.0076	0.0912	0.0001	4.198
1-2-1	~~0.1342~~	~~0.7778~~	0.0001	0.0032	~~0.0799~~	0.3361	2.561	0.0075	0.1428	0.0012	4.247
1-2-2	0.1232	0.7400	0.0001	0.0022	0.0288	0.3466	2.333	0.0087	0.1170	0.0014	4.380
1-2-3	0.1193	0.7397	0.0001	0.0023	0.0516	0.3416	2.397	0.0104	0.1265	0.0005	4.233
1-2-4	0.1216	0.7095	0.0000	0.0019	0.0204	0.2987	2.112	0.0098	0.1043	0.0002	3.690
1-2-5	0.1165	0.7077	0.0000	0.0018	0.0283	0.2974	2.100	0.0100	0.1126	0.0002	3.692
1-2-6	0.1281	0.7105	0.0001	0.0020	0.0264	0.3124	2.183	0.0101	0.1291	0.0001	3.699
1-2-7	0.1269	0.7598	-0.0001	0.0019	0.0026	0.3127	2.149	0.0113	0.1230	0.0002	4.055
9-3-1	9.652	0.8267	0.0001	0.0031	0.0480	0.4335	3.034	0.0085	0.1466	0.0017	4.514
9-3-2	9.826	0.8397	0.0000	0.0030	0.0541	0.4271	3.092	0.0072	0.1580	0.0005	4.560
9-3-3	9.616	0.8239	0.0001	0.0031	0.0608	0.4459	3.237	0.0079	0.1553	0.0008	4.540
9-3-4	9.437	0.8242	-0.0001	0.0027	0.0022	0.3974	2.747	0.0088	0.1266	0.0002	4.190
3-3-1	0.3562	0.8841	0.0001	0.0030	0.0498	0.3579	2.151	0.0058	0.1247	0.0007	4.609
3-3-2	0.3445	0.8820	0.0001	0.0030	0.0493	0.3407	2.078	0.0047	0.1069	0.0007	4.471
3-3-3	0.3391	0.8835	0.0000	0.0029	0.0557	0.3602	2.176	0.0053	0.1121	0.0007	4.492
3-3-4	0.3278	0.8997	-0.0001	0.0025	0.0010	0.3058	1.862	0.0064	0.1209	0.0003	4.210
1-3-1	0.1185	0.7301	0.0001	0.0024	0.0440	0.3404	2.614	0.0100	0.1085	0.0032	4.215
1-3-2	0.1193	0.7301	0.0000	0.0023	0.0378	0.3434	2.620	0.0083	0.1793	0.0011	4.284
1-3-3	0.1217	0.7416	0.0000	0.0025	0.0369	0.3916	2.822	0.0086	0.1300	0.0014	4.307
1-3-4	~~0.1437~~	0.7591	-0.0001	0.0021	0.0024	0.3274	2.459	0.0109	0.2023	0.0007	4.140
6-3-1	1.925	0.6129	0.0001	0.0028	0.0541	0.3422	2.476	0.0072	0.1234	0.0019	4.425
6-3-2	2.043	0.6472	0.0001	0.0032	0.0708	0.3604	~~3.847~~	0.0068	0.1352	0.0003	4.592
6-3-3	1.985	0.6419	0.0001	0.0029	0.0529	0.3427	2.399	0.0074	0.1284	0.0008	4.618
6-3-4	1.984	0.6577	0.0000	0.0027	0.0012	0.3331	~~2.218~~	0.0100	0.1092	0.0000	4.228
8-3-1	8.149	0.6605	0.0001	0.0034	0.0878	0.3711	2.621	0.0061	0.1300	0.0010	4.437
8-3-2	7.786	0.6428	0.0001	0.0029	0.0397	0.3853	2.550	0.0062	0.1381	0.0020	4.280
8-3-3	8.034	0.6653	0.0001	0.0031	0.0399	0.4038	2.591	0.0063	0.1302	0.0010	4.446
8-3-4	7.847	0.6485	-0.0001	0.0025	0.0051	0.3294	2.170	0.0067	0.1056	0.0002	3.951
2-3-1	0.2833	0.5361	0.0001	0.0040	0.0616	~~0.4512~~	2.817	0.0056	0.1051	0.0008	4.504
2-3-2	0.2758	0.5390	0.0001	0.0039	0.0512	0.3517	2.321	0.0052	0.1006	0.0007	4.321
2-3-3	0.2818	0.5456	0.0001	0.0039	0.0513	0.3761	2.378	0.0052	0.0948	0.0011	4.350
2-3-4	0.2588	0.5248	0.0000	0.0035	0.0089	0.3605	2.380	0.0063	0.1461	0.0000	4.011
7-3-1	6.108	0.6844	0.0000	0.0031	0.0284	0.4311	3.151	0.0070	0.1439	0.0008	4.501
7-3-2	6.213	0.7025	0.0001	0.0032	0.0391	0.4414	3.279	0.0078	0.1377	0.0018	4.649
7-3-3	5.774	~~0.6619~~	0.0002	0.0032	0.0625	0.4611	3.325	0.0074	0.1912	0.0021	4.545
7-3-4	5.871	0.6521	0.0000	0.0024	0.0353	0.3859	2.913	0.0079	0.1038	0.0006	3.884
7-3-5	5.866	0.6486	0.0000	0.0027	0.0495	0.4136	3.072	0.0080	0.1711	0.0007	3.983
7-3-6	5.915	0.6498	0.0000	0.0026	0.0335	0.4557	3.224	0.0079	0.1242	0.0006	3.984
7-3-7	5.882	0.6765	0.0000	0.0030	0.0671	0.3910	3.244	0.0085	0.1446	0.0003	4.193

* Measurements of Cd, Co and Pb were below LOQ.

Deviations (%) between replicates in salmon samples

The calculated deviations (%) between the technical replicates measured in the salmon samples are shown in Table C3. For the whole-body samples, deviations above 25% are marked in blue, while deviations above 10% are marked in pink for the fillet samples. Table C4 shows calculated deviations for reanalyzed fillet samples. The deviations were (%) calculated using equation (5).

Table C3: Deviations (%) between replicates in whole-body and fillet samples (n = 3), calculated using equation (5). Deviations above the limit for whole-body samples at 25% are marked in blue, and deviations above the limit for fillet samples at 10% are marked in pink

Deviations (%) in whole-body samples							
Sample code	**Se**	**As**	**Cu**	**Fe**	**Hg**	**Mn**	**Zn**
1-1	10.0	5.3	3.9	24.9	2.6	37.7	25.8
1-2	18.9	2.0	6.6	4.6	7.6	58.0	16.7
1-3	4.1	2.3	3.5	1.6	1.8	29.2	11.2
2-1	12.9	11.8	13.8	15.5	19.3	6.3	7.4
2-2	10.1	4.4	6.3	5.9	8.8	31.6	13.6
2-3	7.1	1.4	8.8	3.4	5.1	35.7	22.9
3-1	6.5	3.5	1.5	6.7	9.5	47.4	18.5
3-2	11.7	0.8	3.1	3.7	7.5	15.2	25.1
3-3	2.7	2.7	7.6	4.6	7.5	25.5	15.5
6-1	2.2	1.6	5.2	2.0	10.1	29.3	13.2
6-2	1.1	2.7	3.6	7.4	12.2	19.3	7.5
6-3	3.9	17.1	15.5	2.6	5.9	21.8	37.4
7-1	5.5	3.7	4.5	5.3	5.0	32.3	6.1
7-2	0.6	3.8	14.7	5.0	3.6	38.9	28.7
7-3	5.3	3.6	5.1	20.4	16.1	13.3	9.5
8-1	7.8	4.3	6.0	6.8	11.5	15.2	32.6
8-2	5.2	2.1	3.3	4.9	7.2	29.2	14.6
8-3	5.2	5.1	2.9	3.8	9.6	49.1	2.1
9-1	4.1	2.5	1.5	4.4	8.5	4.5	20.7
9-2	1.5	2.0	4.8	2.8	9.4	12.1	7.8
9-3	5.0	7.7	7.4	3.7	5.0	31.3	55.3

Deviations (%) in fillet samples							
Sample code	**Se**	**As**	**Cu**	**Fe**	**Hg**	**Mn**	**Zn**
1-1	8.3	2.7	8.3	11.3	7.0	37.3	3.4
1-2*	11.8	5.1	3.1	9.4	32.8	20.0	3.4
1-3	2.7	1.6	14.3	7.8	19.2	50.9	2.2
2-1	5.9	1.1	3.8	5.7	4.6	51.9	3.8
2-2	5.0	1.9	9.9	5.5	35.2	58.4	8.5
2-3	2.7	1.8	25.3	19.8	6.9	10.3	4.2
3-1*	8.5	3.7	4.2	6.5	11.4	68.6	5.1
3-2*	4.8	0.6	22.0	3.8	20.7	85.1	6.7
3-3	4.9	0.2	5.5	4.6	21.7	15.5	3.0
6-1	3.6	3.5	9.1	1.5	6.0	43.6	2.7
6-2	1.8	1.4	4.0	5.8	11.9	76.1	4.9
6-3	6.0	5.4	5.2	49.8	8.2	9.2	4.2
7-1	2.0	4.3	15.1	11.7	3.2	54.1	3.9
7-2	1.1	0.4	2.2	17.9	4.5	19.1	1.3
7-3*	7.3	5.9	6.8	5.3	11.5	34.0	3.2
8-1	3.2	2.0	6.2	6.5	26.7	26.6	1.4
8-2	1.1	1.1	6.8	6.0	28.8	25.2	3.5
8-3	4.5	3.4	8.5	2.7	2.7	6.1	3.8
9-1	2.4	1.4	8.9	7.1	5.6	48.0	1.9
9-2*	5.5	4.7	5.2	2.0	9.4	56.4	1.8
9-3	2.2	1.9	4.3	6.5	16.3	7.4	1.0

* Selected for reanalysis.

Table C4: Calculated deviations (%) between replicates (n = 3) in reanalyzed fillet samples, using equation (5). Deviations above 10% are marked in pink.

Sample code	Se	As	Cu	Fe	Hg	Mn	Zn
1-2	9.4	0.4	5.0	3.9	3.0	21.5	0.2
3-1	1.6	2.2	17.4	11.8	32.4	59.1	3.9
3-2	7.3	5.6	5.8	5.5	7.9	34.4	4.3
7-3	0.8	0.5	16.7	10.2	1.1	50.6	2.5
9-2*	0.3	1.9	4.0	0.5	4.8	23.8	1.7

* n = 2.

Normalized values for measurements in salmon samples

Normalized values calculated using equation (6) for data polishing, are shown in Table C5 for measurements in whole-body samples (n = 4) and fillet samples (n = 4 to 7).

Table C5: Normalized values of the measurements in whole-body and fillet samples, calculated using equation (6).

Normalized values for whole-body samples								
Date	Sample code	Se	As	Cu	Fe	Hg	Mn	Zn
4.11.19	1-1-1	0.94	0.96	0.90	0.92	0.98	0.86	0.74
4.11.19	1-1-2	1.02	1.01	0.91	1.16	0.99	1.27	0.96
4.11.19	1-1-3	0.93	1.02	0.88	0.91	1.01	1.10	0.82
24.3.17	1-1-4	1.11	1.01	1.31	1.01	1.03	0.77	1.48
4.11.19	1-2-1	1.05	0.99	1.02	1.02	1.01	0.98	1.05
4.11.19	1-2-2	1.02	0.97	0.96	0.97	0.96	0.79	1.04
4.11.19	1-2-3	0.99	0.98	0.96	1.01	0.94	1.41	0.99
24.3.17	1-2-4	0.93	1.05	1.06	1.00	1.08	0.82	0.91
4.11.19	1-3-1	1.00	0.97	1.03	1.00	0.97	0.99	0.93
4.11.19	1-3-2	1.00	0.99	1.05	0.99	0.97	0.87	0.95
4.11.19	1-3-3	1.04	0.98	1.01	1.00	0.99	1.16	1.14
24.3.17	1-3-4	0.95	1.06	0.91	1.01	1.07	0.98	0.98
4.11.19	2-1-1	1.07	1.03	1.01	1.04	1.03	1.18	1.18
4.11.19	2-1-2	0.98	0.91	0.89	0.89	0.85	1.11	1.09
4.11.19	2-1-3	1.11	1.02	1.02	1.05	0.95	1.15	1.18
24.3.17	2-1-4	0.84	1.03	1.08	1.02	1.16	0.56	0.55
4.11.19	2-2-1	1.04	0.97	0.92	0.96	0.95	1.02	1.16
4.11.19	2-2-2	0.98	1.02	0.98	1.01	1.04	0.82	0.84
4.11.19	2-2-3	0.96	1.01	0.97	1.02	1.01	0.74	0.91
24.3.17	2-2-4	1.02	0.99	1.12	1.01	1.00	1.43	1.09
4.11.19	2-3-1	1.01	1.00	1.03	1.02	0.97	1.24	1.13
4.11.19	2-3-2	0.99	0.99	0.95	1.00	0.96	0.85	1.02
4.11.19	2-3-3	1.06	1.01	0.99	1.03	0.92	1.20	1.23
24.3.17	2-3-4	0.94	1.00	1.04	0.95	1.14	0.71	0.62
4.11.19	3-1-1	1.02	1.02	0.98	0.99	1.02	0.78	1.19
4.11.19	3-1-2	1.00	0.98	0.96	0.96	0.93	0.97	1.18
4.11.19	3-1-3	1.01	1.02	0.98	1.03	0.98	1.25	1.04
24.3.17	3-1-4	0.98	0.98	1.08	1.02	1.07	1.00	0.59
4.11.19	3-2-1	0.96	1.00	1.05	0.98	1.01	1.09	0.99
4.11.19	3-2-2	1.04	1.01	1.05	0.99	0.94	0.97	1.11
4.11.19	3-2-3	0.94	1.00	1.08	1.02	1.00	0.94	0.97
24.3.17	3-2-4	1.06	0.98	0.82	1.01	1.06	1.00	0.93
4.11.19	3-3-1	1.01	1.00	1.03	0.99	1.03	1.10	0.98
4.11.19	3-3-2	0.98	1.00	1.00	0.98	0.96	0.86	0.93

4.11.19	3-3-3	1.03	1.02	1.08	1.03	1.00	0.88	1.08
24.3.17	3-3-4	0.99	0.98	0.89	1.01	1.00	1.16	1.02
4.11.19	6-1-1	1.01	1.00	1.07	1.02	0.94	0.85	1.20
4.11.19	6-1-2	1.03	0.99	1.02	1.04	1.04	1.13	1.11
4.11.19	6-1-3	1.02	0.99	1.03	1.03	0.99	0.92	1.20
24.3.17	6-1-4	0.95	1.02	0.88	0.91	1.03	1.10	0.49
4.11.19	6-2-1	1.01	0.98	1.03	0.98	1.00	0.94	1.07
4.11.19	6-2-2	1.02	1.01	1.02	1.05	1.05	1.08	1.14
4.11.19	6-2-3	1.01	1.00	0.99	0.98	0.93	1.14	1.16
24.3.17	6-2-4	0.96	1.02	0.96	0.99	1.02	0.84	0.63
4.11.19	6-3-1	1.02	0.95	1.10	1.01	1.01	1.10	0.97
4.11.19	6-3-2	1.09	0.97	0.96	1.01	1.02	1.06	1.16
4.11.19	6-3-3	0.97	1.12	1.13	0.99	0.97	0.88	0.90
24.3.17	6-3-4	0.93	0.95	0.81	0.99	1.00	0.97	0.97
4.11.19	7-1-1	1.00	1.02	1.04	1.02	1.02	1.00	0.85
4.11.19	7-1-2	1.00	1.01	0.99	1.00	0.98	1.25	0.96
4.11.19	7-1-3	1.01	0.99	1.01	0.97	1.03	0.91	1.14
24.3.17	7-1-4	0.99	0.98	0.96	1.01	0.98	0.84	1.05
4.11.19	7-2-1	1.12	1.01	1.14	1.02	1.00	1.19	1.04
4.11.19	7-2-2	0.96	1.00	1.08	0.97	1.00	1.04	0.88
4.11.19	7-2-3	0.93	1.03	0.98	1.01	1.03	0.80	0.92
24.3.17	7-2-4	0.99	0.96	0.79	1.00	0.98	0.97	1.16
4.11.19	7-3-1	1.02	1.00	1.03	1.14	1.04	0.90	0.76
4.11.19	7-3-2	0.98	1.02	0.98	0.98	1.01	1.03	1.17
4.11.19	7-3-3	0.97	0.98	1.02	0.93	0.88	0.95	0.69
24.3.17	7-3-4	1.02	0.99	0.97	0.95	1.06	1.12	1.39
4.11.19	8-1-1	0.99	1.02	0.94	1.00	1.06	0.97	0.98
4.11.19	8-1-2	1.00	0.98	0.89	0.96	0.94	1.08	1.08
4.11.19	8-1-3	1.02	0.98	0.90	0.94	0.96	1.14	0.92
24.3.17	8-1-4	0.99	1.01	1.26	1.10	1.05	0.81	1.02
4.11.19	8-2-1	0.98	1.00	1.08	1.06	0.98	1.12	1.12
4.11.19	8-2-2	0.98	0.99	1.07	1.01	0.94	1.25	1.10
4.11.19	8-2-3	1.02	1.01	1.04	1.02	1.01	0.93	1.23
24.3.17	8-2-4	1.02	1.01	0.81	0.91	1.06	0.71	0.56
4.11.19	8-3-1	1.03	0.99	1.06	0.98	0.97	0.87	1.31
4.11.19	8-3-2	1.01	1.04	1.03	1.02	1.04	0.86	0.90
4.11.19	8-3-3	0.99	0.99	1.04	1.00	0.94	1.37	1.08
24.3.17	8-3-4	0.97	0.99	0.87	0.99	1.04	0.89	0.70
4.11.19	9-1-1	0.99	0.98	1.09	1.05	0.99	1.18	1.05
4.11.19	9-1-2	1.03	1.00	1.08	1.04	1.01	1.13	1.03
4.11.19	9-1-3	0.98	1.00	1.10	1.00	0.92	1.13	1.04
24.3.17	9-1-4	1.00	1.02	0.73	0.91	1.08	0.55	0.87
4.11.19	9-2-1	1.00	1.00	1.02	1.01	1.03	0.95	1.16
4.11.19	9-2-2	0.95	1.01	1.01	1.03	1.00	1.08	0.93
4.11.19	9-2-3	1.02	0.99	0.97	1.00	0.94	1.01	1.18
24.3.17	9-2-4	1.03	1.00	1.00	0.96	1.04	0.96	0.72
4.11.19	9-3-1	1.01	1.04	1.04	1.01	1.02	0.93	1.05
4.11.19	9-3-2	1.03	0.96	0.96	0.98	0.97	0.87	1.16
4.11.19	9-3-3	0.98	0.99	0.99	0.99	0.99	1.19	1.08
24.3.17	9-3-4	0.99	1.01	1.02	1.02	1.02	1.01	0.71

Normalized values for fillet samples

Date	Sample code	Se	As	Cu	Fe	Hg	Mn	Zn
10.6.20	1-1-1	1.05	0.98	0.99	1.02	0.93	0.70	0.99
10.6.20	1-1-2	1.01	1.00	1.04	1.09	0.88	1.04	1.03
10.6.20	1-1-3	0.97	0.99	0.96	0.97	0.87	1.04	0.99
24.3.17	1-1-4	0.97	1.03	1.02	0.92	1.32	1.22	0.99
10.6.20	1-2-1	1.08	1.06	1.05	1.13	0.78	1.17	1.06
10.6.20	1-2-2	0.99	1.01	1.08	1.03	0.90	0.96	1.10
10.6.20	1-2-3	0.96	1.01	1.06	1.06	1.08	1.04	1.06
5.8.20	1-2-4	0.98	0.97	0.93	0.93	1.01	0.85	0.92

5.8.20	1-2-5	0.94	0.96	0.93	0.93	1.03	0.92	0.92
5.8.20	1-2-6	1.03	0.97	0.97	0.97	1.04	1.06	0.92
24.3.17	1-2-7	1.02	1.03	0.97	0.95	1.17	1.01	1.01
10.6.20	1-3-1	0.94	0.99	0.97	0.99	1.06	0.70	0.99
10.6.20	1-3-2	0.95	0.99	0.98	1.00	0.87	1.16	1.01
10.6.20	1-3-3	0.97	1.00	1.12	1.07	0.91	0.84	1.02
24.3.17	1-3-4	1.14	1.03	0.93	0.94	1.16	1.31	0.98
10.6.20	2-1-1	1.04	0.99	1.04	1.04	0.92	1.28	1.04
10.6.20	2-1-2	0.98	0.99	1.00	1.01	0.88	1.06	1.00
10.6.20	2-1-3	1.00	1.00	1.03	0.98	0.92	0.75	1.00
24.3.17	2-1-4	0.98	1.01	0.93	0.97	1.28	0.91	0.96
10.6.20	2-2-1	1.03	1.01	0.99	1.00	0.68	0.93	0.98
10.6.20	2-2-2	1.01	0.99	0.98	1.01	0.93	0.80	1.00
10.6.20	2-2-3	0.98	0.99	1.07	1.06	0.98	1.41	1.07
24.3.17	2-2-4	0.99	1.01	0.96	0.93	1.42	0.86	0.95
10.6.20	2-3-1	1.03	1.00	1.17	1.14	1.00	0.94	1.05
10.6.20	2-3-2	1.00	1.00	0.91	0.94	0.94	0.90	1.01
10.6.20	2-3-3	1.03	1.02	0.98	0.96	0.94	0.85	1.01
24.3.17	2-3-4	0.94	0.98	0.94	0.96	1.13	1.31	0.93
10.6.20	3-1-1	1.08	1.22	1.01	1.02	0.84	1.08	1.07
10.6.20	3-1-2	0.99	1.00	0.97	0.97	0.78	0.71	1.02
10.6.20	3-1-3	1.02	1.01	0.97	0.96	0.75	1.46	1.06
5.8.20	3-1-4	0.96	0.99	1.03	1.03	1.38	0.73	0.95
5.8.20	3-1-5	0.96	0.97	0.95	0.98	1.11	0.81	0.94
5.8.20	3-1-6	0.98	0.97	1.13	1.10	1.00	1.29	0.97
24.3.17	3-1-7	1.00	1.03	0.93	0.94	1.14	0.91	1.00
10.6.20	3-2-1	1.04	1.03	1.04	1.05	0.74	1.05	1.08
10.6.20	3-2-2	1.08	1.03	1.00	1.04	0.84	1.71	1.14
10.6.20	3-2-3	1.03	1.03	1.24	1.01	0.91	0.72	1.06
5.8.20	3-2-4	1.01	0.98	0.95	1.00	1.16	0.69	0.93
5.8.20	3-2-5	0.98	0.96	0.92	0.96	1.14	0.96	0.92
5.8.20	3-2-6	0.94	0.93	0.90	0.94	1.07	0.73	0.89
24.3.17	3-2-7	0.92	1.04	0.95	1.01	1.13	1.14	0.98
10.6.20	3-3-1	1.04	1.00	1.05	1.04	1.05	1.07	1.04
10.6.20	3-3-2	1.01	0.99	1.00	1.01	0.84	0.92	1.01
10.6.20	3-3-3	0.99	1.00	1.06	1.05	0.95	0.96	1.01
24.3.17	3-3-4	0.96	1.01	0.90	0.90	1.16	1.04	0.95
10.6.20	6-1-1	1.02	1.00	0.95	1.01	0.89	0.85	1.00
10.6.20	6-1-2	1.02	1.00	1.01	1.00	0.86	0.71	1.01
10.6.20	6-1-3	0.99	0.97	1.04	1.01	0.84	1.09	0.98
24.3.17	6-1-4	0.96	1.03	0.99	0.98	1.40	1.35	1.00
10.6.20	6-2-1	1.02	1.00	1.06	1.04	1.00	0.83	1.02
10.6.20	6-2-2	1.00	1.01	1.02	0.98	0.91	0.81	1.00
10.6.20	6-2-3	1.00	1.00	1.04	1.01	0.89	1.64	1.05
24.3.17	6-2-4	0.98	1.00	0.87	0.97	1.20	0.72	0.92
10.6.20	6-3-1	0.97	0.96	0.99	0.91	0.92	0.99	0.99
10.6.20	6-3-2	1.03	1.01	1.05	1.41	0.87	1.09	1.03
10.6.20	6-3-3	1.00	1.00	0.99	0.88	0.94	1.03	1.03
24.3.17	6-3-4	1.00	1.03	0.97	0.81	1.27	0.88	0.95
10.6.20	7-1-1	1.01	0.97	0.92	0.94	0.94	1.30	1.02
10.6.20	7-1-2	1.00	1.00	1.04	1.02	0.92	0.71	0.99
10.6.20	7-1-3	1.02	1.01	1.07	1.05	0.91	1.25	1.03
24.3.17	7-1-4	0.98	1.01	0.97	0.99	1.23	0.74	0.96
10.6.20	7-2-1	1.02	1.00	1.01	1.14	0.89	1.01	1.03
10.6.20	7-2-2	1.00	1.01	1.03	1.01	0.91	1.14	1.03
10.6.20	7-2-3	1.01	1.01	1.03	0.96	0.93	0.94	1.01
24.3.17	7-2-4	0.97	0.98	0.94	0.89	1.28	0.91	0.94
10.6.20	7-3-1	1.03	1.02	1.01	0.99	0.90	0.99	1.06
10.6.20	7-3-2	1.04	1.05	1.04	1.03	1.01	0.95	1.09
10.6.20	7-3-3	0.97	0.99	1.08	1.05	0.96	1.32	1.07
5.8.20	7-3-4	0.99	0.98	0.91	0.92	1.01	0.71	0.91

5.8.20	7-3-5	0.99	0.97	0.97	0.97	1.02	1.18	0.94
5.8.20	7-3-6	0.99	0.97	1.07	1.02	1.01	0.86	0.94
24.3.17	7-3-7	0.99	1.01	0.92	1.02	1.09	1.00	0.99
10.6.20	8-1-1	1.00	0.98	1.04	1.00	0.81	1.05	1.00
10.6.20	8-1-2	1.03	1.00	0.97	0.97	0.84	0.81	1.01
10.6.20	8-1-3	1.02	1.00	1.03	1.04	1.05	0.91	1.01
24.3.17	8-1-4	0.95	1.01	0.96	1.00	1.29	1.22	0.98
10.6.20	8-2-1	1.00	1.01	1.08	1.07	0.99	1.10	1.03
10.6.20	8-2-2	1.01	1.00	1.03	1.05	0.92	1.00	1.05
10.6.20	8-2-3	1.02	1.00	1.01	1.01	0.74	0.85	1.01
24.3.17	8-2-4	0.97	0.98	0.88	0.87	1.36	1.05	0.90
10.6.20	8-3-1	1.02	1.01	1.00	1.06	0.97	1.03	1.04
10.6.20	8-3-2	0.98	0.98	1.03	1.03	0.98	1.10	1.00
10.6.20	8-3-3	1.01	1.02	1.08	1.04	0.99	1.03	1.04
24.3.17	8-3-4	0.99	0.99	0.88	0.87	1.06	0.84	0.92
10.6.20	9-1-1	1.01	1.00	0.98	0.97	0.91	0.82	1.00
10.6.20	9-1-2	1.03	1.01	1.04	1.04	0.95	0.93	1.02
10.6.20	9-1-3	1.01	0.99	0.95	1.00	0.96	1.31	1.01
24.3.17	9-1-4	0.96	1.00	1.04	0.99	1.17	0.94	0.97
10.6.20	9-2-1	0.98	0.99	1.05	1.02	0.86	1.34	1.05
10.6.20	9-2-2	1.01	1.02	1.09	1.01	0.89	0.77	1.04
10.6.20	9-2-3	1.04	1.03	1.03	1.03	0.81	0.91	1.06
5.8.20	9-2-4	0.99	0.97	0.91	0.95	1.15	0.92	0.92
5.8.20	9-2-5	0.99	0.95	0.94	0.94	1.09	1.16	0.93
24.3.17	9-2-6	0.99	1.04	0.98	1.04	1.19	0.91	0.99
10.6.20	9-3-1	1.00	1.00	1.02	1.00	1.05	1.00	1.01
10.6.20	9-3-2	1.02	1.01	1.00	1.02	0.89	1.08	1.02
10.6.20	9-3-3	1.00	0.99	1.05	1.07	0.97	1.06	1.02
24.3.17	9-3-4	0.98	0.99	0.93	0.91	1.09	0.86	0.94

Histograms and boxplots for identification of outliers

Boxplots used for identification of outliers of the each subset before and after outlier exclusions are shown in Figure C1 for whole-body samples and in Figure C2 for fillet samples. The distribution of the normalized measurements of each element in the whole-body samples are shown as histograms before and after outlier exclusions in Figure C3. For the fillet samples, the histograms showing the distribution of the normalized measurements of each element before and after outlier exclusions can be found in Figure C4.

Normality is given in the histogram headings for normal distributed subsets of elements, based on p-values from Shapiro-Wilk's normality test. Boxplots of normalized measurements of each element before and after outlier exclusions in the whole-body samples are shown in Figure C1, and for fillet samples in Figure C2.

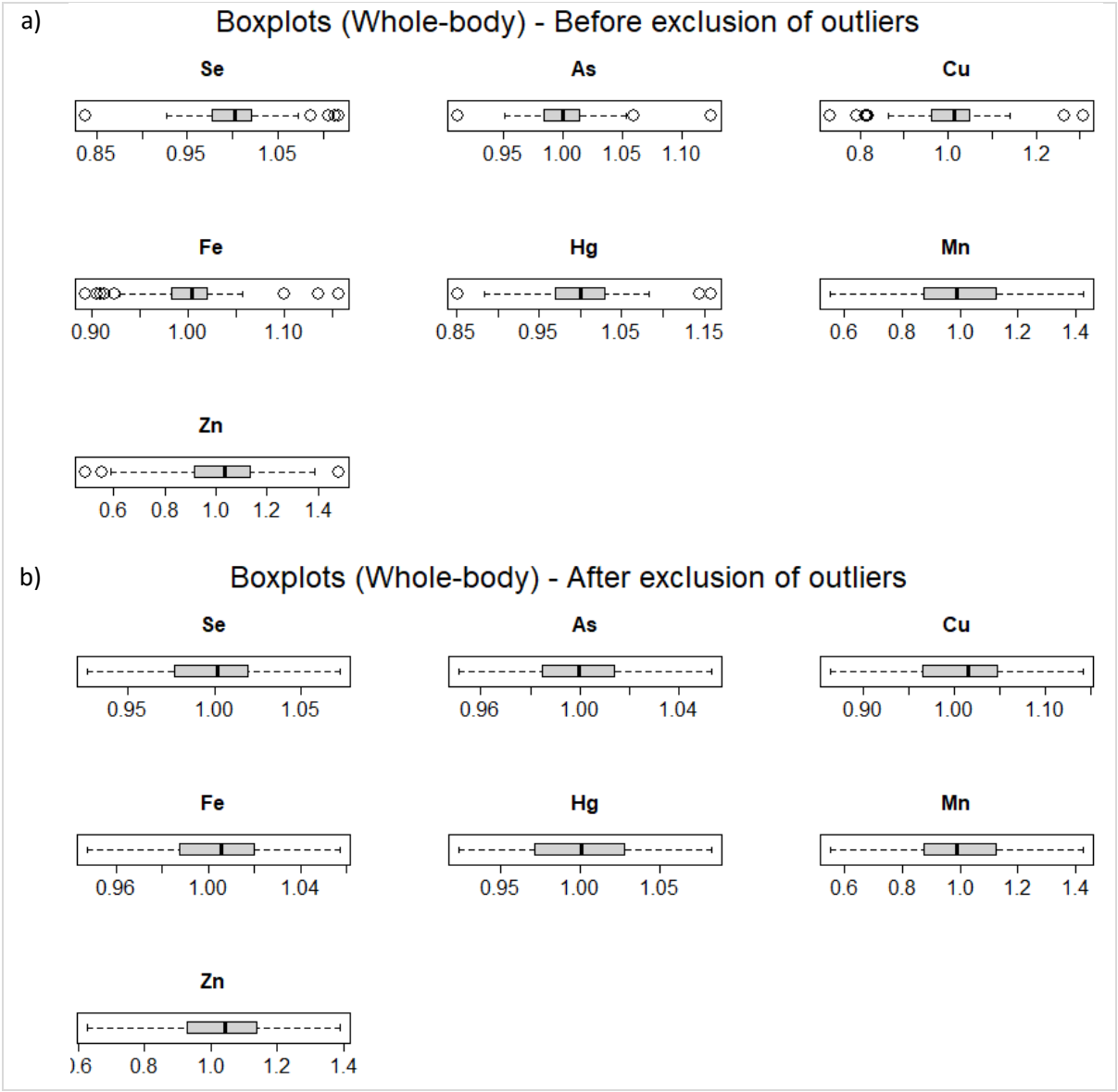


Figure C1: Boxplots of element subsets in whole-body samples a) before and b) after outlier exclusions.

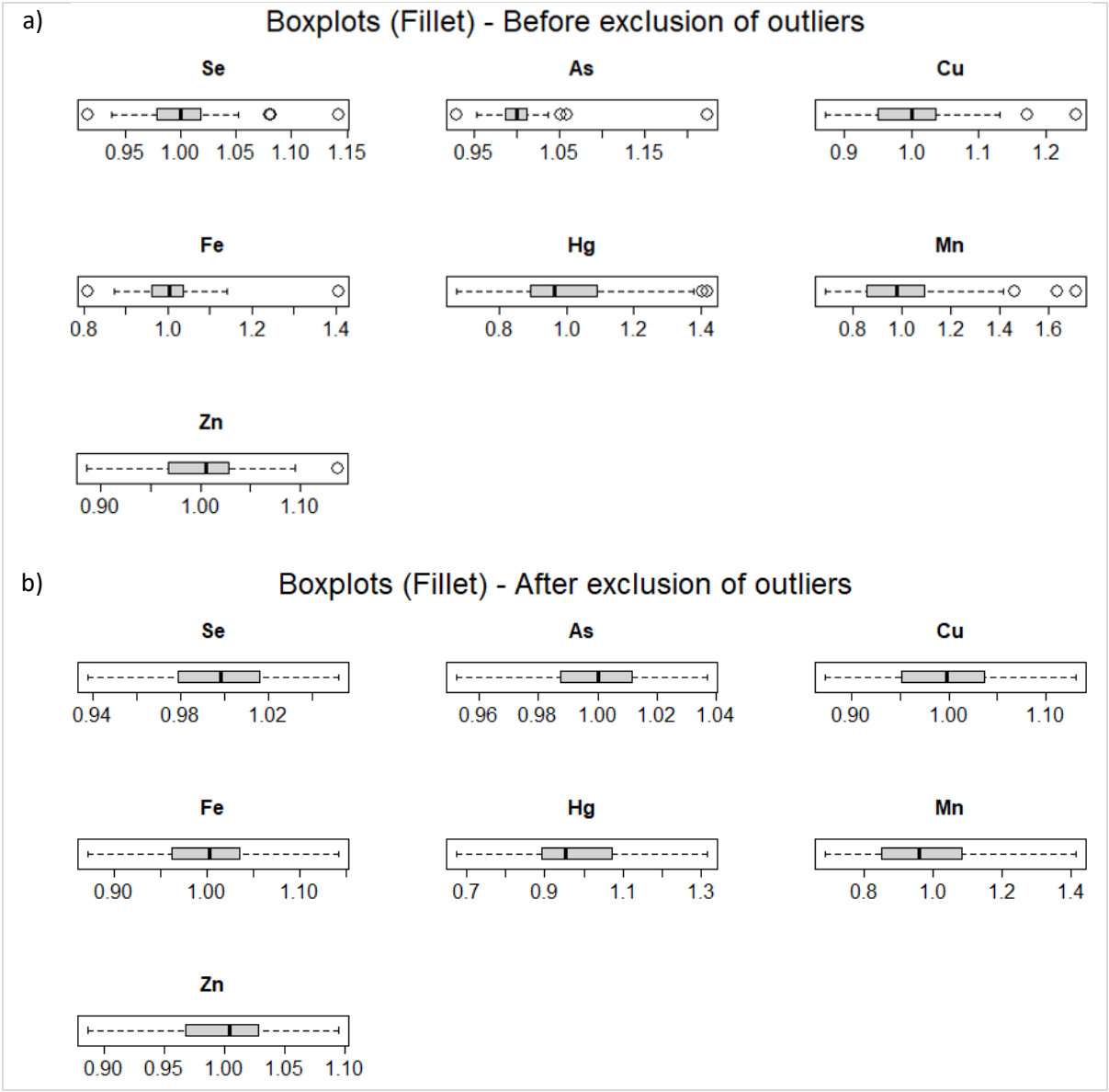


Figure C2: Boxplots of element subsets in fillet samples a) before and b) after outlier exclusions.

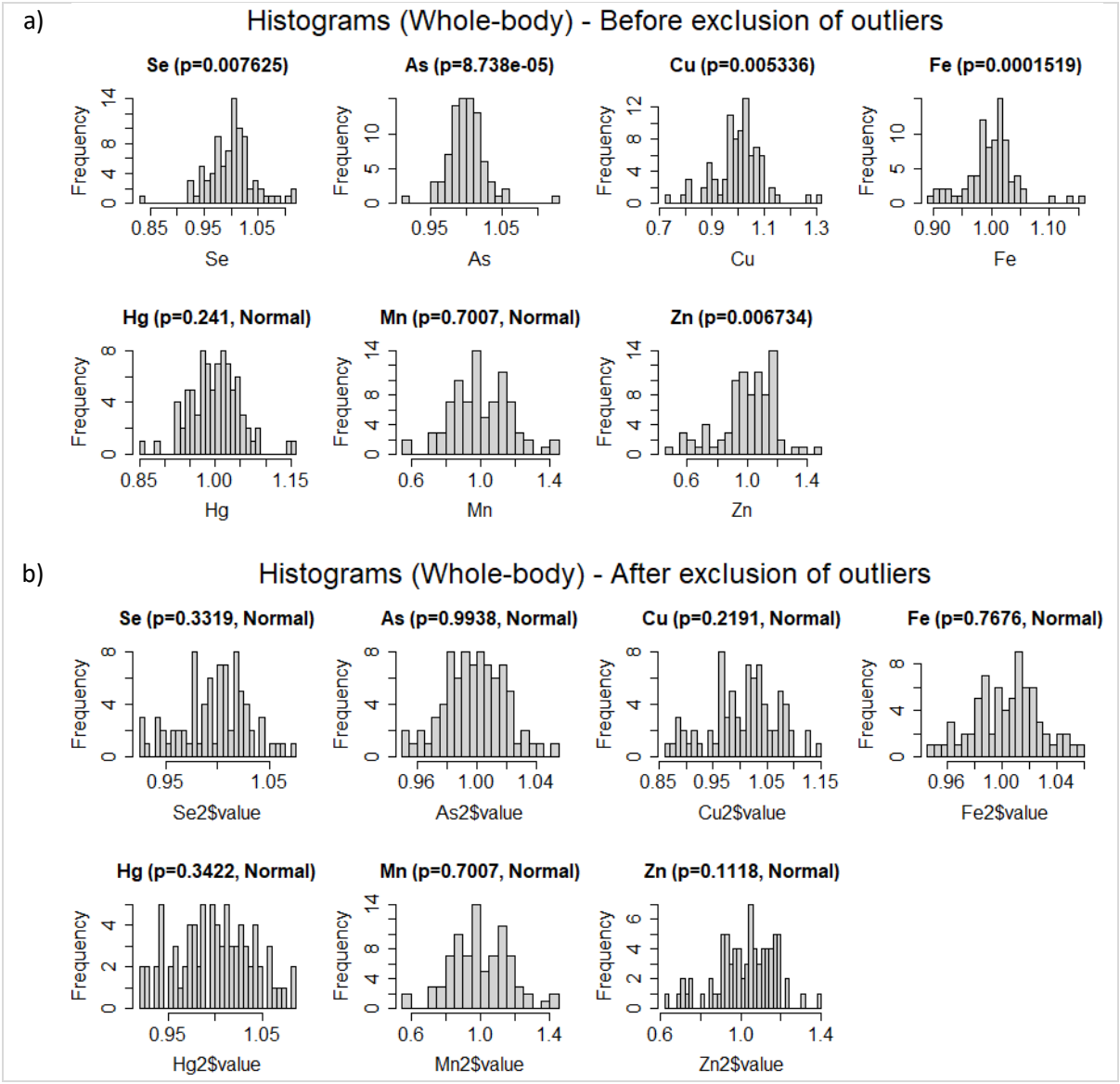


Figure C3: Histograms showing the distribution of the normalized measurements of each element in the whole-body samples a) before and b) after exclusion of outliers, with p-values from Shapiro-Wilk's normality test.

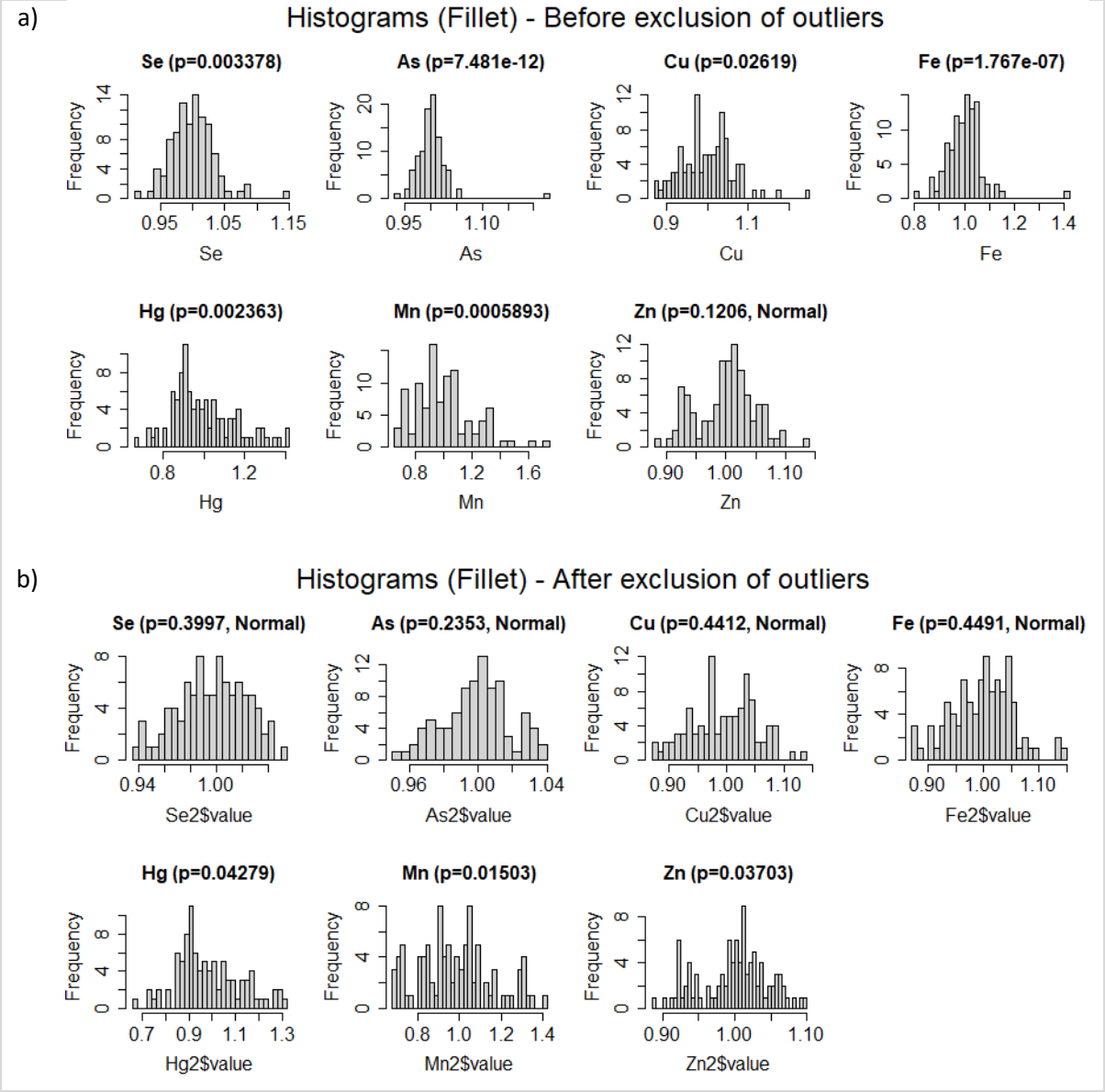


Figure C4: Histograms showing the distribution of the normalized measurements of each element in the fillet samples a) before and b) after exclusion of outliers, with p-values from Shapiro-Wilk's normality test.

Polished datasets used in R for correlation coefficients

Table C6 shows the organized datasets of element concentrations (mg/kg) for whole-body and fillet samples with excluded data points. These datasets were imported to R for correlations studies. Excluded data are marked as "not available (NA)" for compatibility in R.

Table C6: Dataset i, ii, iii and iv containing element concentrations (mg/kg ww) in technical replicates. Excluded values are marked as "not available (NA)".

Dataset i) Whole-body from fish fed with inorganic Se supplemented feed							
Diet - Bio.rep - Tech.rep	**Se**	**As**	**Cu**	**Fe**	**Hg**	**Mn**	**Zn**
1-1-1	0.1338	0.5597	1.2634	NA	0.0084	1.0339	18.0519
1-1-2	0.1450	0.5859	1.2831	NA	0.0085	1.5202	23.3230
1-1-3	0.1314	0.5904	1.2343	NA	0.0086	1.3196	19.9099
1-1-4	NA	0.5893	NA	11.2191	0.0088	0.9294	NA
1-2-1	NA	0.6490	1.5021	11.3179	0.0092	1.0126	18.8632
1-2-2	0.1483	0.6364	1.4117	10.8059	0.0087	0.8160	15.9970
1-2-3	0.1430	0.6408	1.4067	11.2618	0.0085	1.4493	16.5910
1-2-4	0.1534	0.6885	1.5561	11.1188	0.0098	0.8413	20.9319
1-3-1	0.1529	0.6426	1.7513	12.1910	0.0093	1.2532	29.8984
1-3-2	0.1535	0.6575	1.7831	12.0616	0.0093	1.1000	29.4791
1-3-3	0.1593	0.6499	1.7209	12.2519	0.0094	1.4728	32.9287
1-3-4	0.1592	NA	1.5507	12.2856	0.0102	1.2454	NA
2-1-1	0.7556	0.5312	1.3193	11.0594	0.0054	1.3260	28.2920
2-1-2	0.6880	NA	NA	NA	NA	1.2447	26.3029
2-1-3	NA	0.5291	1.3316	11.1301	0.0050	1.2870	28.3584
2-1-4	NA	0.5345	1.3997	10.8098	NA	0.6223	NA
2-2-1	0.8247	0.4646	1.4380	10.4613	0.0040	1.0307	22.1333
2-2-2	0.8964	0.4855	1.5316	10.9887	0.0044	0.8275	24.7991
2-2-3	0.8115	0.4841	1.5211	11.0967	0.0043	0.7550	21.6878
2-2-4	0.9132	0.4746	1.7505	10.9256	0.0042	1.4464	20.7908
2-3-1	0.7621	0.5004	1.4708	10.4781	0.0041	1.2264	22.5342
2-3-2	0.7187	0.4981	1.3464	10.2790	0.0041	0.8394	18.0851
2-3-3	0.7719	0.5050	1.4045	10.6369	0.0039	1.1815	22.9436
2-3-4	0.7847	0.5010	1.4750	NA	NA	0.7001	14.0504
3-1-1	1.3343	0.6926	1.6003	10.8290	0.0054	0.9665	34.9804
3-1-2	1.3112	0.6700	1.5758	10.5119	0.0049	1.2102	31.7586
3-1-3	1.3982	0.6940	1.5922	11.2378	0.0052	1.5568	38.2315
3-1-4	1.2447	0.6698	1.7635	11.2024	0.0057	1.2425	NA
3-2-1	1.5982	0.5931	2.2773	9.7923	0.0049	1.2287	28.8698
3-2-2	NA	0.5979	2.2823	9.8082	0.0046	1.0958	34.4500
3-2-3	1.5184	0.5941	2.3494	10.1552	0.0049	1.0570	26.9095
3-2-4	1.4593	0.5798	NA	10.0661	0.0051	1.1229	28.8556
3-3-1	1.3509	0.6429	2.0618	10.2236	0.0051	1.5123	25.6924
3-3-2	1.3660	0.6396	2.0017	10.1475	0.0048	1.1815	28.2010
3-3-3	1.3885	0.6571	2.1585	10.6213	0.0050	1.2032	24.1799
3-3-4	1.3574	0.6285	1.7850	10.4063	0.0050	1.5874	26.6622

Dataset ii) Whole-body from fish fed with organic Se supplemented feed							
Diet - Bio.rep - Tech.rep	**Se**	**As**	**Cu**	**Fe**	**Hg**	**Mn**	**Zn**
1-1-1	0.1338	0.5597	1.2634	NA	0.0084	1.0339	18.0519
1-1-2	0.1450	0.5859	1.2831	NA	0.0085	1.5202	23.3230
1-1-3	0.1314	0.5904	1.2343	NA	0.0086	1.3196	19.9099
1-1-4	NA	0.5893	NA	11.2191	0.0088	0.9294	NA
1-2-1	NA	0.6490	1.5021	11.3179	0.0092	1.0126	18.8632
1-2-2	0.1483	0.6364	1.4117	10.8059	0.0087	0.8160	15.9970
1-2-3	0.1430	0.6408	1.4067	11.2618	0.0085	1.4493	16.5910
1-2-4	0.1534	0.6885	1.5561	11.1188	0.0098	0.8413	20.9319
1-3-1	0.1529	0.6426	1.7513	12.1910	0.0093	1.2532	29.8984

1-3-2	0.1535	0.6575	1.7831	12.0616	0.0093	1.1000	29.4791
1-3-3	0.1593	0.6499	1.7209	12.2519	0.0094	1.4728	32.9287
1-3-4	0.1592	NA	1.5507	12.2856	0.0102	1.2454	NA
6-1-1	1.9585	0.6384	1.5041	11.7862	0.0076	1.3148	45.5984
6-1-2	1.9168	0.6281	1.4290	12.0258	0.0084	1.7546	45.2406
6-1-3	1.9314	0.6312	1.4403	11.9808	0.0079	1.4357	39.8501
6-1-4	1.8784	0.6461	1.2340	NA	0.0082	1.7162	NA
6-2-1	1.9909	0.6528	1.4059	10.5485	0.0087	1.0461	41.2072
6-2-2	2.0133	0.6708	1.3932	11.3499	0.0091	1.1985	38.2050
6-2-3	1.9945	0.6663	1.3567	10.5840	0.0080	1.2722	41.2035
6-2-4	1.8564	0.6778	1.3190	10.6402	0.0089	0.9348	NA
6-3-1	2.1087	0.6244	1.7268	11.3277	0.0084	1.4403	38.7786
6-3-2	2.0692	0.6369	1.5135	11.4026	0.0085	1.3905	26.6637
6-3-3	2.0283	0.7384	1.7722	11.1115	0.0080	1.1508	31.8420
6-3-4	1.9828	0.6258	NA	11.1196	0.0083	1.2697	20.7579
7-1-1	5.9592	0.6084	1.3092	11.4227	0.0071	1.3661	30.9878
7-1-2	5.7840	0.6001	1.2521	11.1998	0.0068	1.7057	30.6201
7-1-3	5.6395	0.5864	1.2666	10.8272	0.0071	1.2407	29.1481
7-1-4	5.2984	0.5852	1.2107	11.3387	0.0068	1.1547	26.8483
7-2-1	5.9034	0.6353	1.7890	11.5335	0.0070	1.0613	21.1328
7-2-2	5.9073	0.6263	1.6961	10.9708	0.0070	0.9222	23.6855
7-2-3	5.9364	0.6502	1.5423	11.4305	0.0073	0.7115	28.1195
7-2-4	5.8202	0.6036	NA	11.2950	0.0069	0.8623	26.1059
7-3-1	5.9281	0.6018	1.5659	NA	0.0067	1.2280	39.8814
7-3-2	6.0566	0.6132	1.4872	12.3979	0.0066	1.4026	43.8093
7-3-3	5.7423	0.5912	1.5525	NA	NA	1.2952	40.9949
7-3-4	5.8022	0.5956	1.4711	11.9924	0.0068	1.5232	27.0095
8-1-1	7.1676	0.6779	1.2110	12.1451	0.0079	1.0057	39.5518
8-1-2	6.7293	0.6505	1.1409	NA	0.0071	1.1101	28.7448
8-1-3	6.6324	0.6497	1.1614	NA	0.0072	1.1723	31.1528
8-1-4	7.0169	0.6716	NA	NA	0.0078	0.8410	37.0567
8-2-1	6.7542	0.6087	1.9191	14.2609	0.0068	1.5896	28.4776
8-2-2	6.5508	0.5998	1.8950	13.5846	0.0065	1.7766	27.0589
8-2-3	6.9026	0.6128	1.8574	13.8004	0.0070	1.3205	31.2936
8-2-4	6.6553	0.6122	NA	NA	0.0073	1.0131	29.5275
8-3-1	6.6829	0.6634	1.7747	11.9248	0.0076	0.9104	21.2901
8-3-2	6.9732	0.6977	1.7244	12.3839	0.0082	0.9020	20.8534
8-3-3	6.6232	0.6639	1.7471	12.0967	0.0074	1.4334	21.0583
8-3-4	6.8036	0.6680	1.4478	12.0359	0.0082	0.9257	17.6764
9-1-1	11.3527	0.6119	2.8744	14.3973	0.0058	1.5505	42.3095
9-1-2	11.3195	0.6257	2.8599	14.3392	0.0059	1.4828	43.2939
9-1-3	11.7949	0.6276	2.9021	13.7714	0.0054	1.4874	51.7752
9-1-4	10.7915	0.6384	NA	NA	0.0063	0.7243	44.5950
9-2-1	9.7759	0.6650	1.8016	12.5800	0.0076	0.9520	28.6813
9-2-2	9.8961	0.6710	1.7961	12.7106	0.0074	1.0749	30.3665
9-2-3	9.7529	0.6574	1.7162	12.3624	0.0069	1.0143	31.0253
9-2-4	9.2917	0.6608	1.7812	11.9421	0.0077	0.9575	16.8186
9-3-1	9.9746	0.6896	1.6987	12.1328	0.0068	1.1505	25.9449
9-3-2	9.5302	0.6386	1.5775	11.6879	0.0064	1.0831	39.9761
9-3-3	9.4895	0.6538	1.6147	11.8915	0.0065	1.4688	23.4835
9-3-4	9.9397	0.6685	1.6651	12.2040	0.0068	1.2503	47.5268

Dataset iii) Fillet from fish fed with inorganic Se supplemented feed

Diet - Bio.rep - Tech.rep	Se	As	Cu	Fe	Hg	Mn	Zn
1-1-1	0.1282	0.6703	0.3451	2.3360	0.0078	0.1061	4.0379
1-1-2	0.1236	0.6887	0.3636	2.4818	0.0074	0.1586	4.1771
1-1-3	0.1180	0.6771	0.3346	2.2178	0.0072	0.1580	4.0464

1-1-4	0.1176	0.7053	0.3559	2.1111	0.0110	0.1850	4.0389
1-2-1	NA	NA	0.3361	2.5608	0.0075	0.1428	4.2473
1-2-2	0.1232	0.7400	0.3466	2.3328	0.0087	0.1170	4.3798
1-2-3	0.1193	0.7397	0.3416	2.3970	0.0104	0.1265	4.2330
1-2-4	0.1216	0.7095	0.2987	2.1119	0.0098	0.1043	3.6901
1-2-5	0.1165	0.7077	0.2974	2.1003	0.0100	0.1126	3.6921
1-2-6	0.1281	0.7105	0.3124	2.1831	0.0101	0.1291	3.6992
1-2-7	0.1269	0.7598	0.3127	2.1486	0.0113	0.1230	4.0546
1-3-1	0.1185	0.7301	0.3404	2.6136	0.0100	0.1085	4.2152
1-3-2	0.1193	0.7301	0.3434	2.6204	0.0083	0.1793	4.2835
1-3-3	0.1217	0.7416	0.3916	2.8222	0.0086	0.1300	4.3072
1-3-4	NA	0.7591	0.3274	2.4589	0.0109	0.2023	4.1403
2-1-1	0.2624	0.5370	0.3500	2.0893	0.0044	0.1658	4.1950
2-1-2	0.2474	0.5385	0.3371	2.0274	0.0042	0.1361	4.0572
2-1-3	0.2539	0.5430	0.3447	1.9730	0.0044	0.0968	4.0403
2-1-4	0.2483	0.5480	0.3105	1.9563	0.0061	0.1173	3.8962
2-2-1	0.2945	0.5253	0.3638	2.4424	0.0026	0.1222	4.1551
2-2-2	0.2902	0.5153	0.3594	2.4679	0.0036	0.1058	4.2144
2-2-3	0.2800	0.5177	0.3962	2.5801	0.0038	0.1865	4.5213
2-2-4	0.2828	0.5249	0.3548	2.2635	NA	0.1136	4.0213
2-3-1	0.2833	0.5361	NA	2.8173	0.0056	0.1051	4.5044
2-3-2	0.2758	0.5390	0.3517	2.3209	0.0052	0.1006	4.3214
2-3-3	0.2818	0.5456	0.3761	2.3775	0.0052	0.0948	4.3500
2-3-4	0.2588	0.5248	0.3605	2.3799	0.0063	0.1461	4.0108
3-1-1	NA	NA	0.3692	2.4231	0.0046	0.1693	4.3988
3-1-2	0.3315	0.7940	0.3540	2.3105	0.0042	0.1116	4.1778
3-1-3	0.3390	0.8041	0.3544	2.2720	0.0041	NA	4.3486
3-1-4	0.3217	0.7880	0.3750	2.4496	NA	0.1139	3.8929
3-1-5	0.3210	0.7727	0.3463	2.3183	0.0060	0.1269	3.8467
3-1-6	0.3263	0.7709	0.4120	2.6090	0.0054	0.2009	4.0004
3-1-7	0.3340	0.8189	0.3404	2.2273	0.0062	0.1426	4.1036
3-2-1	0.3407	0.8567	0.3521	2.0156	0.0039	0.1787	4.6664
3-2-2	NA	0.8583	0.3406	2.0085	0.0045	NA	NA
3-2-3	0.3388	0.8618	NA	1.9409	0.0048	0.1233	4.5775
3-2-4	0.3335	0.8211	0.3229	1.9166	0.0062	0.1172	3.9835
3-2-5	0.3228	0.8044	0.3141	1.8481	0.0061	0.1636	3.9729
3-2-6	0.3099	NA	0.3047	1.8153	0.0057	0.1244	3.8133
3-2-7	NA	0.8639	0.3213	1.9357	0.0060	0.1941	4.2006
3-3-1	0.3562	0.8841	0.3579	2.1512	0.0058	0.1247	4.6085
3-3-2	0.3445	0.8820	0.3407	2.0775	0.0047	0.1069	4.4710
3-3-3	0.3391	0.8835	0.3602	2.1763	0.0053	0.1121	4.4917
3-3-4	0.3278	0.8997	0.3058	1.8624	0.0064	0.1209	4.2101

Dataset iv) Fillet from fish fed with organic Se supplemented feed							
Diet - Bio.rep - Tech.rep	**Se**	**As**	**Cu**	**Fe**	**Hg**	**Mn**	**Zn**
1-1-1	0.1282	0.6703	0.3451	2.3360	0.0078	0.1061	4.0379
1-1-2	0.1236	0.6887	0.3636	2.4818	0.0074	0.1586	4.1771
1-1-3	0.1180	0.6771	0.3346	2.2178	0.0072	0.1580	4.0464
1-1-4	0.1176	0.7053	0.3559	2.1111	0.0110	0.1850	4.0389
1-2-1	NA	NA	0.3361	2.5608	0.0075	0.1428	4.2473
1-2-2	0.1232	0.7400	0.3466	2.3328	0.0087	0.1170	4.3798
1-2-3	0.1193	0.7397	0.3416	2.3970	0.0104	0.1265	4.2330
1-2-4	0.1216	0.7095	0.2987	2.1119	0.0098	0.1043	3.6901
1-2-5	0.1165	0.7077	0.2974	2.1003	0.0100	0.1126	3.6921
1-2-6	0.1281	0.7105	0.3124	2.1831	0.0101	0.1291	3.6992
1-2-7	0.1269	0.7598	0.3127	2.1486	0.0113	0.1230	4.0546

1-3-1	0.1185	0.7301	0.3404	2.6136	0.0100	0.1085	4.2152
1-3-2	0.1193	0.7301	0.3434	2.6204	0.0083	0.1793	4.2835
1-3-3	0.1217	0.7416	0.3916	2.8222	0.0086	0.1300	4.3072
1-3-4	NA	0.7591	0.3274	2.4589	0.0109	0.2023	4.1403
6-1-1	2.1602	0.6758	0.3041	2.1383	0.0058	0.1116	4.2547
6-1-2	2.1574	0.6714	0.3242	2.1061	0.0056	0.0921	4.2885
6-1-3	2.0844	0.6528	0.3333	2.1333	0.0055	0.1424	4.1756
6-1-4	2.0294	0.6941	0.3166	2.0687	NA	0.1762	4.2450
6-2-1	2.0775	0.7365	0.3652	2.4494	0.0094	0.1430	4.3031
6-2-2	2.0395	0.7435	0.3510	2.3120	0.0086	0.1386	4.2457
6-2-3	2.0537	0.7330	0.3593	2.3707	0.0083	NA	4.4562
6-2-4	2.0098	0.7332	0.3005	2.2824	0.0113	0.1244	3.9010
6-3-1	1.9249	0.6129	0.3422	2.4763	0.0072	0.1234	4.4251
6-3-2	2.0433	0.6472	0.3604	NA	0.0068	0.1352	4.5919
6-3-3	1.9850	0.6419	0.3427	2.3992	0.0074	0.1284	4.6175
6-3-4	1.9840	0.6577	0.3331	NA	0.0100	0.1092	4.2278
7-1-1	6.0387	0.7028	0.3014	2.1311	0.0066	0.1783	4.2986
7-1-2	5.9697	0.7240	0.3402	2.3207	0.0065	0.0976	4.1662
7-1-3	6.0888	0.7335	0.3515	2.3979	0.0064	0.1719	4.3308
7-1-4	5.8800	0.7333	0.3196	2.2433	0.0087	0.1008	4.0448
7-2-1	6.3169	0.6486	0.3738	2.8681	0.0053	0.1005	4.6008
7-2-2	6.2476	0.6503	0.3820	2.5426	0.0054	0.1135	4.6048
7-2-3	6.3074	0.6514	0.3802	2.4019	0.0055	0.0939	4.5431
7-2-4	6.0092	0.6322	0.3474	2.2342	0.0076	0.0912	4.1981
7-3-1	6.1084	0.6844	0.4311	3.1513	0.0070	0.1439	4.5011
7-3-2	6.2134	0.7025	0.4414	3.2793	0.0078	0.1377	4.6493
7-3-3	5.7736	NA	0.4611	3.3251	0.0074	0.1912	4.5449
7-3-4	5.8711	0.6521	0.3859	2.9127	0.0079	0.1038	3.8836
7-3-5	5.8665	0.6486	0.4136	3.0719	0.0080	0.1711	3.9825
7-3-6	5.9149	0.6498	0.4557	3.2243	0.0079	0.1242	3.9840
7-3-7	5.8817	0.6765	0.3910	3.2439	0.0085	0.1446	4.1929
8-1-1	7.3733	0.7224	0.3911	2.3630	0.0054	0.1194	4.1188
8-1-2	7.6164	0.7369	0.3676	2.2987	0.0056	0.0916	4.1756
8-1-3	7.5206	0.7353	0.3876	2.4521	0.0070	0.1036	4.1584
8-1-4	7.0350	0.7429	0.3628	2.3608	0.0086	0.1384	4.0468
8-2-1	8.0016	0.7078	0.4050	2.6266	0.0055	0.1337	4.6443
8-2-2	8.0311	0.7030	0.3884	2.5736	0.0051	0.1223	4.7245
8-2-3	8.0879	0.7003	0.3786	2.4725	0.0041	0.1036	4.5625
8-2-4	7.7511	0.6897	0.3294	2.1378	NA	0.1284	4.0678
8-3-1	8.1490	0.6605	0.3711	2.6213	0.0061	0.1300	4.4370
8-3-2	7.7861	0.6428	0.3853	2.5504	0.0062	0.1381	4.2795
8-3-3	8.0342	0.6653	0.4038	2.5913	0.0063	0.1302	4.4460
8-3-4	7.8466	0.6485	0.3294	2.1699	0.0067	0.1056	3.9508
9-1-1	11.3226	0.8198	0.3219	2.2327	0.0057	0.1191	4.1880
9-1-2	11.5874	0.8287	0.3399	2.3967	0.0060	0.1344	4.2661
9-1-3	11.3148	0.8168	0.3110	2.3133	0.0061	0.1901	4.1972
9-1-4	10.7695	0.8221	0.3398	2.2735	0.0074	0.1369	4.0379
9-2-1	11.5741	0.7244	0.3512	2.4028	0.0046	0.1703	4.2247
9-2-2	11.8371	0.7471	0.3616	2.3791	0.0048	0.0980	4.1899
9-2-3	12.2233	0.7591	0.3434	2.4267	0.0044	0.1162	4.2675
9-2-4	11.5992	0.7121	0.3019	2.2249	0.0062	0.1168	3.6886
9-2-5	11.6310	0.6989	0.3143	2.2131	0.0059	0.1483	3.7506
9-2-6	11.6534	0.7609	0.3250	2.4547	0.0064	0.1157	3.9901
9-3-1	9.6518	0.8267	0.4335	3.0341	0.0085	0.1466	4.5138
9-3-2	9.8255	0.8397	0.4271	3.0916	0.0072	0.1580	4.5598
9-3-3	9.6157	0.8239	0.4459	3.2373	0.0079	0.1553	4.5403
9-3-4	9.4374	0.8242	0.3974	2.7474	0.0088	0.1266	4.1897

Appendix D – Element concentrations in biological replicates of salmon samples

The first number of the codes, i.e. "1-1", in the tables of Appendix D represents the samples and replicates of samples of both whole-body and fillet of Atlantic salmon. The first digit of the codes represents the diet fed to the salmon and the second digit is the number of a biological replicate.

The salmon fed with basal diet starts with the code "1". The salmon fed with selenite supplemented diets with Se concentration 5.4 mg Se/kg and 11 mg Se/kg starts with the codes "2" and "3", respectively. The salmon fed with SeMet supplemented diets starts with the codes "6" for 6.2 mg Se/kg, "7" for 16 mg Se/kg, "8" for 21 mg Se/kg and "9" for 39 mg Se/kg.

The concentrations of Se, As, Cu, Fe, Hg, Mn and Zn in biological replicates (pooled are shown in Table D1 for whole-body samples and in Table D2 for fillet samples of Atlantic salmon. The concentrations in biological replicates calculated from polished datasets of technical replicates.

Table D1: Element concentrations in biological triplicates (mean measurements of technical replicates) of whole-body samples (mg/kg ww, n = 4). Extreme values identified as outliers are marked with strikethrough. Mean measurements (n = 3) are marked in blue, while mean measurements (n< 3) are marked in pink.

Diet - Bio.rep	Se mean	As mean	Cu mean	Fe mean	Hg mean	Mn mean	Zn mean
1-1	0.1367	0.5813	1.260	11.22	0.0086	1.201	20.428
1-2	0.1482	0.6537	1.469	11.13	0.0090	1.030	18.096
1-3	0.1562	0.6500	1.701	12.20	0.0095	1.268	30.769
2-1	0.7218	0.5316	1.302	11.00	~~0.0052~~	1.120	27.297
2-2	0.8614	0.4772	1.560	10.87	0.0042	1.015	22.353
2-3	0.7594	0.5011	1.424	10.29	0.0040	0.987	19.403
3-1	1.322	0.6816	1.633	10.95	0.0053	1.244	34.990
3-2	1.525	0.5912	2.303	9.96	0.0049	1.126	29.771
3-3	1.366	0.6420	2.002	10.35	0.0050	1.371	26.184
6-1	1.921	0.6360	1.402	11.93	0.0080	1.555	43.563
6-2	1.964	0.6669	1.369	10.78	0.0086	1.113	40.205
6-3	2.047	0.6290	1.671	11.24	0.0083	1.313	29.511
7-1	5.670	0.5950	1.260	11.20	0.0069	1.367	29.401
7-2	5.892	0.6288	1.676	11.31	0.0071	0.8893	24.761
7-3	5.882	0.6004	1.519	12.20	0.0067	1.362	37.924
8-1	6.887	0.6624	1.171	11.89	0.0075	1.032	34.127
8-2	6.716	0.6084	1.891	13.88	0.0069	1.425	29.089
8-3	6.771	0.6732	1.674	12.11	0.0078	1.043	20.220
9-1	11.31	0.6259	2.879	14.17	0.0059	1.311	45.493
9-2	9.679	0.6636	1.774	12.40	0.0074	1.000	26.723
9-3	9.734	0.6626	1.639	11.98	0.0066	1.238	34.233

Table D2: Element concentrations in biological replicates (mean concentrations of technical replicates) of fillet samples (mg/kg ww, n = 4 - 7). Extreme values identified as outliers are marked with strikethrough. Mean measurements (n = 3) are marked in blue, while mean measurements (n < 3) are marked in pink. Mean measurements (n > 4) are marked in grey.

Diet - Bio.rep	Se mean	As mean	Cr mean	Cu mean	Fe mean	Hg mean	Mn mean	Zn mean
1-1	0.0030	0.6854	0.0023	0.0478	0.350	2.2867	0.0083	0.1218
1-2	0.1226	0.7279	0.0340	0.3208	2.262	0.0097	0.1222	3.999
1-3	0.1199	0.7402	0.0303	0.3507	2.629	0.0095	0.1550	4.237
2-1	0.2530	0.5416	0.0175	0.3356	2.011	0.0048	0.1290	4.047
2-2	0.2869	0.5208	0.0592	0.3686	2.438	0.0033	0.1320	4.228
2-3	0.2749	0.5364	0.0432	0.3628	2.474	0.0056	0.1116	4.297
3-1	0.3289	0.7915	0.0224	0.3645	2.373	0.0051	0.1442	4.110
3-2	0.3291	0.8443	0.0247	0.3259	1.926	0.0053	0.1502	4.202
3-3	0.3419	0.8873	0.0389	0.3412	2.067	0.0056	0.1162	4.445
6-1	2.108	0.6735	0.0167	0.3196	2.112	0.0057	0.1306	4.241
6-2	2.045	0.7366	0.0285	0.3440	2.354	0.0094	0.1353	4.227
6-3	1.984	0.6399	0.0448	0.3446	2.438	0.0078	0.1240	4.466
7-1	5.994	0.7234	0.0162	0.3282	2.273	0.0071	0.1372	4.210
7-2	6.220	0.6456	0.0330	0.3709	2.512	0.0059	0.0998	4.487
7-3	5.947	0.6690	0.0451	0.4257	3.173	0.0078	0.1452	4.248
8-1	7.386	0.7344	0.0137	0.3772	2.369	0.0066	0.1132	4.125
8-2	7.968	0.7002	0.0387	0.3753	2.453	0.0049	0.1220	4.500
8-3	7.954	0.6543	0.0431	0.3724	2.483	0.0063	0.1260	4.278
9-1	11.25	0.8218	0.0241	0.3281	2.304	0.0063	0.1451	4.172
9-2	11.75	0.7337	0.0280	0.3329	2.350	0.0054	0.1276	4.019
9-3	~~9.633~~	0.8286	0.0413	0.4260	~~3.028~~	0.0081	0.1466	4.451

Appendix E – Correlation coefficients and p-values for elements in salmon samples

Table E1 shows Pearson's correlation coefficients, sample size and p-values extracted from R for i) whole-body of salmon fed with inorganic Se and ii) whole-body of salmon fed with inorganic Se. Pearson's correlations coefficients and Spearman's rank-order correlations, sample size and p-values are shown in Table E2 for iii) fillet of salmon fed with inorganic Se, are in Table E3 for iv) fillet of salmon fed with organic Se.

Table E1: Pearson's correlation coefficients, sample size and p-values for i) whole-body of salmon fed with inorganic Se and ii) whole-body of salmon fed with organic Se.

Pearson's correlations for whole-body salmon

i) Whole-body of salmon fed with inorganic Se

```
Call:corr.test(x = whole_inorg, method = "pea
rson")
Correlation matrix
      Se   As    Cu    Fe    Hg   Mn    Zn
Se  1.00 0.07  0.64 -0.74 -0.80 0.09  0.45
As  0.07 1.00  0.34  0.24  0.49 0.31  0.43
Cu  0.64 0.34  1.00 -0.39 -0.26 0.17  0.44
Fe -0.74 0.24 -0.39  1.00  0.74 0.10  0.05
Hg -0.80 0.49 -0.26  0.74  1.00 0.00 -0.21
Mn  0.09 0.31  0.17  0.10  0.00 1.00  0.39
Zn  0.45 0.43  0.44  0.05 -0.21 0.39  1.00
Sample Size
   Se As Cu Fe Hg Mn Zn
Se 31 29 29 26 29 31 29
As 29 34 32 30 32 34 31
Cu 29 32 33 29 31 33 30
Fe 26 30 29 31 30 31 27
Hg 29 32 31 30 33 33 30
Mn 31 34 33 31 33 36 32
Zn 29 31 30 27 30 32 32
Probability values (Entries above the diagona
l are adjusted for multiple tests.)
     Se   As   Cu   Fe   Hg   Mn   Zn
Se 0.00 1.00 0.00 0.00 0.00 1.00 0.22
As 0.72 0.00 0.63 1.00 0.07 0.78 0.22
Cu 0.00 0.06 0.00 0.45 1.00 1.00 0.22
Fe 0.00 0.21 0.04 0.00 0.00 1.00 1.00
Hg 0.00 0.00 0.15 0.00 0.00 1.00 1.00
Mn 0.65 0.08 0.36 0.58 0.98 0.00 0.35
Zn 0.01 0.02 0.01 0.79 0.25 0.03 0.00
```

ii) Whole-body of salmon fed with organic Se

```
Call:corr.test(x = whole_org, method = "pears
on")
Correlation matrix
      Se    As    Cu    Fe    Hg    Mn    Zn
Se  1.00  0.08  0.52  0.57 -0.84 -0.11  0.27
As  0.08  1.00  0.06 -0.16  0.25 -0.36 -0.01
Cu  0.52  0.06  1.00  0.77 -0.44  0.18  0.31
Fe  0.57 -0.16  0.77  1.00 -0.49  0.43  0.29
Hg -0.84  0.25 -0.44 -0.49  1.00 -0.19 -0.37
Mn -0.11 -0.36  0.18  0.43 -0.19  1.00  0.28
Zn  0.27 -0.01  0.31  0.29 -0.37  0.28  1.00
Sample Size
   Se As Cu Fe Hg Mn Zn
Se 58 57 53 47 57 58 55
As 57 59 53 48 58 59 56
Cu 53 53 54 46 53 54 51
Fe 47 48 46 49 49 49 46
Hg 57 58 53 49 59 59 55
Mn 58 59 54 49 59 60 56
Zn 55 56 51 46 55 56 56
Probability values (Entries above the diagona
l are adjusted for multiple tests.)
     Se   As   Cu   Fe   Hg   Mn   Zn
Se 0.00 1.00 0.00 0.00 0.00 1.00 0.47
As 0.56 0.00 1.00 1.00 0.47 0.08 1.00
Cu 0.00 0.68 0.00 0.00 0.02 1.00 0.35
Fe 0.00 0.28 0.00 0.00 0.01 0.03 0.47
Hg 0.00 0.05 0.00 0.00 0.00 1.00 0.08
Mn 0.42 0.01 0.20 0.00 0.16 0.00 0.43
Zn 0.05 0.94 0.03 0.05 0.01 0.04 0.00
```

Table E2: Pearson's correlations and Spearman's rank-order correlations, sample size and p-values for iii) fillet of salmon fed with inorganic Se.

iii) Fillet of salmon fed with inorganic Se

Pearson's correlations

```
Call:corr.test(x = fillet_inorg, method =
"pearson")
Correlation matrix
      Se    As    Cu    Fe    Hg    Mn    Zn
Se  1.00  0.20  0.22 -0.27 -0.82 -0.06  0.32
As  0.20  1.00 -0.21 -0.33  0.17  0.16  0.18
Cu  0.22 -0.21  1.00  0.67 -0.39  0.22  0.50
Fe -0.27 -0.33  0.67  1.00  0.14  0.08  0.24
Hg -0.82  0.17 -0.39  0.14  1.00  0.07 -0.40
Mn -0.06  0.16  0.22  0.08  0.07  1.00  0.10
Zn  0.32  0.18  0.50  0.24 -0.40  0.10  1.00
Sample Size
   Se As Cu Fe Hg Mn Zn
Se 40 39 38 40 38 39 40
As 39 42 40 42 40 40 41
Cu 38 40 43 43 41 41 42
Fe 40 42 43 45 43 43 44
Hg 38 40 41 43 43 41 42
Mn 39 40 41 43 41 43 43
Zn 40 41 42 44 42 43 44
Probability values (Entries above the
diagonal are adjusted for multiple tests.)
     Se   As   Cu   Fe   Hg   Mn   Zn
Se 0.00 1.00 1.00 1.00 0.00 1.00 0.67
As 0.23 0.00 1.00 0.54 1.00 1.00 1.00
Cu 0.19 0.19 0.00 0.00 0.19 1.00 0.02
Fe 0.09 0.03 0.00 0.00 1.00 1.00 1.00
Hg 0.00 0.30 0.01 0.38 0.00 1.00 0.14
Mn 0.73 0.32 0.17 0.60 0.68 0.00 1.00
Zn 0.04 0.27 0.00 0.11 0.01 0.52 0.00
```

Spearman's rank-order correlations

```
Call:corr.test(x = fillet_inorg, method =
"spearman")
Correlation matrix
      Se    As    Cu    Fe    Hg    Mn    Zn
Se  1.00  0.51  0.23 -0.28 -0.66 -0.06  0.31
As  0.51  1.00 -0.24 -0.42  0.10  0.16  0.18
Cu  0.23 -0.24  1.00  0.60 -0.45  0.09  0.45
Fe -0.28 -0.42  0.60  1.00  0.12  0.06  0.26
Hg -0.66  0.10 -0.45  0.12  1.00  0.11 -0.39
Mn -0.06  0.16  0.09  0.06  0.11  1.00  0.01
Zn  0.31  0.18  0.45  0.26 -0.39  0.01  1.00
Sample Size
   Se As Cu Fe Hg Mn Zn
Se 40 39 38 40 38 39 40
As 39 42 40 42 40 40 41
Cu 38 40 43 43 41 41 42
Fe 40 42 43 45 43 43 44
Hg 38 40 41 43 43 41 42
Mn 39 40 41 43 41 43 43
Zn 40 41 42 44 42 43 44
Probability values (Entries above the
diagonal are adjusted for multiple tests.)
     Se   As   Cu   Fe   Hg   Mn   Zn
Se 0.00 0.02 1.00 1.00 0.00 1.00 0.70
As 0.00 0.00 1.00 0.09 1.00 1.00 1.00
Cu 0.16 0.14 0.00 0.00 0.06 1.00 0.05
Fe 0.08 0.01 0.00 0.00 1.00 1.00 1.00
Hg 0.00 0.55 0.00 0.43 0.00 1.00 0.17
Mn 0.72 0.33 0.58 0.69 0.48 0.00 1.00
Zn 0.05 0.25 0.00 0.09 0.01 0.92 0.00
```

Table E3: Pearson's correlations and Spearman's rank-order correlations, sample size and p-values for iii) fillet of salmon fed with organic Se.

iv) Fillet of salmon fed with organic Se

Pearson's correlations

```
Call:corr.test(x = fillet_org, method = "pea
rson")
Correlation matrix
      Se    As    Cu   Fe    Hg   Mn    Zn
Se  1.00  0.38  0.24 0.21 -0.63 0.00  0.10
As  0.38  1.00  0.02 0.06  0.14 0.24 -0.03
Cu  0.24  0.02  1.00 0.87 -0.15 0.13  0.55
Fe  0.21  0.06  0.87 1.00  0.01 0.21  0.45
Hg -0.63  0.14 -0.15 0.01  1.00 0.18 -0.37
Mn  0.00  0.24  0.13 0.21  0.18 1.00  0.10
Zn  0.10 -0.03  0.55 0.45 -0.37 0.10  1.00
Sample Size
   Se As Cu Fe Hg Mn Zn
Se 66 65 66 64 64 65 66
As 65 66 66 64 64 65 66
Cu 66 66 68 66 66 67 68
Fe 64 64 66 66 64 65 66
Hg 64 64 66 64 66 65 66
Mn 65 65 67 65 65 67 67
Zn 66 66 68 66 66 67 68
Probability values (Entries above the diagon
al are adjusted for multiple tests.)
     Se   As   Cu   Fe   Hg   Mn   Zn
Se 0.00 0.03 0.77 1.00 0.00 1.00 1.00
As 0.00 0.00 1.00 1.00 1.00 0.77 1.00
Cu 0.05 0.88 0.00 0.00 1.00 1.00 0.00
Fe 0.10 0.66 0.00 0.00 1.00 1.00 0.00
Hg 0.00 0.27 0.23 0.96 0.00 1.00 0.04
Mn 0.99 0.05 0.29 0.09 0.14 0.00 1.00
Zn 0.43 0.82 0.00 0.00 0.00 0.44 0.00
```

Spearman's rank-order correlations

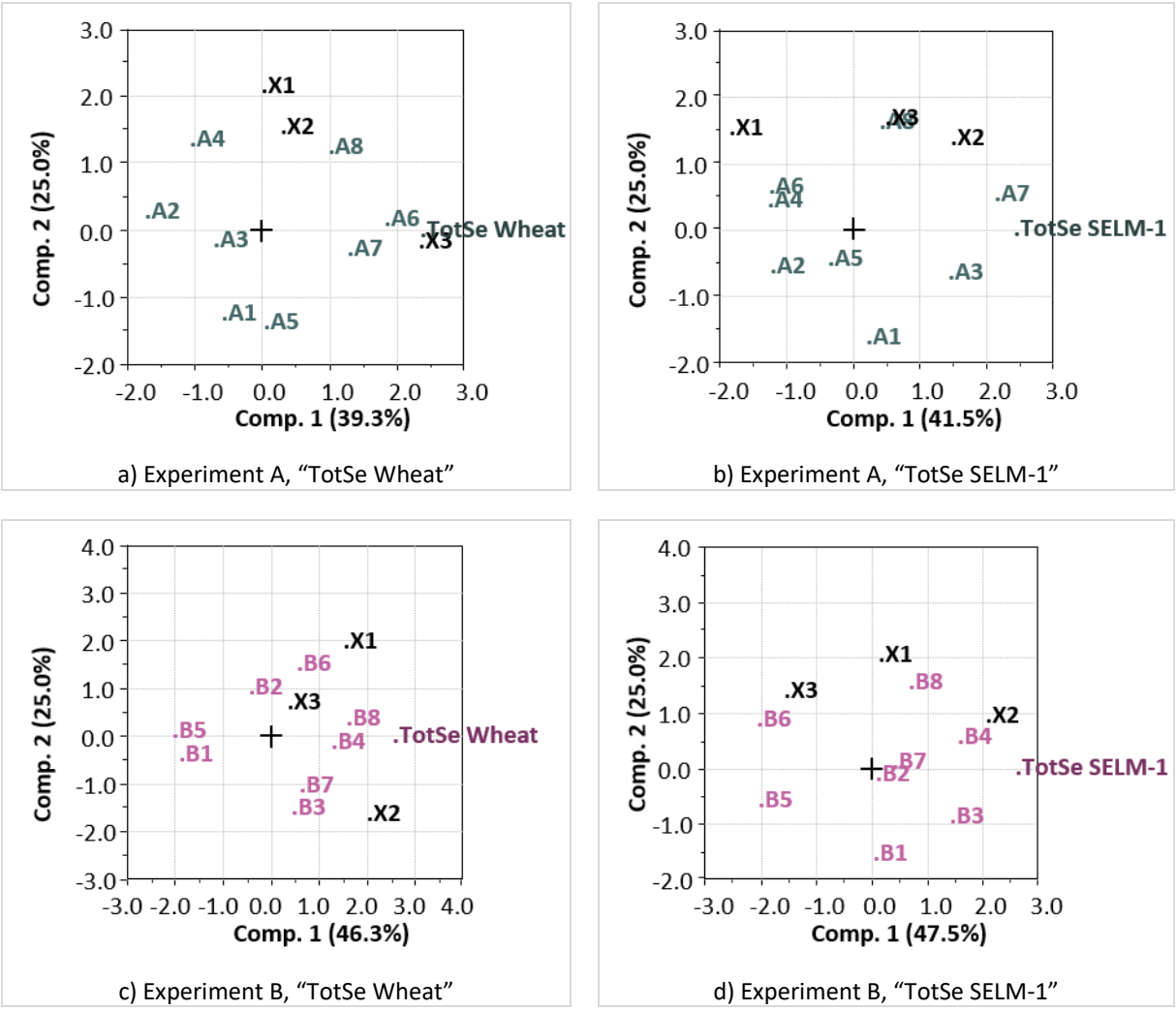
```
Call:corr.test(x = fillet_org, method = "spe
arman")
Correlation matrix
      Se    As    Cu    Fe    Hg    Mn    Zn
Se  1.00  0.29  0.22  0.21 -0.65 -0.03  0.11
As  0.29  1.00 -0.07  0.00  0.20  0.19 -0.09
Cu  0.22 -0.07  1.00  0.82 -0.14  0.14  0.51
Fe  0.21  0.00  0.82  1.00 -0.02  0.20  0.51
Hg -0.65  0.20 -0.14 -0.02  1.00  0.20 -0.33
Mn -0.03  0.19  0.14  0.20  0.20  1.00  0.14
Zn  0.11 -0.09  0.51  0.51 -0.33  0.14  1.00
Sample Size
   Se As Cu Fe Hg Mn Zn
Se 66 65 66 64 64 65 66
As 65 66 66 64 64 65 66
Cu 66 66 68 66 66 67 68
Fe 64 64 66 66 64 65 66
Hg 64 64 66 64 66 65 66
Mn 65 65 67 65 65 67 67
Zn 66 66 68 66 66 67 68
Probability values (Entries above the diagon
al are adjusted for multiple tests.)
     Se   As   Cu   Fe   Hg   Mn   Zn
Se 0.00 0.33 1.00 1.00 0.00 1.00 1.00
As 0.02 0.00 1.00 1.00 1.00 1.00 1.00
Cu 0.08 0.56 0.00 0.00 1.00 1.00 0.00
Fe 0.09 0.99 0.00 0.00 1.00 1.00 0.00
Hg 0.00 0.11 0.27 0.87 0.00 1.00 0.11
Mn 0.80 0.13 0.27 0.10 0.11 0.00 1.00
Zn 0.36 0.49 0.00 0.00 0.01 0.27 0.00
```

Appendix F – PCA for development of Se speciation method

Screening (Experiment A and B)

PCA including total Se recoveries (%) as individual responses for screening (Experiment A and B)

The biplots from including the individual responses "TotSe Wheat" and "TotSe SELM-1", for screening the factors buffer (X1), pre-extraction (X2) and enzyme (X3), with eight experiments in experiment A and experiment B, are shown in Figure F1. Bar graphs of regression coefficients for the factors and interactions between the factors for screening with individual responses "TotSe Wheat" and "TotSe SELM-1", are shown in Figure F2 for experiment A and in Figure F3 for experiment B. Figure F2 and Figure F3 are also included variable importance plots (VIP) for the same factors and factor interactions.



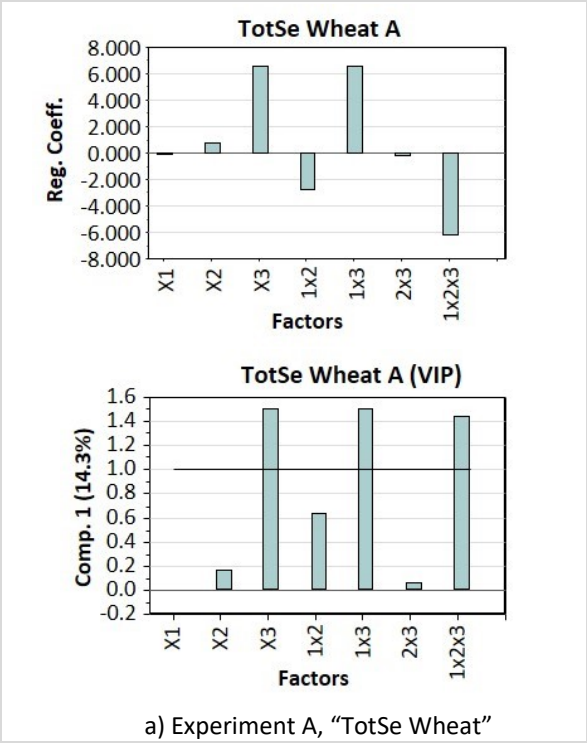
a) Experiment A, "TotSe Wheat"

b) Experiment A, "TotSe SELM-1"

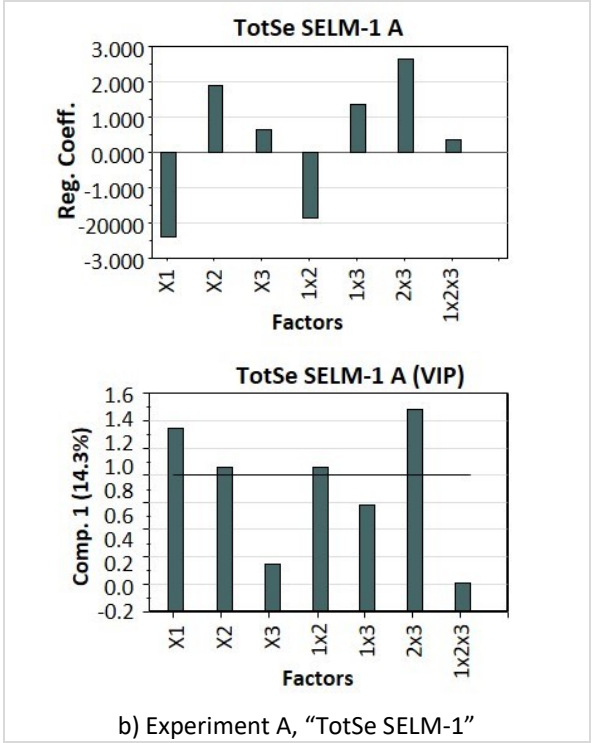
c) Experiment B, "TotSe Wheat"

d) Experiment B, "TotSe SELM-1"

Figure F1: Biplots (Comp. 1 vs 2) showing the relations between experiments (1-8) and variables for factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3), and the individual responses for a) "TotSe Wheat" (total Se recovery in ERM BC210a) in experiment A, b) "TotSe SELM-1" (total Se recovery in SELM-1) in experiment A, c) "TotSe Wheat" in experiment A and d) "TotSe SELM-1" in experiment B for screening .

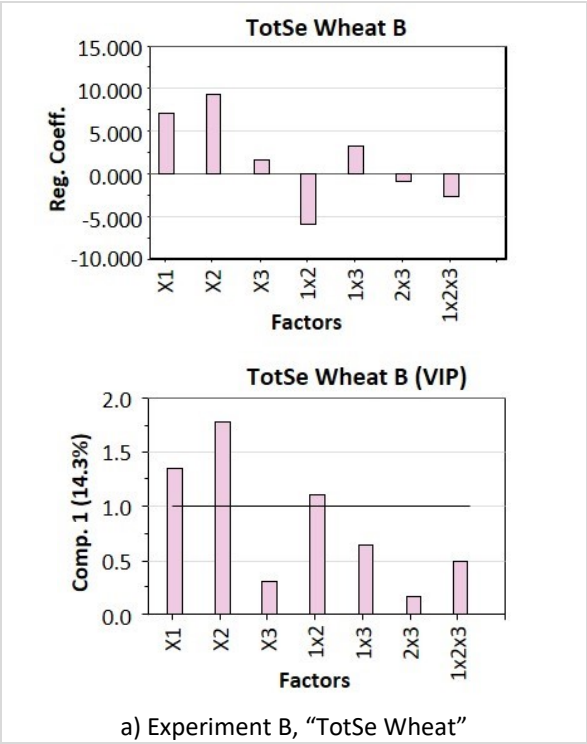


a) Experiment A, "TotSe Wheat"

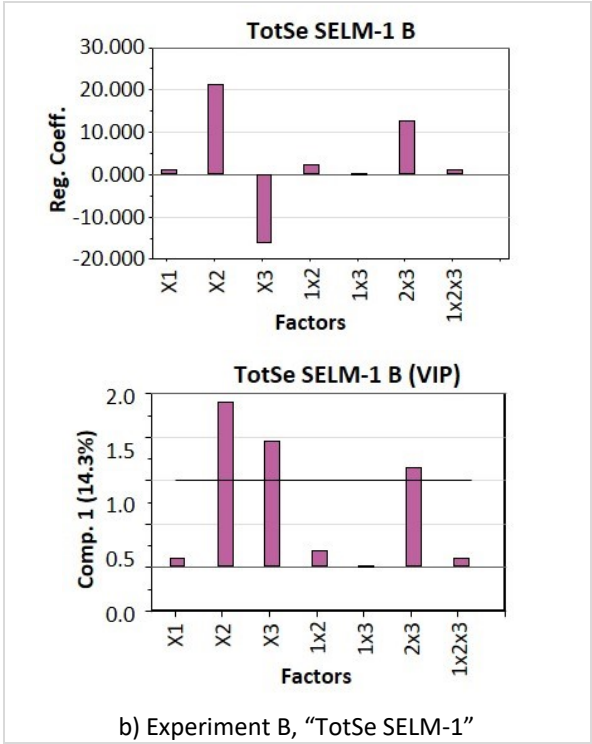


b) Experiment A, "TotSe SELM-1"

Figure F2: Bar graphs of regression coefficients and variable importance plot (VIP, comp. 1) of the factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3), and factor interactions for screening (experiment A) with one response a) "TotSe Wheat" (total Se recovery in ERM BC210a) and b) "TotSe SELM-1" (total Se recovery in SELM-1).



a) Experiment B, "TotSe Wheat"



b) Experiment B, "TotSe SELM-1"

Figure F3: Bar graphs of regression coefficients and variable importance plot (VIP, comp. 1) of the factors "buffer" (X1), "pre-extraction" (X2) and "enzyme" (X3), and factor interactions for screening (experiment B) with one response a) "TotSe Wheat" (total Se recovery in ERM BC210a) and b) "TotSe SELM-1" (total Se recovery in SELM-1).

Optimization (Experiment C)

PCA including four responses (Experiment C)

Biplot with all four responses, "TotSe Wheat", "TotSe SELM-1", "SeMet Wheat" and "SeMet SELM-1", included in the analysis for optimization of the factors in experiment C, are shown in Figure F4.

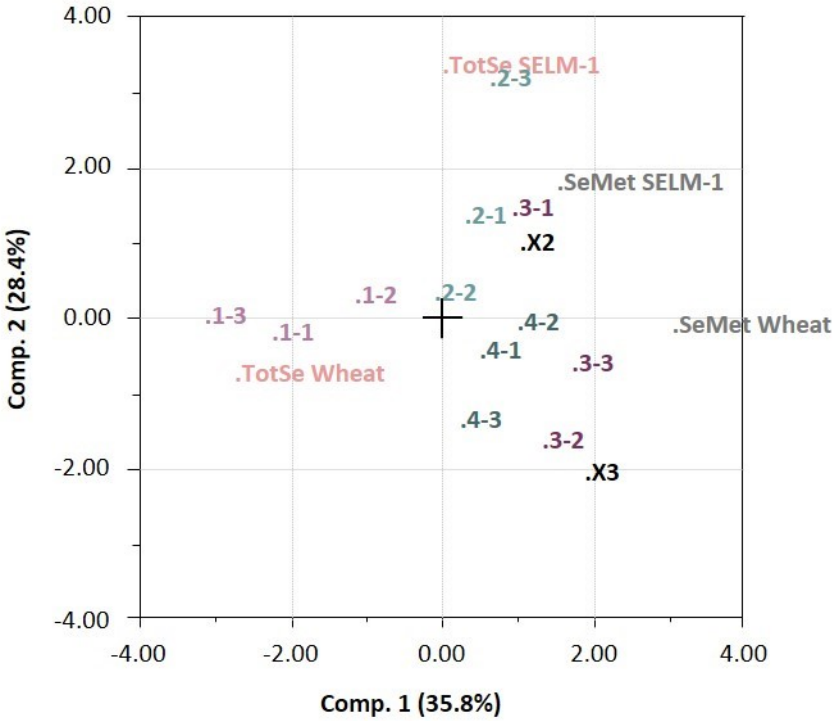
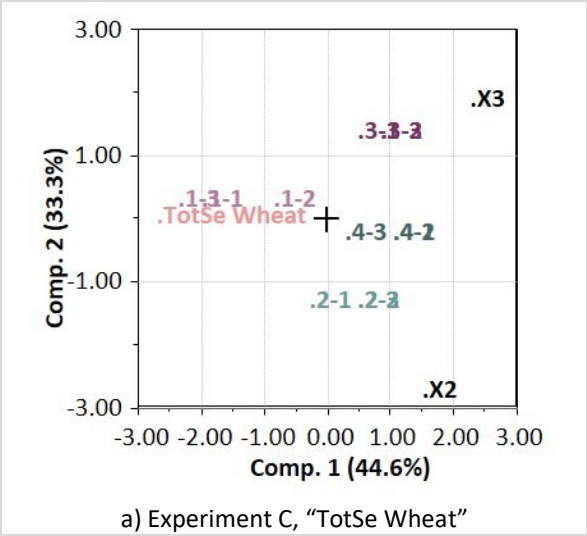


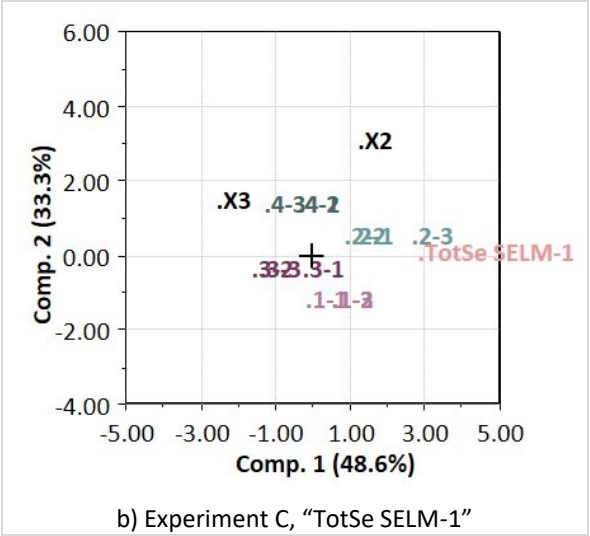
Figure F4: Biplots (Comp. 1 vs 2) showing the relations between experiments (1-8) and variables for factors "pre-extraction" (X2) and "enzyme" (X3), and all responses "TotSe Wheat" (total Se recovery in ERM BC210a), "TotSe SELM-1" (total Se recovery in SELM-1), "SeMet Wheat" (SeMet recovery in ERM BC210a) and "SeMet SELM-1" (SeMet recovery in SELM-1) for optimization.

PCA including total Se recoveries (%) as individual responses (Experiment C)

The biplots from including individual response, "TotSe Wheat" and "TotSe SELM-1 for optimization using the factors pre-extraction (X2) and enzyme (X3) with triplicates of four experiments are shown in Figure F5. Bar graphs of regression coefficients for the factors and interactions between the factors and VIP plots for total Se recoveries are shown in Figure F6.

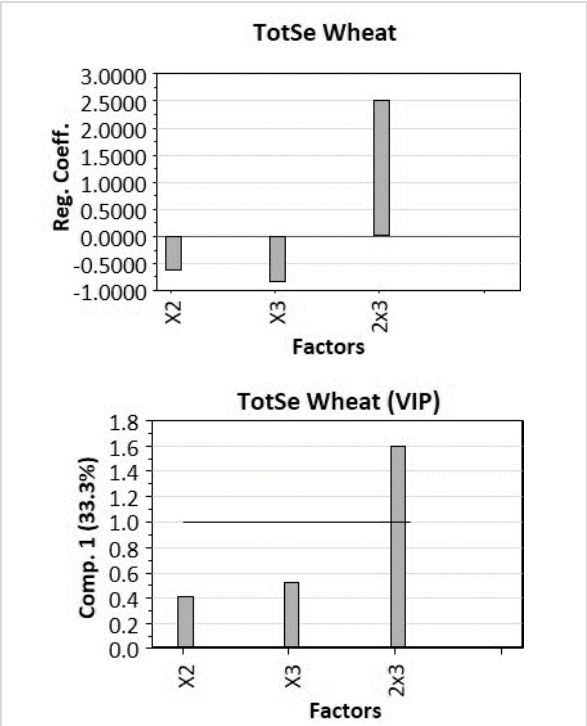


a) Experiment C, "TotSe Wheat"

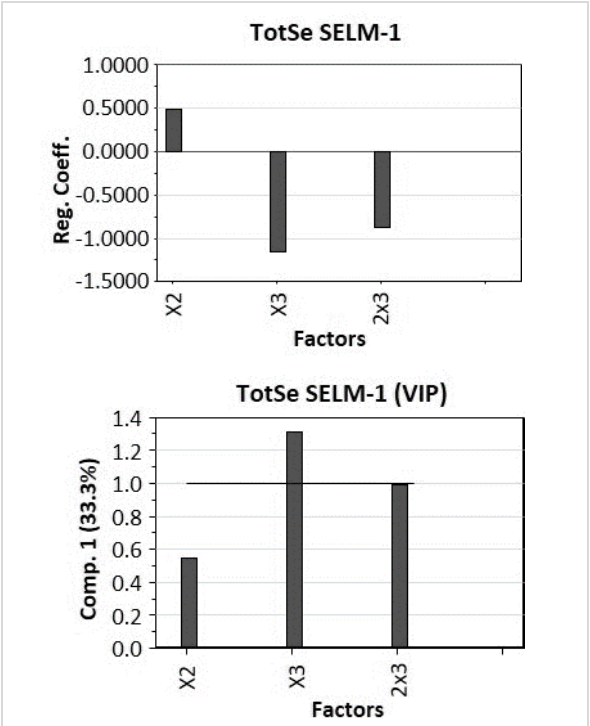


b) Experiment C, "TotSe SELM-1"

Figure F5: Biplots (Comp. 1 vs 2) showing the relations between object (replicates of exp 1-4) and variables for factors "pre-extraction" (X2) and "enzyme" (X3), and individual responses a) "TotSe Wheat" (total Se recovery in ERM BC210a) and b) "TotSe SELM-1" (total Se recovery in SELM-1) for optimization.



a) Experiment C, "TotSe Wheat"



b) Experiment C, "TotSe SELM-1"

Figure F6: Bar graphs of regression coefficients and variable importance plot (VIP, comp. 1) of the factors "pre-extraction" (X2) and "enzyme" (X3), and factor interactions for optimization with individual responses a) "TotSe Wheat" (total Se recovery in ERM BC210a) and b) "TotSe SELM-1" (total Se recovery in SELM-1) for optimization.

Median concentrations and recoveries (%) for total Se and SeMet (Experiment C)

The median total selenium and SeMet concentrations (median ± SD, n = 3, mg/kg ww) from experiment C on ERMBC210a (wheat flour) and SELM-1 (selenized yeast) and the calculated recoveries (median ± SD, n = 3, %) are shown in Table F1, with experimental design in standard order.

Table F1: Experiment C; 2^2 full factorial design for optimization, number of experiments, factors and levels with codes, and concentrations of SeMet (median ± SD, n = 3, mg/kg ww) and total Se (median ± SD, n = 3, mg/kg ww) in ERM BC210a (wheat flour) and SELM-1 (selenized yeast), n = 3.

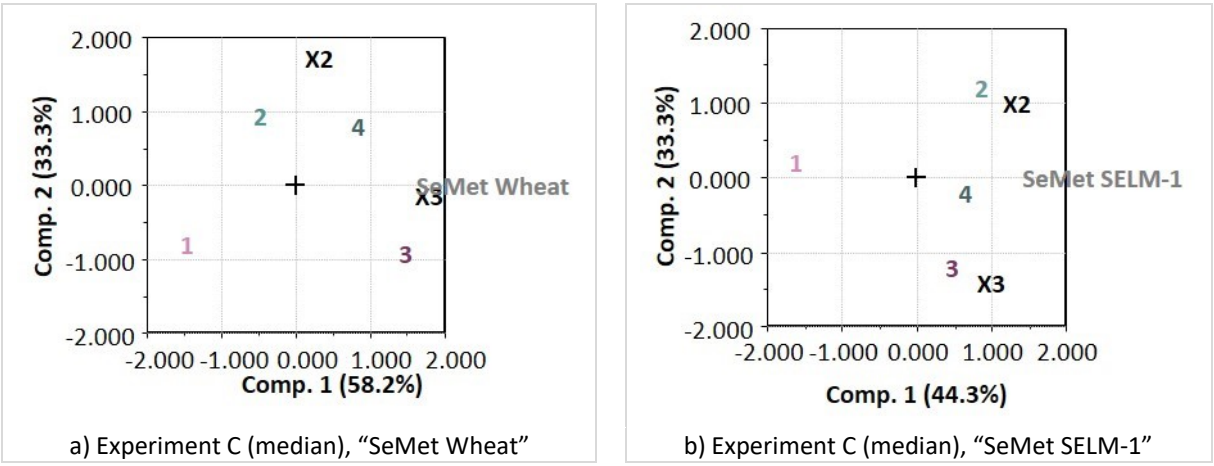
Exp	X2 Pre-extraction	X3 Enzyme	Median concentrations			
			Wheat flour		SELM-1	
			Total Se (mg/kg ww)	SeMet (mg/kg ww)	Total Se (mg/kg ww)	SeMet (mg/kg ww)
1	- (No)	- (Protease)	19.1 ± 0.3	4 ± 1	2193 ± 53	2437 ± 50
2	+ (Yes)	- (Protease)	17.6 ± 0.4	5.8 ± 0.8	2190 ± 89	2168 ± 92
3	- (No)	+ (Combined)	17.6 ± 0.2	7.5 ± 0.7	2132 ± 66	2544 ± 83
4	+ (Yes)	+ (Combined)	18.1 ± 0.4	6.0 ± 0.5	2166 ± 43	2480 ± 33

Exp	X2 Pre-extraction	X3 Enzyme	Median responses as recoveries (%)			
			TotSe Wheat	SeMet Wheat	TotSe SELM-1	SeMet SELM-1
1	- (No)	- (Protease)	111 ± 4	36 ± 7	108 ± 2	76 ± 1
2	+ (Yes)	- (Protease)	102 ± 2	52 ± 3	108 ± 4	80 ± 3
3	- (No)	+ (Combined)	102 ± 1	68 ± 3	105 ± 3	80 ± 3
4	+ (Yes)	+ (Combined)	105 ± 2	54 ± 2	107 ± 2	78 ± 1

* Recoveries (%) were calculated using equation (7).

PCA including median recoveries for total Se and SeMet as individual responses (Experiment C)

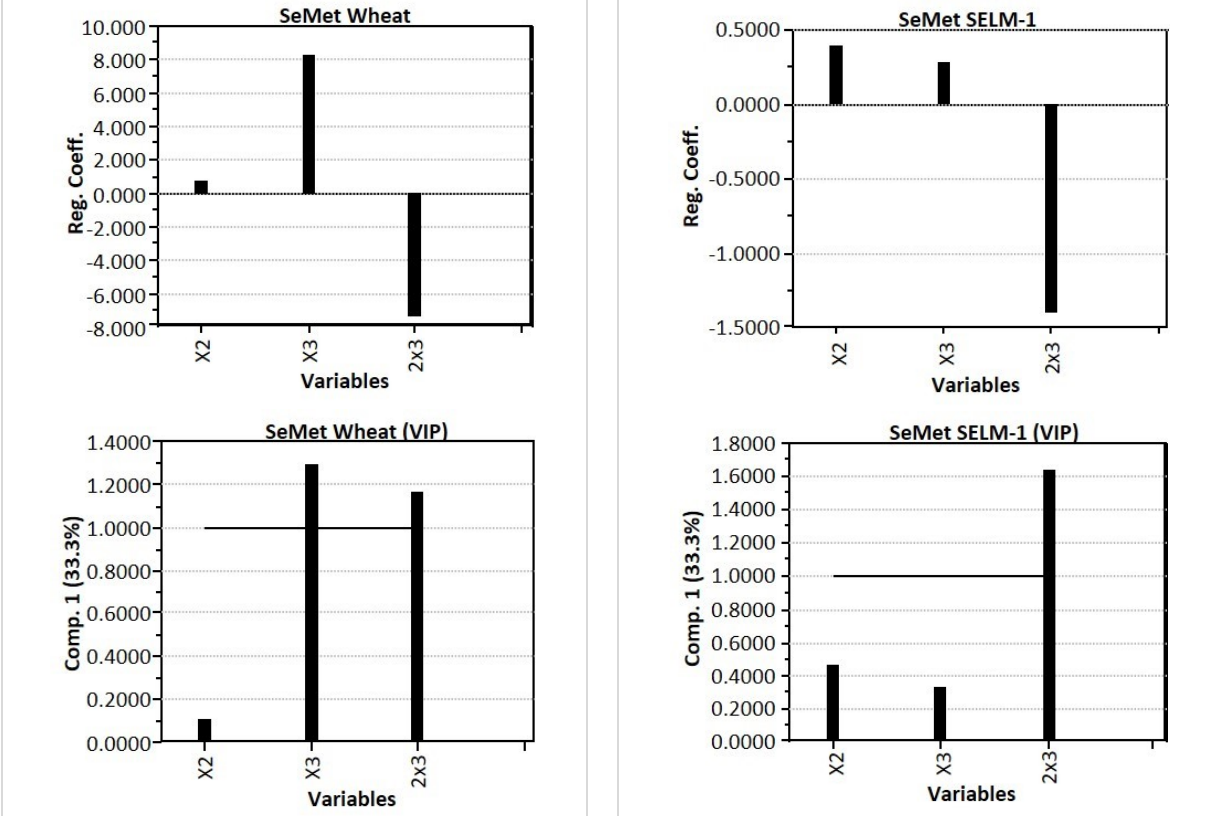
The biplots for individual response, “SeMet Wheat” and “SeMet SELM-1”, included in experiment C for optimization of the factors “pre-extraction” (X2) and “enzyme” (X3),four experiments (median, n = 3), are shown in Figure F7. Bar graphs of regression coefficients and VIP plots for the factors and interactions between the factors in experiment C are shown in Figure F8.



a) Experiment C (median), "SeMet Wheat"

b) Experiment C (median), "SeMet SELM-1"

Figure F7: Biplots (Comp. 1 vs 2) showing the relations between objects (exp 1-4) and the factors "pre-extraction" (X2) and "enzyme" (X3), and individual responses a) "SeMet Wheat" (SeMet recovery in ERM BC210a) and b) "SeMet SELM-1" (SeMet recovery in SELM-1) for optimization.



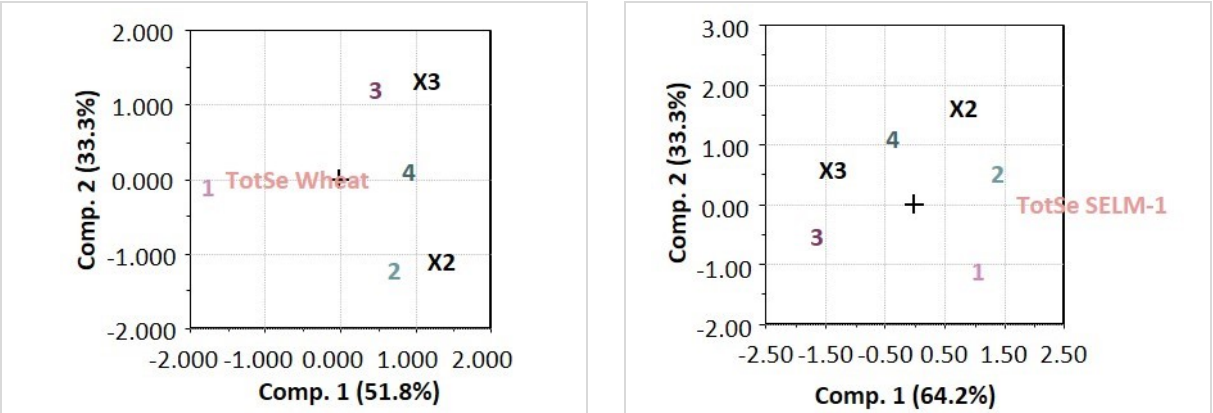
a) Experiment C (median), "SeMet Wheat"

b) Experiment C (median), "SeMet SELM-1"

Figure F8: Bar graphs of regression coefficients and variable importance plots (VIP, comp. 1) of the factors "pre-extraction" (X2) and "enzyme" (X3) and factor interactions with individual responses a) "SeMet Wheat" (SeMet recovery in ERM BC210a) and b) "SeMet SELM-1" (SeMet recovery in SELM-1) for optimization.

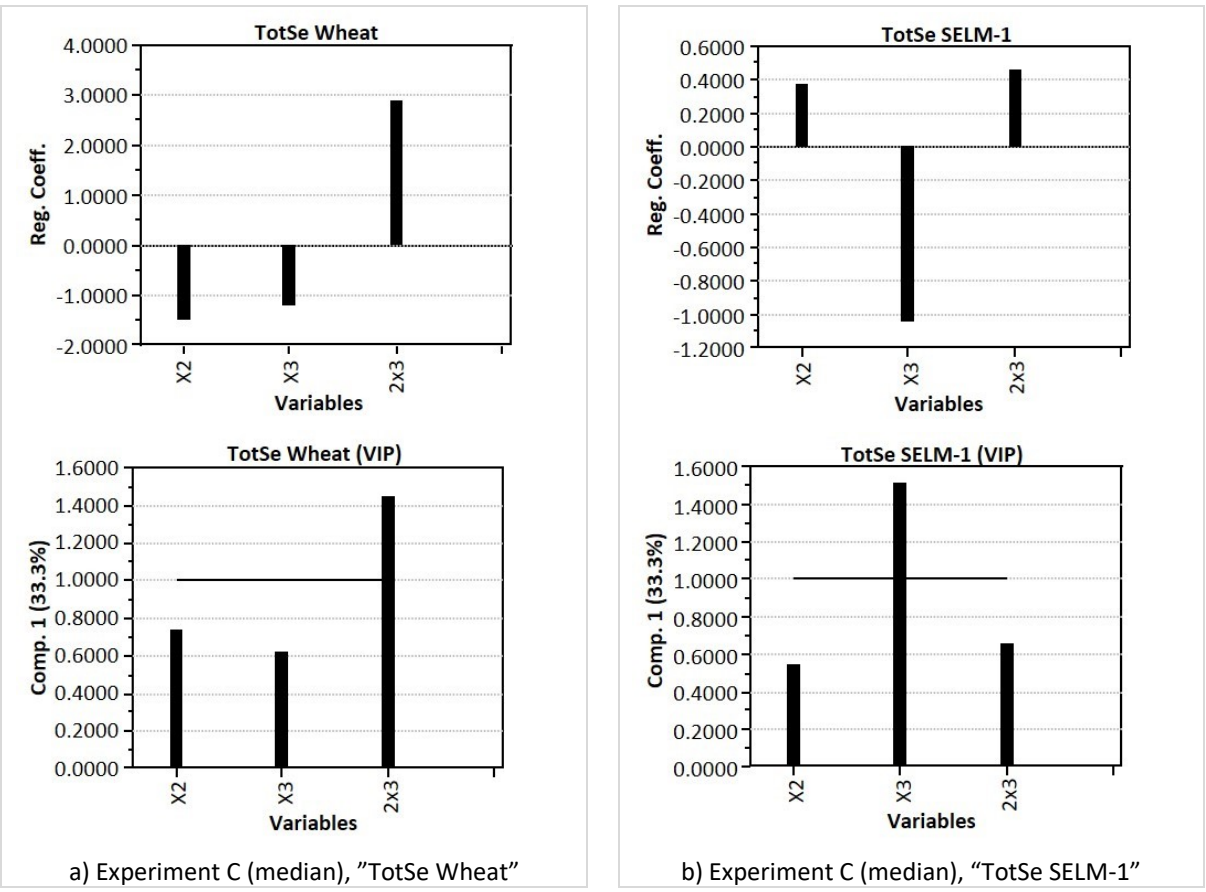
The biplots for individual response, "TotSe Wheat" and "TotSe SELM-1", included in experiment C for optimization of the factors pre-extraction (X2) and enzyme (X3) four experiments (median, n = 3), are shown

in Figure F9. Bar graphs of regression coefficients and VIP plots for the factors and interactions between the factors in experiment C are shown in Figure F10.



a) Experiment C (median), "TotSe Wheat" b) Experiment C (median), "TotSe SELM-1"

Figure F9: Biplots (Comp. 1 vs 2) showing the relations between objects (exp 1-4) and the "pre-extraction" (X2) and "enzyme" (X3) and individual responses a) "TotSe Wheat" (total Se recovery in ERM BC210a) and b) "TotSe SELM-1" (total Se recovery in SELM-1) for optimization.



a) Experiment C (median), "TotSe Wheat" b) Experiment C (median), "TotSe SELM-1"

Figure F10: Bar graphs of regression coefficients and variable importance plots (VIP, comp. 1) of the factors "pre-extraction" (X2) and "enzyme" (X3) and factor interactions with individual responses a) "TotSe Wheat" (total Se recovery in ERM BC210a) and b) "TotSe SELM-1" (total Se recovery in SELM-1) for optimization.

Appendix G – Se speciation data and chromatograms

Replicated measurements and deviations (%)

Replicated measurements (mg Se/kg ww) and recovery (%) for total Se in soluble and non-soluble fractions and for SeMet in soluble fractions in experimental diets, and calculated deviations (%) are shown in Table G1. Replicated SeMet measurements (mg Se/kg ww) and calculated deviations (%) are shown in Table G2 for commercial fish feed and feed ingredients extracted using the optimized and initial method. For CRMs, ERM BC210a and SELM-1, replicated SeMet measurements (mg Se/kg ww) and deviations (%) are shown in Table G3. Trueness for the replicates measurements for the CRMs are also shown in Table G3. Deviations were calculated using equation (5).

Table G1: Total Se (mg Se/kg ww) and recovery of Se in soluble and non-soluble fractions, SeMet (mg Se/kg ww) and recovery of SeMet in non-soluble fraction, and calculated deviations (%) for replicates of experimental diets.

Total Se in soluble fraction			
Diet	**Total Se (mg/kg ww) in soluble fraction**	**Recovery of Se in soluble fraction (%)**	**Deviation (%)**
Diet A	0.4336	115	3.1
	0.4205	112	
Diet B	0.7274	115	4.0
	0.6988	111	
Diet C	0.4087	91	8.4
	0.4441	99	
	0.4209	94	
Diet D	6.191	100	3.3
	6.400	103	
Diet E	16.63	103	2.3
	16.38	101	
	16.25	100	
Diet F	3.542	66	5.7
	3.747	69	
	3.566	66	

Total Se in non-soluble fraction			
Diet	**Total Se (mg/kg ww) in non-soluble fraction**	**Recovery of Se in non-soluble fraction (%)**	**Deviation (%)**
Diet A	0.0626	17	4.0
	0.0601	16	
Diet B	0.0844	13	4.0

	0.0811	13	
Diet C	0.1536	34	0.5
	0.1529	34	
Diet D	0.829	13	3.3
	0.802	13	
Diet E	1.80	11	4.3
	1.88	12	
Diet F	1.489	28	1.9
	1.461	27	
	1.481	27	

SeMet in soluble fraction

Diet	SeMet (mg Se/kg ww) in soluble fraction	SeMet recovery of supplemented SeMet (%)	Deviation (%)
Diet A	0.0880	59	33
	0.0630	42	
Diet B	0.2563	64	4.9
	0.2495	62	
	0.2441	61	
Diet C	0.0132	>100	34
	0.0186	>100	
Diet D	3.344	67	1.6
	3.344	67	
	3.396	68	
Diet E	10.63	71	7.3
	9.880	66	
	10.38	69	
Diet F	0.0031	>100	55
	0.0054	>100	

Table G2: SeMet (mg Se/kg ww) and deviations (%) for replicated samples of commercial fish feed and feed ingredients using the optimized method and the initial method.

	Optimized method		Initial method	
Sample	**SeMet (mg Se/kg ww)**	**Deviation (%)**	**SeMet (mg Se/kg ww)**	**Deviation (%)**
FF1	0.134 0.141	5.3	0.165 0.167 0.159	4.6
FF2	0.3799		0.394 0.408 0.391	4.3
FF3	0.1192		0.149 0.148 0.143	4.1
FF4	0.1069 0.1094	2.3	0.0974	
FF5	0.0908 0.0915 0.0979	7.6	0.1423	
FF6	0.4476 0.4449 0.4506	1.3	0.421 0.413	1.9
FM1	0.3076 0.3201 0.3134	4.0	0.2784	
FM2	0.2841 0.2803 0.3005	7.0	0.2877	
FM3	0.4522 0.4132 0.4098	9.99	0.4485 0.4514	
PM1	0.0913 0.0878 0.0858	6.2	0.0776	
PM2	0.3951 0.4181	5.7	0.4320 0.4328	0.2
IM1	0.0491 0.0489	0.41	0.0388	
IM2	0.088 0.086 0.080	9.6	-0.037 -0.035 -0.036	-5.4

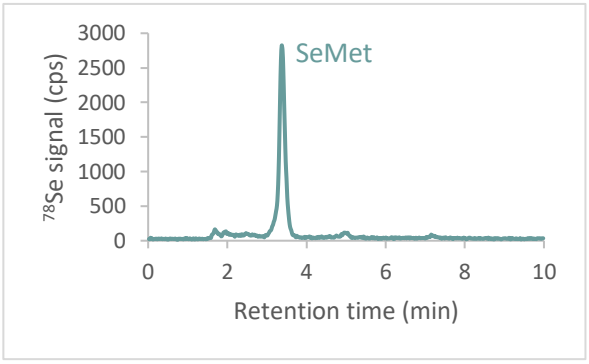
Table G3: SeMet (mg Se/kg ww), trueness (%) and deviations (%) for replicated measurements of CRMs ERM BC210a and SELM-1 from five subsequent days.

ERM BC210a			
Day	**SeMet (mg Se/kg ww)**	**Trueness (analytical recovery, %)**	**Deviation (%)**
	7.46	68	
1	7.47	68	7.2
	6.94	63	
2	8.01	73	-
3	7.49	68	2.3
	7.66	69	
4	8.19	74	-
	8.36	76	
5	8.25	75	4.8
	7.97	72	

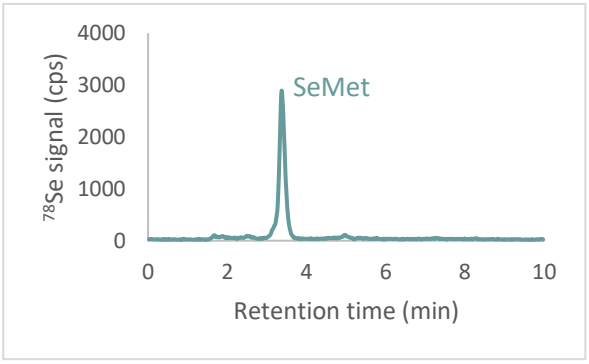
SELM-1			
Day	**SeMet (mg Se/kg ww)**	**Trueness (analytical recovery, %)**	**Deviation (%)**
	1024	80	
1	971.7	76	6.1
	1033	80	
2	985.4	77	-
3	918.5	72	-
4	899.6	70	-
	976.6	76	
5	1038	81	9.9
	1079	84	

Chromatographic profiles for Se speciation

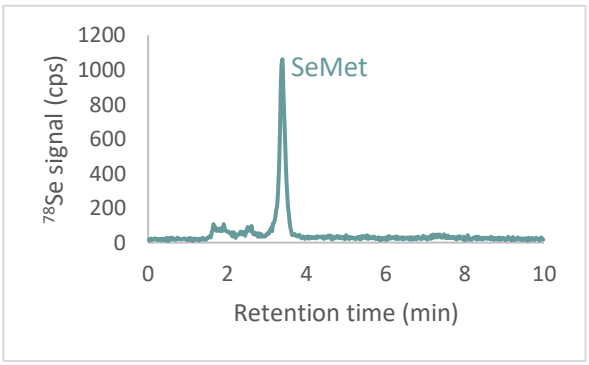
The HPLC-ICP-MS chromatograms of experimental diets, diet A (supplemented with 0.15 mg Se/kg SeMet), diet B (supplemented with 0.4 mg Se/kg), diet C (basal diet for diet D-F) and diet E (supplemented with 15 mg Se/kg) are shown in Figure G1. The chromatograms of commercial fish feed, FF2, FF3, FF4 and FF5, are shown in Figure G2, while chromatograms of feed ingredients, FM3, PM2 and IM1, are shown in Figure G3. Figure G4 shows an overlay of SeMet spiked samples for FM2, PM1 and IM2.



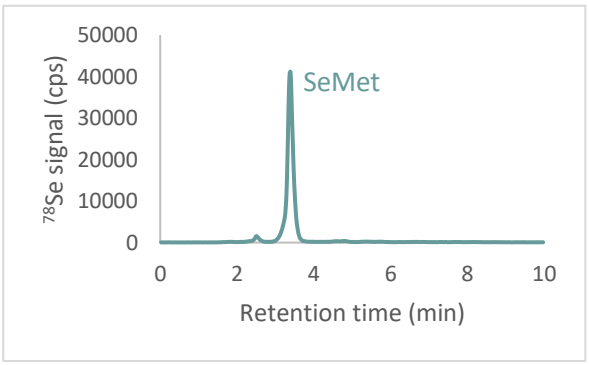
a) Diet A (SeMet supplemented, 0.15 mg Se/kg) (4x dilution)



b) Diet B (SeMet supplemented, 0.4 mg Se/kg) (4x dilution)

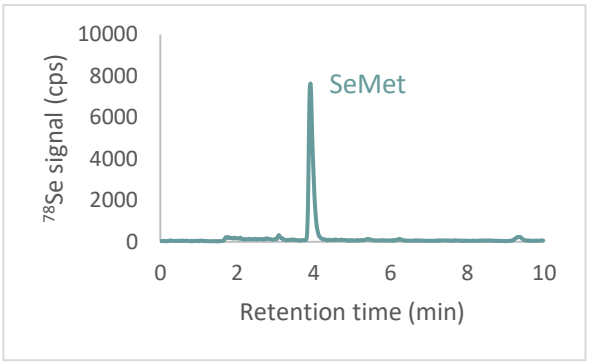


c) Diet C (Basal for diet D-F. Not supplement) (4x dilution)

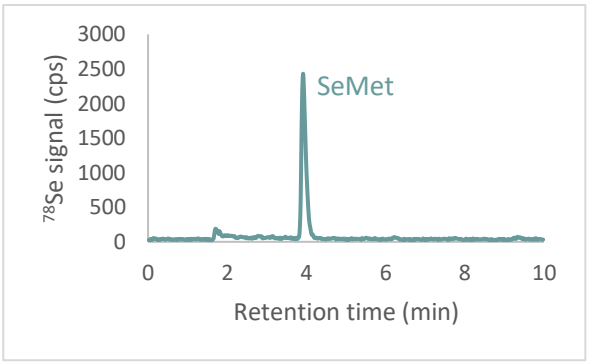


Diet E (SeMet supplemented, 15 mg Se/kg) (8x dilution)

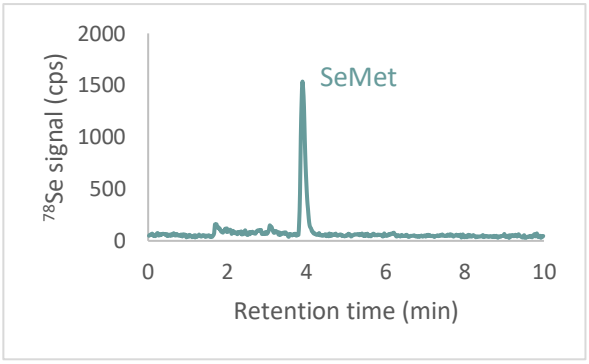
Figure G1: Chromatograms of experimental diets a) diet A, b) diet B, c) diet C and d) diet E, with SeMet eluting at retention time of 3.4 min, when analyzed by HPLC-ICP-MS.



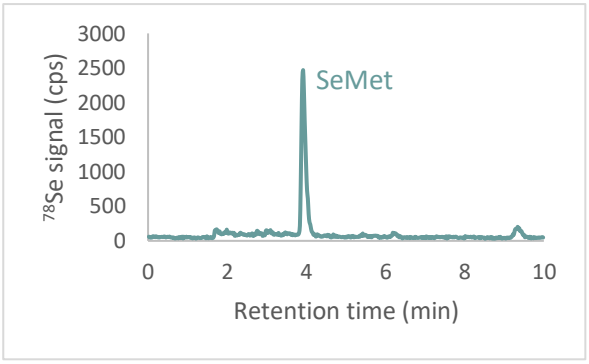
a) FF2 (4x dilution)



b) FF3 (4x dilution)

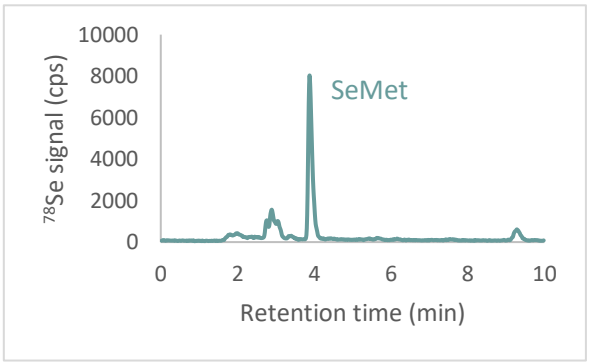


c) FF4 (4x dilution)

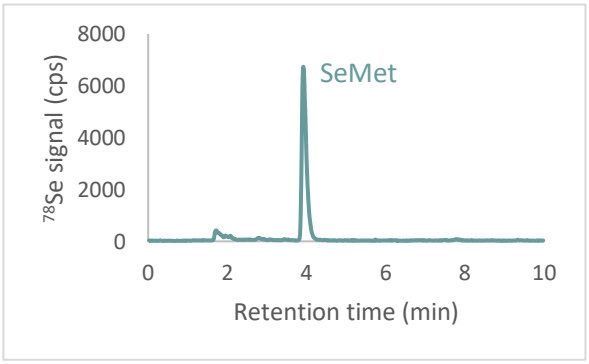


d) FF5 (4x dilution)

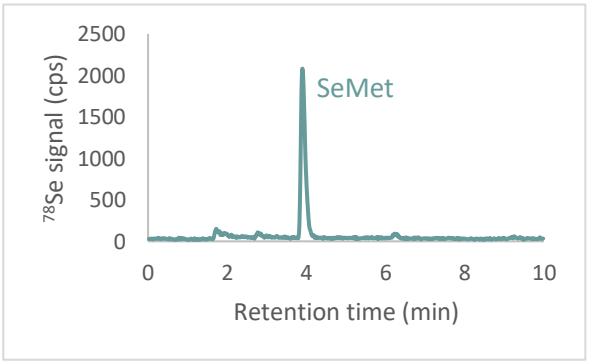
Figure G2: Chromatograms of fish feed a) FF2, b) FF3, c) FF4 and d) FF5, with SeMet eluting at retention time of 3.9 min, when analyzed by HPLC-ICP-MS.



a) FM3 (4x dilution)

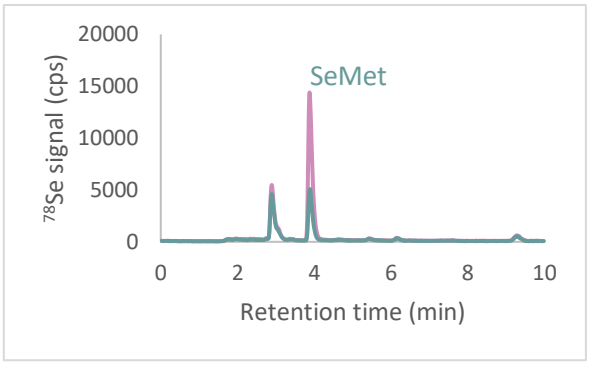


b) PM2 (4x dilution)

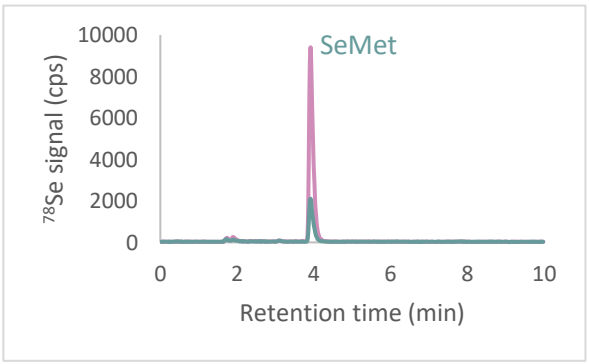


c) IM2 (4x dilution)

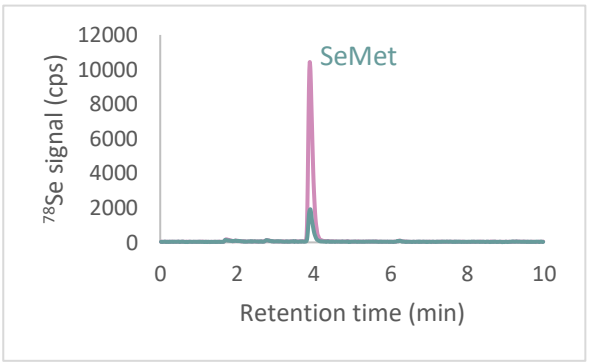
Figure G3: Chromatograms of feed ingredients a) FM3, b) PM2 and c) IM2, with SeMet eluting at retention time of 3.9 min, when analyzed by HPLC-ICP-MS.



a) FM2 (4x dilution)



b) PM1 (4x dilution)



c) IM2 (4x dilution)

Figure G4: Chromatograms of selected feed ingredients a) FM2, b) PM1 and c) IM2 spiked with 5 µg/L SeMet standard solution. SeMet eluting at retention time of 3.9 min, when analyzed by HPLC-ICP-MS.