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Selenium Speciation in Animal Feeds and Fish Tissues by HPLC ICP-MS – Validation and Application of an Analytical Method

By

Kashif Hanif

Department of Chemistry

University of Bergen

A thesis submitted for the degree of

Erasmus Mundus Master in Quality in Analytical Laboratories (EMQAL)

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Kashif Hanif

Department of Chemistry
University of Bergen

Supervisors

Dr. Veronika Sele, PhD
Marine Toxicology, Institute of Marine Research

Dr. Marta Silva, PhD
Marine Toxicology, Institute of Marine Research

Dr. Antony Philip, PhD
Marine Nutrition, Institute of Marine Research

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ABSTRACT

Selenium (Se) is essential for human and animal health and crucial for selenoprotein synthesis with diverse biological roles. It's mainly sourced from animal feed, particularly fish feed, with fishmeal and marine by-products ensuring adequate Se levels. However, recent shifts from marine to plant-based feed ingredients have decreased Se levels in fish feed and whole-body fish, highlighting the need for Se supplementation. It can be added as inorganic (selenite) or organic (SeMet) forms. In Europe, the maximum limit is 0.5 mg/kg feed for total Se in animal feeds. The European Food Safety Authority (EFSA) approved organic feed additives like selenized yeast (organic Se), limited to 0.2 mg/kg feed due to its higher bioavailability than inorganic Se. Thus, the difference in the legislative limit between supplemented total Se and organic Se emphasizes the need for precise analytical methods for Se speciation.

In this thesis, the main aim was to validate an in-house method of Se speciation for animal feed and fish tissue samples. In addition, one aim was to study total Se content in different feeds, including fish feed, land animal feed, feed ingredients and fish tissues using ICP-MS. The application of the validated method for the determination of organic Se (SeMet) in rainbow trout feeds and whole-body samples using HPLC ICP-MS.

This study examined total Se concentrations in salmonid commercial feeds (n = 27), salmonid experimental feeds (n = 6), zebrafish feed (n = 3), rainbow trout feed (n = 12), land animal feeds for ruminants (n = 10), swine (n = 2), and chicken (n = 3), feed materials (n = 9), and rainbow trout whole-body samples (n = 39). The results showed that fish feed has more total Se than land animal feeds. Several animal feed exceeded the legal limit of 0.5 mg/kg total Se. Pig and poultry feeds lower total Se contents. Tuna meal-based rainbow trout feed has more total Se than plant meal-based feeds. Tuna and fish meals have much more total Se than plant and insect meals. In rainbow trout whole-body samples, tuna meal-fed trout showed greater total Se concentrations than plant meal-fed trout.

The validation procedure for the Se speciation (SeMet) was performed by analyzing feed and tissue samples over six days using Eurachem and NMKL guidelines. The validation procedure revealed good selectivity for SeMet in several sample types, where SeMet was baseline separated from other Se peaks. Zebrafish, land-animal, and blue mussel meal samples exhibited unknown Se species peaks. Linearity (r = 0.99) between concentration and response indicated a linear fit model. Method LOD and LOQ were estimated at 0.02 and 0.06 mg/kg, respectively. Method trueness indicated 53% to 80% recovery for wheat-based CRM (ERM BC210a) and 72% to 83% for selenized yeast-based CRM (SELM-1). CRM recovery, especially ERM BC210a, was low and not in acceptable range. Method precision was good, notably for SELM-1. Fish tissue and land-animal feed samples showed the lowest and highest within-day variance. Fish feed, tuna tissue, salmon tissue, and SELM-1 exhibited better precision. The analytical method's precision was acceptable because the Horwitz ratio was within the required range of 0.5–2. Salmon tissue had the lowest MU (13%), while land-animal feed had the greatest (64%). Fish feed, zebrafish feed, tuna tissue, blue mussel, and SELM-1 had acceptable MU (%).

Application of method on rainbow trout experimental feeds demonstrated that plant meal-based diets recovered more SeMet than tuna meal-based diets. Tuna based-meal feed had a greater total Se concentration than vegetal based-meal feed. These data may suggest that this study's enzymatic extraction method works better with vegetal meal-based feed than tuna meal-based feed. SeMet recovery from rainbow trout raised on vegetable and tuna meal-based diets was 18% and 14%, respectively, for whole-body samples. The group that fed on vegetal meal-based diet with organic Se and Hg had the greatest SeMet concentration (0.44 mg/kg) and recovery (25%). A rainbow fish sample fed a tuna meal-based diet with organic Se had a SeMet level of 0.43 mg/kg and a recovery of 17%.

The present study presented an overview of total Se content in commercial and experimental feeds of marine and land animals. Higher Se concentrations in commercial feeds raised concerns for compliance to current legislative requirements and animal and public health risks. Moreover, the method validation parameters showed good potential of this in-house developed method for Se speciation in feed and tissue samples. The application of the method also proved its potential applicability on diverse sample matrices. However, further improvements will be needed, particularly for its application on land animal and fish meal-based feeds.

CONTENTS

ABBREVIATIONS

CRM	Certified Reference Material
Dw	Dry weight
EFSA	European Food Safety Authority
EMQAL	Erasmus Mundus Master in Quality in Analytical Laboratories
FF	Fish Feed
FM	Fish Meal
GPx	Glutathione peroxidase
Hg	Mercury
HPLC	High-performance liquid chromatography
ICP-MS	Inductive coupled plasma mass spectrometry
IM	Insect Meal
IMR	Institute of Marine Research
IP	Intermediate precision
kDa	Kilodalton
LC	Liquid Chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MeOH	Methanol
ML	Maximum limit
MS	Mass spectrometry
MU	Measurement of uncertainty
n	Sample size
p-value	Probability value
PM	Plant Meal
QA	Quality assurance
QC	Quality control
r	Repeatability
RP	Reversed phase
rpm	Revolutions per minute
RSD	Relative standard deviation
SD	Standard deviation
Se	Selenium
SeCys	Selenocysteine
$SeCys_2$	Selenocystine
SeMet	Selenomethionine
SeMetSeCys	Selenomethylselenocysteine
ww	Wet weight

1. INTRODUCTION

1.1 Background

Selenium (Se) is an essential and naturally occurring trace element that is required in small amounts in animal diet. Selenium is an integral component required for the synthesis of selenoproteins, which have numerous biological functions in redox homeostasis, immunity, reproduction, and thyroid hormone metabolism [1, 2, 3]. On the other hand, Se is only thought to be beneficial at specific concentrations, with a narrow range between the level of deficiency and intoxication in animals [3, 4].

In animal feed, fishmeal and marine by-products employed as feed ingredients provide the selenium concentrations to maintain physiological functions [5, 6]. In recent years, plant-based components have become more prevalent in feed for farmed fish in Norway. In the 1990s, fish meal and fish oil made up 90% of the ingredients in fish feed, which has traditionally been mostly composed of marine-based feed ingredients [7]. When compared to ingredients for a plant-based diet, fish meal is reported to naturally contain a higher concentration of selenium [8]. Also, Se is less readily available in plant-based feed ingredients. This deficiency can be fulfilled by supplementing Se to commercial feed, which is important for normal physiological functions in fish [9].

Selenium can be added as a supplement to an animal diet in mainly two form inorganic and organic forms. Selenium metabolic pathway, bioavailability, physiological functions, and toxicity are highly dependent on the element's chemical forms [10]. The bioavailability and toxicity of Se depend on the species in which it is present, according to several studies [5, 10, 11]. Consequently, the chemical forms, environmental distribution, and physiological levels of the various forms of Se are crucial. In general, organic forms of Se, including selenomethionine (SeMet), selenocysteine (SeCys), and methylselenocysteine (MeSeCys), are more bioavailable and less toxic than inorganic forms (selenite and selenate) [10, 12, 13]. Therefore, bioavailability, total Se content, and antioxidant capacity in animals can be drastically altered by supplementing their diets with various Se sources and levels, according to studies [14 – 17].

In the European Union (EU), Se is regulated as a feed additive through the European feed legislation. A maximum content for total Se in animal feeds, including fish feed, has been set at 0.5 mg/kg feed (Council Directive 70/524/EC and revisions) to protect animal and human health. Organic Se is more bioactive than the inorganic forms via promoting growth and the activity of antioxidant enzymes in animals [5, 10, 18]. Based on the apparent increased bioavailability of organic selenium compared to inorganic selenium, the European Food Safety Authority (EFSA) has approved supplementation of Se using organic selenium-yeast forms as feed additives [19, 20]. The European Union (EU) then developed the required regulations for using several Se feed additives, primarily selenised yeasts, at a supplementation level of no more than 0.2 mg Se/kg feed [20]. The legislative difference between total Se and supplemented organic Se forms endorse a suitable analytical method for Se speciation by differentiating organic Se forms and not only determining total Se.

Hyphenated techniques are typically used for the quantitative and qualitative identification of elemental species in biological materials, where high-performance liquid chromatography (HPLC) coupled to inductively coupled plasma mass spectrometry (ICP-MS) is the method most frequently employed [21]. Several analytical methods for selenium speciation are described in the literature, but in contrast to actual matrices [22, 23], several approaches have only been tested on commercially available standards [23, 24]. An in-house method for the speciation of selenium was developed for fish feed and tissues of Atlantic salmon [25]. The extraction procedures were optimized to improve the applicability of this method for fish feed and feed ingredients [26].

In order to further explore the applicability of the developed method on a variety of samples, a method validation study was planned in the current project. Method validation and quality assurance are important for analytical methods to acquire reliable data and know the data's uncertainties [27, 28]. Data validity is a prerequisite for fundamental hypotheses about analytical methods to prove that method is fit for purpose or intended use [27]. In this master project, a validation procedure was applied to the methodology developed for Se speciation analysis. Furthermore, in this master project, the validated method was applied to a set of rainbow trout feed and whole-body samples to study the content of organic selenium (SeMet) and its recovery through HPLC ICP-MS.

1.2 Research Aims

This thesis aimed to validate an in-house method of selenium speciation for animal feed and fish tissue samples. In addition, to determine total selenium content and organic Se species (i.e. SeMet) in animal feed, feed ingredients and fish tissue samples.

1.3 Objectives

The following objectives for this project are described below.

a. Determination of total selenium content in fish tissues, land-animal feed, fish feed, and feed ingredients.
b. Validation of Se speciation method, with determination of quality parameters, i.e., selectivity, linearity, LOD, LOQ, trueness, recovery, precision, and measurement of uncertainty for the method.
c. Determination of SeMet in fish feed and fish tissues where feeds have been supplemented with Se and Hg.

1.4 Hypotheses

a. Determination of total Se can give relevant information about the current levels of total Se in different animal feeds and it will be similar or different.
b. Validation of method can provide valuable information about the suitability of the method for Se speciation.
c. Selenium speciation data can give relevant information the chemical forms of selenium in feed and tissue.

2. THEORY

2.1 Selenium – Chemistry, sources, and occurrence

Selenium (Se) is a naturally occurring metalloid element which is essential to human and animal health in trace amounts [29]. Selenium was first identified and discovered by the Swedish chemist Jöns Jakob Berzelius in 1817 and the name derives from the Greek word '*selene'* for moon, in contract to the Latin word *'tellus'* for earth [29, 30]. Selenium belongs to group VIA (chalcogen group) of the periodic table with atomic number 34 along with oxygen, sulfur, and tellurium [30]. The selenium has physical and chemical properties that are intermediate between the metals and non-metals [29 - 32]. In nature, Se occurs as six stable isotopes but the ^{82}Se is generally regarded as a stable isotope. The chemical behavior of selenium mostly resembles sulfur, and it can exist in the 2^-, 0, 4^+ and 6^+ oxidation state [29]. The physical and chemical properties of Se presented in *Table 1*.

Table 1: Physical and chemical properties of selenium [30, 31, 32]

Element name and symbol	Selenium (Se)
Atomic number	34
Atomic mass	78.96 Da
Density	4,808 kg m^{-3}
Melting point	220°C
Boiling point	685°C
Vapor pressure	1 mmHg at 356°C
Natural isotopes: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{82}Se	Abundance: 0.87%, 9.02%, 7.58%, 23.52%, 49.82%, 9.19%
Se^{2-}	Selenide
Se^{0}	Elemental selenium
Se^{4+}, Se^{6+}	Selenite, Selenate
SeMet, SeCys	Selenomethionine, Selenocysteine

2.1.1 Natural sources of selenium

The primary sources of Se in the environment are natural and human-made [29]. Selenium is present in all naturally occurring substances on Earth, including rocks, soils, waters, air, and plants and animal tissues [29, 33]. Selenium is continuously recycled in the environment via the atmospheric, marine, and terrestrial systems on a global scale. According to estimates of Se flux, anthropogenic (human-made) activities are the primary source of Se release in the cycle, whereas the marine system is the primary natural channel to supply it in cycle [29, 34]. Selenium is cycling in the atmosphere (*Figure 1*) is significant due to the velocity of transit, but the terrestrial system is the most significant in terms of animal and human health [29, 34] due to the direct links between agricultural activities and the food chain. *Table 2* provides an overview of the Se concentrations in different natural matrices.

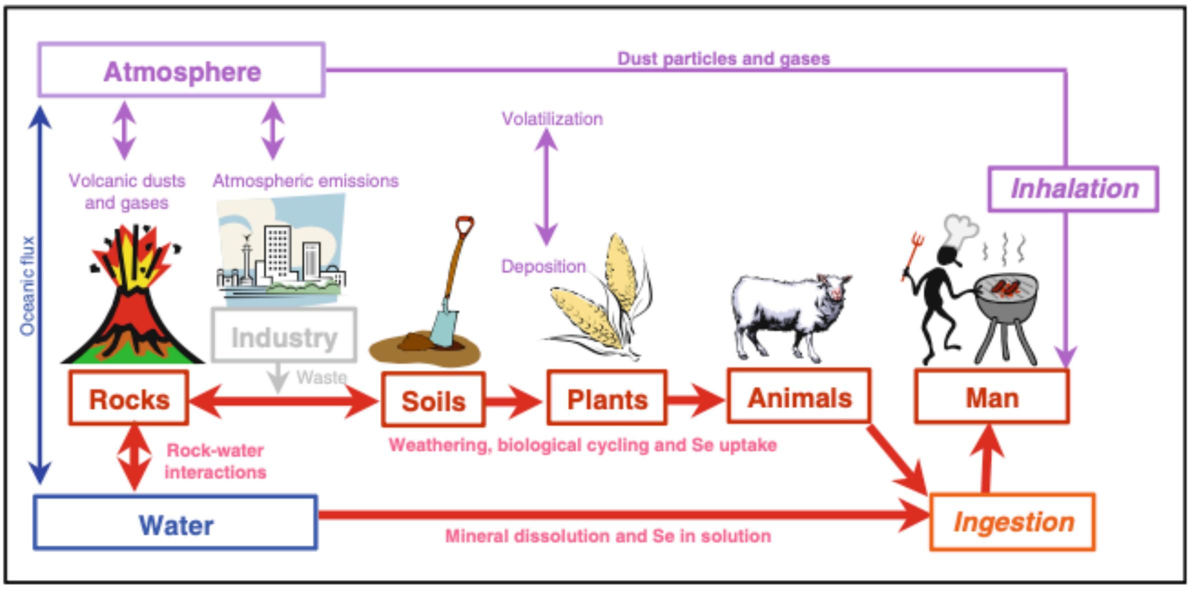


Figure 1: A simplified schematic diagram of the cycling of selenium in environment [29]

2.1.2 Selenium in the environment

Selenium is the 30th most prevalent metalloid element in the Earth's crust. It is present in the crust of the world at an estimated average concentration of 0.05 mg Se kg^{-1} [35] (*Table 2*). Among these rocks, sandstone, quartzite, and limestone contain the highest concentrations of selenium [36]. Geology (*Figure 1*) has a fundamental influence over selenium concentrations in the soils on which we cultivate the plants and animals that comprise the human food chain [29]. In seleniferous soil regions, such as the United States, Canada, Colombia, the United Kingdom, China, and Russia, concentrations of Se can reach 1200 mg Se kg^{-1} [29].

Selenium is mobilized by a wide range of man-made activities that often involve the contact of Se-containing molecules with water. According to estimates, 14,000 tonnes of Se flow annually from land to the oceans through surface and groundwaters (*Figure 1*), a significant conduit for selenium loss from land in the Se cycle [37]. Most rivers are believed to contain 85% more particulate selenium than aqueous Se [38]. Seawater's average concentration of Se (*Table 2*) is predicted to be 0.09 g/L [39], and the oceans are a significant environmental sink for Se [38].

Table 2: Selenium concentration in selected natural matrices [29, 31, 38, 40]

Material	**Total Se (mg kg^{-1})**	**Material**	**Total Se (mg kg^{-1})**
Earth's crust	0.05	Groundwater (Norway)	0.01 – 4.82
Volcanic rocks	0.35	Forage (Norway)	0.05 – 0.042
Limestone	0.03 – 0.08	Moss (Norway)	0.8 – 1.23
Marine carbonates	0.17	Grain (Norway)	0.006 – 0.042
Soil (World)	0.4	Algae (Marine)	0.04 – 0.24
Soil (seleniferous)	1 – 5000	Algae (freshwater)	<2
Soil (Norway)	3 – 6	Fish (marine)	0.3 – 2
Soil (Finland)	0.005 – 1.241	Fish (freshwater)	0.42 – 0.64
Fresh water (μg L^{-1})	0.02	Animal tissues (general)	0.4 - 4
Seawater (general)	0.09		

2.1.3 Selenium in the feed and feed ingredients

The Se concentration of foods varies by geographical origin, both within and between nations [41]. The provision of enough selenium in the diet is undeniably essential but it is made more difficult by the fact that selenium contents in feeds and forages vary considerably on the plant species, sampling season, and soil selenium status [41, 42, 43]. Literature showed that the average values of total Se for wheat cultivated in the United Kingdom, the United States, and Canada are 0.03, 0.37–0.46, and 0.76 mg per kg DM, respectively [43]. Although differences in term of total Se amongst grains are quite minor, they have been classified as follows: wheat > rice > corn > barley > oats [44]. In general, linseed meal contains more Se than extracted soybean meal and rapeseed meal (0.82, 0.30, and 0.14 mg kg^{-1} DM, respectively) [45]. There are several cereal by-products that are rich sources of Se, including maize gluten feed, ricebran meal, and distillers' dark grains (0.24, 0.16, and 0.18 mg/kg DM, respectively) [45, 46]. Salmon and herring meals are also enriched sources of Se (1.9 mg Se/kg DM) while tuna-fish meal contains high content of Se (5.1–6.2 mg Se/kg DM) [46, 47], making them the only protein concentrates of marine origin widespread in use that add significant amounts of Se to compounded rations [47]. In comparison, animal protein source of non-marine origin (meat meal) contains less Se and can be considerably variable (0.11–1.14 mg Se/kg DM) [45, 47]. However, in the United Kingdom, figures for two products, blood, and feather meals, have been generally stable (0.60 and 0.76 mg Se/kg) [45].

2.2 Metabolism and biological role of selenium

The majority of Se consumed by an animal is absorbed in the small intestine. However, the rates and processes of Se metabolism vary depending on Se species present and also vary among animal species [29]. The common intermediate, selenide or its equivalent, can be formed from both inorganic and organic sources of Se. It is converted to selenide for subsequent utilisation or excretion, selenite and selenate. In contrast, selenomethionine is converted to selenocysteine via the trans-selenation pathway, identical to the trans-sulfuration process for methionine to cysteine and then lysed to selenide [48]. In general, ruminants absorb less Se than single-stomached animals, because in ruminants the rumen microbes convert selenite to insoluble forms [29]. Inorganic Se is mostly absorbed by other tissues of the body while selenomethionine, the primary form which transfer from plant to animal, binds to protein tissues in the body [49 - 52]. Red blood cells absorb selenite, bind it to albumin, and transport it to the liver. The majority of ingested Se is rapidly eliminated in urine, breath, perspiration, and bile, with the remaining bound or integrated into blood and proteins [29]. In mono-gastric animals, Se is primarily eliminated in the urine (70–80%); however, in ruminants, Se is primarily excreted in the feces [49, 50]. The primary metabolic process converts both inorganic and organic Se to mono, di, or trimethylated forms in animals and humans [53].

2.2.1 Biological role of selenium in animals

There are presently 25 essential selenoproteins have been recognized in animals and humans that contain SeCys, and several of them are engaged redox reactions [51, 54, 55]. The glutathione peroxidase (GPx), thioredoxin reductase, iodothyronine deiodinase, and selenophosphate synthetase groups are responsible for enzyme function in living systems. Selenium serves a crucial biological role in complicated interactions with vitamin E and polyunsaturated fatty acids as a component of the enzyme GPx, which protects tissues from peroxidative damage by accelerating the reduction of lipid hydrogen peroxide or organic hydroperoxides [54 - 55].

One of the primary antioxidant defense mechanisms in both animals and human is formed by the combination of GPx, vitamin E, and superoxide dismutases [55]. Moreover, it has been associated with enzyme activation, immune system function, pancreatic function, DNA repair, and the detoxification of xenobiotic agents including herbicides [49 - 51, 54]. In most cases, Se appears to provide shield to animals from the toxicity of arsenic because Se is present in the prosthetic groups of several metalloenzymes. Although this is not always the case, and the biological response relies on the ratio of selenium/metal involved [50]. Moreover, Se also plays a crucial role in reproduction, thyroid hormone metabolism, and cancer prevention [29, 50, 55].

2.2.2 Selenium deficiency in animals

Se deficiency in animals is prevalent and pervasive around the globe, affecting several countries. Thus, Se deficiency considerably reduces animal productivity in these regions [49, 56]. Selenium deficiency is linked to diseases, which include mulberry heart disease, dietary hepatic apoptosis in pigs, exudative diathesis, embryonic mortality, and bird pancreatic fibrosis [29, 49, 57]. Ruminants and other species may also experience nutritional muscular dystrophy, white muscle disease (WMD), and retained placenta [49, 57]. The clinical manifestations of selenium insufficiency in animals include decreased appetite, growth, production, reproductive fertility, lethargy, and muscle weakness [49, 50, 51, 56]. Selenium deficiency in aquaculture, particularly Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*), results in stunted growth, lethargy, loss of appetite, diminished muscular tone, and death [58]. Muscular dystrophy in Atlantic salmon, Hitra disease in farmed salmon, and exudative diathesis in rainbow trout are illnesses related to Se deficiency [58, 59, 60]. These disorders are responsive to adding vitamin E and Se to feed [49, 50, 51, 56].

2.2.3 Selenium toxicity in animals

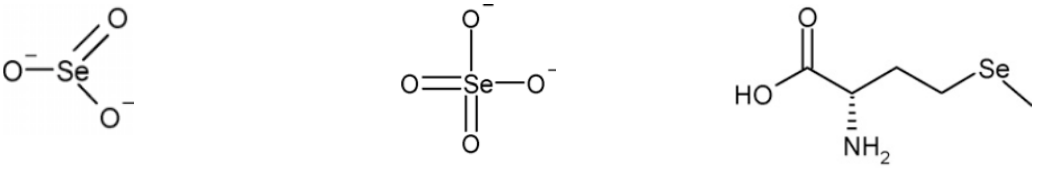
It is noteworthy that there is a narrow boarderline between Se essentiality and toxicity in animals [61]. Studies on laboratory animals (rats and mice) have shown that hydrogen selenide is the most lethal Se compound when inhaled, and sodium selenite is the most poisonous when ingested. But elemental selenium in the diet has low toxicity due to its insoluble nature [32, 50]. Acute Se poisoning is unusual because animals are rarely exposed to selenium-rich food resources and prefer to avoid the consumption of selenium-accumulating plants. [49, 50, 51]. While chronic Se intoxication is more common for animals caused by ingestion of plants containing 5–40 mg kg^{-1} over a long period (weeks or months). This chronic condition is characterized by lethargy, lack of vigour, anorexia, rough skin, shedding of the hooves, deterioration of the joints and bones, lameness, anemia, hepatic cirrhosis, and decreased reproductive performance [29, 49, 50]. Selenium level of 47–53 mg L^{-1} in surface waters cause anemia and decreased hatchability in trout, whilst selenium concentrations of 70–760 mg L^{-1} are lethal to the majority of aquatic species [29, 31, 50].

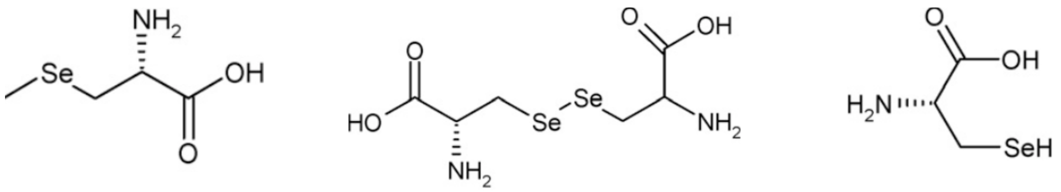
2.3 Selenium species in food and feed

Selenium naturally occurs in different chemical forms, or Se species. Chemical species are defined by the International Union of Pure and Applied Chemistry (IUPAC) as: *"a specific form of an element defined as to isotopic composition, electronic or oxidation state, and/or complex or molecule structure of an element"* [62]. Selenium can be found as inorganic and organically bound species [63, 64]. The most relevant Se species in terms of food and feed are presented in Figure 2.

Selenium is primarily found in nature in four inorganic forms, including elemental Se (Se^0), selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}), and selenide (SeO_3^{2-}) [63]. It is mainly salts of sodium selenite and sodium selenate that are relevant in food and feed samples. There are more than 20 different forms of organic Se in food, but the most prevalent species are selenomethionine (SeMet), selenocysteine (SeCys2) and methyl-selenocysteine (SeMeCys) (Figure 2) [64]. SeMet and SeCys are organic Se species known to produce Se-containing proteins, while SeCys forms selenoproteins essential for biological processes [65]. SeMet, conversely, results in the formation of Se-containing proteins that are not considered as selenoproteins [66]. Compared with sodium selenite, SeMet is the best Se reservoir in living systems [67].

The chemical forms and levels of these species in food or feed determine whether they are beneficial or detrimental for consumers [68, 69]. Organic Se has higher bioavailability, lower toxicity, and lower selenium content than inorganic selenium [49]. In animals, Se is known to be more accessible in organic forms, such as Se amino acids like SeMet, than in inorganic forms like selenite and selenate [70, 71]. In fish and other animals, the feed supplemented with organic forms of Se is absorbed and retained in their tissues more quickly than animals supplemented with inorganic forms of Se [70, 72, 73].


Selenite Selenate Selenomethonine


Selenomethylselenocysteine Selenocystine Selenocysteine

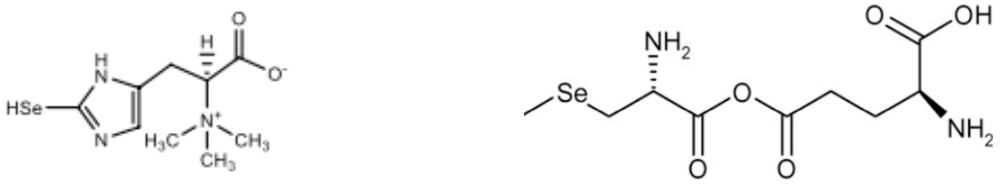

Selenoneine ϒ-Glutamyl selenomethylselenocysteine

Figure 2: Structure of selected Se species in living organisms [61]

Numerous scientific opinions from the European Food Safety Authority (EFSA) stated that the supplementation level of organic Se from the organic Se sources should be limited in diet to ensure consumer safety [19, 20].

2.4 Selenium and mercury interaction

Studies have revealed that Se can be involved in the detoxification of the heavy metal mercury (Hg), reducing the exposure of humans to methyl mercury (MeHg) poisoning [74, 75]. According to the current research, the protective effect of Se against Hg may be proportional to the amount of Se present. In general, the molar concentrations of Se and Hg in protected tissues are more than 1:1 stoichiometry of Se:Hg to be effective [76]. It is proposed that Se protection occurs through Hg sequestration, which is the mechanism underpinning the basic hypothesis that Hg exposure is less dangerous when tissue Se is excessive. Mercury has a strong affinity for Se, which causes the precipitation of the physiologically inert molecule HgSe [77].

A recent study on rainbow trout (*Oncorhynchus mykiss*) discovered that Se interacts with Hg bioaccumulation and has an antagonistic interaction between the two [78]. According to the findings, when the fish were individually exposed to Hg and Se species during the period, there was a continuous (linear pattern) bioaccumulation of MeHg in the fish muscle. When fish are exposed to SeMet first, then MeHg afterwards, the bioaccumulation of MeHg is reduced, indicating that SeMet supplementation has helped detoxify MeHg [78]. Similar findings were found in another study, which suggested that MeHg's binding to glutathione and Se may have shielded beluga whales (*Delphinapterus leucas*) from the damaging effects of high MeHg quantities in their bodies [79].

2.5 Selenium supplementation in fish feed and animal feed

There is a great deal of interest in producing Se-enriched dietary supplements [80, 81], which serve as an alternative way to enhance Se intake. Several dietary supplements contain inorganic Se salts, most notably sodium selenate. Some selenium-enriched supplements, however, are produced using selenium-enriched *Saccharomyces cerevisiae* yeast. Depending on the culture's growth conditions, yeast can accumulate large amounts of Se (up to 3000 mg kg^{-1}) and convert it into SeMet [82, 83].

2.5.1 Aquaculture feed

The contribution of aquaculture has significantly increased along with the recent surge in global fish consumption, increasing the demand for formulated aquaculture feeds [84]. Aquaculture feed is generally composed of various feed ingredients to meet fish growth and health requirements. Fish meal and fish oil, made from pelagic fisheries, have traditionally been the two main components of aquaculture feeds [85] but are now replaced with more plant-based ingredients [86]. However, there are issues related to the supply of adequate amounts of micronutrients, particularly Se, in feed derived from plant-based sources [6, 87]. Whereas fish meal and marine byproducts are known to be relatively rich in Se, the amount of Se in various plant components varies greatly (Table 2) [29].

According to a study by Berntssen *et al.*, dietary supplementation of organic and inorganic Se in Atlantic salmon did not have a negative impact on the health of the fish at Se levels of 1 and 2.1 mg kg^{-1} of selenite and SeMet-yeast, respectively. SeMet-yeast at the same dose did not affect health, while sub-lethal toxic levels of 15 mg kg^{-1} selenite induced lower feed intake and growth in salmon [88]. Another study on the safe level of Se supplementation found that the safe feed limit for selenite was set at 1-2 mg kg^{-1} feed w/w, whereas the safe feed limit for SeMet was higher at 2.8 mg kg^{-1} w/w. According to the study, Atlantic salmon appeared to tolerate the addition of selenite or SeMet to a level of total Se of 1-2 or 3 mg kg^{-1} feed, respectively, in a high plant-based salmon feed with background levels of 0.45 mg Se kg^{-1} [89]. Sele *et al.*, reported the transfer of Se species from feed to muscles of Atlantic salmon, where a greater retention of Se was seen in the muscle of salmon fed organic Se diets [25].

2.5.2 Land animal feed

Trace elements, including Se, are mostly obtained by land animals via plant-based resources, particularly cereals like maize, soybean meal, wheat, and barley. The typical daily Se intake is insufficient to meet animal health needs due to the low Se contents in plant-based meals [90, 91]. Thus, it is now quite popular for swine, poultry, and cattle production to use Se-containing mineral supplements in formula diets [21, 92]. Selenium absorption differed substantially in ruminants compared to single-stomached animals, 48% greater in monogastric animals [93].

It has been hypothesized that ruminal microorganisms convert inorganic Se species into insoluble selenides in ruminants, thus decreasing their bioavailability [94].

2.5.3 Legislative aspects of Se supplementation in relation to feed safety

In Europe Union (EU), the use of feed additives is governed by the EU feed legislation. The current limit for total Se in animal feeds is 0.5 mg kg^{-1} (Council Directive 70/524/EC and amendments). Scientific opinions on the use of organic Se-enriched yeast as feed additives have been issued by the European Food Safety Authority (EFSA) [19, 20]. These opinions are based on the apparent higher bioavailability of organic Se compared to inorganic Se, stating that the supplementation level should be limited to a maximum of 0.2 mg kg^{-1} of feed to ensure consumer safety [19, 20, 95]. Hence, the European Commission has controlled the use of numerous Se feed additives, primarily selenised yeasts, at a maximum supplementation of 0.2 mg Se kg^{-1} feed [96–98]. There is a strong demand for accurate methods for measuring Se species in various matrices due to the expanding use of a wide range of mineral supplements (Se enriched yeast) and advancements in legislation [19 - 20, 95, 99].

2.6 Analytical techniques for trace element speciation in food and feed

The knowledge of an element's chemical species can offer details on potential chemical and biochemical reactions and, as a result, help in deeper understanding of toxicity or essentiality [99]. The study of element speciation has grown significantly during the last decade [62]. The results of toxicological studies clearly demonstrated that several species of the same element can have significantly positive or negative effects [62]. Thus, determining the element concentration alone is insufficient for a meaningful and reliable risk assessment [99].

The analytical process of species identification and measurement entails precise quantification in representative samples, as well as quality control [99]. The International Union of Pure and Applied Chemistry (IUPAC) defines speciation analysis as *"analytical activities of identifying and/or measuring the quantities of one or more individual chemical species in a sample"* [62].

2.6.1 Selenium speciation analysis – overview

To comprehend Se species bioaccumulation, metabolic pathways, toxicity, and to determine dietary intake, Se species quantities must be measured [61]. Whereas organic Se forms like SeMet and SeCys, which are bonded inside proteins, are mostly obtained from plant and animal sources, the inorganic Se forms (selenite and selenate) are found in water and dietary supplements [100]. The elemental concentration and particular Se species affect Se's bioavailability and toxicity [61].

Se speciation provides quantitative and qualitative information about Se forms [101]. Several steps are involved, i.e., sample pre-treatment, separation, detection, identification, and quantification of the species [22, 102]. Sample pre-treatment includes extraction, pre-concentration, and derivatization. Each of these stages will have an impact on how accurate and high-quality the results are in the end [61].

2.6.2 Sampling, storage, and processing

The stability of Se species in samples and standards is crucial for obtaining reliable results in a speciation analysis for Se. Sampling and storage have a significant impact on the validity of the findings because they can alter the Se content in a sample by contamination, adsorption, inter-species conversion, precipitation, and volatilization [103]. Se species in extracts can also alter as a result of factors such species interaction with the container material, microbial metabolites, temperature, pH, and light action [104, 105].

According to several investigations, biological tissues that are frozen at -10 °C or freeze-dried retain Se and maintain species [104, 105]. Total Se concentrations in the freeze-dried material are claimed to be consistent and independent of the container’s material or the storage temperature for measurements up to 365 days [105]. Another study revealed that Se species in freeze-dried or frozen (-20 °C) materials are likely to be permanently stable. Extracts and standards can be kept stable if they are kept at -20 °C or in the dark, but also suggested that the storage time must be determined for each individual Se species and sample matrix [102].

2.6.3 Extraction of selenium species

Most often, Se appears in biological materials as selenoamino acids or is integrated into proteins [100]. Selenoamino acids can be extracted from samples using a variety of techniques, including aqueous leaching, acid-base hydrolysis, and enzymatic methods, with the latter being the method preferred by the majority of researchers [61]. For the correct measurement of Se species, extraction techniques that display great extraction efficiency while maintaining the integrity of Se species are necessary [102]. Peptide bonds must be broken in order to leave intact amino acids or their derivatives that contain Se, and the preferred technique for extracting Se species from food and biological samples is enzymatic digestion [61]. Proteolytic enzymes are frequently used in this procedure, and the type of enzyme and extraction conditions used have a significant impact on extraction efficiency [61, 102].

Although different enzymes have varying activity that can affect the extraction efficiency, it is crucial to optimize both the quantity of enzyme utilized, or the enzyme to sample ratio, as well as the incubation duration [26, 106]. Literature showed that for the hydrolysis of selenoamino acids in food matrices, proteolytic enzymes such as proteinase K, subtilisin, pepsin and protease, or mixtures of enzymes protease and lipase, have been utilized [61, 102], however protease type XIV is the most commonly used enzyme [102]. Cellulase, a non-proteolytic enzyme, has been used to extract selenium species that has been chemically or physically trapped within the cell wall of plant material [26, 61]. The most effective way to extract Se from environmental and biological materials is to incubate samples with enzymes at controlled temperatures for extended periods (17-24 h) [102, 106].

2.6.4 Separation techniques - Liquid chromatography

The most popular method for Se speciation is high-performance liquid chromatography (HPLC), which has a variety of separation modes [22, 99]. It can be easily interfaced to ICP-MS through conventional nebulizers, doesn't require derivatization to produce volatile species (which is necessary for GC analysis), and has good reproducibility [99, 102]. The most significant benefits of liquid chromatography (LC) are the wide range of separation modes, with the application of different mobile and stationary phases [22, 99].

Size exclusion chromatography (SEC), ion-exchange chromatography (IEC), reversed-phase liquid chromatography (RPLC), and ion-pairing chromatography (IPC) are the most frequently used LC separation methods in elemental and Se speciation [22, 99]. The most common HPLC mode is reverse phase, which uses a polar liquid mobile phase and a non-polar stationary phase (octadecyl C_{18} or octyl C_8 chains) [61]. By introducing an organic modifier, the polarity is decreased in order to carry out the elution (methanol, acetonitrile, isopropanol). According to the polarity of the compounds, the separation occurs [61, 99, 102]. The primary advantage is the broad analytical spectrum accessible, in addition the efficient separation technique enables excellent resolution of species, and the flexibility given by the numerous mobile phases permits the insertion of ion-pairing reagents for analysis of ionized molecules [99].

2.6.5 Detection system – Inductively coupled plasma-mass spectrometry (ICP-MS)

Inductively coupled plasma-mass spectrometry (ICP-MS) is a common technique for trace element analysis, both general and speciation analyses. These benefits include the ability to detect several elements and isotopes as well as having high sensitivity and a large linear dynamic range [99, 94]. For a variety of samples with element concentrations in the mg g^{-1} to ng g^{-1} range, ICP-MS is presently the preferred approach since it has high sensitivity, multiple-element capabilities, and detection selectivity. The selective detector can discriminate between elements using isotopic information [99, 107].

Theoretically, an inductively linked plasma with a temperature of between 5,000 and 9,000 K serves as the ionization source. The gas flows through three concentric quartz tubes that make up the quartz torch, where the plasma (mainly argon) is created (Figure 3). The nebulized samples are transported to the plasma by the inner gas flow, also known as the "nebulizer" gas flow. The argon plasma supplies energy for the plasma to transfer to the analyte(s) for excitation and ionization (Figure 3) [22, 99, 107].

Polyatomic interferences might a occur in ICP-MS analysis [99], created when chlorine and carbon are entered to the argon plasma from the source of sample and buffer components, respectively. The spectrum interferences caused by cluster ions can be minimized using reaction or collision cell gases. For Se speciation, hydrogen is normally used to avoid major interferences on the major isotopes of Se (^{80}Se, ^{78}Se, ^{76}Se) from argon [108]. The ions are broken up, and prevented from moving farther in the direction of the mass analyzer [22, 99, 107].

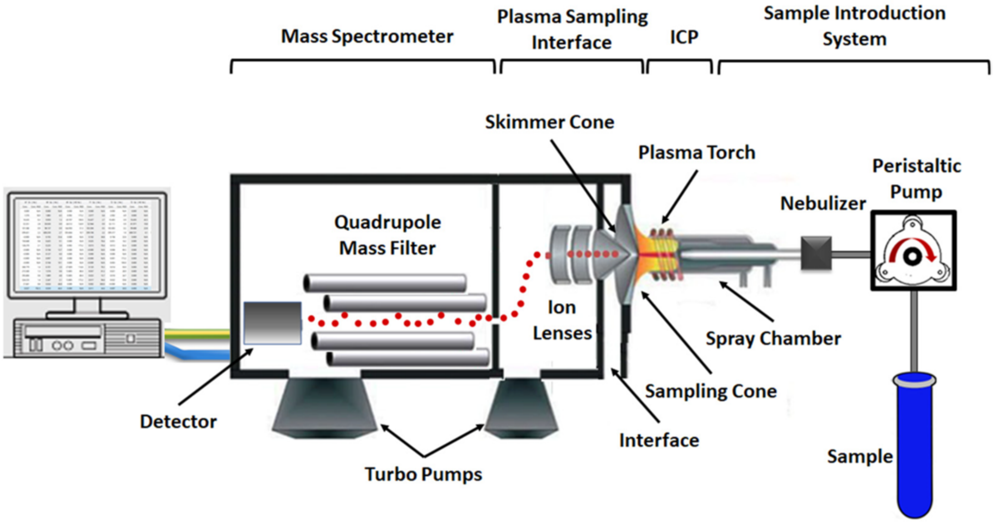


Figure 3: Schematic diagram of Inductively coupled plasma-mass spectrometry (ICP-MS) [109]

2.6.6 Hyphenation of HPLC and ICP–MS

In Se speciation, separation techniques (gas chromatography, liquid chromatography, and electrophoretic techniques) have been combined with a wide range of atomic detectors (ICP-AES, AFS, ICP-MS, and MIP-AES) for determining Se species [61, 102]. Nowadays, coupling of HPLC with ICP-MS (Figure 4) is the most practical, well-liked, and effective analytical method for determining the Se speciation in biological materials [61, 107].

Se speciation is a difficult task because to the intricacy and heterogeneity of analyte matrixes and the normally low level of Se compounds present samples. A potent strategy is the combining of separation procedures with Se-specific detection. High sensitivity is required to operate as an element-specific detector, and mass spectrometry using an atmospheric pressure ionization source, like the ICP, has proved successful hyphenation approach with HPLC [22, 99].

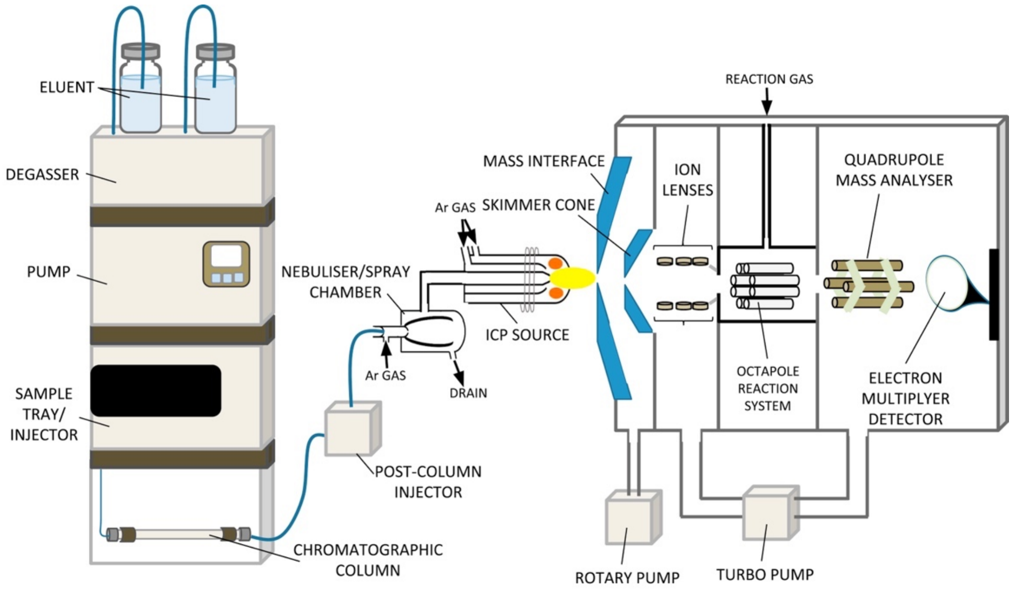


Figure 4: Schematic diagram of hyphenated set-up of HPLC ICP-MS [110]

2.7 Quality assurance and quality control in speciation analysis

Quality control (QC) is essential to the process of element speciation. Quality control is required at the planning stages of an experiment because, in reality, this is rarely guaranteed, and the range of errors is very wide [27]. Clean workspaces and ongoing use of suitable control materials are prerequisites during all trace and ultra-trace analytical activities [99]. The determination of the blank level will be more exact under such conditions. The following are additional requirements [27, 99].

2.7.1 Identification of species:

Correct species identification is a key requirement for reliable analytical work. Because of matrix-dependent changes in retention times and the possibility of present (unknown) species in the sample that could co-elute with the available standards, identification based on the available standards is vital when using separation techniques with element selective detection. Spiking studies can be carried out to make sure that the species in the sample and the standard are the same [27, 99].

2.7.2 Calibration standards:

Correct calibration using calibrants of each examined species with known stoichiometry is another essential condition. High purity of chemicals is required and ensure the that fresh lots of calibrants must be confirmed [27, 99].

2.7.3 Quantitative speciation: The appropriate calibration curve must be used for quantification of analyte. Quantification is not achievable while unidentified compounds are being monitored. Only by correlating peak area calibration graphs to those of closely eluting recognized species, the species can be determined [27, 99].

2.7.4 Blanks: They are employed to identify the portion of a measurement result that is not attributable to the parameter being tested. The blank will have a composition that is as similar to the sample as feasible, but it will be free of analytes [28].

2.7.5 Certified reference materials: The use of reference materials (RMs) or certified reference materials (CRMs) is one of the preferred approaches for quality control, despite the unfortunate shortage of RMs that correspond to practical situations in some analyses [27, 99]. Each speciation analysis procedure should incorporate appropriate RMs and CRMs as early as practicable. Although confirming the certified value helps demonstrate the trustworthiness of findings for unknown samples [27], it never provides complete proof of the accuracy of results because sample matrices are rarely the same as the CRM matrix. The method validation should, whenever possible, incorporate certified reference materials for the target species in the same or at least close matrices. [27, 99].

2.8 Method validation

According to ISO/IEC 17025 standard, the definition of method validation is, *“the confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled”* [111]. A designed set of trials to access these values is known as method validation. Selectivity, sensitivity, precision, bias, linearity, working range, limit of detection, limit of quantitation, calibration, and ruggedness are the technique performance parameters that are frequently examined during method validation [28, 112]. Figure 5 depicts the practical approach during the process of method validation for an analytical testing method [28].

2.8.1 Process of method validation

The specifics of the problem and the information currently at hand determine the scope of validation, or the amount of work that must be put forward [28, 112]. The modified or developed technique will need to be optimized and the necessary controls defined before the method validation procedure can proceed, ensuring that the method can be used regularly in the laboratory [28]. During the initial stages of method validation (Figure 5), the laboratory establishes the analytical requirement, which outlines the performance characteristics the analytical method must adhere to fulfil certain needs. Then, the laboratory will find an appropriate current methodology or create or adapt a methodology if deemed necessary. Subsequently, the laboratory will discover and assess pertinent performance attributes, comparing them to the analytical criteria [28, 113].

The validation procedure concludes with a declaration about the fulfilment or non-fulfilment of the analytical requirement (Figure 5). If the analytical requirement is not satisfied, additional method development is required [113]. The process mentioned above of creation and evaluation persists till the method is determined to possess the capability to fulfil the specified need. The method is then proven to be "fit for purpose" by gathering evidence [28, 113]. The next section explains the most common validation parameters involved in the method validation process.

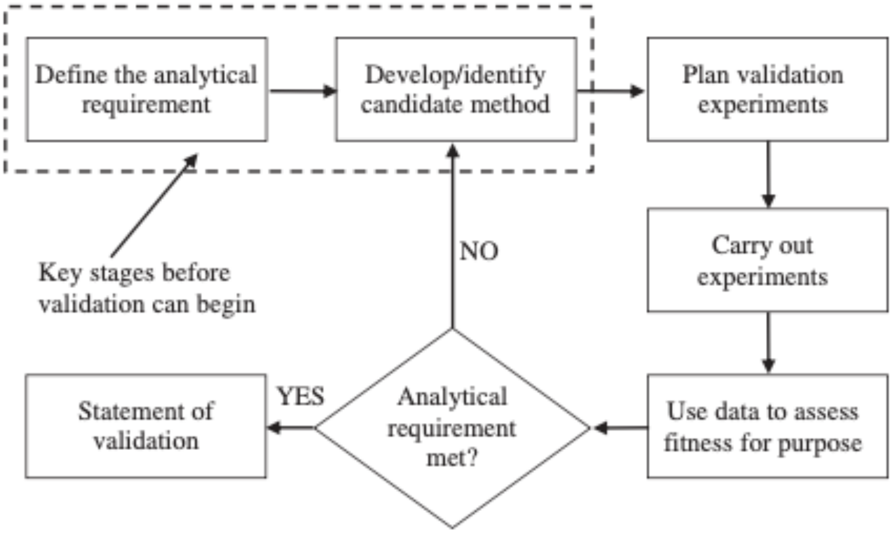


Figure 5: A simplified schematic diagram of method validation process [28]

2.8.2 Selectivity

The analytical selectivity is defined as *“the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behavior present in mixtures or matrices”* [114]. The analysis of blank reagents should be devoid of peaks that correlate to the retention periods of the analytes. Alternately, selectivity can be justified using scientific arguments based on the principle of the method, indicating that the method measures only what it is intended to measure.

2.8.3 Linearity

Linearity refers to the analytical procedure's capacity to provide concentration estimates that are directly proportional to the actual concentration. In other words, linearity examines whether concentration estimates produce a straight line when plotted against actual concentrations [28]. The working range is the *“the interval over which the method provides results with an acceptable uncertainty”* [113]. In working range, its lower end is bounded by the limit of quantification and the upper end is defined by the concentrations at which significant anomalies in the analytical sensitivity are observed [113].

2.8.4 Limit of detection (LOD) and limit of quantification (LOQ)

When measurements are made at low concentrations, there are two general concepts need to be considered. First is the limit of detection (LOD), it is *“the detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which be detected but not necessarily quantified as an exact value”* [115]. While the limit of quantification (LOQ) can be defined as *“the quantification limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy”* [115].

2.8.5 Trueness and recovery

The trueness refers to the presence of systematic error in a measurement outcome. The trueness of an analytical method is defined as *“measurement trueness is an expression of how* *close the mean of an infinite number of results is to reference value"* [113]. Practically, it is not possible to take an infinite number of measurements, trueness cannot be measured. However, the practical assessment of the trueness can be made, and this assessment is normally expressed quantitatively in terms of 'bias' [113].

2.8.6 Precision (repeatability and intermediate precision)

Precision (measurement precision) is the degree to which results are similar. It is typically expressed by statistical parameters that characterize the dispersion of results, such as the standard deviation (or relative standard deviation) calculated from replicate measurements conducted on a suitable material under specified conditions [28, 112, 113]. Repeatability, which is assumed to create the smallest variation in results, is a measure of the variation in results when a measurement is performed by a single analyst using the same equipment over a short period of time. Reproducibility, which is supposed to produce the greatest variation in results, is a measurement of the variation in results between laboratories [113, 114].

Between these two extremes, 'intermediate (measurement) precision' provides an estimate of the variation in results when measurements are performed in a single laboratory under conditions that are more variable than repeatability conditions. In each instance, the exact conditions used should be specified. The objective is to obtain a precision estimate that incorporates all sources of variation that will occur under routine laboratory conditions (different analysts, extended timescale, different pieces of equipment) [113, 114, 116].

2.8.7 Measurement of uncertainty

Uncertainty is the interval associated with a measurement result that represents the reasonable range of values that can be attributed to the quantity being measured. A measure of uncertainty should account for all recognized effects operating on the outcome. According to well-established procedures, the uncertainties associated with each effect are combined [28, 113]. There are several approaches to estimate the measurement of uncertainty for the results obtained for chemical measurements. But the main approach used for analytical methods based on overall, long-term precision of the method that includes the intermediate precision or reproducibility of the measurements [113, 114].

2.8.8 Robustness

The robustness (ruggedness) of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters. Robustness provides an indication of the method's reliability during normal usage [113].

2.9 Challenges with selenium speciation in real matrices

With reference to selenium speciation results, validation is not a straightforward activity since some of the analytical techniques mentioned above are unavailable in most laboratories, and the number of certified reference materials (CRMs) and intercomparison exercises are still limited [61]. Several CRMs containing selenium have been developed through international intercomparison efforts in recent years. The majority of them are dependent on Se-enhanced foods and supplements [61], including the selenised yeast reference material (SELM-1) created by the National Research Council (NRC) [23].

Selenium is one of the most frequently used elements for speciation, as seen by the large number of research articles on the subject, but the validity of the result is rarely reported [61]. For instance, due to its high Se concentration, selenized yeast is one of the most widely used and "simple" matrix for selenium speciation [23, 117]. Yet, the inherent challenges of producing correct data on SeMet are well known [61]. Lack of selenocompound standards and approved reference materials is one of the major issues in evaluating accuracy [61]. The number of CRM for Se speciation is quite limited and largely concentrated in Se-enriched food supplements being not suited for other types of matrices (for instance animal tissues) when selenoamino acids concentration is at the low parts per million range [61, 27]. The situation becomes much more complex due to the dependence of extraction efficiency on the matrix. Because of this, Se speciation researchers are employing a variety of techniques for Se speciation [22, 61, 102] thus there is great need to further studies these techniques to achieve high-quality, reliable and reproducible results.

3. MATERIALS AND METHODS

3.1 Samples

In this study, the samples included for analysis were fish feed, land animal feed, feed ingredients and fish tissues (*Table 3*). All samples were determined for total Se and other elements, a selected number of samples and together with certified reference materials were used for the method validation procedure for Se speciation. Subsequently, a set of samples were chosen for application of the validated method for determining Se species.

3.1.1 Fish feed samples

Fish feed samples for Salmonids (n = 27) were chosen randomly from the commercial fish feed retrieved through the Norwegian National Feed Surveillance program (2021), which was led by the Institute of Marine Research (IMR), Bergen on behalf of the Norwegian Food Safety Authority (NFSA) [8]. Along with the commercial fish feed, different experimental diets (n = 21) were also added in this study (*Table 3*). The experimental fish diets included the zebrafish fish feed samples (n = 3), salmon feed (n = 6) and rainbow trout feed samples (n = 12). The experimental diets for Salmonids were provided by the Foods of Norway, a Centre for Research-based innovation at the Norwegian University of Life Sciences (NMBU), Norway. The rainbow trout experimental diets were collected from a previous study (TuneSel, Project no. 15745). It consisted of two group of experimental diets: i) feed samples (n = 6) with plant-based ingredients and ii) feed samples (n = 6) with tuna meal-based ingredients (Table A 1 and Table A 2, Appendix-A). All fish feed samples were stored in appropriately labeled plastic container at 2-8 °C till further use. The details of fish feed samples are shown in the *Table 3*.

3.1.2 Land animal feed samples

The land animal feed samples (n = 10) were chosen randomly from the commercial ruminant feed. These samples were retrieved from the Norwegian National Feed Surveillance program (2021), which was led by the Institute of Marine Research (IMR), Bergen on behalf of the Norwegian Food Safety Authority (NFSA) [8]. In addition, different experimental feed samples (n = 6) were also included in this study (*Table 3*). The experimental diets were comprised of the piglet feed samples (n = 2), and chicken feed samples (n = 4) provided by the Foods of Norway, a Centre for Research-based innovation at the Norwegian University of Life Sciences

(NMBU), Norway. The details of feed samples are presented in *Table 3*. All the feed samples were stored in appropriately labeled plastic container at 2-8 °C till further use.

3.1.3 Feed ingredients samples

For the feed ingredients, fish meal samples (n = 3), insect meal samples (n = 3), tuna meal sample (n = 2) and plant meal sample (n = 1) were included in this study (*Table 3*). The fish meal and insect meal samples were retrieved from the Norwegian National Feed Surveillance program (2021), which was led by the Institute of Marine Research (IMR), Bergen on behalf of the Norwegian Food Safety Authority (NFSA) [8]. While tuna meal and plant meal retrieved from the previous study (TuneSel, Project no. 15745). The details of feed ingredients samples are shown in *Table 3*. All the samples were stored in appropriately labeled plastic container at 2-8 °C till further use.

Table 3: Overview of samples of fish feed, land animal feed and feed ingredients

Fish feed samples	**Sample ID**
Salmonid commercial feed (SF)	SCF1 – SCF27
Salmonid experimental feed (SEF)	SEF1 – SEF6
Zebrafish feed (ZFF)	ZFF1 – ZFF3
Rainbow trout feed (RTF)	RTF1 – RTF12
Land animal feed sample	**Sample ID**
Ruminant feed (RF)	RF1 – RF10
Piglet feed (PF)	PF1 – PF2
Chicken feed (CF)	CF1 – CF4
Feed ingredients samples	**Sample ID**
Fish meal (FM)	FM1 – FM3
Insect meal (IM)	IM1 – IM3
Tuna meal (TM)	TM1 – TM2
Plant meal (PM)	PM1
Fish tissue samples	**Sample ID**
Rainbow trout (whole) – Initial (Day 0)	RTI1 – RTI3
Rainbow trout (whole) – Day 21	RT1 – RT36
Salmon fillet (SFT)	SFT1 – SFT2
Tuna fillet (TFT)	TFT1
Blue mussel (BMT)	BMT1

3.1.4 Fish tissue samples

Whole fish samples (n = 39) were retrieved from a previous research project (TuneSel, Project no. 15745). In this project, rainbow trout juveniles were fed on different experimental diets (n = 12), composed of plant-based and tuna meal-based diets (Table A 1 and Table A 2) and supplemented with different levels of selenium, either organic SeMet or inorganic selenite, and mercury, either organic methylmercury or inorganic Hg. The description of fish tissues samples is shown in Table A 1 and Table A 2 (Appendix A).

In addition, samples of salmon fillet (n = 2), tuna fillet (n = 1) and blue mussel (n = 1) were included in this study. All the fish tissue samples were stored in appropriately labeled plastic container at -20 °C till further use.

3.2 Chemicals and reagents

All the chemicals and reagents used in this study were of analytical grade quality. For the sample preparation, reagent preparation and analysis, ultrapure water (18.2 MW^{cm}) was used. The ultrapure water was obtained from an in-house Milli-Q water purification system (Merck Millipore, Burlington, MA, USA). The details of chemicals and reagents used during the experimental work were given in *Table 4*.

Cellulase from *Aspergillus niger* with enzymatic activity ^{3}0.3 units/mg powder used for breakdown of cellulose (fibrous content) present in plant material. While protease type XIV from *Streptomyces griseus* with enzymatic activity ^{3}3.5 units/mg powder served as proteolytic enzyme (*Table 4*). The combination of these enzymes used to digest the sample matrix to extract the Se species from the sample.

Table 4: List of chemicals and reagents used during the experimental work

Name of product	Catalog No.	Supplier / Manufacturer
Nitric acid (HNO_3) ^{3} 69% Suprapur®	CAS 7697-37-2	Merck (Darmstadt, Germany)
Hydrogen peroxide (H_2O_2) 30%, Perhydrol®	CAS 7722-84-1	Merck (Darmstadt, Germany)

Multi-element standard	SS 6083S	Spectrascan, Teknolab (Ski, Norway)
Mercury (Hg) 1000mg/mL	SS 1532	Spectrascan, Teknolab (Ski, Norway)
Gold (Au) 1000mg/mL ICP standard	170321 (Supelco)	Merck (Darmstadt, Germany)
Rhodium (Rh) 1000mg/mL	SS 1550	Spectrascan, Teknolab (Ski, Norway)
Germanium (Ge) 1000mg/mL	SS 1130	Spectrascan, Teknolab (Ski, Norway)
Thulium (Tm) 1000mg/mL ICP standard	170361 (Supelco)	Merck (Darmstadt, Germany)
Stock tuning solution of Li, U, Ba, Bi, Ce, Co, and In	SS 6088SS	Spectrascan, Teknolab (Ski, Norway)
ICP-MS stock tuning solution	5188-6564	Agilent (Waldbronn, Germany)
Ammonium phosphate dibasic ((NH$_4$)$_2$HPO$_4$) 99%	CAS 7783-28-0	Merck (Darmstadt, Germany)
Gemini® 5mm C6-Phenyl 110 Å, LC Column 150 x 4.6 mm, Ea	00F-4444-E0	Phenomenex (California, USA)
Ammonia (NH$_3$) 25% solution	CAS 1336-21-6	Merck (Darmstadt, Germany)
Ortho-Phosphoric acid (H$_3$PO$_4$) 85% solution	CAS 7664-38-2	Merck (Darmstadt, Germany)
Ammonium formate (HCO$_2$NH$_4$) ^{3}97% purity	CAS 540-69-2	VWR Chemicals (Bergen, Norway)
Seleno-DL-Methionine, 99% purity	CAS 1464-42-2	Sigma-Aldrich (Oslo, Norway)
Seleno-L-cystine, 95% purity	CAS 29621-88-3	Sigma-Aldrich (Oslo, Norway)
Se-Methyl-seleno-L-cystein ^{3}98%	CAS 26046-90-2	Fluka (Oslo, Norway)
Sodium selenite, 99% purity	CAS 10102-18-8	Sigma-Aldrich (Oslo, Norway)
Methanol (CH$_3$OH), 99.9% purity, LC-MS grade	CAS 67-56-1	Sigma-Aldrich (Oslo, Norway)
Cellulase from *Aspergillus niger*	C1184	Sigma-Aldrich (Oslo, Norway)
Protease type XIV from *Streptomyces griseus*	P5147	Sigma-Aldrich (Oslo, Norway)

3.4 Certified reference materials (CRMs)

Certified reference materials (CRMs) were used in this study to ensure the quality of the analytical measurements/analysis. The necessary information of CRMs and their producers are presented in *Table 5*. The certified values for total Se and SeMet (mg/kg) in SELM-1 (selenized yeast) and ERM BC210a (wheat flour) are mentioned in *Table 6*.

Table 5: Certified reference materials (CRMs), their producers and use

Name	Producer	Use
SRM 1566b (Oyster tissue)	National Institute of Standards and Technology (Gaithersburg, MD, USA)	Multi-element and total Se determination
TORT-3 (Lobster hepatopancreas)	National Research Council Canada (Ottawa, ON, Canada)	Multi-element and total Se determination
SELM-1 (Selenized yeast)	National Research Council Canada (Ottawa, ON, Canada)	Se speciation
ERM BC210a (Wheat flour)	LGC (Middlesex, UK)	Se speciation

Table 6: Certified values for total Se and SeMet (mg/kg) in CRMs, SELM-1 (selenized yeast) and ERM BC210a (wheat flour) *

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

* Values were calculated from the certificates provided with the certified reference materials by using the equation (11) in section (12).

3.5 Instrumentation and software

The routine instruments such as pipettes, incubator, refrigerators, freezers, and food processors were not mentioned in *Table 7*. Similarly, the common glassware and plastic ware such as cylinders, beakers, Pasteur pipettes, glass bottles and volumetric were not included in the list (*Table 8*). All these instruments were well managed, controlled and monitored by the laboratory staff. All the necessary instruments/equipment and consumables items used during the experimental work mentioned in the *Table 7* and *Table 8*, respectively.

Table 7: Equipment and software used during experimental work

Equipment / Instrument	Manufacturer
Ultra centrifugal mill ZM 100	Retsch (Haan, Germany)
Cryogenic miller 6875D (Freezer/Mill$^{\text{Ò}}$)	SPEX SamplePrep (New Jersey, USA)
Knife mill GM 200	Retsch (Haan, Germany)
FreeZone 18L console -50 °C freeze dry system	Labconco (Kansas, USA)
Weigh balance XPR204	Mettler Toledo (Greifensee, Switzerland)
Milli-Q Water purification system	Merck Millipore (Burlington, MA, USA)
UltraWAVE Microwave acid digestion system	Milestone UltraWAVE (Sorisole, Italy)
TraceCLEAN acid steam cleaning system	Milestone UltraWAVE (Sorisole, Italy)
Thermo Scientific iCAP-Q ICP-MS	Thermo Scientific (Waltham, MA, USA)
SC-4 DX autosampler	Elemental Scientific (Omaha, NE, USA)
HPLC 1260 Infinity II Bio-Inert LC System	Agilent Tech. (Santa Clara, CA, USA)
ICP-MS 7900	Agilent Tech. (Santa Clara, CA, USA)
WTWÔ SenTixÔ 81 pH electrode	Xylem (New York, USA)
WTW InoLab pH meter	Xylem (New York, USA)
Grant OLS200 Water bath	Grant Technologies (Cambridge, UK)
Centrifuge 5702	Eppendorf (Hamburg, Germany)
Centrifuge 5424 R	Eppendorf (Hamburg, Germany)
Vortex mixer MS1 Minishaker	IKA (Staufen, Germany)
LABOPORT$^{\text{Ò}}$ N816.3 KT. 18 Vacuum pump	KNF Neuberger GmbH (Germany)
Qtegra iCap Q software (v. 2.10, 2018)	Thermo Scientific (Waltham, MA, USA)
MassHunter$^{\text{Ò}}$ 4.5 Workstation software	Agilent Tech. (Santa Clara, CA, USA)

Table 8: Consumables items used during experimental work

Consumable item	Manufacturer
Digestion vials (TFM / quarts) with TFM caps	Milestone (Sorisole, Italy)
FalconÔ 15 mL centrifuge tubes	Thermo Fischer (Waltham, USA)
FalconÔ 50 mL centrifuge tubes	Thermo Fischer (Waltham, USA)
13 ml sterile centrifuge tubes (PP)	Sarstedt (Nümbrecht, Germany)
Amicon Ultra 0.5 mL centrifugal filter-10 kDa cutoff	Merck (Darmstadt, Germany)

2 mL micro centrifuge tubes	Merck (Darmstadt, Germany)
Filter membrane, 47 mm, 0.45 µm (5191-4337)	Agilent (Waldbronn, Germany)
Syringe filters, 25 mm, 0.45 µm (5190-5093)	Agilent (Waldbronn, Germany)
HPLC vials (PP), snap top, 1 mL (5182-0567)	Agilent (Waldbronn, Germany)
HPLC vials (PP), snap top, 250 µL (9301-0977)	Agilent (Waldbronn, Germany)
HPLC vials snap caps with silicone septa (5182-0550)	Agilent (Waldbronn, Germany)

3.6 Sample preparation

3.6.1 Feed and feed ingredients

Commercial feed (fish and land animal) and feed ingredients samples were homogenized prior to grinding by using a laboratory mill (Retsch, Germany). All grinded samples of commercial feed and feed ingredients were stored in clean and labelled container at 2-8°C prior to analysis.

All experimental diets were homogenized and processed during the previous study of method optimization [26] by using a cryogenic mill (6875D). The freezer mill pre-cools multiple samples with liquid nitrogen (LN_2) and grinds the samples by an impact rod in each vial (polycarbonate). The mill settings for a complete process used for pellets (~ 3 g) was pre-cool 15 min, run time 2 min, cool time 1 min, and cycles 3. The processed samples of experimental diets were stored in clean containers at 2-8°C prior to analysis.

3.6.2 Fish tissues samples

The fish tissue samples were taken from previous studies in ready-to-use form [26, 124]. Briefly, the whole-body of rainbow trout juveniles, fillet samples of Atlantic salmon and blue mussel were freeze-dried (lyophilization) prior to analysis. The purpose of applying this technique was to convert the sample in dry form (powder). After lyophilization (Labconco®), the samples were grounded, homogenized, and weighed again for calculation of moisture content. The dry samples were stored in clean containers at -20 °C prior to analysis.

3.7 Overview of analytical work

An overview of the analytical work conducted in this project is shown in *Figure 6*. The analytical work was divided in to three main sections; 1) estimation of total Se through ICP-MS, 2) method validation of the speciation technique by HPLC-ICP-MS, 3) application of the speciation method on selected feed and fish tissue samples. The *Figure 6* provides a comprehensive explanation of the samples and essential procedures involved.

Key steps	Samples		Instrument	
- Sample preparation - Microwave assisted acid digestion - Analysis by ICP-MS - Data analysis & interpretation	- Salmon feed - Rainbow trout feed - Zebra fish feed - Piglet feed - Ruminants feed - Chicken feed - Feed ingredients - Salmon fillet - Tuna fillet - Rainbow trout whole - Blue Mussel	 	ICP-MS 	**Total Se/Multielement Analysis**
- Validation protocol - Enzymatic digestion - Analysis by HPLC ICP-MS - Data analysis & interpretation	- CRMs - Salmon feed - Zebra fish feed - Ruminants feed - Salmon fillet - Tuna fillet - Blue Mussel	  	HPLC ICP-MS 	**Method Validation**
- Enzymatic digestion - Analysis by HPLC ICP-MS - Data analysis & interpretation	- Rainbow trout feed - Rainbow trout whole body tissue	 	HPLC ICP-MS 	**Application of Method**

Figure 6: Overview and flow of analytical work in this study, explaining key steps, samples analyzed and the instrumentation applied.

3.8 Determination of total selenium in feed and fish tissues by ICP-MS

3.8.1 Digestion of samples

The determination of total Se was performed as outlined in internal standard procedure at the Institute of Marine Research (IMR, 2020b) which is based on the method described by Julshamn *et al.* (2007). In this method, micro-wave assisted acid digestion was performed on the samples using an UltraWAVE digestion system (Milestone). In this study, two replicates of each sample, three replicates of each CRM along with blank sample was run. In brief, approximately 0.2 g of homogenized sample was weighed in digestion vials containing 0.5 mL Milli-Q water. Then 2 mL of concentrated HNO_3 was added to each vial, including blank samples. The vials were then capped and placed in carousel (15 slots) in a specific pattern. Finally, the carousel with samples was placed a container with 130 mL Milli-Q water and 5 mL H_2O_2 in the UltraWAVE system, digested for ~2 hour under a pressure of 40 bar. Prior to analysis by ICP-MS, the digested samples were cooled down and then the samples were diluted to 25 mL with Milli-Q water by 25 mL volumetric flask. The diluted extracts were stored in 50 mL polypropylene tubes at room temperature.

3.8.2 Analysis of extract by ICP-MS

The concentration of total Se in the acid digests was determined by using ICP-MS. The analysis was performed using a iCAP Q ICP-MS (Thermo Scientific, Waltham, MA, USA). The ICP-MS was connected with a FAST SC-4Q DX autosampler from Elemental Scientific (Omaha, NE, USA). The whole integrated system was controlled and operated with Qtegra iCap Q software (Thermo Scientific, MA, USA). This method is accredited for the elements Cu, Zn, As, Se, Cd, Hg and Pb in different samples matrices (Institute of Marine Research, 2020a) [118].

In brief, an external calibration curve was established by using a multi-elements solution, including Se (Spectrascan, Oslo, Norway, see *Table 4* for details). A Hg single-element solution, containing gold for stabilization of Hg ions, were added to the multi-element calibration standard. The standards were freshly prepared by diluting to appropriate concentrations with 5% HNO_3 (v/v). Internal standards of Ge, Rh and Tm (Spectrascan) were used during run, calibration standards and blank samples, for correction of possible matrix effects or changes in operating conditions in the analyses (*Table 4*). The instrument was tuned prior to analysis with a freshly prepared tuning solution (1 ppb, Spectrascan) to ensure the monitoring of possible interferences from oxide species and double charged ions. The instrumental settings for iCAP Q ICP-MS are shown in *Table 9*.

Table 9: The instrumental settings for iCAP Q ICP-MS

Parameter	Value
Plasma power (Argon, 99.99% purity)	1550 W
Nebulizer gas flow (Microflow PFTA-ST)	1.05 L/min
Auxiliary gas flow	0.8 L/min
CCT1 gas flow (Helium, 99.99% purity)	4.6 L/min
Integration time	0.1 s
Isotopes monitored	^{78}Se, ^{111}Cd, ^{52}Cr, ^{55}Mn, ^{202}Hg, ^{59}Co, ^{63}Cu, ^{56}Fe, ^{208}Pb, ^{66}Zn, ^{75}As

3.9 Determination of organic Se species by HPLC ICP-MS

3.9.1 Procedure for extraction of organic Se species

In this study, the extraction procedure for organic species of Se is based on the method described by Sele *et. al.* (2018) [25] and Vaksdal (2021) [26]. Briefly, the organic Se species were extracted from samples through enzymatic digestion and filtration. For the extraction procedure, ~ 0.2 g of sample material or CRM (two replicate) was weighed in 13 mL centrifuge tube (Sarstedt, Germany). Then freshly prepared solution of cellulase (2.5 mL) and protease enzyme (2.5 mL) (Sigma-Aldrich, Norway) with enzymatic strength of ≥28 U/mL was added in tube. Each sample in tube was mixed thoroughly using a vortex mixer. After mixing, the samples were incubated in a water bath at 37 °C for 20 h, shaking at 100 rpm/min in the dark. In next step, the enzymatic activity was stopped by placing the sample tubes in a water bath at 100 °C for 10 min. After inactivation step, cooled down the tubes and then all tubes were centrifuged at 3500 rpm for 10 min at 4 °C (Eppendorf, Germany).

In next step, the supernatant from each tube passed through the 0.45 µm syringe filter (Merck, Germany) and transferred to new tube (soluble fraction). For separation of 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 cutoff filter (Merck, Germany). The samples were filtered by centrifugation at 14,000 g for 20 min at 4 °C (Eppendorf, Germany). The filtered samples were transferred to 2 mL centrifuge tubes and stored at -20 °C prior to analysis on HPLC ICP-MS. Unfiltered soluble extracts and non-soluble fractions were also stored at 20 °C prior to microwave assisted digestions and analysis by ICP-MS. The samples were thawed at room temperature prior to analysis.

3.9.2 Speciation analysis of organic Se species with HPLC ICP-MS

The speciation analysis of Se species in feed and tissues extracts was performed through the hyphenated technique by combining HPLC with ICP-MS. In this study, the chromatographic separation was carried out by HPLC 1260 Infinity II Bio-Inert LC System (Agilent technologies, CA, USA) combined with an autosampler. After separation, the Se species were detected by using an ICP-MS 7900 (Agilent technologies, CA, USA) which was equipped with Mira Mist PEEK nebulizer (Agilent, Germany). The hyphenated instrument settings and data processing was carried out through a computer software MassHunter$^{\text{Ò}}$ (Agilent technologies, CA, USA).

For separation of organic Se species a Gemini® C6-Phenyl HPLC column (150 x 4.6 mm, 5 µm), (Phenomenex, USA) was applied. In this column, the stationary phase was C6 linked phenyl with TMS end capping which is recommended for separation of polar or basic compounds under high pH conditions. The mobile phase comprised of ammonium formate (NH_4HCO_2) solution with ionic strength of 20 mM in an aqueous solution of MeOH (1%, v/v). The pH of the mobile phase was adjusted to 9 with help of solution of formic acid (HCOOH) and ammonia (NH_3) 25% solution (Merck, Germany). Prior to analysis, the mobile was filtered through a 0.45 µm membrane filter placed in a filtration assembly (Merck, Germany) connected with a vacuum pump (KNF, Germany). Prior to the analysis, the RP column was cleaned and prepared by flushing with deionized water (Milli-Q).

An external calibration curve of SeMet from a standard solution of Seleno-DL-methionine (Sigma-Aldrich, Norway) was made. For preparation of calibration curve, concentrations of 0, 0.5, 1, 5, 10, 25, 50 mg Se/L were prepared from a standard solution of Seleno-DL-methionine diluted in water. The sample extracts were diluted with Milli-Q water based on the content of total selenium to ensure that their concentrations felled within the range of the calibration curve. Briefly, following dilution rates were applied to achieve desired concentration: blanks, tuna fillet and blue mussel samples were diluted 4 times, salmon fillet, fish and land animals feed samples were diluted 2 times, ERM BC210a and SELM-1 reference materials were diluted 10 times and 2000 times, respectively.

As a pre-requisite, a performance check of the ICP-MS was performed, prior to analysis. The ICP-MS was tuned by using a tuning solution of Lithium (Li), Yttrium (Y), and Thallium (TI) on their respective counts / second of their selected isotopes (^{7}Li > 3,000 counts/sec, ^{89}Y > 15,000 counts/sec, and ^{205}TI > 9,000 counts/sec with RSD < 5% at pump velocity of 0.12 rps [104]. In addition, a solution containing ^{78}Se was monitored with an internal (*in-house* laboratory) limit of ^{78}Se > 1500 counts / second for tuning of ICP-MS. All the necessary instrumental settings applied during the speciation analysis are shown in *Table 10*.

Once the HPLC ICP-MS set-up was ready for speciation analysis, the nebulizer of the ICP-MS was connected to the HPLC column through a PEEK tubing (Agilent, Germany). The ICP-MS system was equipped with a collision reaction cell (CRC), hydrogen (H_2) gas was supplied as an octopole reaction gas at a flow rate of 2.5 mL/min (*Table 10*). The provision of H_2 gas prevent the interferences produced from the polyatomic argon to the monitored isotope (^{78}Se) of selenium. As explained in theoretical background, the most abundant selenium isotope ^{80}Se was not monitored due to high signal interreferences from argon isotopes ^{40}Ar^{40}Ar. But ^{76}Se isotope was also monitored with an integration time at 0.1 second. All the instrumental settings and data management was monitored through a computer software MassHunter$^{\text{Ò}}$ Agilent technologies, CA, USA).

Table 10: The instrumental settings for hyphenated set-up of HPLC ICP-MS

Chromatographic conditions (Reversed phase HPLC)	
Parameter	**Value**
Elution mode	Isocratic
Column	Gemini® 5mm C6-Phenyl 150 x 4.6 mm
Mobile phase	20mM NH_4HCO_2 + 1% MeOH – pH 9
Mobile phase flow rate	1.0 mL/min
Operating pressure	< 200 bars
Injection volume	25 mL
Detection system setting (ICP-MS)	
Parameter	**Value**
Carrier gas flow (Argon, 99.99% purity)	1.15 – 1.25 L/min
Plasma power	1550 W
Plasma gas flow	15 L/min
Auxiliary gas flow	0.12 L/min
H_2 gas flow (CRC)	2.5 mL/min
Integration time	0.1 s
Temperature (spray chamber)	2 °C
Isotopes monitored	^{78}Se, ^{111}Cd, ^{52}Cr, ^{55}Mn, ^{202}Hg, ^{59}Co, ^{63}Cu, ^{56}Fe, ^{208}Pb, ^{66}Zn, ^{75}As

3.10 Method validation

The method for Se speciation in fish feed and tissues was based on previous work [25] and further optimization of the extraction procedure by Vaksdal (2021) using a fractional factorial design for testing several enzyme-combinations for SeMet extraction [26]. As a result, the extraction procedure was optimized.

To demonstrate the applicability of in-house laboratory developed method, a single-laboratory validation was decided to be carried out following the guidelines from Eurachem and NMKL [113, 116]. The validation was applied to determine the selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), trueness, recovery, precision, repeatability, and measurement of uncertainty (MU) of the method for determination of organic SeMet (*Table 11*) using HPLC ICP-MS.

During method validation, it was targeted that the method must quantify the SeMet. Furthermore, the resolution of Se species was demonstrated by using a standard mixture containing the selenite, SeMet, SeMetSeCys, and SeCys at one of a day during the validation activities (*Table 11*). The performance characteristics for SeMet are shown in *Table 12*. In validation plan, two certified reference materials were used, namely SELM-1 (selenized yeast), and ERM BC210a (wheat flour) along with seven samples, namely one sample of fish feed, one sample of zebrafish feed, one sample of land-animal feed, two samples of salmon tissues, one sample of tuna tissues and one sample of blue mussel (*Table 11*). In addition, selected spiked and non-spiked samples were also included. The overview of the validation plan is presented in *Table 11* with necessary details.

3.11 Method validation parameters

An evaluation of some parameters of method validation such as selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), trueness, recovery, precision, repeatability, and measurement of uncertainty (MU) were evaluated (*Table 11*) as described by Eurachem and NMKL guidelines [113, 116].

Table 11: Overview of design for the validation process

Day	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**		**Purpose**
Analyst	**A**	**A**	**B**	**A**	**A**	**C**		
Analytical run							**Total measurements**	
Standard solution / Calibration curve (1 blank, 6 points)	7	7	7	7	7	7 + A standard mixture	5	Linear range
Reagent blind	2	2	2	2	2		10	LOD/LOQ
CRM 1 (ERM BC210a)	2	2	2	2	2		10	Trueness
CRM 2 (SELM-1)	2	2	2	2	2		10	Trueness
SMPL 1 (Fish feed)	2	2	2	2	2		10	Selectivity / Repeatability
SMPL 2 (Zebrafish feed)	2	2	2	2	2		10	Selectivity / Repeatability
SMPL 3 (Salmon tissue low)	2	2	2	2	2		10	Selectivity / Repeatability
SMPL 4 (Salmon tissue high)	2	2	2	2	2		10	Selectivity / Repeatability
SMPL 5 (Land-animal feed)	2	2	2	2	2		10	Selectivity / Repeatability
SMPL 6 (Tuna fish tissues)	2	2	2	2	2		10	Selectivity / Repeatability
SMPL 7 (Blue mussel)	2	2	2	2	2		10	Selectivity / Repeatability
Spiked samples (SMPL 1, SMPL 7) – 3 spiked and 3 non-spiked						12 + calibration curve		Selectivity + linear range
Sum of extracts analyzed	**20**	**20**	**20**	**20**	**20**	**19**		

3.11.1 Selectivity

For determination of the selectivity, the chromatograms of the CRMs, samples , reagent blank, buffer solution, protease and cellulase enzymes were evaluated (*Table 11*). The respective chromatograms were evaluated in terms of signals at the retention time of the analyte SeMet and other Se species, providing information of possible interferences originated from the sample matrix, reagents, or similar analytes [28, 112, 113].

3.11.2 Linearity

For calculation of linear range, a plot response (y-axis) against concentration (x-axis) was made, visually examined for the outliers which may not be reflected in the regression. The appropriate regression statistics was performed to calculate and plot the residuals. The random distribution of residuals was observed to confirm the linearity. In addition, systematic trends were also evaluated to find out any non-linearity or a change in variance with level [113].

Here, each day six different concentrations of standard solutions were introduced into the HPLC ICP-MS (*Table 11*). Each concentration was determined thrice during analysis. A plot was drawn between the response (y-axis) and the concentration (x-axis) and necessary regression statistics was performed to calculate plot residuals.

The approach used for determining method working range was to examine blank plus reference materials or spiked blanks 2 to 3 times at 6-10 evenly spaced concentrations within the range of interest (*Table 11*). If data from bias and precision studies covering the range of interest are available, a separate technique working range may not be necessary.

3.11.3 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ of the analytical method was estimated based on analysis of reagent blanks. The approach was based on the standard deviation of the blank, in which the measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples (n = 10) and calculating the standard deviation of these responses.

According to this approach, the following formulas were used to calculate the LOD and LOQ [113, 115]. The necessary details of the replicates and performances characteristics are given in *Table 11* and *Table 12*.

$$LOD = 3 \times s'_0 \qquad \text{[Equation 1]}$$

$$LOQ = 10 \times s'_0 \qquad \text{[Equation 2]}$$

$$LOQ = 3.3 \times LOD \qquad \text{[Equation 3]}$$

where, s'_0 = standard deviation of the replicates

3.11.4 Trueness and recovery

For the determination of the trueness of the method, the estimated values of SeMet measured in the CRMs were compared with reference values (*Table 6*). In addition, the trueness was also estimated in experimental diets (TunSel project) which were supplemented with different concentration of the SeMet (Table A 1 and Table A 2). The approach of analytical recoveries (%) was used for assessment of trueness of the CRMs and experimental diets as given in later section of spiking recovery.

The recovery of spiked samples, i.e., fish feed (n = 1) and blue mussel (n = 1) was calculated to evaluate the trueness of the method. These samples were spiked with 5mg Se/L of SeMet standard before the extraction to evaluate the extraction recovery for SeMet and alternatively in the final extract (in other set of sample's extract) to evaluate the chromatography recovery (*Table 11* and *Table 12*). In addition, a mixture of Se standards (sodium selenite, SeMet, Se-Methyl-seleno-L-cysteine and seleno-L-cystine) was also spiked at a concentration of 5mg Se/L in selected samples. The spiked samples were analyzed with un-spiked set of samples to measure the concentrations which were used to calculate the trueness in term of spiked recovery as mentioned in later section of spiking recovery (*Table 11* and *Table 12*).

3.11.5 Precision (repeatability and intermediate precision)

For calculation of precision, in situations where the measured concentration is well above the detection limit, it is frequently observed that the precision is proportional to the analyte concentration. In such scenarios, it may be better to represent precision as a relative standard deviation (RSD), since this is approximately constant across the range of interest. The Eurachem guideline [105] recommends performing six to fifteen groups of duplicate measurements on separate days (**Table *11***). The precision quality was evaluated on basis of the Horwitz ratio [105] (*Table 11* and *Table 12*). By using the one-way ANOVA technique, the intermediate precision (IP) and repeatability (r) were estimated through following equations [113, 120],

$$u(r) = \sqrt{MS_E} \qquad \text{[Equation 4]}$$

where, repeatability variance was estimated by the within-day variance, MS_E with degrees of freedom.

$$u(IP) = \sqrt{u^2(r) + u^2(b)} \qquad \text{[Equation 5]}$$

where, IP variance, $u^2(IP)$ was calculated on basis of both the within-day $u^2(r)$ and between-day variance $u^2(b)$.

$$u^2(b) = \frac{MS_M - MS_E}{J} \qquad \text{[Equation 6]}$$

where, for the estimation of u2(b), with vb = I – 1 degree of freedom.

$$RSD_{IP,h} = 2C^{-0.15} \qquad \text{[Equation 7]}$$

The above equation was used to for estimation of Horwitz ratio.

3.11.6 Measurement of uncertainty

Measurement uncertainty (MU) was estimated using the simplified approach mainly based on precision studies, if properly planned it covered as many of the uncertainty sources as possible (*Table 11* and *Table 12*), Intermediate precision provide the necessary data required to calculate measurement uncertainty [120, 121, 122].

$$u^2(c) = \frac{u^2(r)}{I.J} + \frac{u^2(b)}{I} \qquad \text{[Equation 8]}$$

where, $u^2(c)$ is the standard measurement of uncertainty, I is the number of days and J is the number of replicates per day. This expression will be simplified with expression given in equation 5 if single measurement was carried out on a day [120]. The standard measurement uncertainty often expressed as,

$$u(c) = u(IP) \qquad \text{[Equation 9]}$$

Assuming normal distribution the measurement of uncertainty (MU) is given as,

$$MU = 2 * u(c) = 2 * u(IP) \qquad \text{[Equation 10]}$$

3.11.7 Robustness

At the first phase of method optimization by previous study, a fractional factorial design was utilized to identify the key components to evaluate the robustness of this method [26].

3.12 Application of validated method on rainbow trout feed and tissue samples

The validated method was applied on fish feed (experimental diets) and fish tissues samples for speciation analysis of organic selenium species (SeMet). The samples were randomly selected from the TuneSel project (*Table 3*), and mainly comprised of rainbow trout experimental diets (n = 13) and tissue samples (n = 13). For application of this method, one sample was selected from each group of diets and tissue samples. The description of these experimental diets and tissues samples have already been given in Table A1 and Table A2 (Appendix-A), respectively.

For extraction of selenium species, the enzymatic extraction was performed on the selected samples, according to method already explained in section 3.4.1. In this study, two replicates of each sample, CRM along with reagent blank samples were extracted. Later, the digested extracts were subjected to Se speciation analysis through HPLC ICP-MS as described in section 3.9.2. All the instrumental settings and data management was monitored through a computer software MassHunter® Agilent technologies, CA, USA).

Table 12: Target performance characteristics of organic Se species (SeMet) in feed and fish tissue samples

Sr. No.	Performance characteristics	Target
1.	Selectivity	- The method allows quantification of the analytes without being affected by matrix components and possible interferences.
2.	Limit of detection (LOD)	- For SeMet, the LOD should be ≤ 0.03 mg/kg.
3.	Limit of quantification (LOQ)	- For SeMet, LOQ ≤ ½ of the legislative limit. - For feedstuff, it should be demonstrated that SeMet is below 0.2 mg/kg.
4.	Linearity	- Linear fit is appropriate for calibration model. - Correlation coefficient, r ≥ 0.99 - Residuals are randomly distributed
5.	Trueness and recovery	- Recovery should be within 75-100% of the certified values for the CRMs. - For real samples (feed, tissues), a recovery of 70-120% can be accepted, since it will be based on spiked amounts, and it is challenging to know the correct or real value for SeMet.
6.	Precision	- Precision should be from 80-120%
7.	Repeatability	- Assessment based on Horwitz ratio.
8.	Intermediate precision	- For SeMet, Horwitz ratio should be less than 2.
9.	Measurement uncertainty (MU)	- MU should be less than 30%.

3.13 Statistics and data analysis

Estimation of recovery of total Se and Se species

The analytical recoveries (%) for total selenium and SeMet in certified reference materials and experimental diets were calculated using the following equation.

$$\text{Analytical recovery (\%)} = \frac{\text{C measured}}{\text{C target}} \times 100 \qquad \text{[Equation 11]}$$

where:

- C *measured* = is the measured concentration of Se (mg Se/kg) or SeMet (mg SeMet/kg)
- C *target* = is the target concentration for Se (mg Se/kg) or SeMet (mg SeMet/kg)

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

The certified values for SeMet stated in mg SeMet/kg must be converted to mg Se/kg because the original measurements of SeMet were reported in mg Se/L. The following equation was used to convert SeMet values to mg Se/kg.

$$\text{C Se} = \text{C SeMet} \times \frac{\text{M Se}}{\text{M SeMet}} \qquad \text{[Equation 12]}$$

where:

- C Se = is the concentration of Se in SeMet (mg Se/kg)
- C SeMet = is the 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)

Estimation of extraction recovery (%)

For estimation of extraction recover (%) of organic Se (SeMet) from the total Se content in a sample, following equation was used.

$$\text{Extraction recovery (\%)} = \frac{\text{C organic Se}}{\text{C total Se}} \times 100 \quad \text{[Equation 13]}$$

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)

Estimation of spike recovery (%)

The SeMet concentrations in the spiked samples (commercial fish feed and blue mussel), the SeMet concentrations in the unspiked samples, and the supplemented amounts of SeMet were used to estimate the spike recovery (%). The following equation was used to estimate the spike recovery (%).

$$\text{Spike recovery (\%)} = \frac{\text{C spiked sample} - \text{C unspiked sample}}{\text{C added}} \times 100 \quad \text{[Equation 14]}$$

where:

- C *spiked sample* = concentration in spiked sample
- C *unspiked sample* = concentration in unspiked sample
- C *added* = added concentration

Statistical software

The statistical analysis of results was performed using the Microsoft Excel software (Microsoft 365, 2021). The data is presented in the form of the mean and standard deviation. A two-sided t-test was conducted to examine significant differences between means, with a confidence level of 95%. Precision components were estimated by means of one-way of variance (ANOVA). The threshold for statistical significance was established at a p-value of 0.05 or less.

4. RESULTS AND DISCUSSION

4.1 Total selenium in feed and feed materials

4.1.1 Commercial feed samples

In this study, in total 27 commercial feed for salmonids, 10 feeds for ruminants, 4 for chicken, and 2 feeds for piglets were analysed for total Se through ICP-MS.

Fish feed for Salmonids

The results of salmonids commercial feed samples are presented in Figure 7. The results of salmonids commercial feeds showed a range from 0.38 to 2.14 mg/kg (n = 27), presented in Figure 7. Several of the feeds examined (n = 22) contained concentrations above the upper limit for total Se in animal feeds, including fish feed, at 0.5 mg kg^{-1} (Council Directive 70/524/EC and amendments). These results were similar to findings reported by feed surveillance program (0.3 – 1.5 Se mg/kg, n = 93) [8], 0.35 – 0.89 Se mg/kg [25] and total Se in plant-based salmon feed with 0.45 mg/kg [89].

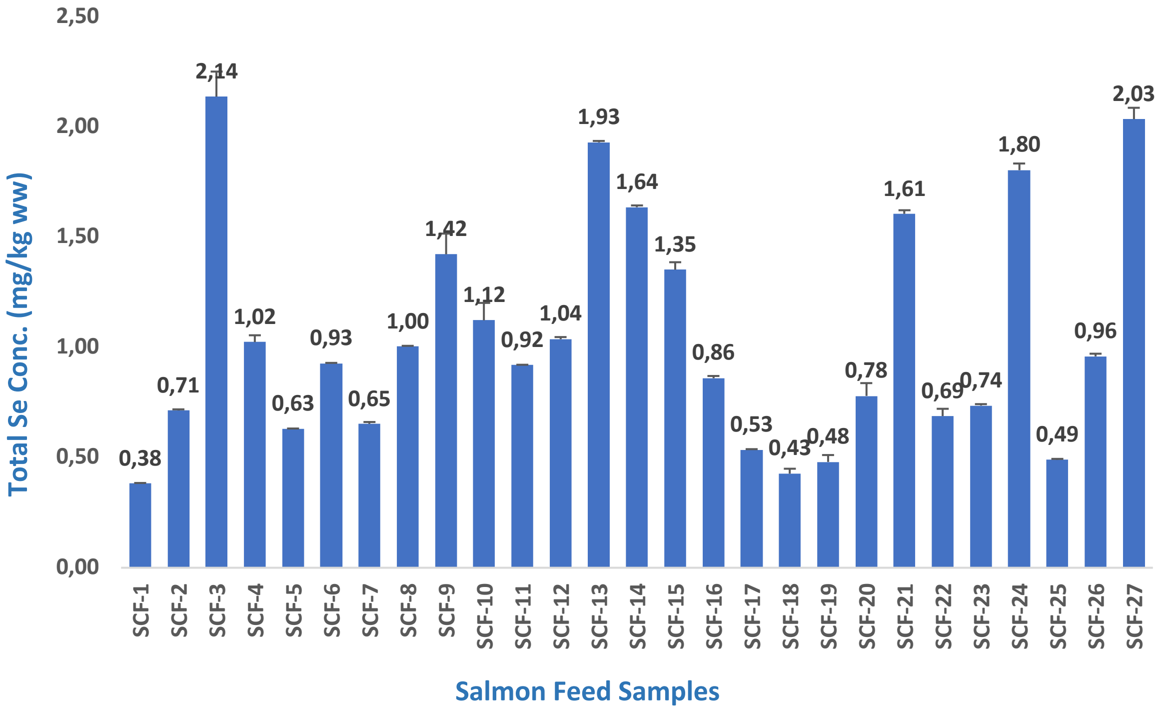


Figure 7: Total Se concentration (mg/kg, n = 2 replicates) in salmon commercial feeds

Land-animal feed

For the land-animal feeds for ruminants (RF), the results showed the range from 0.45 mg/kg to 1.70 mg/kg with a mean of 0.65 mg/kg. The possible reason of high concentration (1.70 mg/kg) could be a supplementation of Se to this feed sample. Previous studies have shown that supplementation with live yeast culture can result in high Se content [19-20]. While all other feed samples from ruminants reported the total Se concentration approximately around 0.5 mg/kg (Figure 8), which is maximum limit for Se in animal feed according to EU legislation for feed additives. In ruminants feed samples, a considerable variation was found between replicates thus those samples were repeated for acid digestion after appropriate homogenization during sampling process. The possible reason could be fibrous nature of ruminants feed which make it more challenging to homogenize even after grinding of samples. The boxplot presented in Figure B 1 (Appendix-B) showed an overall lower level of total Se in land-animal feeds.

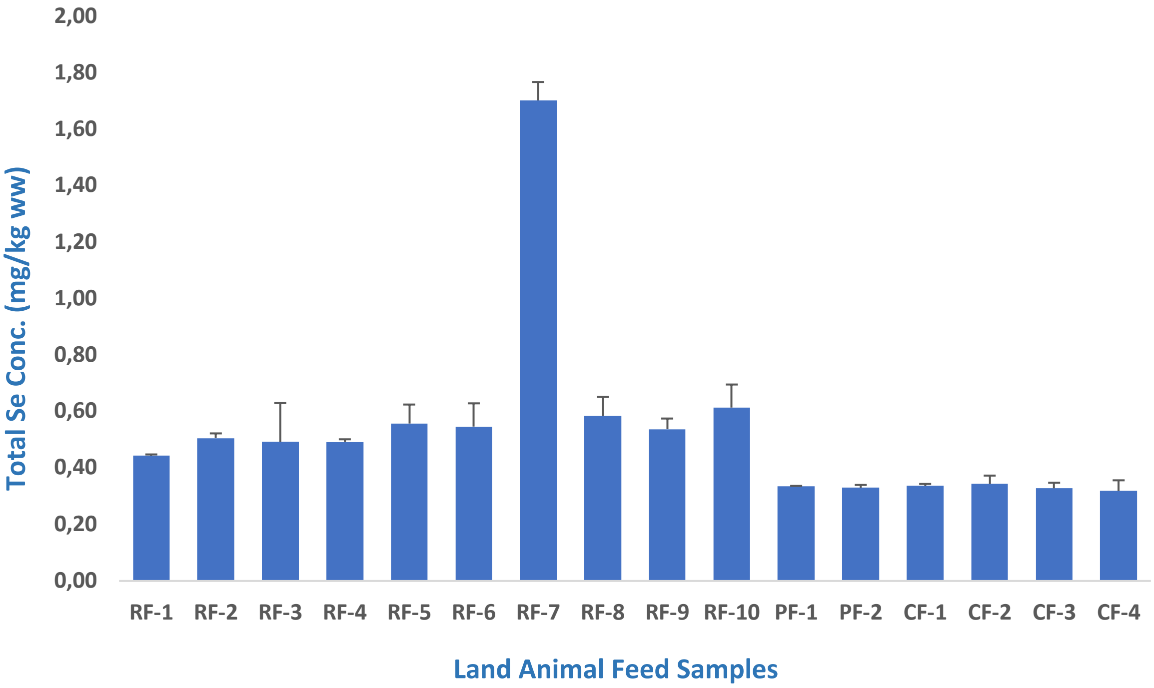


Figure 8: Total Se concentration (mg/kg, n =2 replicates) in land animals feed samples (RF = Ruminant feed, PF = Pig feed and CF = Chicken feed)

Feed materials

The following feed materials of fish meal (FM), insect meal (IM), plant meal (PM) and tuna meal (TM) samples were analyzed for total Se, with results presented in Table 13. For fish meal samples (n = 3), the results showed the concentration of total Se ranged from 1.70 to 4.55 mg/kg. For insect meal samples (n = 3), results were ranging from 0.14mg/kg to 0.33 mg/kg. For tuna meal (n = 2) and plant meal (n = 1), results showed in range of 8.32 – 11.9 mg/kg and 0.47 mg/kg, respectively. These tuna meal and plant meal were used in formulation of rainbow trout feed mentioned in Table A 1 and Table A 2 (Appendix-A). These results were similar to findings reported by feed surveillance program for fish meal (1.7 – 3.2 mg/kg, n = 10), plant meal (0.01 – 1.40 mg/kg) and insect meal (0.1 – 0.4 mg/kg) [8].

Literature showed that in plant-based meals, linseed meal contains more selenium than extracted soybean meal and rapeseed meal (0.82, 0.30, and 0.14 Se mg/kg DM, respectively) [45]. Salmon and herring meals are high sources of selenium (1.9 mg Se/kg DM) while tuna-fish meal contains high content of selenium (5.1–6.2 mg Se/kg DM) [47], making them the only protein concentrates of marine origin widespread in use animal feed [47]. In comparison, animal protein source of non-marine origin contains less selenium and can be considerably variable (0.11–1.14 mg Se kg^{-1} DM) [45, 47].

Table 13: Total Se concentration (mg/kg ± SD, n = 2 replicates) in feed material samples

Feed material sample	Total Se conc. (mg/kg) ± SD
Fish meal	
FM-1	4.6 ± 0.2
FM-2	3.6 ± 0.3
FM-3	1.70 ± 0.07
Insect meal	
IM-1	0.16 ± 0.01
IM-2	0.143 ± 0.006
IM-3	0.33 ± 0.01
Tuna meal	
TM-1	11.9 ± 0.3
TM-2	8.32 ± 0.13
Plant meal	
PM-1	0.47 ± 0.01

4.1.2 Experimental feed samples

Salmon fish feed

In total, six *Salmonoid* experimental feed samples were analyzed for total Se. The experimental feed samples showed the total Se content from a 0.78 to 1.52 mg/kg Se range (Figure 9). These experimental feeds were based on soybean meal supplemented with yeast as a novel source of protein [123]. Interestingly, instead of traditional *Saccharomyces* yeasts, these feeds were supplemented at the rate of 10% of different *non-Saccharomyces* yeasts: *Cyberlindnera jadinii* (CJ), and *Wickerhamomyces anomalus* (WA). The aim of the study was to investigate the effect of novel source of yeasts on intestinal health and results showed a positive response on reducing the inflammation in salmon [123]. The Figure 9 shows the variable amount of Se content in each sample, and all the samples contained the Se concentration over the upper limit of Se supplementation (0.5 mg/kg). The possible explanation for the high Se content relates to supplementing these experimental diets with novel yeast species [123].

Other studies showed that in a high plant-based salmon feed with background levels of 0.45 mg Se/kg, Atlantic salmon appeared to tolerate the supplementation of selenite or SeMet to a level of total selenium of 1-2 or 3 mg/kg feed [89]. At higher Se levels (15 mg/kg), oxidative stress and changes in lipid metabolism seemed to be toxic processes for both inorganic and organic Se, with inorganic Se being more harmful [88].

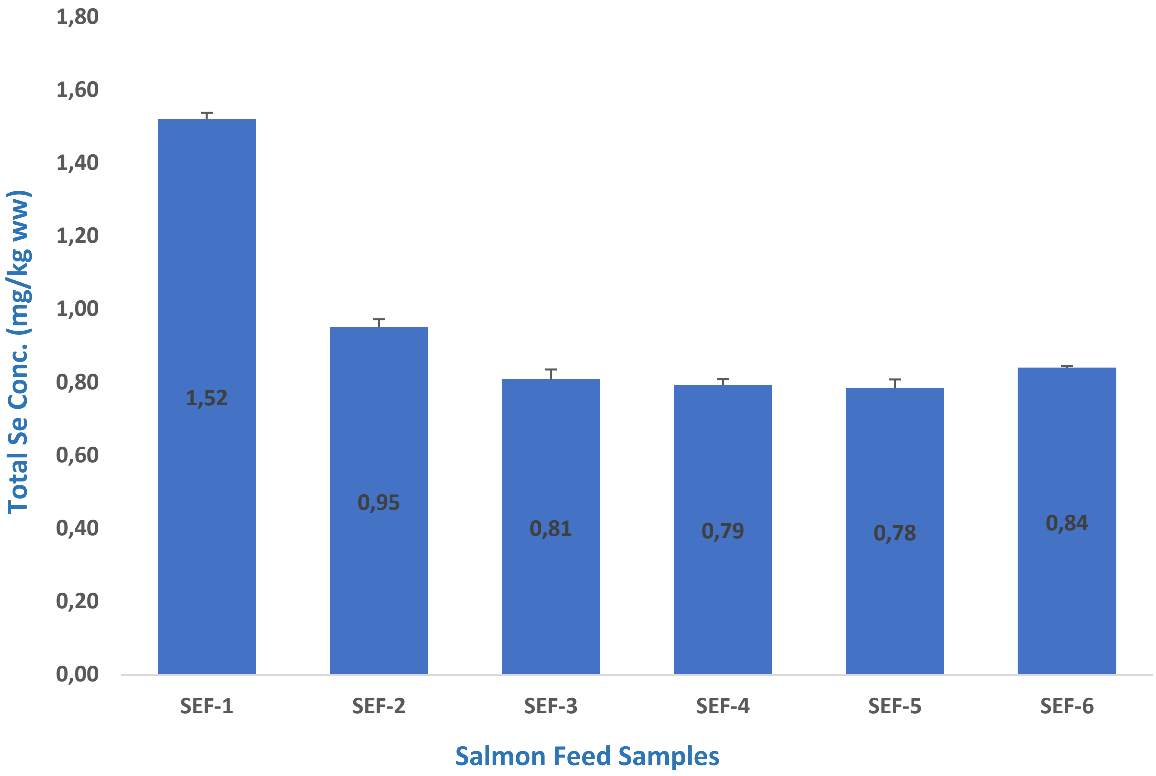


Figure 9: Total Se content (mg/kg, n = 2 replicates) in salmon experimental feed (SEF) samples

Zebrafish feed

A total of three samples of experimental zebrafish fish feed were analyzed, and the total Se content was ranging from 0.46 to 0.70 mg/kg. The Figure 10 showed the slight variable amount of Se content in each sample and two samples contained the Se content beyond the set limit of Se supplementation (0.5 mg/kg) in feed. The results showed that there was not significant deviation in values among the two replicates of sample.

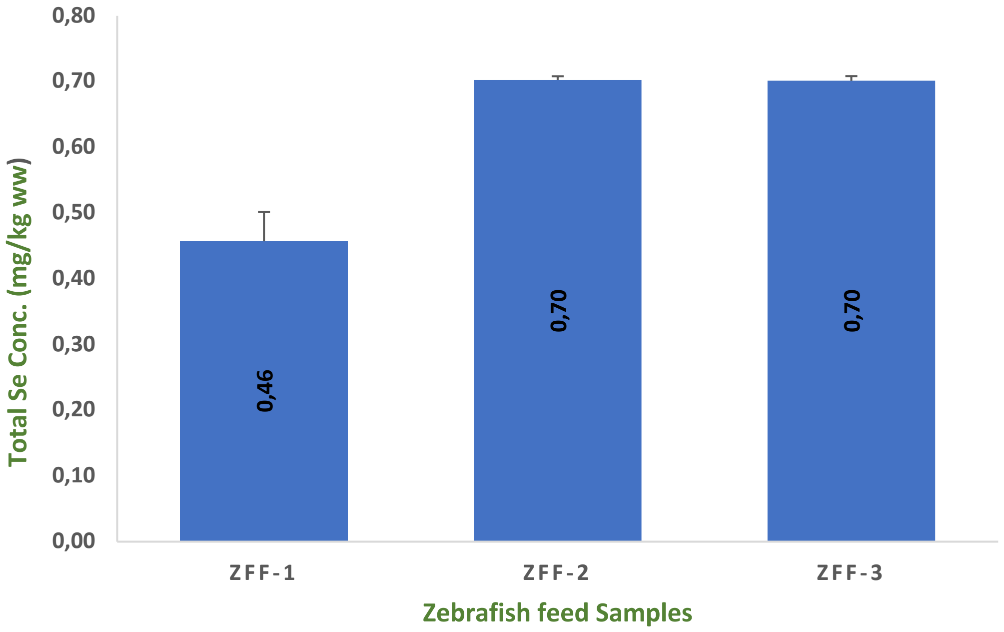


Figure 10: Total Se content (mg/kg, n = 2 replicates) in zebrafish feed (ZFF) samples

Rainbow trout feed

In total, 12 rainbow trout diets (n = 12) comprised of vegetal meal-based (n = 6, RTF 1-6) and tuna meal-based (n = 6, RTF 7-12) were analyzed (Table A 1 and Table A 2) with RTF 1 and RTF 7 were control samples for vegetal meal and tuna meal-based feeds. The results showed that overall, the tuna meal-based feed contained a higher concentration of the total Se, and an opposite trend was observed in vegetal meal-based feeds (Figure 11). The total Se concentration was 7.35 – 9.51 mg/kg and 0.46 – 3.17 mg/kg for tuna meal and vegetal meal-based feeds, respectively. These experimental feeds were retrieved from a study to investigate the effects of food sources and Se and mercury (Hg) levels on growth performance, Se and Hg accumulation, and general health indices in juvenile rainbow trout [124]. A 6-month feeding trial was carried out with a control plant-based diet (containing analyzed Se level 0.3 mg/kg diet) or a control tuna-based diet (containing analyzed Se level 7.5 mg/kg diet) supplemented with 0 or 1.5 mg Se/kg diet supplied either as sodium selenite or selenomethionine (SeMet) [124]. The result of current study is consistent with the previous study [124]. All feeds except one feed sample contained the total Se content exceeding the maximum content for Se (0.5 mg/kg) in feed and the possible reason is the supplementation of experimental feeds with Se (Figure 11).

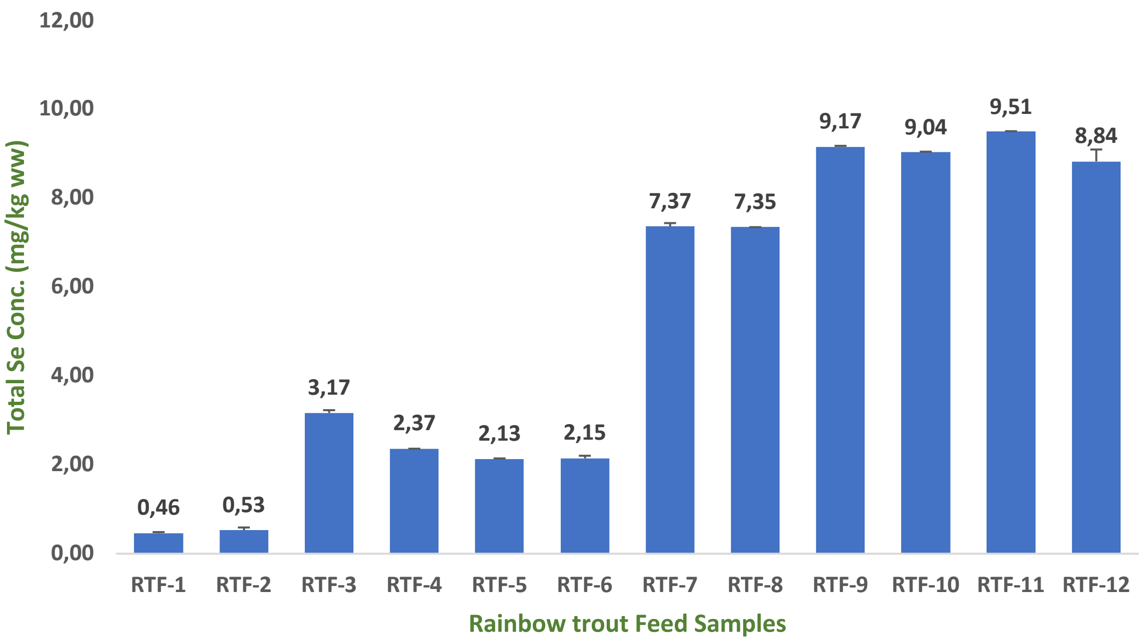


Figure 11: Total Se content (mg/kg, n = 2 replicates) in rainbow trout feed (RTF) samples

Land animals feed

Piglet feed (PF) samples (n = 2) and chicken feed (CF) samples (n = 4) were analyzed in experimental feed for land animals. In piglet feed samples, Figure 8 showed the concentration of total Se was 0.33 mg/kg in both samples. The chicken feed samples were ranging from 0.32 to 0.34 Se mg/kg. The piglet feed samples were derived from a study, and it aimed to examine the impact of administering *Cyberlindnera jadinii* yeast on the maturation of gastrointestinal function and overall health in pigs during the weaning [125]. The boxplot presented in the Figure B 1 (Appendix-B) showed a generally lower level of total Se in land animal feeds.

In comparison to legislative upper limit of Se supplementation (0.5 mg kg^{-1}) in animal feed by EU (Council Directive 70/524/EC and amendments). The national research council set a maximum Se tolerable level in animal diets of 2 mg/kg for the main livestock species, without taking the element's chemical forms into account [126]. Similarly, the distinctions between ruminant and monogastric animals are not taken into consideration. Selenium absorption differed substantially in ruminants as compared with single-stomached animals [93].

4.2 Total selenium in rainbow trout (*Oncorhynchus mykiss*) whole-body

The rainbow trout (whole body) samples (n = 39) which were fed on experimental diets (n = 12) in triplicate tanks were analyzed for multi-element analysis including total Se. The samples were grouped in biological replicates (n = 13) and each biological replicate had technical replicates (n = 3). Each technical replicate was analyzed in two replicates (Table 3). As described in earlier section, a 6-month feeding trial was carried out with a control and formulated plant-based diet and tuna-based diet supplemented with either with Se or Hg or both [124]. The details about the composition of the diets and supplementation of Se and Hg are mentioned in Table A 1 and Table A 2 in Appendix-A. The Figure 12 presented the biological groups of the rainbow trout in relation to different formulation of rainbow trout feeds. The results shows that the highest concentration of 2.41±0.19 mg/kg of total Se was found in group in which fish fed on a diet supplemented with organic selenium in tune meal-based feed (Figure 12). Similarly, a concentration of 2.16±0.25 mg/kg Se was observed in group, which was fed on diet supplemented with organic Hg and organic Se.

In general, all the whole-body tissue samples of trout fed on tuna meal-based diets contained the total Se concentration towards higher side (Figure 12). In comparison of groups fed on tuna meal-based diets, most of the groups of trout fed on vegetal meal-based diets contained lower concentration of Se except the group RTW-3 and RTW-6 which contained 1.54±0.15 and 1.61±0.17 Se mg/kg, respectively. Among these two groups, one group (RTW-3) was supplemented with inorganic selenium while second group (RTW-6) was supplemented with organic Hg and organic Se (Figure 12). A boxplot presented in the Figure C 1 (Appendix-C) showed a comparative overview of total Se in whole-body samples of rainbow trout fed with vegetal and tuna meal-based experimental feeds.

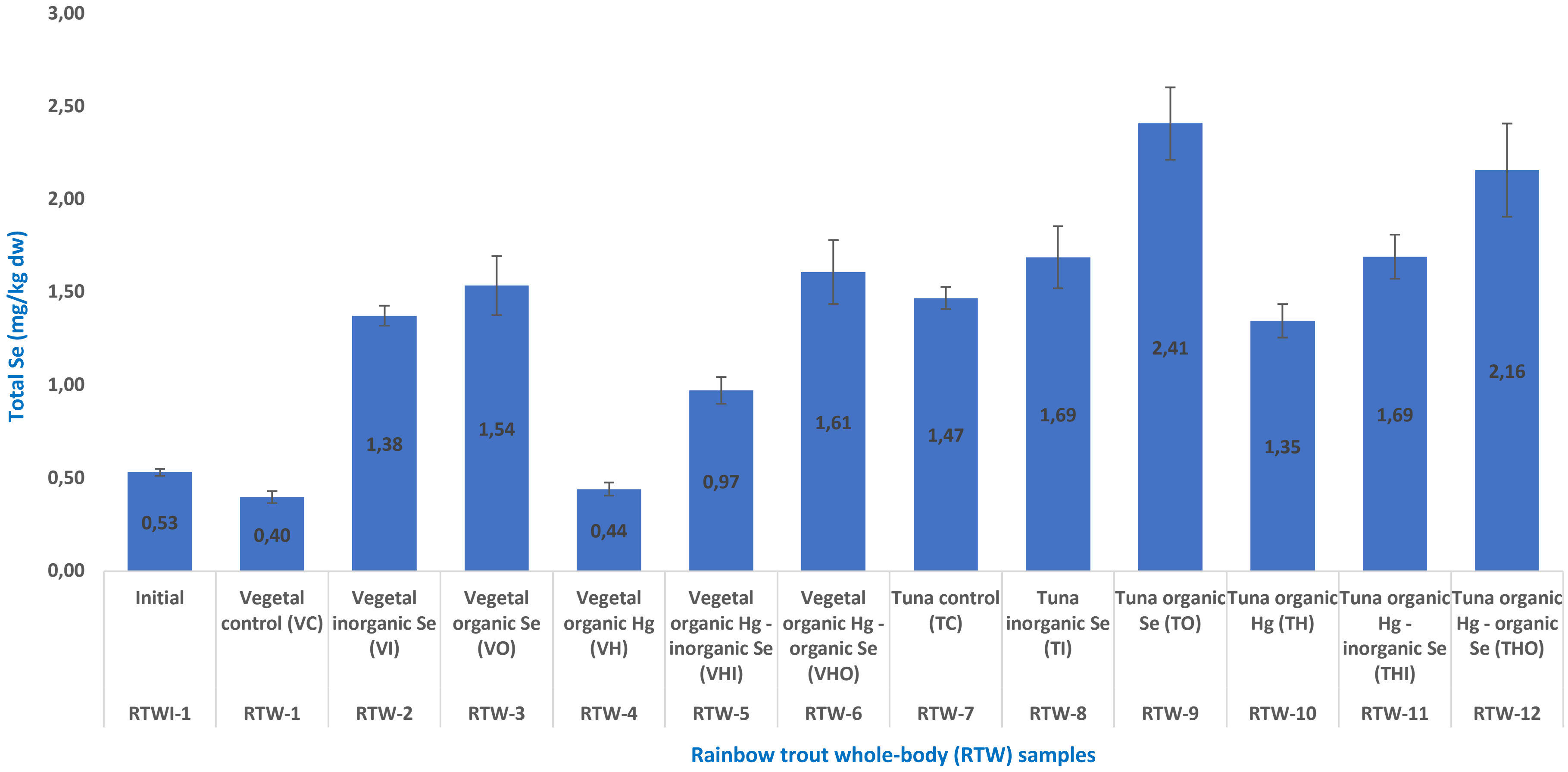


Figure 12: Total Se concentration (mg/kg dw, mean ± SD, n = 6) in rainbow trout (whole body) fed on initial feed (control) different vegetal and tuna meal-based diets, both control and supplemented with inorganic Se (selenite) or organic Se (SeMet) combined with organic Hg (MeHg).

4.3 Method validation

Evaluation of performance characteristics of the method

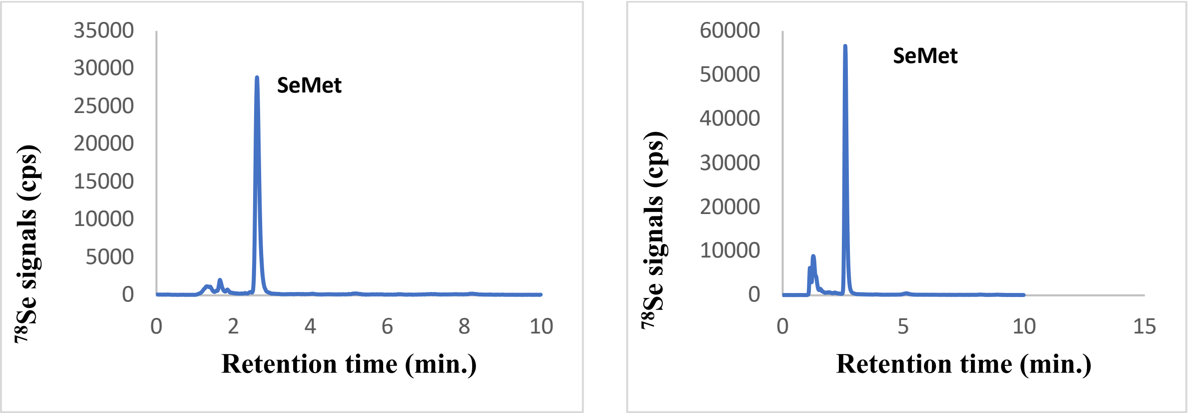
4.3.1 Selectivity

The selectivity was determined based on the chromatographs of the CRMs, feed and fish tissue samples, reagent blank, buffer solution, protease and cellulase enzymes. The chromatograms of CRMs showed good resolution of SeMet chromatographic peak with a baseline separation of SeMet at retention time of 2.6 min, with no interferences from other Se species (Figure 13). Similarly, most chromatograms of samples such as fish feed (Figure 15a), salmon tissue with low Se (Figure 16a) and high Se level (Figure 16b) and tuna tissue (Figure 16c) also showed good resolution of SeMet chromatographic peak, except for a few chromatograms related to zebrafish feed (Figure 15b), land-animal feed (Figure 15c), and blue mussel (Figure 16d). For the blue mussels peaks of unknown Se species were observed (Figure 16d), however, Se peaks were not overlapping SeMet and since they were eluting at retention time of 1–2 min before the SeMet. The signal intensity for unknown Se species were observed higher than the signal for SeMet. The results also showed that the extraction method did not contribute to additional interferences (other than SeMet) during the chromatographic analysis. The selectivity for SeMet in different sample types and concentration ranges of SeMet was found to be good.

The SeMet signal in the reagent blank samples was also examined in each sequence. For all reagent blanks analysed it was observed a Se peak in the retention time of 2.6 min of SeMet (Figure 14b). Initially, it was assumed that this signal for SeMet in reagent blank (Figure 14b) may result from the combined enzyme of protease and cellulase or the ammonium phosphate buffer. After further investigation and analysis the individual samples of buffer (Figure 14a), cellulase (Figure 14c) and protease (Figure 14d), it was found that the protease enzyme was the mainly causing this interference in the blank samples. The same enzyme digestion solution was used for both samples and blank samples, so in theory the samples were having similar amount of SeMet from the enzyme as the blank samples. This may impact the concentrations found in the samples. To correct this interference, based on the results from the blank samples, all sample measurements were corrected for the blank signals.

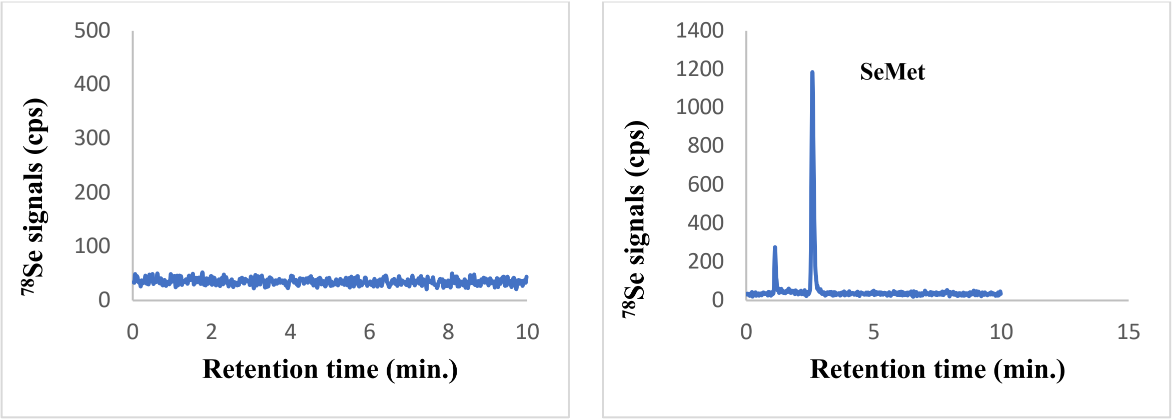
The analytical selectivity is *"the extent to which the method can be used to determine particular analytes in mixtures or matrices without interferences from other components of similar behavior present in mixtures or matrices"* [114]. The method demonstrated the capability to specifically determine the SeMet in CRMs, different types of animal feeds and tissues samples.

The concentration of an analyte is typically not directly measured at the measurement stage. Instead, a particular attribute (like signal) is quantified. So, it is essential to prove that the measured attribute exclusively arises from the analyte and not from anything physically or chemically similar or by coincidence, which would skew the measurement result [113]. The current method demonstrated in chromatograms the clear and well separated peaks at specific retention time. To increase the selectivity of the measuring system, an isolation stage may need to come before the measurement stage. The capacity of a method to measure the analyte of interest in samples that have certain interferences is typically used to determine how selective a method is for its intended use [113, 114]. In this study, enzymatic extraction with cellulase and protease followed by filtration removed most of the interferences from the mixture which was evident in the chromatograms.

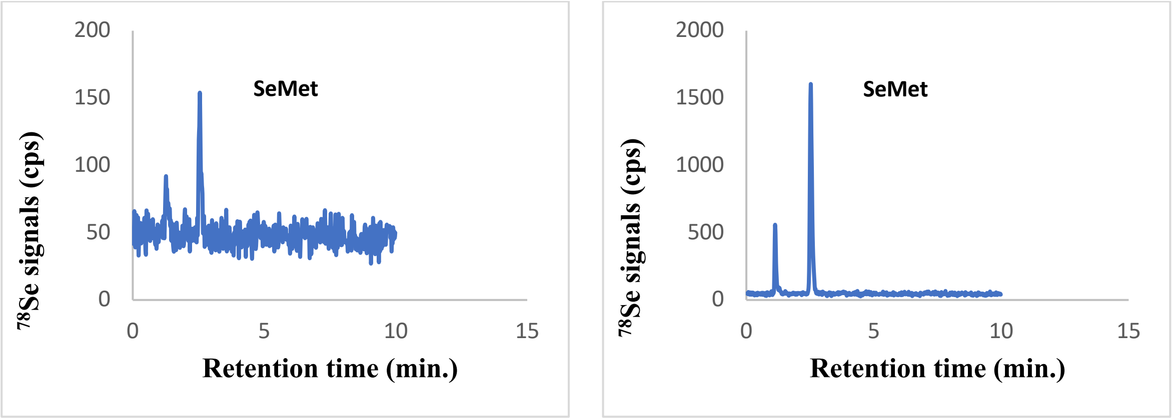


(a) CRM SELM-1 (2000x dilution) (b) CRM ERM BC210a (10x dilution)

Figure 13: Chromatogram of CRM (a) SELM-1 (Selenized yeast) and (b) CRM ERM BC210a (Wheat) with SeMet eluting at retention time of 2.6 min.

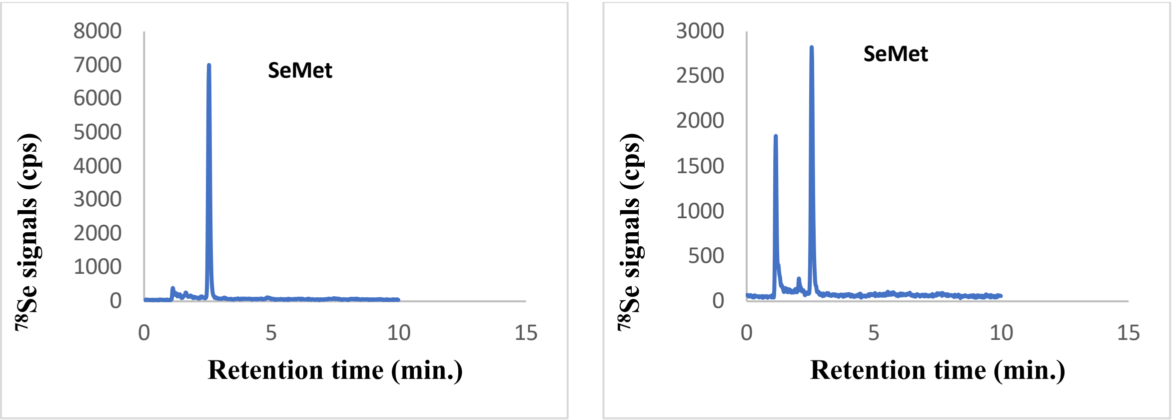


(a) Ammonium phosphate buffer (b) Reagent blank (2x dilution)

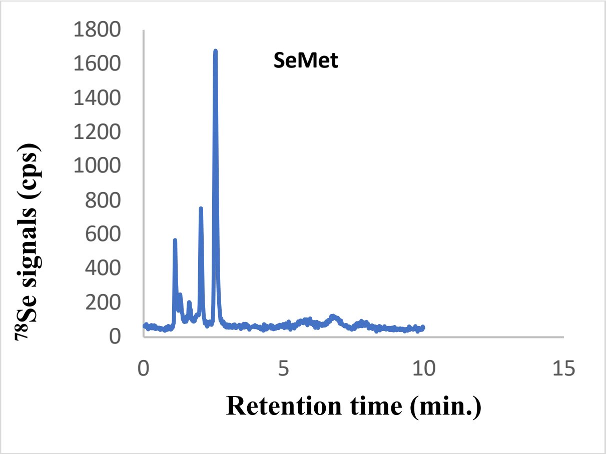


(c) Cellulase enzyme (2x dilution) (d) Protease enzyme (2x dilution)

Figure 14: Chromatograms of Se signal for (a) buffer, (b) reagent blank (extraction solution), (c) cellulase enzyme, and (d) protease enzyme, with Se signal in the void of chromatogram (R.T. 1-2 min) and at R.T. 2.6 min, interfering with SeMet.

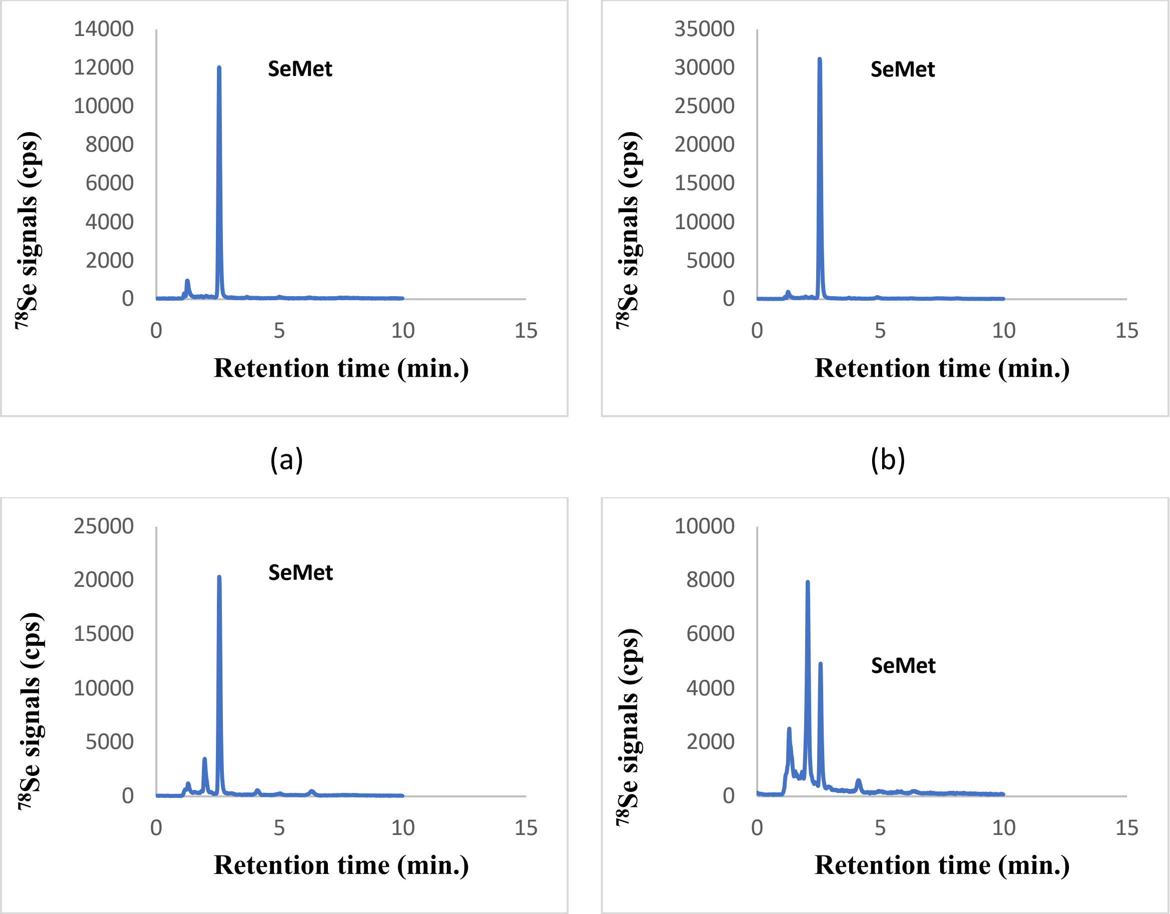


(a) Fish feed (2x dilution) (b) Zebrafish feed (2x dilution)



(c) Land-animal feed (2x dilution)

Figure 15: Chromatograms of fish feed, zebrafish feed and land-animal feed samples with SeMet eluting at retention time of 2.6 min.



(a) (b)

(c) (d)

Figure 16: Chromatograms of tissue samples (a) salmon tissue with low concentration of Se (2x dilution), (b) salmon tissue with high concentration of Se (2x dilution), (c) tuna fillet (4x dilution) and (d) blue mussel (4x dilution) with SeMet eluting at retention time of 2.6 min.

4.3.2 Limit of detection (LOD) and limit of quantification (LOQ)

The reagent blanks (n = 10) were analyzed for five consecutive days to determine LOD and LOQ (Table D 1, Appendix-D). From the area signals of the blanks, the estimated LOD and LOQ were 0.7 µg/L and 2.5 SeMet mg/L, respectively. Considering weight of sample and dilution factors, this corresponds to a LOD of 0.02 mg/kg, and a LOQ of 0.06 mg/kg.

There are several ways of determining LOD/LOQ for analytical methods, e.g. based on standard deviation or signal to noise ratio [113, 115, 127]. In the previous method for Se speciation, which had another chromatographic principle, LOD was determined to be 0.03 mg/kg for SeMet when using the S/N -ratio of the blank signal [25]. Also, in that method a signal for Se was observed for SeMet. The LOD found in this validation procedure is hence in good agreement with earlier results.

Based on the signal intensity for Se in the calibration standards, where the lowest point was at 0.05 µg/L, the Se signal by the ICP-MS is higher than reflected from the calculated LOD/LOQ values. Moreover, if Milli-Q water or ammonium phosphate buffer were used as blanks, we would get lower LOD and LOQ. Hence, in this case, the LOD/LOQ did not reflect the sensitivity capacity of the instrument.

4.3.3 Linearity

For the estimation of linearity, a plot between response (y-axis) and concentration (x-axis) was plotted and visually examined for outliers [113, 116]. Table 14 shows the signals (response) monitored for the six standard concentrations, ranging from 0.5 to 50 µg/L. The visual examination of the calibration graph showed a linear trend line between the concentration and the response (Figure 17). The regression coefficient (r) for the linear curve was at least 0.99, which shows a linear fit model. In addition, the plots between normal concentration (Figure 18) and log concentration (Figure E 1, Appendix-E) versus residuals showed a random distribution of the residuals. The random distribution of residuals confirms the linearity of the method, while systematic trends indicate non-linearity or a change in variance with level [113, 116]. This information represents that this method is satisfactory in terms of linearity.

Table 14: Concentrations of SeMet standards (µg/L) and signals for SeMet peaks (area of peak, ^{78}Se)

Concentration (µg/L)	**Signals (area of peak, ^{78}Se)**				
	Run 1	**Run 2**	**Run 3**	**Run 4**	**Run 5**
0.5	5195.46	5301.97	4356.39	4704.05	4652.00
1	10009.96	10712.78	9932.31	8943.83	9713.54
5	56956.69	51001.11	52672.35	46840.01	45897.46
10	110158.15	101397.56	102197.11	93562.37	65479.48
25	28185009	237547.32	258466.84	206930.94	227543.21
50	559589.06	520104.30	526320.45	472122.85	449947.97

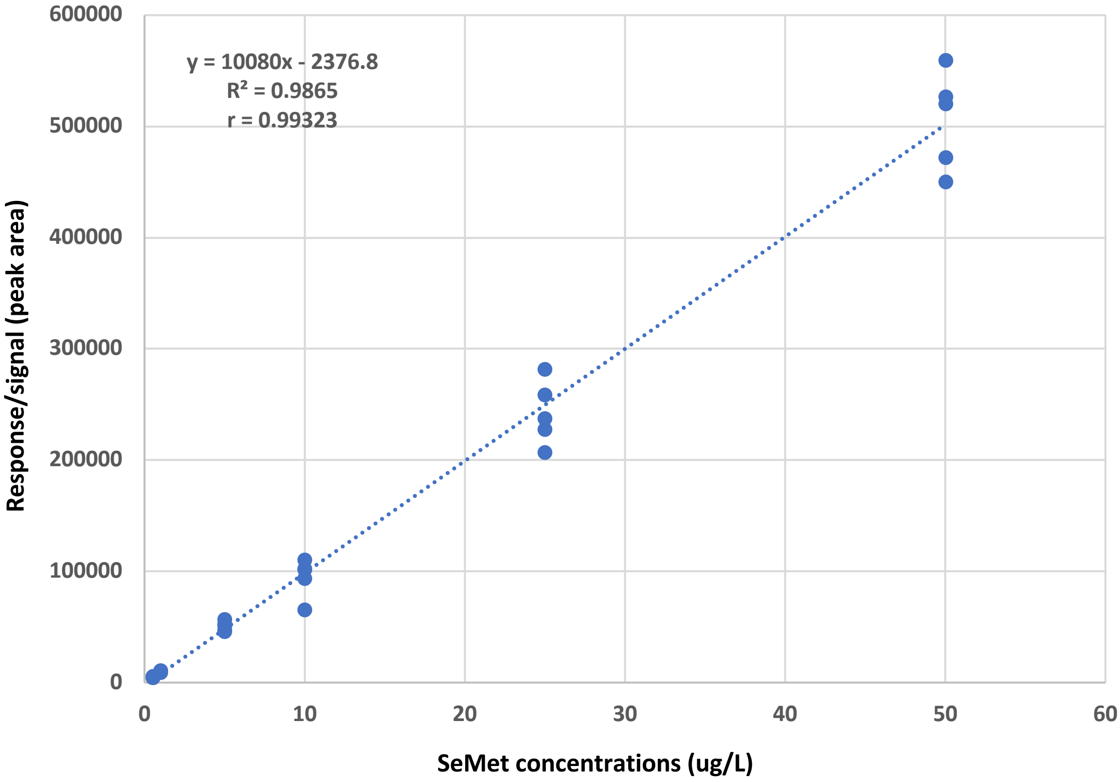


Figure 17: Calibration graph for SeMet concentrations (µg/L) and responses (peak area), with regression statistics.

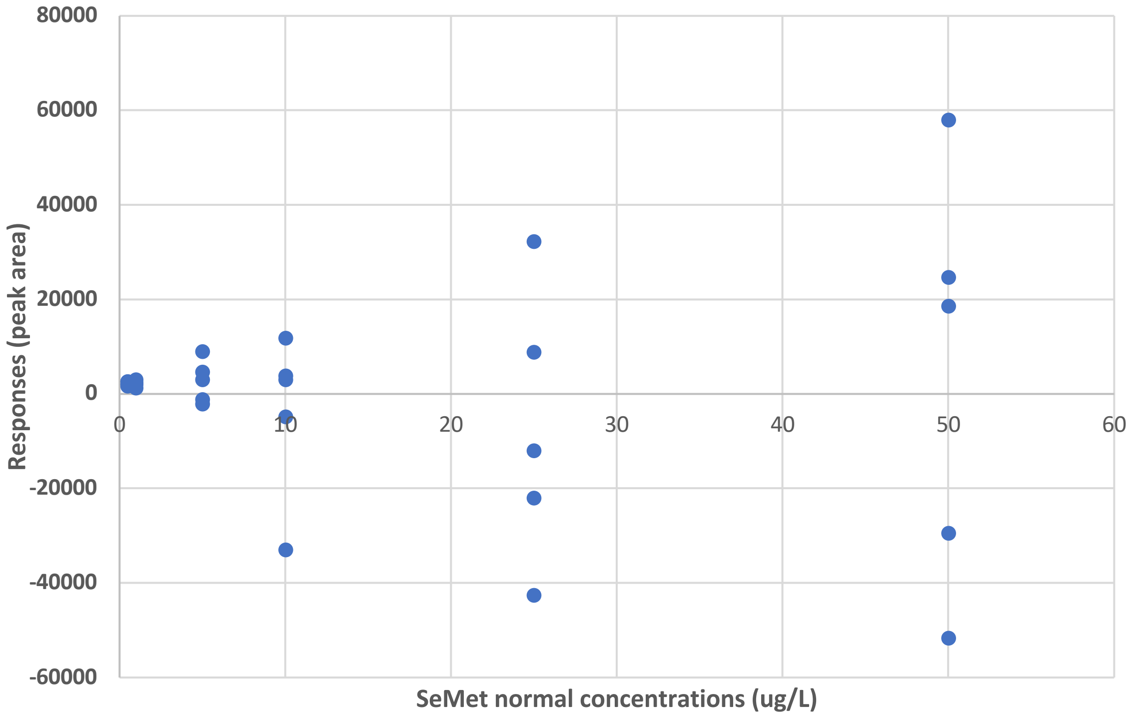


Figure 18: Residual plot for normal concentration of SeMet (μg/L), responses (peak area), with residuals.

4.3.4 Trueness and recovery

The trueness of the method was determined by analyzing the two CRMs, ERM BC210a (wheat) and SELM-1 (selenized yeast). To our knowledge, these are the only available CRMs with certified values for SeMet. Two analysts were involved in analyzing each CRM ten times over five days to calculate the total concentration of SeMet and the corresponding recovery (Table 15). For ERM BC210a, the manufacturer reported a concentration of 27.4 ± 2.6 mg/kg of SeMet in this CRM which was calculated to concentration of Se in SeMet being 11.03 ± 1.04 mg/kg (Table 6). The obtained concentration of SeMet from the validation ranged from 5.8 ± 0.2 mg/kg to 8.8 ± 0.1 mg/kg, and the recovery ranged from 53% to 80% (Table 15). The average concentration of SeMet from these analyses was 7.08 ± 0.21 mg/kg (n = 10) with an average recovery of 64% from ERM BC210a (Table 15). This CRM was used in this study because it is based on wheat and is relevant in terms of plant-based ingredients are a major component in aquatic and land-animal feeds [72, 112]. The observed recovery for the CRM was under the certified values, and hence not satisfactory.

For SELM-1, the concentration of 3190 ± 260 mg/kg of SeMet was the certified values of this CRM, which were calculated to Se in SeMet at 1284 ± 105 (Table 6). The analyzed concentration of SeMet ranged from 928 ± 3 mg/kg to 1062 ± 69 mg/kg, and the recovery ranged from 72% to 83% (Table 15). The overall average concentration of SeMet in SELM-1 was calculated to be 972 ± 43 mg/kg (n = 10) with an average recovery of 76% from SELM-1 (Table 15). SELM-1 is mainly based on the selenized yeast culture of *Saccharomyces cerevisiae* [19-20, 23]. The selenium-enriched yeast can be a source of organic Se supplementation in aquatic and land-animal feeds [19-20, 117]. Overall, the observed recovery seems better with this CRM than with ERM BC210a (Table 15). This could maybe be explained by some matrix effects on the recovery of SeMet from samples. Also, the concentration of Se and SeMet in SELM-1 are higher than the ERM-BC210, which may influence the results. Overall, with a recovery ranging from 72 to 83% is considered low but will be accepted and followed-up with more data points in following analysis using this method.

Table 15: Concentration and recovery of SeMet (mg/kg) ± SD in CRM ERM BC 210a (wheat) and CRM SELM-1 (selenized yeast)

Day	**Analyst**	**CRM 1 (ERM BC 210a)**		**CRM 2 (SELM-1)**	
		Mean conc. (mg/kg) ± SD	**Avg. recovery (%)**	**Mean conc. (mg/kg) ± SD**	**Avg. recovery (%)**
1	**A**	5.8 ± 0.2	53 ± 1	928 ± 3	72 ± 0.2
2	**A**	6.9 ± 0.3	63 ± 2	973 ± 58	76 ± 5
3	**B**	8.8 ± 0.1	80 ± 1	1062 ± 69	83 ± 5
4	**A**	6.7 ± 0.3	61 ± 2	934 ± 51	73 ± 4
5	**A**	7.1 ± 0.3	65 ± 2.6	962 ± 35	75 ± 3
Average recovery (%)		7.1 ± 0.2	64 ± 1.91	972 ± 43	76 ± 3

4.3.5 Precision (repeatability and intermediate precision)

The precision is the degree to which results are similar. The precision of laboratory results is represented in terms of repeatability, reproducibility and intermediate precision based on the results. It is typically expressed by statistical parameters that characterize the dispersion of results, such as the standard deviation (or relative standard deviation, RSD) calculated from replicate measurements conducted on a suitable material under specified conditions [105, 108]. Repeatability, which is assumed to create the smallest variation in results, is a measure of the variation in results when a measurement is performed by a single analyst using the same equipment over a short period of time [113]. Intermediate precision (within-laboratory reproducibility) showed the agreement between the results from within-laboratory variations due to random events or errors that might occur. In the case of intermediate precision, more effects are accounted for over a more extended period [113, 116].

In the case of CRMs, the RSD for repeatability and intermediate precision were observed as 4.4% and 15.7% for ERM BC210a, while 7.1% and 7.5% for SELM-1, respectively (Table 16). For feed samples, the RSD for repeatability and intermediate precision were recorded as 3.6% and 8.8% for fish feed, 5.4% and 11.2% for zebrafish feed and 32.2% and 32.2% for land-animal feed, respectively (Table 16). In the case of salmon tissue samples, the RSD for repeatability and intermediate precision were reported (Table 16) as 5.2% and 6.6% for a fillet with a low Se concentration and 4.9% and 6.6% for a fillet with a high Se concentration, respectively. The repeatability and intermediate precision (RSD) for tuna and blue mussel samples as 7.6% and 9.6% for tuna fillet and 5.1% and 11.6% for blue mussel (Table 16), respectively.

Regarding repeatability, the lowest RSD of 3.6% was observed in the fish feed sample, while the highest RSD of 32% of measurements was found in the land-animal feed sample (Table 16). The deviation trend is consistent in feed (Table G 1and Table G 2, Appendix-G) and tissue samples (Table G 4, Table G 5, Table G 6 and Table G 7, Appendix-G) except for land-animal feed samples. The data (Table G 3, Appendix-G) showed that the results for SeMet in the land-animal feed had larger variations in measurements within the day and between the days. The concentration of SeMet in the land-animal feeds is low, and close to the LOD/LOQ. Furthermore, the coarse nature of the feed material with high fiber content could explain the high RSD for these results.

Regarding intermediate precision, among the different CRMs, feed, and tissue samples (Table 16), the lowest RSD of 6.63% and 6.64% were observed for both salmon tissue samples with high and low concentrations of Se, respectively. In comparison, tuna fish tissue showed higher

RSD of 9.59% for intermediate precision. But overall, the highest RSD for intermediate precision was found in land-animal feed sample at 32% (Table 16). As explained earlier, this variation could be due to high fiber content in the feed which made it a bit harder to take representative sample for quantification of SeMet.

Based on the precision, the Horwitz ratio ranged from 0.12 to 0.47 for the CRMs, feed, and tissue samples (Table 16) analyzed during the validation study. The Horwitz ratio was below the value of 2 for all the samples and CRMs. However, the highest ratio of 0.47 was noted for CRMs and land-animal feed samples. In contrast, the Horwitz ratio for other samples is mentioned in Table 16. The ratio is found within the requirement range of 0.5–2 (Table 12), which reflects the acceptability of the analytical method in terms of the precision of the method.

4.3.6 Measurement uncertainty

The measurement of uncertainty (MU) was calculated from the estimation of SeMet in feed and tissue samples and are presented in Table 16. The table represented the mean value of SeMet (mg/kg) and calculated MU for each CRMs and sample. The MU (%) was calculated to range from 13% to 64% (Table 16), which showed a large range in MU. The highest MU is observed in land-animal feed sample, but this sample also contained low concentrations of SeMet. Salmon tissue samples showed lower levels of MU, with 13% (MU). Among the two CRMs, the ERM BC210a showed a relatively high MU of 32% compared to SELM-1, with a MU of 15%. These observations also reflect the strong influence of matrix effects on the MU of the reported results. The MU (%) for the fish feed, zebrafish feed, tuna fish tissue and blue mussel was 18%, 23%, 20% and 23% (Table 16).

A measure of uncertainty should account for all recognized effects operating on the outcome [113]. There are several approaches to estimate the measurement of uncertainty for the results obtained for chemical measurements. But the main approach used for this method was based on overall, long-term precision of the method that includes the intermediate precision of the measurements [113].

Table 16: Results of repeatability, intermediate precision, Horwitz ratio and measurement uncertainty for estimation of the organic Se species (SeMet) in feed and fish tissue samples

Sample	Mean conc. of SeMet (mg/kg) ± SD	Repeatability RSD (%)	Intermediate Precision RSD (%)	HorRat	Measurement Uncertainty (%)	Mean conc. of SeMet (mg/kg) ± MU
CRM 1 (ERM BC210a)	7.1 ± 0.3	4.4	15.7	0.47	32	7.1 ± 2.2
CRM 2 (SELM-1)	972 ± 69	7.1	7.5	0.47	15	972 ± 145
SMPL 1 (Fish feed)	0.158 ± 0.005	3.6	8.8	0.15	18	0.16 ± 0.02
SMPL 2 (Zebrafish feed)	0.073 ± 0.003	5.4	11.2	0.17	23	0.73 ± 0.02
SMPL 3 (Land-animal feed)	0.06 ± 0.02	32.2	32.2	0.47	64	0.06 ± 0.04
SMPL 4 (Salmon tissue low Se)	0.29 ± 0.01	5.2	6.6	0.12	13	0.29 ± 0.04
SMPL 5 (Salmon tissue high Se)	0.76 ± 0.03	4.9	6.6	0.14	13	0.76 ± 0.10
SMPL 6 (Tuna fish tissues)	1.12 ± 0.08	7.6	9.6	0.22	20	1.12 ± 0.21
SMPL 7 (Blue mussel)	0.24 ± 0.01	5.1	11.6	0.21	23	0.24 ± 0.06

4.4 Spiking recovery of selenium species

4.4.1 Spiking recovery of SeMet from feed and tissue samples

For assessing the trueness of method for real samples, a set of spiking experiments with SeMet was conducted. One sample of fish feed and one sample of blue mussel were spiked with known SeMet concentrations. The spiking of the samples was performed before the enzymatic extraction to assess the extraction recovery of SeMet, and and after the enzymatic extraction to assess the chromatographic recovery. The results are shown in Table 17.

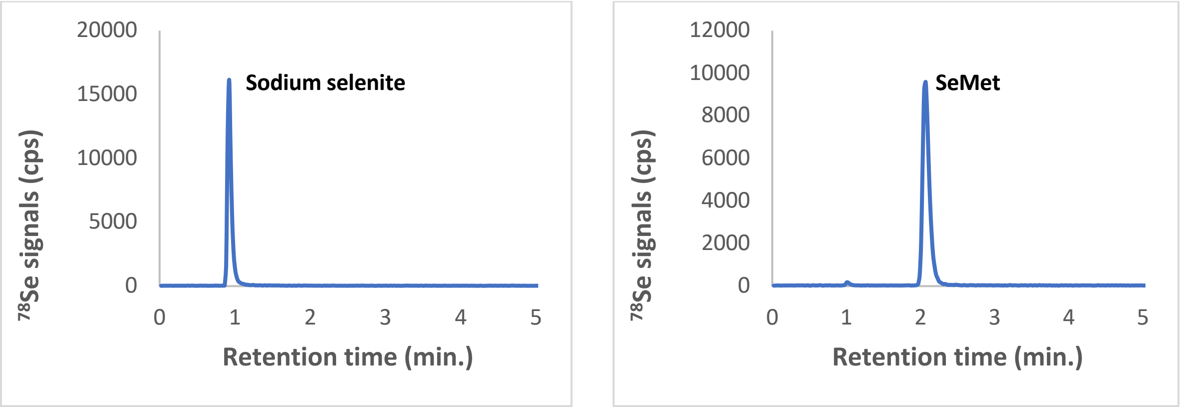
In the case of fish samples, the results showed that the extraction recovery of SeMet before extraction ranges from 104% to 120%, with an average recovery of 110%. While in case of spiking after the extraction showed recovery in the range of 98% to 123% with an average recovery of 114% (Table 17). Overall, the average recovery of SeMet before and after the extraction was at 112%, suggesting a good recovery and trueness for a sample matrix of fish feed.

In the case of the blue mussel sample, the results were slightly different. The results showed that the extraction recovery of SeMet before extraction ranges from 80% to 117%, with an average recovery of 98%. In contrast, spiking after the extraction recovered the SeMet in the 87% to 97% range, with an average recovery of 93% (Table 17). The overall recovery of SeMet before and after the extraction procedure was 96%, indicating also a good recovery for this sample matrix.

4.4.2 Spiking recovery of Sodium Selenite, SeMet, SeMetSeCys and SeCys

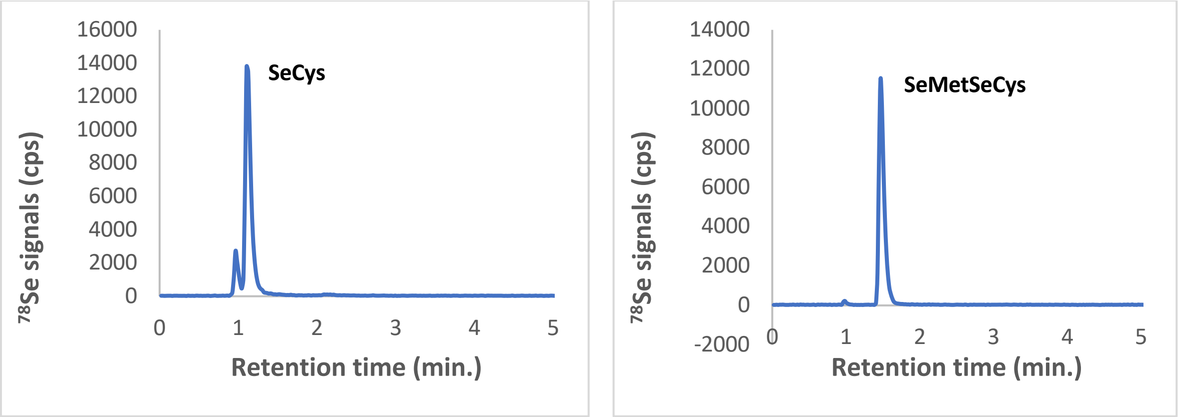
For the chromatographic separation of selected selenium species, a spike mix (10 ppb) was prepared with inorganic (sodium selenite) and organic (SeMet, SeMetSeCys and SeCys) species of Se. The spiking was performed after the extraction procedure with this spike mix to the fish muscle, kidney, and spleen samples. The chromatographic separation through HPLC and detection through ICP-MS showed good separation. The chromatograph of spike mix revealed clear and well distinguished peaks of each analyte. The chromatograms of sodium selenite (Figure 19a), SeMet (Figure 19b), SeCys (Figure 19c) and SeMetSeCys (Figure

19d) showed clear and distinguished peaks of each analyte. It shows a satisfactory separation of the organic and inorganic selenium species in a mixture form.



(a) Sodium selenite 10ppb (RT: 1.6)

(b) SeMet 10ppb (RT: 2.6)



(c) SeCys 10ppb (RT: 1.38)

(d) SeMetSeCys 10ppb (RT: 1.8)

Figure 19: Chromatogram of standard solution (10ppb) of sodium selenite, SeMet, SeCys and SeMetSeCys with their peaks and retention time.

Table 17: Concentration and recovery of SeMet (μg/L) from fish feed and blue mussel samples after spiking with SeMet standard

Sample matrix	**Sample conc. (μg/L)**	**Spiked before extraction**			**Spiked after extraction**		
		Measured conc. (μg/L)	**Conc. after blank correction (μg/L)**	**Recovery (%)**	**Measured conc. (μg/L)**	**Conc. after blank correction (μg/L)**	**Recovery (%)**
Fish feed	2.83	8.24	5.28	106	7.88	4.91	98
	3.21	8.18	5.22	104	9.07	6.10	122
	2.85	8.99	6.03	121	9.12	6.15	123
Average fish feed	2.97 ± 0.17	8.47 ± 0.37	5.51 ± 0.37	110 ± 7	8.69 ± 0.57	5.72 ± 0.57	114 ± 12
Blue mussel	2.60	6.60	4	80	7.37	4.77	95
	-	8.44	5.84	117	7.47	4.88	98
	-	-	-	-	6.94	4.34	87
Average blue mussel	2.60	7.51	4.92	98	7.26 ± 0.23	4.66 ± 0.23	93 ± 4.61

4.5 Application of method: A study of selenomethionine in rainbow trout *(Oncorhynchus mykiss)* whole body and feed samples

The recently validated method was applied to samples to determine the concentrations of SeMet in samples from an experimental trial [112]. Rainbow trout feed (n = 12) and rainbow trout tissue (n = 13) samples from an experimental trial (Table A 1 and Table A 2) was used to determine the total Se concentration by ICP-MS (Figure 11 and Figure 12). SeMet concentrations were then determined using the speciation method by HPLC ICP-MS.

4.5.1 Selenium speciation in rainbow trout experimental feed samples

The results presented in Table 18 showed the concentration of SeMet and the extent of recovery of SeMet in rainbow trout experimental feed samples (n = 12). For vegetal meal-based experimental feeds, the SeMet concentration ranged from 0.09 to 0.72 mg/kg with an average concentration of 0.31 mg/kg. For tuna meal-based experimental feeds, the SeMet concentration ranged from 0.24 to 1.30 mg/kg with an average concentration of 0.54 mg/kg. The recovery of SeMet from vegetal meal-based feeds ranged from 4% to 32%, with an average recovery of 19%. In the case of tuna meal-based feed, the recovery of SeMet ranged from 2.5% to 14%, with an average recovery of 6.2%. Overall, in both groups of experimental feed, SeMet content varied between 0.09 – 1.30 mg/kg, whereas the recovery ranged from 2.5% to 32%.

The tuna meal-based feed supplemented with organic Se exhibited the highest SeMet concentration of 1.30 mg/kg, as observed in Figure H 1 (Appendix-H). This was followed by another sample of tuna meal-based feed supplemented with organic Se and organic Hg, which had a SeMet concentration of 0.96 mg/kg. In contrast, the lowest concentration was 0.09 mg/kg of SeMet was observed in a vegetal meal-based feed without any supplementation. This was followed by another sample from the same feed group, which had a concentration of 0.11 mg/kg after being supplemented with organic Hg (Figure H 1, Appendix-H). The vegetal meal-based feed sample supplemented with organic Se and organic Hg exhibited the highest recovery rate of 32%, as indicated in Table 18. In general, vegetal meal-based feeds supplemented with organic Se showed the higher recovery of SeMet (Table 18).

When comparing the results of tuna meal-based feeds, it was observed that the feed samples exhibited a recovery rate of 2.5% and 2.8% for SeMet when supplemented with inorganic Se. In contrast, the recovery of SeMet in tuna meal-based feed samples was 11% and 14%, which were supplemented with organic Se, as indicated in Table 18. On the other hand, the vegetal control and tuna control samples, exhibited recovery rates of 20% and 3.4%, respectively. Overall, the estimated average content of SeMet (0.54 mg/kg) was higher in experimental feed derived from tuna meal than the vegetal meal-based feeds (0.31 mg/kg).

The results showed that vegetal meal-based meals exhibited a greater recovery of SeMet compared to tuna meal-based diets (Table 18). However, it is noteworthy that the overall total concentration of Se was found towards higher side in feed containing tuna meal compared to those based on vegetal meal, as seen in Table 18. But the previous study could explain these observations as the basal Se levels responsible for 80% of the total Se concentrations found in Se supplemented tuna meal-based feeds [124, 128]. And the suspected primary form in tuna would be selenoneine (organic Se) [124, 128] but our study was focused on specific organic form of Se (SeMet). Moreover, results regarding recovery could imply that the enzymatic extraction method used in this study is more suited and compatible with vegetal meal-based feed than tuna meal-based feeds.

Table 18: Concentration of total Se and SeMet (mg/kg) in Rainbow Trout experimental feed

Rainbow Trout feed	**Mean conc. of total Se (mg/kg ww) ± SD**	**Mean conc. of SeMet (mg/kg ww) ± SD**	**Recovery of SeMet from total Se (%)**
Vegetal control (VC)	0.46 ± 0.03	0.09 ± 0.00	20
Vegetal inorganic Se (VI)	3.17 ± 0.06	0.130 ± 0.003	4
Vegetal organic Se (VO)	2.37 ± 00	0.72 ± 0.03	30
Vegetal organic Hg (VH)	0.53 ± 0.06	0.112 ± 0.002	21
Vegetal organic Hg – inorganic Se (VHI)	2.13 ± 0.01	0.126 ± 0.002	6
Vegetal organic Hg – organic Se (VHO)	2.15 ± 0.06	0.686 ± 0.005	32
Tuna control (TC)	7.37 ± 0.08	0.25 ± 0.02	3
Tuna inorganic Se (TI)	9.17 ± 0.02	0.260 ± 0.001	3
Tuna organic Se (TO)	9.04 ± 0.01	1.30 ± 0.03	14
Tuna organic Hg (TH)	7.353 ± 0.001	0.25 ± 0.01	3
Tuna organic Hg – inorganic Se (THI)	9.51 ± 0.01	0.24 ± 0.01	3
Tuna organic Hg – organic Se (THO)	8.84 ± 0.27	0.965 ± 0.005	11

4.5.2 Rainbow Trout whole-body samples

The validated method was also applied to rainbow trout whole-body samples to determine the concentration of SeMet. The findings presented in Table 19 showed the estimated concentration and recovery of SeMet in whole-body samples (n = 13) of rainbow trout. For the rainbow trout group fed on vegetal meal-based experimental feeds, the SeMet concentration ranged from 0.08 to 0.44 mg/kg with an average concentration of 0.20 mg/kg. The other group of rainbow trout fed on tuna meal-based experimental feeds, the SeMet concentration ranged from 0.15 to 0.43 mg/kg with an average concentration of 0.25 mg/kg. The recovery of SeMet from rainbow trout reared on vegetal meal-based feeds ranged from 6% to 25%, with an average recovery of 18%. In the case of rainbow trout offered the tuna meal-based feed, the recovery of SeMet ranged from 10% to 17%, with an average recovery of 14%. Overall, in both groups of rainbow trout, SeMet content varied between 0.08 – 0.44 mg/kg, whereas the recovery ranged from 6% to 25% (Table 19).

The sample belonging to the group that consumed a vegetal meal-based diet supplemented with organic Se and organic Hg exhibited (Figure I 1, Appendix-I) the highest concentration of SeMet (0.44 mg/kg) and the highest recovery (25%). A similar pattern, as mentioned earlier, was observed in the rainbow trout sample, which exhibited a SeMet content of 0.43 mg/kg and a recovery of 17% (Table 19). This group was fed on a tuna meal-based diet that was supplemented with organic selenium (Figure I 1, Appendix-I).

The rainbow trout samples, which were fed on vegetal meal-based diets supplemented with inorganic Se and inorganic Se with organic Hg, had SeMet recovery of 6% and 12%, respectively (Table 19). The rainbow trout samples, which were given feeds primarily composed of vegetal meal and supplemented with an organic Se and organic Se with organic Hg, exhibited similar recovery of 25% of SeMet. In contrast, the whole-body samples derived from rainbow trout that were provided with diets based on tuna meal and supplemented with inorganic Se with or without organic Hg also exhibited a similar recovery (10%) of SeMet (Table 19). The rainbow trout samples, which were fed tuna meal-based feeds supplemented with organic Se, exhibited a recovery of 17% in whole-body sample.

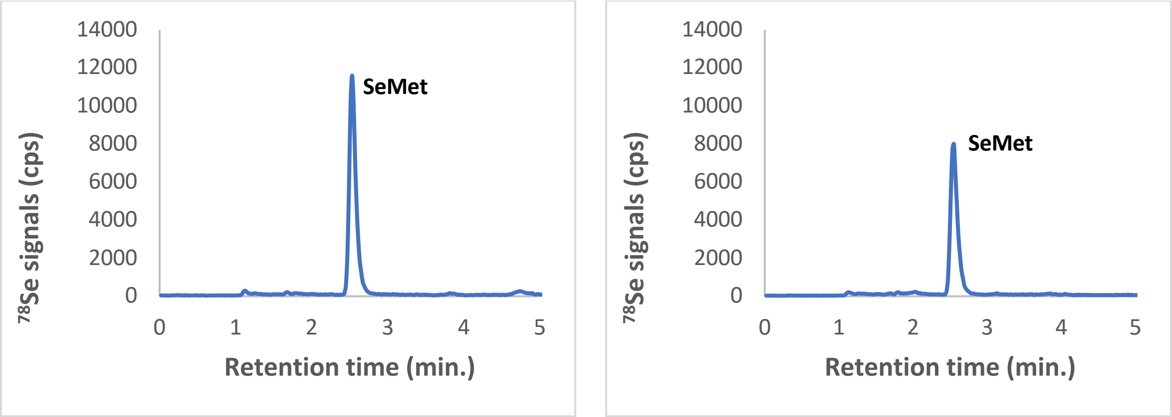
In addition, the chromatograms obtained from the analysis of rainbow trout experimental feeds and whole-body tissue exhibited prominent and well-resolved peaks corresponding to SeMet. These peaks were clearly distinguishable from the baseline in both vegetal meal-based (Figure 20a) and tuna meal-based (Figure 20b) diets. Similarly, the trout fed on vegetal meal-based (Figure 20c) and tuna meal-based (Figure 20d) diets. However, the chromatograms of these samples also revealed the presence of some additional peaks other than the SeMet but still the peak of latter was well separated from other peaks with different retention time. The results of this study demonstrate that the employed extraction process and chromatographic approach are effective in extracting and isolating the organic selenium species (SeMet) from complex matrices.

Literature showed that Se species can be involved in the detoxification of Hg, reducing the exposure of animals and humans to MeHg poisoning [74, 75]. Recent studies on rainbow trout discovered that Se interacts with Hg bioaccumulation and has an antagonistic interaction between the two [78, 79]. In diets based on plants or tuna meal, various kinds of Se supplementation have distinct effects on metabolism and the biological effects of dietary MeHg. Dietary inorganic Se impacted MeHg metabolism by lowering Hg accumulation in fish. However, dietary organic Se showed a more robust capacity to defend against the pro-inflammatory effects of MeHg [124]. Thus, the results obtained from this study on total Se and SeMet concentrations can be further used to elucidate the interaction of Se species (SeMet) with species of Hg.

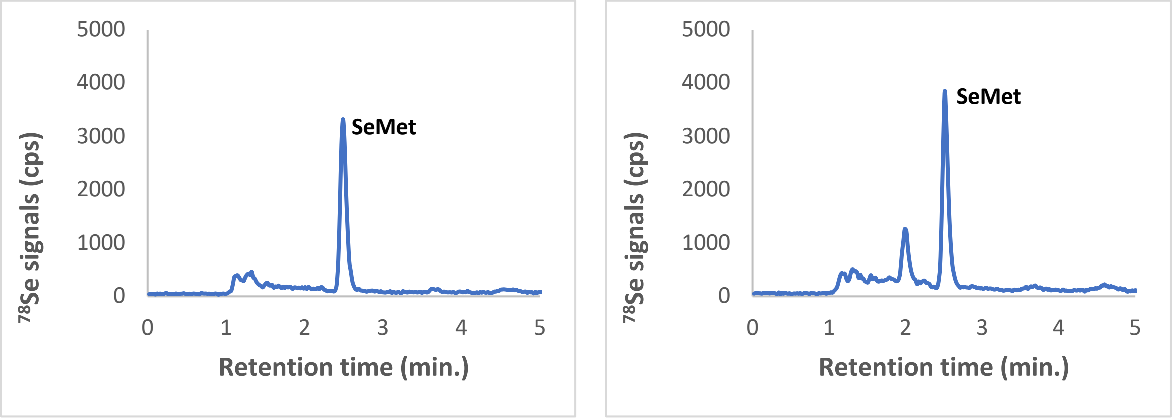
Overall, the percentage recovery of SeMet (organic Se) obtained from rainbow trout is relatively low compared to total Se concentrations in those samples. This finding highlights the presence of other forms of Se (inorganic or organic) in these samples other than SeMet, and this was also witnessed in chromatograms of whole-body samples with additional Se peaks. In addition, literature also showed biotransformation among different species of Se in the body [10, 13, 48, 54, 68, 101, 128]. Thus, further work will be needed to investigate other forms of Se present in the rainbow trout feed and whole-body samples to understand the distribution of total Se.

Table 19**:** Concentration of total Se and SeMet (mg/kg) in Rainbow Trout whole samples

Rainbow Trout whole body simples fed on feed	**Mean conc. of total Se (mg/kg ww) ± SD**	**Mean conc. of SeMet (mg/kg dw) ± SD**	**Recovery of SeMet from total Se (%)**
Initial control	0.553 ± 0.003	0.120 ± 0.001	22
Vegetal control (VC)	0.41 ± 0.04	0.08 ± 0.00	20
Vegetal inorganic Se (VI)	1.34 ± 0.05	0.084 ± 0.001	6
Vegetal organic Se (VO)	1.6 ± 0.1	0.39 ± 0.02	25
Vegetal organic Hg (VH)	0.46 ± 0.02	0.087 ± 0.002	19
Vegetal organic Hg – inorganic Se (VHI)	1.02 ± 0.03	0.127 ± 0.004	12
Vegetal organic Hg – organic Se (VHO)	1.76 ± 0.02	0.44 ± 0.02	25
Tuna control (TC)	1.45 ± 0.00	0.20 ± 0.01	14
Tuna inorganic Se (TI)	1.50 ± 0.06	0.148 ± 0.004	10
Tuna organic Se (TO)	2.46 ± 0.08	0.427 ± 0.007	17
Tuna organic Hg (TH)	1.31 ± 0.07	0.158 ± 0.003	12
Tuna organic Hg – inorganic Se (THI)	1.83 ± 0.03	0.19 ± 0.00	10
Tuna organic Hg – organic Se (THO)	2.17 ± 0.07	0.378 ± 0.009	17



(a) Rainbow trout feed (Vegetal meal-based) (b) Rainbow trout feed (Tuna meal-based)



(c) Rainbow trout tissue (Vegetal meal-based) (d) Rainbow trout tissue (Tuna meal-based)

Figure 20: Chromatograms of Rainbow trout feed and whole-body samples with SeMet eluting at retention time of 2.6 min.

5. CONCLUSIONS

From the total Se determinations in this study, it was seen that Se levels in commercial salmonid feed ranged from 0.38 to 2.14 mg/kg (n = 27). The upper limit (0.5 mg/kg) for total Se in animal feeds was exceeded in 22 feeds. Total Se in salmonid experimental feed ranged from 0.78 to 1.52 mg/kg (n = 6), and all samples exceeded the animal feed maximum of 0.5 mg/kg. Rainbow trout feed contained 7.35–9.51 mg/kg total Se and 0.46–3.17 mg/kg tuna meal and vegetal meal-based feeds. The total Se concentration was higher in tuna meal-based feeds than in vegetal meal-based feeds. Rainbow trout feeds showed higher Se content than 0.5 mg/kg, possibly due to Se supplementation in experimental feeds.

In land animal commercial feeds, ruminant feeds showed 0.45 to 1.70 mg/kg Se (n = 10). Live yeast supplementation for lactating cows possibly resulted into the highest concentration of 1.70 mg/kg in one sample. Piglet feed samples had 0.33 mg/kg total Se (n = 2), and chicken feed samples had 0.32–0.34 Se mg/kg (n = 4). Se concentrations in land animal feed samples were lower, especially in swine and poultry feeds. In feed materials, total Se concentrations ranged from 1.70 to 4.55 mg/kg (n = 3) for fish meal and 0.14 to 0.33 mg for insect meal. Tuna and plant meals had 8–12 mg/kg (n = 2) and 0.47 mg/kg (n = 1), respectively. Tuna meal and fish showed higher total Se than other feed materials.

Rainbow trout whole-body samples had total Se content of 0.40 to 1.61 mg/kg and 1.35 to 2.41 mg/kg, fed on vegetal and tuna meal-based experimental feeds, respectively. Fish fed tuna meal-based feed supplemented with organic Se showed the highest total Se concentration of 2.41 mg/kg and similarly, a group fed tuna meal with organic Hg and Se had 2.16 Se mg/kg. Overall, trout fed tuna meal-based diets had higher total Se concentrations in their whole-body tissue samples than those fed vegetal meal-based diets.

The method was validated for speciation analysis using HPLC and ICP-MS for feed and tissue samples. Validated method showed good selectivity for CRMs, fish feed, tuna, and salmon tissue. These samples exhibited good SeMet chromatographic peak resolution, baseline separation at 2.6 min, and no Se species interference. However, zebrafish feed, land-animal feed, and blue mussel chromatograms showed unknown Se species peaks (eluted 1–2 min before SeMet, so they did not overlap). Additionally, the extraction method did not cause any chromatographic analysis interferences other than SeMet. Each analyte in the spike mix with selected organic and inorganic Se species had clear and distinct peaks on the chromatograph. The method was able to selectively detect SeMet in CRMs, animal feeds, and tissue samples.

Method showed good concentration-response linearity. The regression coefficient (r) for the linear curve was 0.99, indicating a linear fit. Normal and log concentration versus residuals plots showed random residual distributions. From blank signals, LOD and LOQ were estimated at 0.02 and 0.06 mg/kg, respectively. This method improved LOD and LOQ estimates and covered the legislative limit for supplementation of organic Se in feed.

For trueness studies, the method showed recovery rates of 53% to 80% for wheat-based CRM (ERM BC210a). Selenized yeast-based CRM (SELM-1) showed recovery rates from 72% to 83%. However, the observed recovery for the ERM BC210a was not satisfactory as compared with SELM-1. SeMet spiking recovery was 110% and 114% in feed sample before and after extraction while recovery in tissue sample was 98% and 93% before and after extraction.

The validated method's RSD for repeatability and intermediate precision was 4.4% and 15.7% for ERM BC210a and 7.1% and 7.5% for SELM-1. Fish tissue showed the lowest within-day variation and land-animal feed the highest. Overall, fish feed, tuna tissue, salmon tissue, and SELM-1 showed better precision. The Horwitz ratio is found within the range of 0.5–2, which reflects the acceptability of the analytical method in terms of the precision of the method.

MU (%) was highest in land-animal feed and lowest in salmon tissue (13%). Compared to SELM-1, ERM BC210a showed higher MU (%). Fish feed, zebrafish feed, tuna tissue, and blue mussel showed MU (%) within target range. These findings also showed that matrix effects strongly affected the reported results of MU (%).

The validated method was applied on rainbow trout experimental feeds supplemented with organic or inorganic Se and rainbow trout whole-body samples. For rainbow trout experimental feeds, vegetal and tuna meal-based feeds showed SeMet averages of 0.31 and 0.54 mg/kg. The average SeMet recovery in vegetal and tuna meal-based feeds was 19% and 6.2%. Overall, the vegetal meal-based meals exhibited a greater recovery of SeMet compared to tuna meal-based diets. However, the total concentration of Se was found higher in feed containing tuna meal compared to those based on vegetal meal. These findings could imply that the enzymatic extraction method used in this study is more suited and compatible with vegetal meal-based feed than tuna meal-based feeds. But it also highlights the presence of some other inorganic and organic Se species, which contribute to total Se content.

The vegetal and tuna meal-fed rainbow trout whole-body samples showed SeMet average concentrations of 0.20 mg/kg and 0.25 mg/kg, respectively. The average SeMet recovery from rainbow trout fed vegetal or tuna meal was 18% and 14%, respectively. The group fed on vegetal meal-based diet with organic Se and Hg had the highest SeMet concentration (0.44 mg/kg) and recovery (25%). A rainbow trout sample fed a tuna meal-based diet with organic selenium had a SeMet content of 0.43 mg/kg and a recovery of 17%.

The present study provided an overview of the total Se content in feeds used for both commercial and experimental purposes for marine and land animals. Higher Se concentrations in some feeds raised concerns about risks to animal and public health as well as compliance with current legal requirements. The in-house laboratory method for Se speciation in feed and tissue showed good potential in the method validation results. The method's application demonstrated potential applicability on diverse sample matrices. However, further improvements will be needed, especially for feeds made with fish meal and for land animals.

6. FUTURE PERSPECTIVES

Further optimization of extraction method

The current study allowed for extraction method improvements, especially for ruminant and fish meal-based feeds. To improve SeMet recovery, trypsin, proteinase k, flavourzyme, and pancreatin could be studied. It may also be important to compare protease with synergistic enzymes with the addition of various factors like sonication, extraction solutions, extraction time, and extraction temperatures. In addition, considering the other organic forms of Se will be beneficial for better understanding the contribution of different species in total Se content.

Development work on reference materials for feed and feed materials

Trueness and precision studies were showed limitation due to the lack of better reference materials. Thus, developing in-house reference materials from animal feed and tissue may aid method optimisation and validation.

Surveillance study for total Se in land animal's feed

The lack of sufficient current literature on total Se in ruminant, swine, and poultry feed necessitates surveillance studies. These studies will help determine Se levels and feed supplementation options. Such programmes will facilitate risk assessment studies for more rational animal health regulations.

Correlation study between selenium and mercury at species level

Literature showed a significant correlation between Se and Hg, which may help explain their toxicological and biological interactions. However, this interaction can be further narrowed down by species (MeHg, SeMet, SeCys, selenite or selenate) for better understanding and risk assessment.

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8. APPENDIX

Appendix A – Experimental diets for Rainbow trout

Table A 1: Composition of experimental feeds for rainbow trout (TuneSel project) [124]

Vegetal meal-based feeds		Tuna meal-based feeds	
Ingredients	**%**	**Ingredients**	**%**
Plant meals: wheat & corn gluten	73.8	Fish meal from tuna byproducts	60.0
Crystalline amino acid mix	3.2	Dehulled pea meal	10.8
Rapeseed lecithin	2.0	Whole wheat	15.0
Vegetable & fish oil	16.0	Vegetable & fish oil	12.0
Vitamin premix	1.0	Vitamin premix	1.0
Mineral premix without Se	4.0	Mineral premix without Se	1.2

Table A 2: Experimental feeds for rainbow trout with supplementation of Se and Hg and relationship with rainbow trout tissues samples [124]

Experimental diets	Description of diet	Supplementation level (mg/kg)		Rainbow trout tissue samples
		Hg	Se	
Vegetal meal-based feeds				
Diet A	Vegetal control (VC)	-	-	RT1 – RT3
Diet B	Vegetal inorganic Se (VI)	-	1.5	RT4 – RT6
Diet C	Vegetal organic Se (VO)	-	1.5	RT7 – RT9
Diet D	Vegetal organic Hg (VH)	2.0	-	RT10 – RT12
Diet E	Vegetal organic Hg – inorganic Se (VHI)	2.0	1.5	RT13 – RT15
Diet F	Vegetal organic Hg – organic Se (VHO)	2.0	1.5	RT16 – RT18
Tuna meal-based feeds				
Diet G	Tuna control (TC)	-	-	RT19 – RT21
Diet H	Tuna inorganic Se (TI)	-	1.5	RT22 – RT24
Diet I	Tuna organic Se (TO)	-	1.5	RT25 – RT27
Diet J	Tuna organic Hg (TH)	1.6	-	RT28 – RT30
Diet K	Tuna organic Hg – inorganic Se (THI)	1.6	1.5	RT31 – RT33
Diet L	Tuna organic Hg – organic Se (THO)	1.6	1.5	RT34 – RT36

Appendix B – Comparative overview of total Se concentration (mg/kg) in aquatic and land animals feeds

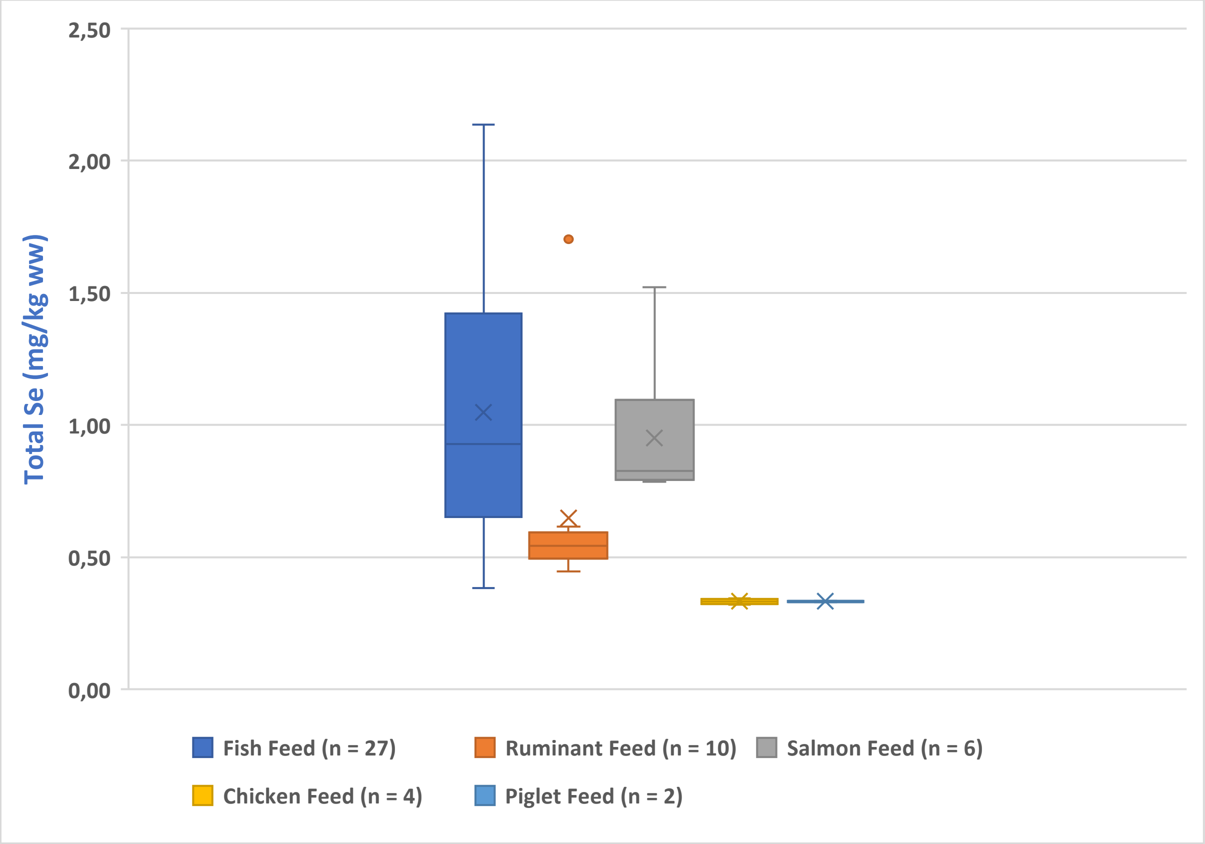

Figure B 1: Boxplot of aquatic and land animals feeds with total Se concentration (mg/kg)

Appendix C – Comparative overview of total Se concentration (mg/kg) in Rainbow trout whole-body samples with vegetal and tuna-meal based diets

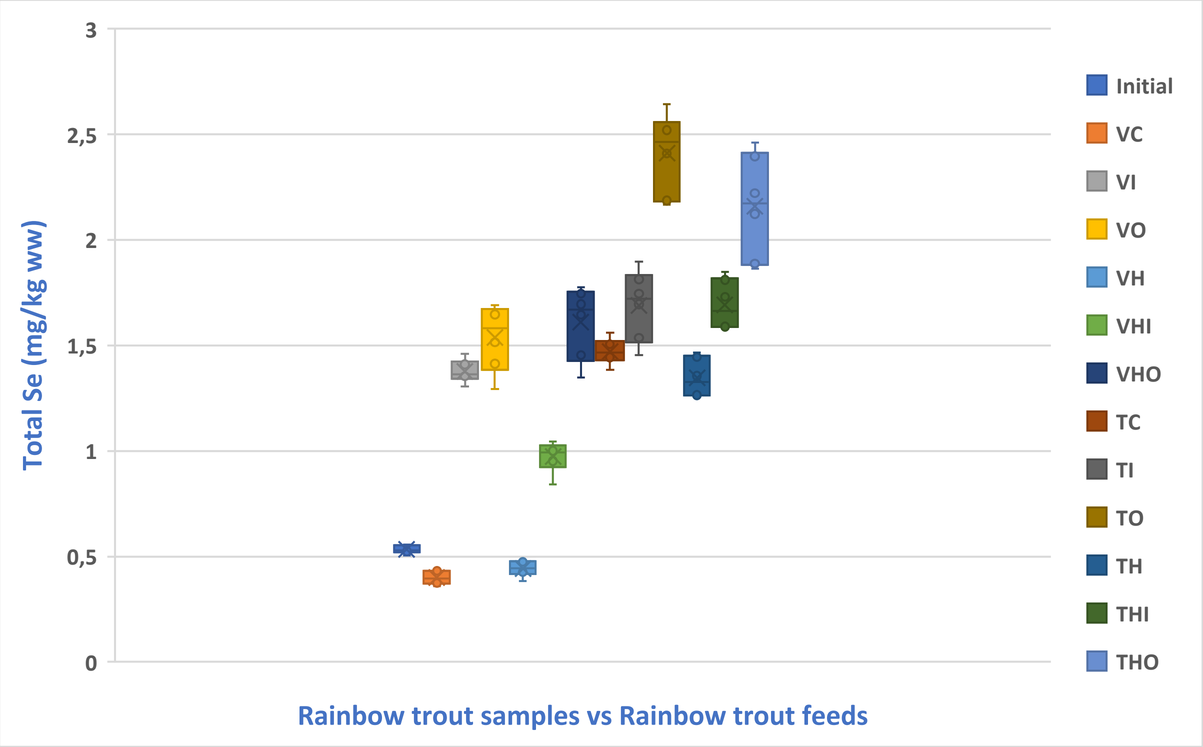

Figure C 1: Boxplot of rainbow trout whole-body samples (biological groups n = 13, n = 6 replicates), with rainbow trout feeds and total Se (mg/kg)

Appendix D – Measurements of reagent blind for estimation of LOD and LOQ

Table D 1: Measurement of signals for SeMet (µg/L) for estimation of LOD and LOQ

Reagent blind	SeMet conc. (µg/L)
1	1.28
2	0.70
3	1.40
4	0.91
5	1,.36
6	1.18
7	1.50
8	1.42
9	1.27
10	1.25
N=	10
Mean=	1.23
SD=	0.25
LOD=	0.74
LOQ=	2.47

Appendix E – Linearity of method with residual plot between log concentration of SeMet (μg/L), responses (peak area), with residuals.

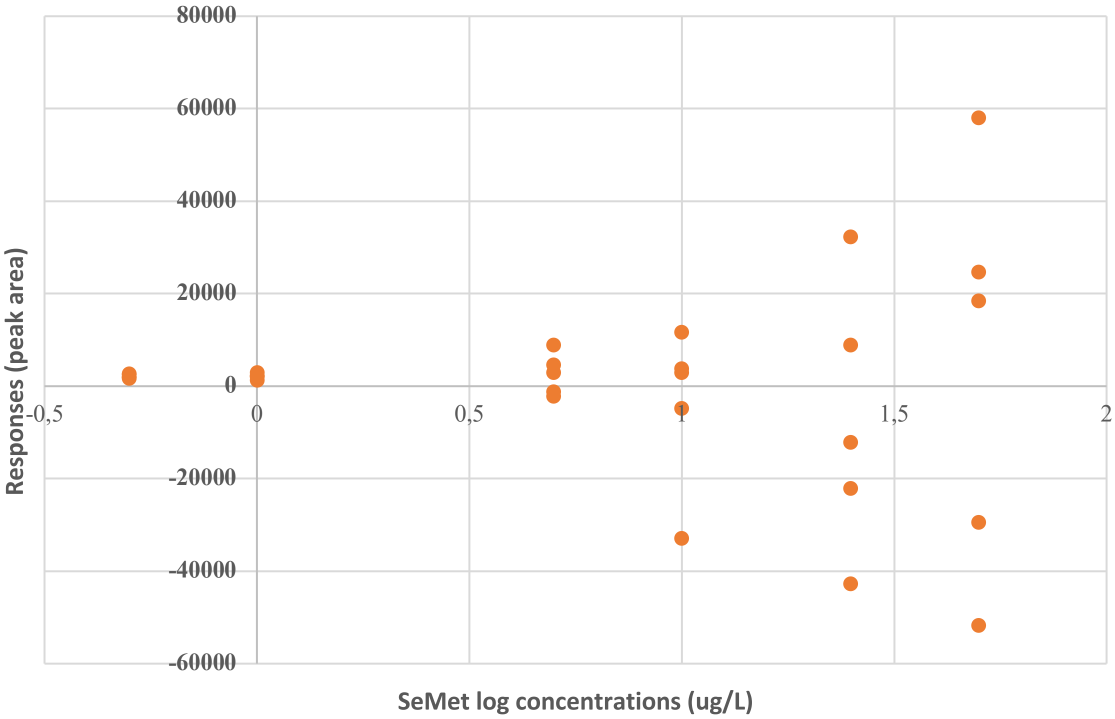

Figure E 1: Residual plot between log concentration of SeMet (μg/L), responses (peak area), with residuals.

Appendix F – Concentrations and recovery of SeMet (mg/kg) in CRMs

Table F 1: Concentration and recovery of SeMet (mg/kg) in CRM ERM BC 210a (wheat)

Day	**Analyst**	**CRM 1 (ERM BC 210a)**			
		Parallel 1 SeMet Conc. (mg/kg)	**Parallel 2 SeMet Conc. (mg/kg)**	**Mean Conc. (mg/kg) ± SD**	**Recovery (%)**
1	**A**	5.95	5.63	5.79 ± 0.16	53
2	**A**	7.16	6.67	6.92 ± 0.25	63
3	**B**	8.66	8.90	8.78 ± 0.12	80
4	**A**	6.45	6.94	6.70 ± 0.25	61
5	**A**	7.42	6.85	7.14 ± 0.29	65

Table F 2: Concentration and recovery of SeMet (mg/kg) in CRM SELM-1 (selenized yeast)

Day	**Analyst**	**CRM 2 (SELM-1)**			
		Parallel 1 SeMet Conc. (mg/kg)	**Parallel 2 SeMet Conc. (mg/kg)**	**Mean Conc. (mg/kg) ± SD**	**Recovery (%)**
1	**A**	924.51	930.57	927.54 ± 3.03	72
2	**A**	1030.49	915.08	972.79 ± 68.55	76
3	**B**	1130.99	993.88	1062.43 ± 0.12	83
4	**A**	985.04	883.52	934.28 ± 50.76	73
5	**A**	996.69	926.90	961.79 ± 34.89	75

Appendix G – Concentrations of SeMet (mg/kg) in feed and tissue samples

Table G 1: Concentrations of SeMet (mg/kg) in fish feed sample (SMPL-1)

Sr. No.	Day	Analyst	Parallel 1 SeMet Conc. (mg/kg)	Parallel 2 SeMet Conc. (mg/kg)	Mean conc. (mg/kg) ± SD
1	1	A	0.1625	0.1782	0.170 ± 0.008
2	2	A	0.1622	0.1687	0.165 ± 0.003
3	3	B	0.1627	0.1626	0.163 ± 0.00
4	4	A	0.1584	0.1593	0.1589 ± 0.0004
5	5	A	0.1392	0.1327	0.136 ± 0.003

Table G 2: Concentration of SeMet (mg/kg) in zebrafish feed sample (SMPL-2)

Sr. No.	Day	Analyst	Parallel 1 SeMet Conc. (mg/kg)	Parallel 2 SeMet Conc. (mg/kg)	Mean conc. (mg/kg) ± SD
1	1	A	0.0689	0.0699	0.069 ± 0.00
2	2	A	0.0737	0.0793	0.076 ± 0.003
3	3	B	0.0783	0.0849	0.082 ± 0.003
4	4	A	0.0723	0.0810	0.077 ± 0.004
5	5	A	0.0626	0.0608	0.062 ± 0.001

Table G 3: Concentration of SeMet (mg/kg) in land-animal feed sample (SMPL-3)

Sr. No.	Day	Analyst	Parallel 1 SeMet Conc. (mg/kg)	Parallel 2 SeMet Conc. (mg/kg)	Mean conc. (mg/kg) ± SD
1	1	A	0.045	0.065	0.055 ± 0.009
2	2	A	0.075	0.076	0.0752 ± 0.0006
3	3	B	0.077	0.055	0.07 ± 0.01
4	4	A	0.021	0.077	0.05± 0.03
5	5	A	0.068	0.068	0.0682 ± 0.0001

Table G 4: Concentration of SeMet (mg/kg) in salmon tissue sample with low Se (SMPL-4)

Sr. No.	Day	Analyst	Parallel 1 SeMet Conc. (mg/kg)	Parallel 2 SeMet Conc. (mg/kg)	Mean conc. (mg/kg) ± SD
1	1	A	0.291	0.284	0.287 ± 0.003
2	2	A	0.292	0.287	0.289 ± 0.003
3	3	B	0.288	0.335	0.31 ± 0.02
4	4	A	0.311	0.308	0.309 ± 0.002
5	5	A	0.271	0.274	0.273 ± 0.001

Table G 5: Concentration of SeMet (mg/kg) in salmon tissue sample with high Se (SMPL-5)

Sr. No.	Day	Analyst	Parallel 1 SeMet Conc. (mg/kg)	Parallel 2 SeMet Conc. (mg/kg)	Mean conc. (mg/kg) ± SD
1	1	A	0.8376	0.7571	0.78 ± 0.04
2	2	A	0.8157	0.7450	0.78 ± 0.04
3	3	B	0.7351	0.7531	0.74 ± 0.01
4	4	A	0.7963	0.7957	0.80 ± 0.00
5	5	A	0.7194	0.6724	0.70 ± 0.02

Table G 6: Concentration of SeMet (mg/kg) in tuna fish tissue sample (SMPL-6)

Sr. No.	Day	Analyst	Parallel 1 SeMet Conc. (mg/kg)	Parallel 2 SeMet Conc. (mg/kg)	Mean conc. (mg/kg) ± SD
1	1	A	1.026	1.025	1,03 ± 0.00
2	2	A	1.261	1.014	1,14 ± 0.12
3	3	B	1.297	1.223	1,26 ± 0.04
4	4	A	1.113	1.083	1,10 ± 0.01
5	5	A	1.109	1.036	1,07 ± 0.04

Table G 7: Concentration of SeMet (mg/kg) in blue mussel tissue sample (SMPL-7)

Sr. No.	Day	Analyst	Parallel 1 SeMet Conc. (mg/kg)	Parallel 2 SeMet Conc. (mg/kg)	Mean conc. (mg/kg) ± SD
1	1	A	0.220	0.209	0.215 ± 0.005
2	2	A	0.235	0.218	0.226 ± 0.009
3	3	B	0.267	0.293	0.28 ± 0.01
4	4	A	0.256	0.237	0.25 ± 0.01
5	5	A	0.224	0.220	0.222 ± 0.002

Appendix H – Concentrations of SeMet (mg/kg) in Rainbow trout feed samples

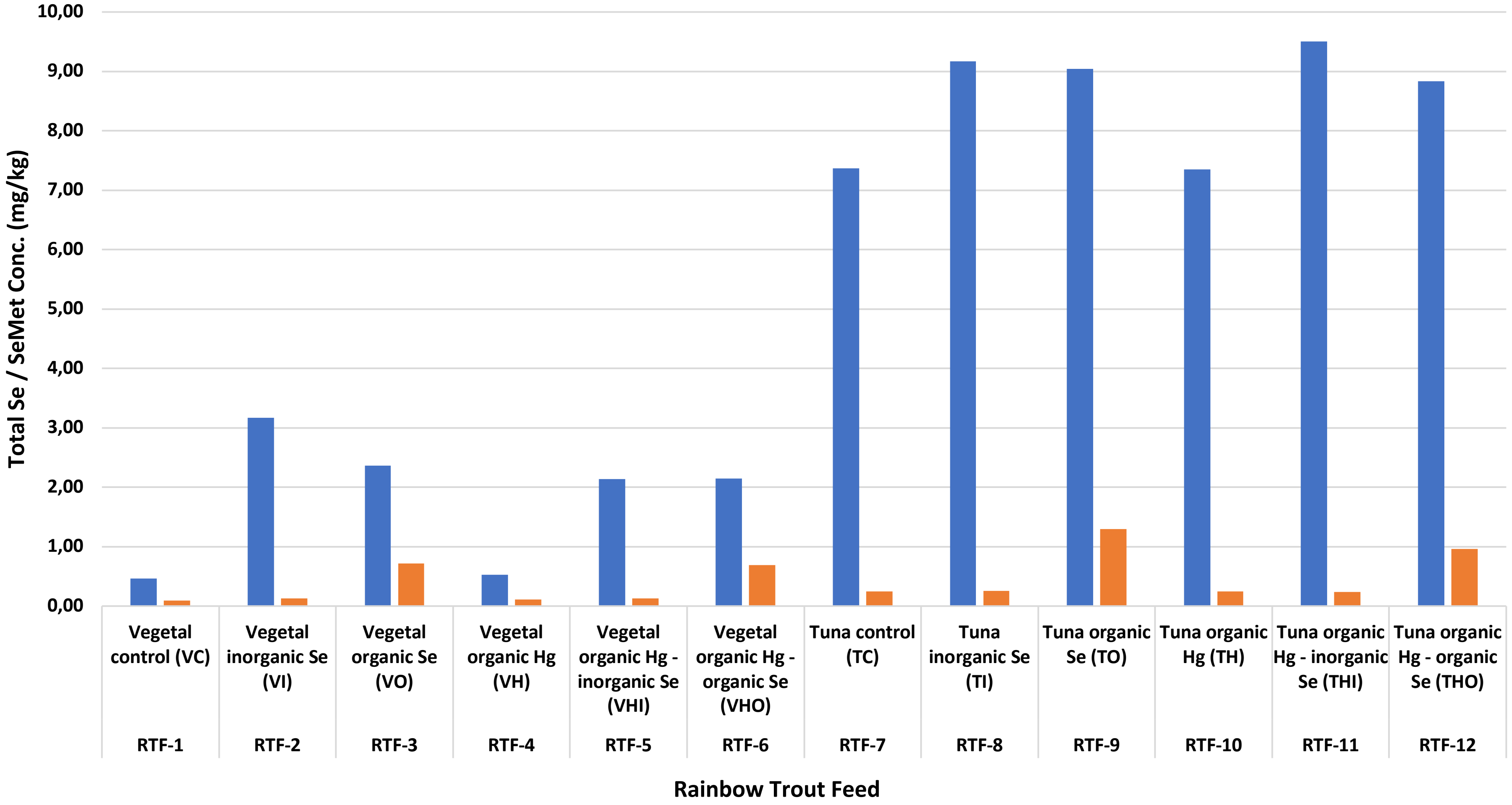

Figure H 1: Concentration of total Se and SeMet (mg/kg) in Rainbow trout experimental feed samples

Appendix I – Concentrations of SeMet (mg/kg) in Rainbow trout whole-body samples

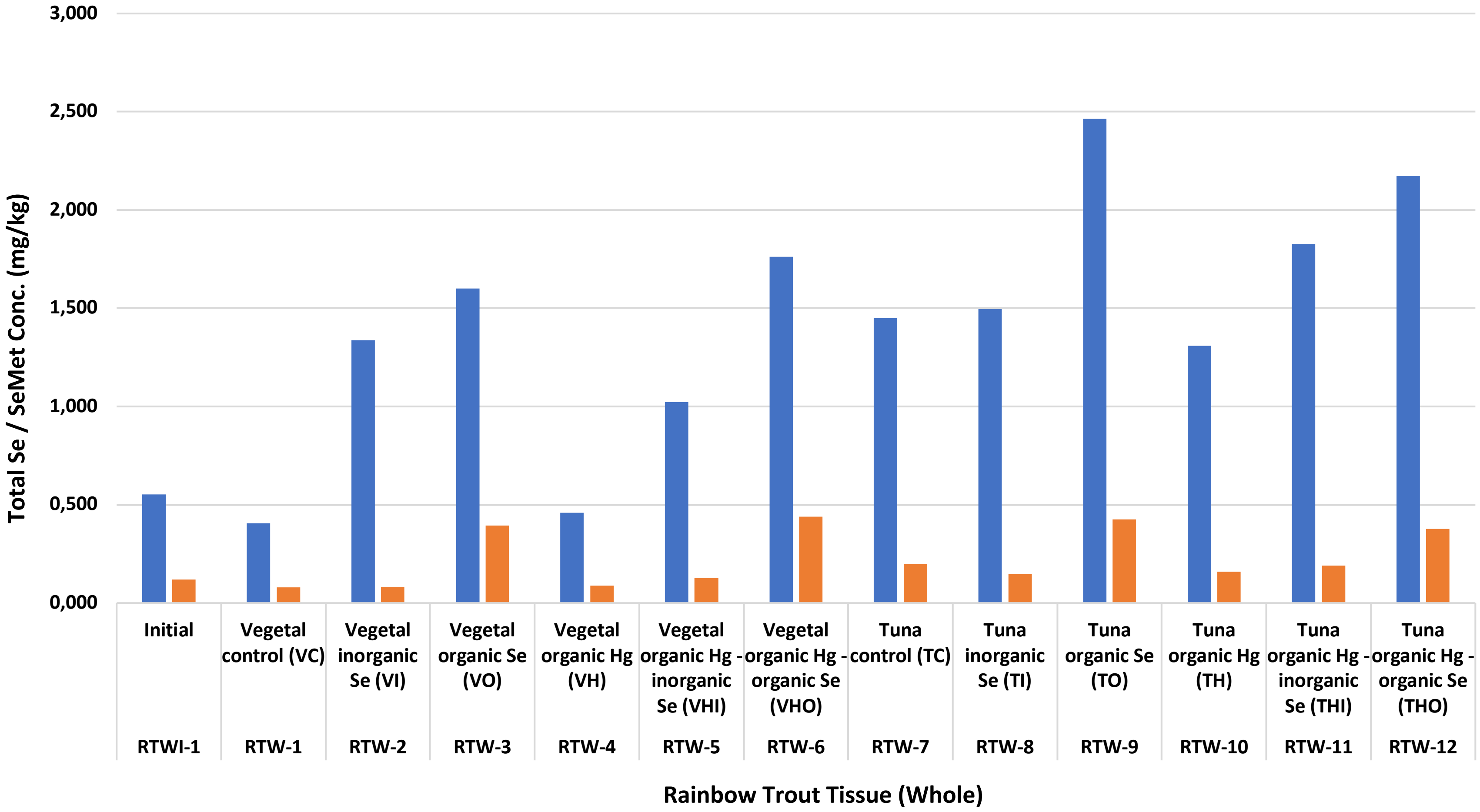

Figure I 1: Concentration of total Se and SeMet (mg/kg) in Rainbow trout whole-body samples