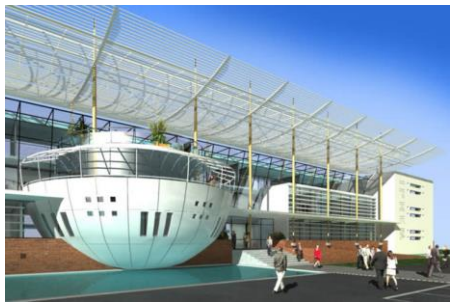

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OPTIMIZATION OF SAMPLE PREPARATION FOR THE ANALYSIS OF SELENIUM METABOLITES IN TROUT

MASTER'S PROJECT

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Contents

1. ABSTRACT

When speciation of ultratrace level compounds is performed, sample preparation often results to be a crucial step in the analysis. The use of high performance liquid chromatography (HPLC) also requires in most cases a sample whose matrix was purified in order to preserve the chromatographic support and thus ensure a proper separation. Analysis of selenium metabolites in blood is a challenge because of the very low concentrations found and the presence of proteins that deteriorate the chromatographic separation.

The aim of this work was to optimize the sample preparation of trout whole blood and blood plasma in order to minimize loss of analytes usually observed during the deproteinization step. In this context, two deproteinization reagents were tested and compared. The losses of analytes were evaluated by monitoring two isotopically enriched Se tracers (^{77}Se-selenite and ^{76}Se-selenomethionine) during the precipitation of proteins. These tracers were quantified after deproteinization of the sample in both the supernatant and the pellet by inductively coupled plasma-mass spectrometry (ICP-MS) and reverse isotope dilution (RID). The results showed that the losses caused by the conventionally used deproteinization reagent were in the order of 20 % higher than those obtained with an alternative reagent in trout blood plasma sample. On the other hand, the losses in trout whole blood sample were slightly higher with the conventionally reagent than with the alternative one. Both samples, trout whole blood and blood plasma with the added Se-enriched tracers, were compared by size exclusion chromatography (SEC) with ultraviolet (UV) detection and SEC-ICP-MS. Moreover, qualitative analysis of selenometabolites by HPLC-ICP-MS in trout tissues (liver, kidney and spleen), using acetonitrile as reagent in the deproteinization step, was carried out.

Keywords: Selenium · Selenium metabolites · Sample preparation · Deproteinization · Trout · Reverse isotope dilution · High performance liquid chromatography-inductively coupled plasma-mass spectrometry

2. INTRODUCTION

2.1. SELENIUM

2.1.1. BRIEF HISTORY OF SELENIUM

Selenium was discovered in 1817 by Jacob Berzelius and Johan Gottlieb Gahn. Both Swedish chemists noted that a reddish sludge (it was the source of selenium), obtained in burning pyrite (an iron sulfide), occurred in the lead chamber used to produce sulfuric acid. When analyzing the reddish sludge, Berzelius discovered the presence of an unknown substance with properties very much like those of tellurium (named for the Earth). Berzelius chose the name Selenium (Greek goddess 'Selene' meaning "Moon") for the new element, noting its resemblance to tellurium [1].

Later, enhanced analysis techniques allowed to detect the presence of this chemical element in different minerals (ferroselite ($FeSe_2$), tiemannite (HgSe), ...), but always in extremely small quantities.

2.1.2. CHEMISTRY OF SELENIUM

Selenium is a chemical element with symbol Se and its standard atomic weight is 78.96 u. Its atomic number is 34 and it is located in group 16, between sulfur and tellurium, and between arsenic and bromine in period 4 [2]. Se is a non-metal (sometimes considered a metalloid) which shares similar chemical properties with especially sulfur and to a lesser extent with tellurium.

The four common oxidation states of Se are: 0 (elemental Se), -2 (Se^{2-}), +4 (SeO_3^{2-}) and +6 (SeO_4^{2-}). Elemental Se is highly insoluble and very stable.

Se has six naturally occurring isotopes, which are listed with their atomic mass and their natural abundance in Table 1 [3]. Five of these are stable: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se and ^{80}Se; whereas that ^{82}Se has a very long half-life (~10^{20} years) and, for practical purposes, can be considered to be stable. Twenty three other unstable isotopes have been characterized.

Table 1 Naturally occurring isotopes of selenium.

Isotope	Atomic mass (u)	Natural abundance (%)
^{74}**Se**	73.922477	0.89
^{76}**Se**	75.919207	9.37
^{77}**Se**	76.919908	7.63
^{78}**Se**	77.917304	23.77
^{80}**Se**	79.916521	49.61
^{82}**Se**	81.916709	8.73

2.1.3. BIOCHEMISTRY OF SELENIUM

Selenium has a major nutritional and biological role in living systems, since it is an essential micronutrient and has been known to be a necessary component of the human diet for many years. It has been recognized as an antioxidant and its presence is related with the reduction of certain types of cancer (chemopreventive agent of cancer) and other diseases.

Se occurs in proteins in the form of the 21st amino acid, selenocysteine (it has a similar structure to cysteine, with an atom of Se taking the place of sulfur); proteins that include selenocysteine are called selenoproteins. In addition, Se occurs in proteins as unspecifically incorporated selenomethionine, which replaces methionine residues; proteins containing such unspecifically incorporated selenomethionine residues are called selenium-containing proteins. Se is present as a necessary component to form the active site, a selenol group (-SeH), in numerous selenoenzymes. In humans, it is a dietary element that functions as a cofactor essential to activity of antioxidant enzymes, such as glutathione peroxidase (GPx), selenoprotein P and certain forms of thioredoxin reductase (TR) found in animals and some plants. Selenium also plays a role in the functioning of the thyroid gland (for example in the iodothyronine deiodinase enzyme (ID)) and in every cell that uses thyroid hormone [4].

2.1.4. SELENIUM TOXICITY AND ITS EFFECTS IN HEALTH

Selenium is an essential trace element with an ambivalent behavior (Fig. 1); it is both essential for most animals as a micronutrient while being highly toxic at higher

concentrations than those required, depending on the species, oxidation state and concentration.

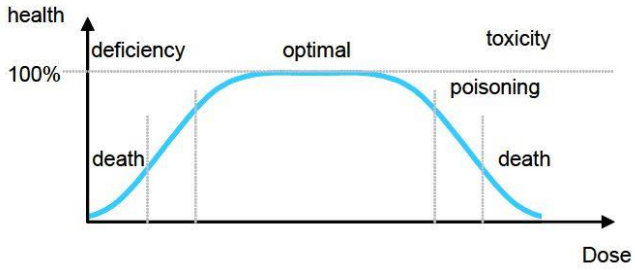

Fig. 1 Dose/health curve of an essential trace element [5].

The beneficial effects of selenium in human health are strongly dependent on its concentration. The concentration range in which selenium is considered toxic or essential is very constricted. It has been estimated that the ingestion of foodstuffs with a selenium content above 1 mg Se kg^{-1} can induce toxicity, meanwhile a concentration below 0.1 mg Se kg^{-1} leads to deficient status. In general, the problems related to selenium toxicity (selenosis) are much lower than those concerning to its deficiency (selenium deficiency).

Besides its concentration, the beneficial effects of selenium on health are also dependent on its chemical form [5,6]. Some selenium compounds or species of interest which are commonly studied in the literature are listed in Table 2.

Table 2 Some inorganic and organic selenium compounds of interest [6].

Chemical name	**Formula**
Hydrogen selenide (selenide)	H_2Se (Se^{2-})
Selenous acid (selenite)	H_2SeO_3 (SeO_3^{2-})
Selenic acid (selenate)	H_2SeO_4 (SeO_4^{2-})
Selenocyanate	HSeCN
Trimethylselenonium cation	$(CH_3)_3Se^+$
Dimethylselenide	$(CH_3)_2Se$
Dimethyldiselenide	$(CH_3)Se\text{-}Se(CH_3)$
Dimethylseleniumsulfide	$(CH_3)Se\text{-}S(CH_3)$
Dimethylseleniumdioxide	$(CH_3)_2SeO_2$
Dimethylselenopropionate	$(CH_3)_2Se^+CH_2CH_2COOH$
Methylselenol	CH_3SeH
Methylseleninic acid	$CH_3Se(O)OH$
Methylselenenic acid	CH_3SeOH
Selenocysteine	$HOOCCH(NH_2)CH_2\text{-}Se\text{-}H$

Table 2 (Continued).

Selenomethylcysteine	$HOOCCH(NH_2)CH_2$-Se-CH_3
Selenocystine	$HOOCCH(NH_2)CH_2$-Se-Se-$CH_2CH(NH_2)COOH$
Selenomethionine	$HOOCCH(NH_2)CH_2CH_2$-Se-CH_3
Selenoethionine	$HOOCCH(NH_2)CH_2CH_2$-Se-CH_2CH_3
γ-Glutamyl-Se-methylselenocysteine	$H_2NCH_2CH_2$-CO-$NHCH(COOH)CH_2$-Se-CH_3
Selenocystathionine	$HOOCCH(NH_2)CH_2CH_2$-Se-$CH_2CH(NH_3)COOH$
Selenohomocysteine	$HOOCCH(NH_2)CH_2CH_2$-Se-H
Se-adenoxylselenohomocysteine	$HOOCCH(NH_2)CH_2CH_2$-Se-$CH_2C_4H_5C_5N_4NH_2$
Selenosugars	Various sugar structures
Selenoproteins	Various proteins and enzymes

2.1.5. SELENIUM IN THE ENVIRONMENT

Selenium is introduced into the environment by both natural processes and by human activity. The chemistry of selenium is complex in both the environment and living systems. Apart from the natural movement of Se, the emission of Se by human activity has to be taken into account and a schematic overview of Se in the environment is given in Fig. 2a.

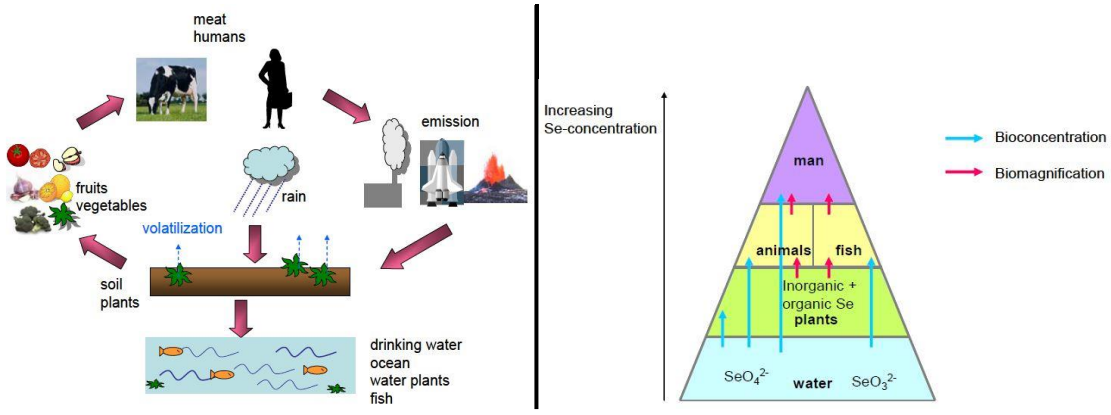

Fig. 2 **(a)** The Se cycle in the environment: Se from soil is utilized by plants, which in turn are consumed by animals and humans; Se can also enter the environment through anthropogenic activity. **(b)** Schematic representation of bioconcentration and biomagnification in the food chain [7].

Notably, selenium can bioaccumulate by means of the food chain (Fig. 2b), and it can be at significant levels for higher animals. Bioaccumulation of Se involves both bioconcentration and the intake of Se via food sources. In the case of aquatic systems,

plankton, plants, algae and invertebrates accumulate selenium, which in turn are eaten by fish and other wildlife. Biomagnification of selenium, which is the accumulation of progressively greater concentrations by successive trophic levels of the food chain, can range from 2 to 6 times between the primary producer (the plants and algae) and the lower consumer (the invertebrates and fish). Ultimately, concentrations in fish that eat contaminated plankton or invertebrates could contain 500 times the selenium concentration of their surrounding water environment [7].

2.2. SELENIUM IN FISH

Selenium is an essential element for fish, since it is a component of several antioxidant enzymes as glutathione peroxidase. Fish can accumulate Se directly from aquatic environment, but the main incorporation pathways are both feeding zoo and phytoplankton through the food chain and diet.

The concentration range for selenium to be considered toxic or beneficial for fish, as for the rest of animals, is quite narrow. Fish require dietary concentrations of 0.1-0.5 mg Se kg^{-1} (dry mass) to maintain their normal growth and physiological functions; however selenium rapidly bioaccumulates and causes toxicity to fish when the dietary concentration reaches ≥3.0 mg Se kg^{-1} (dry mass) [8]. Broadly, liver and kidney are the organs that shown a higher selenium accumulation. Notwithstanding, the metabolic mechanism through which Se induce toxicity in fish is not elucidated, but it has been related with the unspecific incorporation of Se into the proteins.

2.2.1. PHYSIOLOGICAL FUNCTIONS OF SELENIUM IN FISH

Currently, 25 selenoproteins are identified in humans based on a selenoproteome analysis, and their physiological roles and functions are being investigated. However, fish have more selenoproteins than terrestrial animals. The selenoproteomes of fish are greater in number than those of mammals; 30-37 selenoproteins, the selenoproteomes of fish are among the largest known [9].

Selenium in fish, besides its specific functions like being a component of several antioxidant enzymes as glutathione peroxidase, protects cells against oxidation damage from free radicals and reactive oxygen species. In addition to these functions, Se preserves polyunsaturated fatty acids (PUFAs) of the fish thanks to its antioxidant properties. Polyunsaturated fatty acids are fatty acids that contain more than one double bond in their backbone. The more important PUFAs that fish contain, and which are essential for humans (these essential fatty acids are fatty acids that humans and other animals must ingest because the body requires them for good health but cannot synthesize them), are Omega-3 fatty acids (*n*–3 PUFAs) and Omega-6 fatty acids (*n*–6 PUFAs) [10].

2.2.2. FISH IN HUMANS´ DIET

The main source of selenium in human beings is the diet. At present, the recommended safe and adequate daily dietary intake for adults is 55 µg of Se per day for both sexes [4]. Fish are a necessary component of a healthy diet for humans because of their nutritional value in terms of minerals, unsaturated fats, phospholipids, vitamins (A and D) and proteins of high biological value. Fish are also an important source of selenium. It is known that fish contain higher levels of selenium than other foods; therefore, they represent a major dietary source of this essential nutrient for human beings. Also, antioxidant properties of selenium have been emphasized for the preservation of polyunsaturated fatty acids that give to fish their nutritional interest [10].

Due to an increasing awareness of healthy food, the interest in food products enriched with Se or in animals fed with Se-enriched feeds is evidenced by the new development of these foodstuffs. As fish accumulate significant amounts of Se, nowadays in fish farms, fish are fed with Se-enriched foodstuffs in the diet (since the selenium content in fish basically reflects the selenium content of their consumed diet) in order to supplement possible deficiencies of this element or to provide a good dietary source for humans. For its features, aquaculture is a unique tool to control the feed, maturity and weight of fish, which influence the quality of the final product [11]. Hence, adding selenium to fish feed improves their growth in farming conditions and provides a final product that may be a new source for selenium supplementation.

2.3. SELENIUM METABOLISM

Selenium is covalently bound into multiple chemical compounds. As a metalloid, Se is utilized in the metabolic pathways of animals and plants to form Se-containing compounds having carbon–Se covalent bonds. The physiological effect of Se consumption depends on the chemical form. Some forms are preferentially incorporated into selenoproteins (proteins that requires Se for catalytic activity), other are not specifically incorporated into proteins, whereas others are excreted [4].

2.3.1. METABOLIC PATHWAY OF SELENIUM IN ANIMALS

As mentioned above, selenium is an essential micronutrient in animals because it promotes the activities of selenoenzymes, by existing as selenocysteine (SeCys) in their active centers. Se can exist in inorganic and organic forms in nature. For inorganic forms, selenite and selenate can be the nutritional source. On the other hand, organic selenocompounds in nature (selenoamino acids, selenocysteine, …) are also nutritional sources.

The metabolic pathway of Se from its ingestion to excretion has been well studied in animals. Fig. 3 shows the currently proposed Se metabolism in animals [12]. It is supposed that all Se species ingested via the food chain and drinking water are transformed into selenide, and this is utilized for the biosynthesis of SeCys for incorporation into selenoproteins (by means of selenophosphate), or selenosugars and trimethylselenonium ion (TMSe) for excretion. One inorganic Se, selenite, is simply reduced to selenide by endogenous reductants, such as glutathione (GSH). Although the other inorganic Se, selenate, is not reduced directly to selenide by GSH, it is also utilized in selenoprotein synthesis and excreted as selenosugar and TMSe. On the other hand, organic selenocompounds ingested via the food chain, which are mostly selenoamino acids (e.g., SeCys, Se-methylselenocysteine (MeSeCys) and selenomethionine (SeMet)), are transformed into selenide via several pathways. Consequently, even though all Se species are ingested via the food chain and drinking water, Se is utilized to form selenoproteins and excreted as Se-methylseleno-N-acetylgalactosamine (MeSeGalNAc; the major urinary selenosugar) in animals [13].

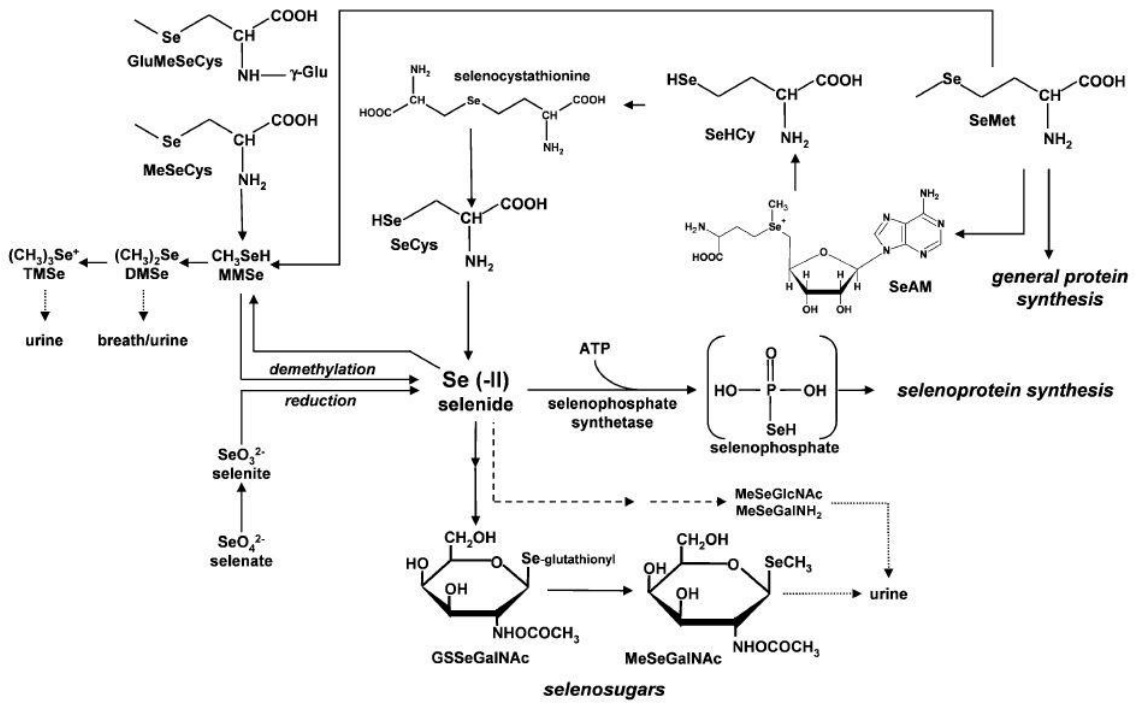

Fig. 3 Metabolic pathway of Se in animals [12]. DMSe, dimethylselenide; SeMet, selenomethionine; MeSeCys, Se-methylselenocysteine; GluMeSeCys, γ-glutamylmethylselenocysteine; SeCys, selenocysteine; GSSeGalNAc, Se-glutathionylseleno-N-acetylgalactosamine; TMSe, trimethylselenonium ion; MeSeGalNAc, Se-methylseleno-N-acetylgalactosamine; MMSe, monomethylselenol; $MeSeGalNH_2$, Se-methylselenogalactosamine; MeSeGlcNAc, Se-methylseleno-N-acetylglucosamine; SeHCy, selenohomocysteine; SeAM, Se-adenosylselenomethionine.

2.3.2. SELENOMETABOLOMICS AND SELENIUM METABOLITES IN FISH

"Selenometabolomics" is a newly coined word that means the metabolomics of Se. As mentioned above, in biological system, Se exists in diverse chemical forms depending on the metabolic pathway. On the other hand, metabolome refers to the complete set of low molecular weight compounds (metabolites), such as metabolic intermediates, hormones and other signaling molecules, and secondary metabolites in a biological system; and metabolomics is the study of the profiles of such metabolites (low molecular weight compounds have typically <1500 Da). Therefore, selenometabolomics refers to the systematic study of Se-containing metabolites (selenium metabolites or selenometabolites) processed in a biological fluid, cell, tissue, organ or organism [14].

The study of selenometabolites in fish offers unique information concerning metabolism, health and nutrition. Due to its chemical properties, selenium can form different selenometabolites through the metabolic pathway in fish, and its biological and toxicological effects are very much dependent on its chemical form. Se is necessary for the synthesis of proteins involved in the regulation of the redox status of the organism; a deficiency in selenium can therefore lead to various diseases. On the other hand, in case of selenium excess, metabolites that are formed may have a prooxidant action with adverse effects.

Some studies suggest that fish and some marine animals have a unique pathway for Se metabolism. However, at present, limited information is available on the Se metabolism in fish and marine animals. As Se forms organoselenium compounds as its metabolites, the identification of unknown selenometabolites can provide important clues and pave the way to elucidate the metabolic pathway of Se. Thus, the speciation of selenium and selenometabolites in fish is crucial to understand its metabolism and assess the biological role of selenium compounds [13,14].

2.4. ANALYTICAL APPROACHES FOR SELENIUM SPECIATION

Speciation of selenium (analytical process for identification and quantification of selenium species in a sample) for the analysis of selenium metabolites implies different steps: sample preparation, separation of species and identification. Each of these steps is going to influence the accuracy and quality of the final result (identification and quantification of species) [15].

As Se is a micronutrient, selenometabolites exist at extremely low concentrations in fish and animals (μg kg^{-1} (ppb) – ng kg^{-1} (ppt)). The difficulty of detecting Se in each selenometabolite after separation on the basis of chemical properties has been overcome with the emergence of inductively coupled plasma-mass spectrometry (ICP-MS) as the most sensitive and robust Se detector available to date. Easily hyphenated with high performance liquid chromatography (HPLC),

HPLC-ICP-MS is the technique of choice for speciation of Se and selenometabolites in biological samples [16].

2.4.1. INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (ICP-MS)

Inductively coupled plasma-mass spectrometry is an excellent technique for determination of trace elements in a diversity of matrices. As with all techniques, it has some major advantages and also some disadvantages. ICP-MS is characterized by low detection limits (high sensitivity), a wide linear dynamic range (the majority of the chemical elements have a dynamic linear detection of 4-11 orders of magnitude using the ICP-MS), its multi-element and multi-isotope capabilities and a high sample throughput. It can, however, in its standard configuration (i.e., equipped with a peristaltic pump) not be used for elemental speciation. ICP-MS can only be used as a detector if it is preceded by a separation technique. In this configuration, it can be used as a sensitive, multi-elemental on-line detector. The most prominent disadvantages are its spectral interferences (polyatomic and isobaric interferences). In ICP-MS, the high efficiency of atomization and ion formation of the inductively coupled plasma is coupled with the specific and sensitive detection capability offered by mass spectrometry [6].

2.4.1.1. PRINCIPLE OF ICP-MS

The sample solution is converted to an aerosol in a pneumatic nebulizer. In order to have a stable plasma, the supply of droplets has to be stable. The larger droplets are hence removed in the spray chamber. The other droplets are carried along by a carrier gas (normally argon (Ar)) into the plasma. The plasma is a mixture of molecules, ions, electrons and atoms at a high temperature (5000-10000 K). It is electrically neutral and is generated at the end of a torch, consisting of three concentric quartz tubes. Around the torch, there is an induction coil, connected to a radiofrequency (RF) generator. By applying a RF current (RF power between 600 and 1800 watts), an alternating magnetic field is generated, resulting in collision of the electrons (produced by a spark via a high voltage Tesla coil) with the Ar atoms, which are finally ionized. When the sample

droplets are introduced in the ICP, they are desolvated and volatilized, the molecules are atomized and finally the atoms are ionized by the high temperature and Ar^+ in the plasma. This occurs when an electron acquires sufficient energy, equal to the first ionization energy of the element. The electron can then escape from the attraction of the atomic nucleus. A schematic overview of the sample introduction system and the ICP is given in Fig. 4. The function of the plasma is to generate an efficient ionization source for the sample, for subsequent detection by the MS [6,17].

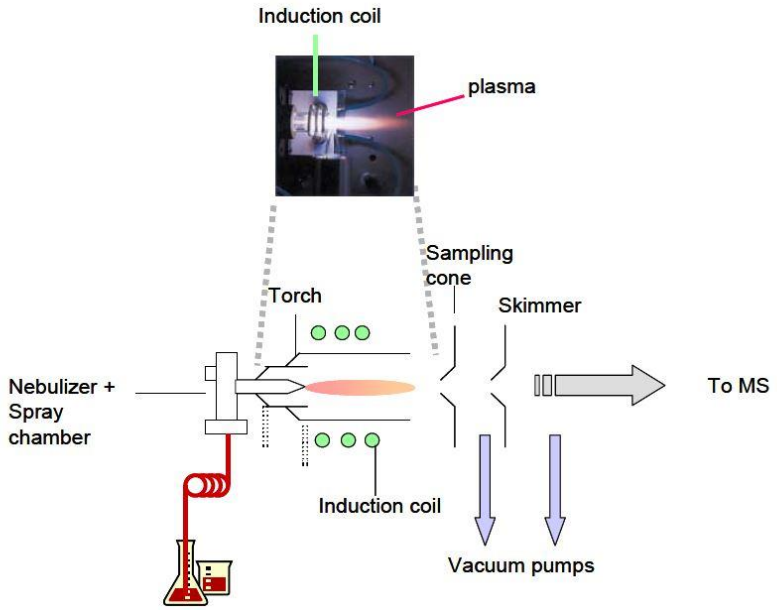

Fig. 4 Schematic overview of the sample introduction system in ICP [17].

The ICP operates at atmospheric pressure, while the MS is operated under high vacuum. Several vacuum stages are hence needed for the proper working of the combination ICP and MS. An interface is placed between the ICP and the MS, consisting of a sampling cone (sampler) allowing the introduction of the ions from the plasma into a higher vacuum stage. The presence of a skimmer allows the introduction of the sample into a subsequent higher vacuum stage. Both the sampling cone and the skimmer are water-cooled and have a small central aperture through which the sample is introduced to the successively higher vacuum stages. Due to the subsequently lower pressure, the sample ions are extracted into the first chamber and are focused by a series of ion lenses into the mass spectrometer [17], which in this research project is a quadrupole mass spectrometer (the quadrupole functions as a “mass filter”).

2.4.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

In speciation analysis several separation techniques (high performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), gel electrophoresis) have been employed to distinguish between the different species. Of all the separation techniques, HPLC is by far the most popular and commonly used technique for trace selenium speciation analysis. HPLC has the advantage of performing separations of non-volatile selenium species; thus, it has generally better versatility than GC which may require a derivatization step before analysis [6].

2.4.2.1. PRINCIPLE OF HPLC

In HPLC compounds are separated by difference in affinity between a liquid mobile phase and a stationary phase. An HPLC system basically consists of a pump which delivers the eluent, an injector which injects the sample into the mobile phase stream (t_0 in Fig. 5), a column where the separation takes place, and a detector. As the compounds in the sample come into contact with the stationary phase (which is actually a bed of particles with a particle size of 3-10 µm), they start to distribute over both phases according to their affinity for the phases (t_1 and t_2 in Fig. 5). The separated species reach the detector (ultraviolet (UV), MS, …) and the resulting chromatogram is generated [18]. In Fig. 5 a schematic overview of the principle is given, the different parts of an HPLC system are indicated together with the HPLC principle and a chromatogram.

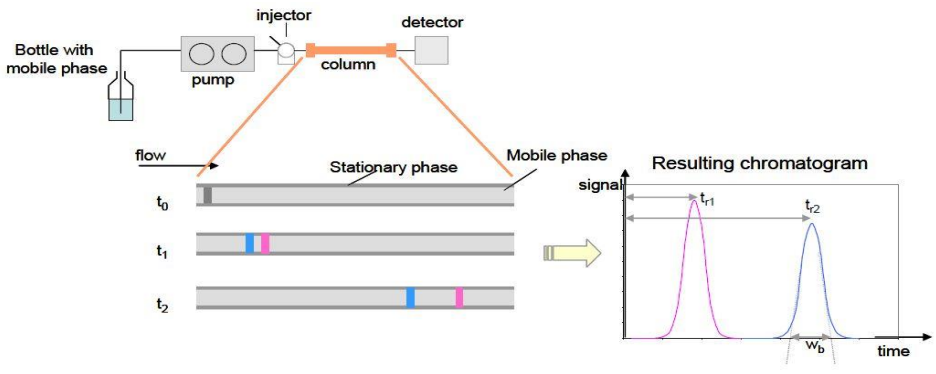

Fig. 5 Schematic overview of the HPLC instrumentation and principle. t_r and w_b are the retention time and peak width at baseline of the respective peaks [18].

The aim of a separation is to obtain well resolved peaks in a minimum of analysis time. The various HPLC techniques are classified according to the distribution mechanism applied for separation. The main modes are: normal phase, reversed phase (RP), ion exchange, ion pairing, size exclusion chromatography (SEC) and affinity chromatography. The methods used throughout this research project are discussed in more detail below.

2.4.2.2. REVERSED PHASE HPLC (RP-HPLC)

Reversed phase HPLC is probably the most extensively method used for the study of selenium compounds. This separation mode uses a non-polar stationary phase and a polar mobile phase (i.e., the separation of analytes is performed using stationary phases which have a surface less polar than the mobile phase) [6]. This makes it suitable for the analysis of organic compounds. The retention of the compounds in RP-HPLC is based on hydrophobic interactions. The more polar the compound is, the less it is retained on the column. The eluent or mobile phase generally consists of mixtures of water or an aqueous buffer and of an organic modifier (methanol, acetonitrile, …). The percentage of organic modifier (RP usually works in gradient) is the most important factor in the retention of non-polar substances. The stronger the retention the more organic modifier is needed to elute the compound [18].

2.4.2.3. SIZE EXCLUSION HPLC (SEC-HPLC)

Size exclusion chromatography (SEC) is the preferred mode for separation of large (bio)molecules. SEC-HPLC is often used in selenium speciation to characterize the various selenometabolites and selenoproteins encountered form different sample matrices. Molecules are separated based on differences in their molecular weights. SEC is a separation mode in which the separation is according to the hydrodynamic volume of the analytes, and the separation takes place in a porous non-absorbing stationary phase with pores of approximately the same size as the analyte's effective solution dimensions. Unlike many of the other modes of HPLC, interaction between the stationary phase and the analyte is minimized for effective size separation (SEC is always performed in isocratic) [6,18].

2.4.3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY HYPHENATED WITH INDUCTIVELY COUPLED PLASMA-MASS SPECTROMETRY (HPLC-ICP-MS)

Trace and ultratrace species can be detected by HPLC-ICP-MS in biological and environmental samples, even when the element is distributed over a variety of compounds. HPLC is a relatively simple technique to couple with ICP-MS because the eluent flow rate is, depending on the column size or diameter, between 0.1 and 1 ml min^{-1}. In the coupling of HPLC to ICP-MS, it is the nebulizer which takes care of the transition from liquid to aerosol. HPLC is coupled to ICP-MS by connecting the outlet of the column to the inlet of the nebulizer, in which tubing is kept as short and small as possible in order to minimize peak broadening and, hence, minimize loss of chromatographic resolution [6]. A schematic presentation of the instrument setup is given in Fig. 6.

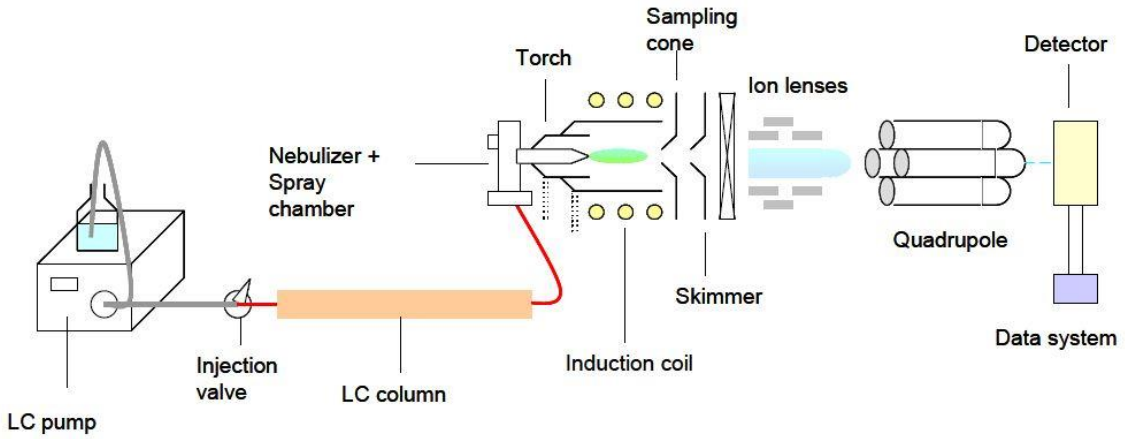

Fig. 6 Schematic presentation of HPLC-ICP-MS [17,18].

HPLC can be directly applied to non-volatile compounds of high and low molecular weight providing a great versatility derived from different separation modes (e.g., reverse phase and size exclusion) and can be easily on-line interfaced to the ICP-MS. Consequently and because of the low volatility of most selenium species (small selenometabolites, selenoamino acids, …) present in biological samples, the use HPLC-ICP-MS is a very powerful and effective tool for selenium speciation analysis at ultratrace levels [4].

2.5. SAMPLE PREPARATION FOR THE ANALYSIS OF SELENIUM METABOLITES: DEPROTEINIZATION

Preparation of the sample is a primary concern in selenium metabolites analysis. Sample preparation step could be understood as any manipulation that modifies the sample matrix and one of the main objectives is to convert the sample to a more suitable condition to the analysis. The selection of sample preparation procedure is going to be dependent on the matrix, chemical form of selenium expected in the sample and the instrumentation selected for further separation and the identification of the species [15].

Sample preparation (which is arguably the most important step when performing any analysis of metabolites) for the further analysis of low molecular weight selenometabolites in biofluids and other complex matrixes has contributed to an enhanced identification of these target analytes and for the exploration of selenometabolomes [19].

The most commonly analyzed biofluids in animals and humans are blood plasma, blood serum, urine and whole blood. Analysis of plasma and serum appears to be the most popular in studies of metabolites [20]. However, these biological matrices are complex mixtures containing numerous components such as proteins which can interfere with analytes during the separation and detection processes. Among these interferences, proteins which are present in a large amount in serum, plasma or whole blood can be irreversibly adsorbed onto the chromatographic support, which causes the deterioration of separation efficiency and a rapid column clogging (reduced lifetime of the column) [21]. Besides, selenium metabolites are in extremely low concentrations (trace levels) in these blood matrixes and, consequently, proteins could dominate the analysis and cause signal suppression of the metabolites [22].

For the above reasons, the analysis of metabolites in serum, plasma and whole blood samples is frequently hindered by the presence of proteins. Thus, the analysis of selenium metabolites requires removal of protein from samples in the sample preparation step [23].

Deproteinization or protein removal is a necessary step especially in blood analysis. A common procedure of deproteinization by protein precipitation is to add a reagent: organic solvent, acids or salts. Normally, deproteinization is carried out using organic solvents such as methanol, ethanol, acetone, acetonitrile or combinations of some of them. In most studies, acetonitrile is the organic solvent of choice to protein precipitation [20-22]. After protein precipitation, the isolation of the deproteinized sample is generally performed by centrifugation [21,23].

In other words, deproteinization allows simplifying the matrix of the blood and plasma samples by means of precipitation of proteins using organic solvents. However, deproteinization solvents or deproteinization agents must not interfere with the target analytes (metabolites) and, besides, they must maintain the sample integrity. On the other hand, protein precipitation procedures suffer from low selectivity and low analyte recovery in some cases; and this can cause that unknown metabolites in low concentrations cannot be detected. Therefore, these limitations must be taken into account during the development of an analytical method using protein precipitation for the sample deproteinization [20,21].

For all mentioned above, in this work, the optimization of the sample preparation (samples of trout whole blood and blood plasma) was investigated and performed to significantly improve analyte recovery, which is critical for the analysis of selenium metabolites with low concentrations.

3. OBJECTIVES

3.1. EXPERIMENTAL OBJECTIVES

When analysis of ultratrace level metabolites is carried out, sample preparation often proves to be a crucial step in the speciation of these compounds. Analysis of selenium metabolites in blood by HPLC-ICP-MS is a challenge because of the very low concentrations found and the presence of proteins that deteriorate the chromatographic separation. Thus, the analysis of metabolites in trout tissues, blood plasma and whole blood samples is frequently hindered by the presence of proteins and, consequently, the analysis of selenium metabolites requires deproteinization or removal of protein from these samples.

So, the main objective of this work is focused in the optimization of the sample preparation, in order to minimize loss of analytes usually observed during the deproteinization step, for the analysis of selenium metabolites in trout. This main objective is approached through the following partial objectives:

- Test and comparison of two deproteinization reagents.

- Evaluation of the losses of analytes by monitoring two tracers isotopically Se-enriched (^{77}Se-selenite and ^{76}Se-selenomethionine) during the precipitation of proteins.

- Quantification of both tracers after deproteinization by reverse isotope dilution (RID) with ICP-MS.

- Comparison of both samples, trout whole blood and blood plasma with the added Se-enriched tracers, by SEC with UV detection and SEC-ICP-MS.

- Besides, qualitative analysis of selenometabolites in trout tissues after deproteinization step, by means of HPLC-ICP-MS, is performed.

3.2. ACADEMIC OBJECTIVES

Within the objectives for participating in the double diploma Master's in Analytical Sciences can be highlighted the following: writing and communication a research work in a foreign language and development and/or improvement of several skills, including leadership, team working ability, flexibility and adaptation to changing situations and interaction with people from other environments and disciplines.

4. EXPERIMENTAL

4.1. SELENIUM TRACER DISTRIBUTION AFTER DEPROTEINIZATION

4.1.1. REAGENTS AND MATERIALS

Analytical reagent grade chemicals purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and ultra-pure water (18 MΩ cm) obtained with a Milli-Q system (Millipore, Bedford, MA, USA) were used throughout all experiments unless stated otherwise. Samples used throughout this experiment were whole blood and blood plasma from rainbow trout (*Oncorhynchus mykiss*).

The two isotopically enriched Se standards used as tracers were ^{77}Se-selenite (^{77}SeIV) and ^{76}Se-selenomethionine (^{76}SeMet). The selenite enriched with ^{77}Se was homemade and it was prepared from the ^{77}Se standard obtained from ISOFLEX (Moscow, Russia). And the selenomethionine enriched with ^{76}Se was purchased from LGC Standards (Teddington, UK). A standard solution of 1000 mg L^{-1} of selenium from PlasmaCAL Standards (SCP Science, Quebec, Canada) was used as spike for the reverse isotope dilution analysis. Hydrogen peroxide from Fisher Scientific (Hampton, NH, USA) and nitric acid (Instra-Analyzed) from J. T. Baker (Center Valley, PA, USA) were used for sample digestions. The two reagents for the deproteinization step were acetonitrile and a mixture of methanol:acetonitrile:acetone (1:1:1, v/v/v).

4.1.2. INSTRUMENTATION

The ICP-MS used was an Agilent 7500ce (Fig. 7) from Agilent Technologies (Tokyo, Japan), consisting of an ICP source with a plasma-shielded torch (grounded metal plate), an octapole reaction system operated in RF only mode and a quadrupole mass analyzer with a secondary electron multiplier operating in dual mode (i.e., either a pulse counting mode or analogue mode, depending on the ion intensity). ICP-MS operating conditions are given in Table 3. DigiPrep MS digestion system from SCP Science (Quebec, Canada) was used for digestion of samples.

Fig. 7 Agilent 7500ce ICP-MS.

Table 3 ICP-MS Agilent 7500ce operating conditions.

Sample introduction	
Nebulizer type	Concentric nebulizer
Spray chamber type	Scott double pass
Plasma parameters	
RF power	1500 W
Carrier gas flow rate	0.95 L min^{-1}
Sampler and skimmer cones	Nickel
Reaction/collision cell parameters	
H_2 gas flow	2.5 mL min^{-1}
He gas flow	1.0 mL min^{-1}
Data acquisition parameter	
Monitored Se isotopes	76, 77, 78, 79, 80, 81, 82 and 83

4.1.3. PROCEDURE

10 µL of 1 ppm ^{77}SeIV tracer solution was added to 100 µL aliquot of trout blood plasma. After that, 200 µL of acetonitrile (ACN) was added on for the precipitation of proteins. The sample was shaken in a vortex and the mixture was centrifuged at 13000 rpm for 10 min. Once centrifuged, supernatant and pellet were separated and, then, both were digested separately in 50 mL polypropylene tube (DigiTube, SCP Science) with 200 µL of H_2O_2 (30 %, w/v) and 400 µL of HNO_3 (70 %, w/v) using the following temperature program: 0.5 h up to 85 ºC and 3 h at 85 ºC. Digested samples were diluted with 15 mL of water and analysed by ICP-MS.

Quantification of tracer after deproteinization was carried out by reverse isotope dilution (RID) with ICP-MS using a standard solution of natural selenium as spike.

Same procedure was performed using ^{76}SeMet solution as tracer (solution of 1 ppm) instead of ^{77}SeIV. Besides, both experiments (one with ^{77}SeIV and the other with ^{76}SeMet) were realized using the mixture of solvents (methanol:acetonitrile:acetone (MAA); 1:1:1, v/v/v) for deproteinization step, as an alternative to the acetonitrile.

The sample of trout whole blood was pretreated before adding the tracer. Whole blood was frozen with liquid nitrogen and then defrosted. This freeze-defrost procedure was repeated 5 times to break the red blood cells for releasing the compounds and substances which are in their inside. After that, defrosted blood was centrifuged at 13000 rpm for 10 min and the supernatant was used for the analysis and the pellet was discarded. The supernatant whole blood was treated and analysed following the same procedures than for the blood plasma (described above).

Standard tracer solutions were first characterized in terms of concentration and isotopic composition by reverse isotope dilution analysis (n = 3):

· ^{77}SeIV: 1010±6 mg kg^{-1}; 98.3 % ^{77}Se and 1.7 % ^{78}Se.

· ^{76}SeMet: 1105±10 mg kg^{-1}; 99.9 % ^{76}Se and 0.1 % ^{78}Se.

4.2. SEC-ICP-MS FOR TROUT SAMPLES WITH TRACER

4.2.1. REAGENTS FOR SEC-ICP-MS ANALYSIS

A buffer solution of 10 mM Tris-HCl at pH 7.0 was prepared from tris(hydroxymethyl)aminomethane powder. This solution was used as mobile phase for size exclusion chromatography.

4.2.2. SEC-ICP-MS INSTRUMENTATION

The HPLC used was a Dionex Ultimate 3000 UHPLC system (Fig. 8) from Thermo Fisher Scientific (Sunnyvale, CA, USA) equipped with a UV detector. The column used for SEC was a Superdex 75 10/300 GL from GE Healthcare (Little Chalfont, UK). Superdex 75 is a prepacked glass column for high performance gel filtration of molecules and biomolecules with molecular weights between 3000 and 70000 Da. For the on-line SEC-ICP-MS measurements, the exit of the column was connected to the nebulizer of the Agilent 7500ce ICP-MS by means of PEEK (polyether ether ketone) tubing. SEC-ICP-MS operating conditions are given in Table 4.

Fig. 8 Dionex Ultimate 3000 UHPLC.

Table 4 SEC-ICP-MS operating conditions.

Chromatographic parameters	
Column	Superdex 75 (300 x 10 mm)
Mobile phase	10 mM Tris-HCl (pH 7.0)
Flow rate	0.6 mL min^{-1}
Injection volume	200 µL
Elution (time)	Isocratic (45 min)
UV detector wavelength	254 nm
Plasma parameters	
RF power	1500 W
Carrier gas flow rate	0.95 L min^{-1}
Sampler and skimmer cones	Nickel
Reaction/collision cell parameters	
H_2 gas flow	4.0 mL min^{-1}
Data acquisition parameter	
Monitored Se isotopes	76, 77 and 78

4.2.3. PROCEDURE

10 µL of 1 ppm ^{77}SeIV tracer solution was added to 100 µL aliquot of trout blood plasma and 10 µL of 1 ppm ^{76}SeMet tracer solution was added to other 100 µL aliquot of trout blood plasma. Samples with tracers were shaken in a vortex and 190 µL of 10 mM Tris-HCl buffer at pH 7.0 was added to each one. After that, the final samples were shaken again and analysed by SEC-ICP-MS.

The sample of trout whole blood was pretreated before adding the tracer. Whole blood was frozen with liquid nitrogen and then defrosted (described above). After centrifugation of defrosted blood, the supernatant was used for the analysis and the pellet was discarded. The supernatant whole blood was processed and analysed by SEC-ICP-MS following the same procedure than for the blood plasma (described in the above paragraph).

4.3. QUALITATIVE ANALYSIS OF SELENIUM METABOLITES IN TROUT TISSUES AFTER DEPROTEINIZATION

4.3.1. STANDARDS AND REAGENTS

Different standard solutions of 1000 mg L^{-1} were used in this experiment: selenite (SeIV) and methaneseleninic acid (MeSeOOH) purchased from Sigma-Aldrich; trimethylselenonium ion (TMSe) and Se-methyl-N-acetylglucosamine (SeGlu) were laboratory made; selenomethionine (SeMet) purchased from Acros Organics (New Jersey, USA); and Se-methyl-N-acetylgalactosamine (SeGal) was kindly provided by Dr. Yasumitsu Ogra (Pharmaceutical University of Showa, Tokyo, Japan). The samples used throughout this experiment were rainbow trout tissues (liver, kidney and spleen), obtained from a local shop. These tissues were previously undergone to a process of cryogenic grinding.

A solution of 2 mM dithiothreitol (DTT) was prepared by dissolving the corresponding reagent in 50 mM Tris-HCl buffer (pH 7.5). A solution of 0.1 % of formic acid was prepared. This solution and acetonitrile were used as mobile phases in the HPLC-ICP-MS analysis. Acetonitrile was also used as deproteinization reagent.

4.3.2. INSTRUMENTATION AND MATERIALS

Agilent 1200 Series HPLC system coupled to Agilent 7700x ICP-MS (Fig. 9) were used for the qualitative analysis of trout tissues, both from Agilent Technologies (Tokyo, Japan). Agilent 7700x ICP-MS was equipped with the organic solvent introduction kit. The column used was a Hypercarb from Thermo Fisher Scientific (Waltham, MA, USA). Hypercarb is a porous graphitic carbon (PGC) column composed of fully porous individual spherical particles. It was used for reversed phase separations. HPLC-ICP-MS operating conditions are given in Table 5.

Fig. 9 Agilent 1200 Series HPLC and Agilent 7700x ICP-MS.

A tissue grind pestle SC 7 mL (Fig. 10) from Kimble-Chase Kontes (Vineland, NJ, USA) was utilized for grinding the three trout tissues.

Fig. 10 Glass tissue grind pestle of 7 mL used.

Table 5 Reversed phase HPLC-ICP-MS operating conditions.

Chromatographic parameters	
Column	Hypercarb, porous graphitic carbon (PGC) (100 x 4.6 mm)
Particle size	5 µm
Mobile phase	A: 0.1 % formic acid B: Acetonitrile (ACN)
Flow rate	1.0 mL min^{-1}
Injection volume	50 µL
Elution (time)	Gradient: 2 % B (0-5 min), 2-75 % B (5-35 min), 75-2 % B (35-40 min), 2 % B (40-45 min)
Plasma parameters	
RF power	1600 W
Carrier gas flow rate	0.67 L min^{-1}
Sampler and skimmer cones	Platinum
Reaction/collision cell parameters	
He gas flow	9.0 mL min^{-1}
Data acquisition parameter	
Monitored Se isotope	78

4.3.3. PROCEDURE

About 500 mg of trout tissue was mixed with 1 mL of 2 mM DTT in order to reduce the S-Se and Se-Se bridges. The mixture was placed in the glass pestle or potter of 7 mL for homogenization and grinding of the tissue. Ground tissue was transferred in a 2 mL Eppendorf vial and centrifuged at 13000 rpm for 5 min. Once centrifuged, 100 µL of the supernatant was mixed with 100 µL of acetonitrile for precipitation of proteins. This mixture was centrifuged at 13000 rpm for 10 min. Supernatant and pellet were separated and, then, the supernatant was concentrated in vacuum in the Concentrator plus (Speed-Vac concentrator) from Eppendorf (Hamburg, Germany) at 30 ºC for 30 min. Finally, the concentrated supernatant was diluted with 100 µL of 0.1 % formic acid (mobile phase) and the resulting solution was analysed by HPLC-ICP-MS in experimental conditions summarized in Table 5. This procedure was carried out for the three tissues of trout (liver, kidney and spleen).

Moreover, solutions of 10 ppb from the six selenium standard solutions were prepared in 0.1 % formic acid and analysed by HPLC-ICP-MS to obtain the corresponding chromatograms of the Se standards.

5. RESULTS AND DISCUSSION

5.1. DISTRIBUTION OF THE ISOTOPIC ENRICHED TRACERS AFTER DEPROTEINIZATION

As explained above, the analysis of selenium metabolites in trout blood and trout tissues requires a deproteinization step due to the presence of proteins which hinder the analysis. For this reason, two deproteinization reagents were tested to optimize the sample preparation step. One of them, acetonitrile (ACN), is a conventional reagent to precipitate protein; while the other, MAA (mixture of methanol:acetonitrile:acetone (1:1:1, v/v/v)) was tested as an alternative of the conventional solvent.

To evaluate the losses of analytes during the precipitation of proteins by the deproteinization reagents, two isotopic enriched selenium tracers were monitored. These tracers were ^{77}Se-selenite (^{77}SeIV) and ^{76}Se-selenomethionine (^{76}SeMet). They were used as representatives of the selenium metabolites during the precipitation of proteins. Their control in the deproteinization step gave an idea about the amount of metabolites which were lost with the proteins depending of the reagent used for the precipitation. Therefore, the monitoring of these tracers gave information about the behaviour of selenometabolites during the deproteinization and, also, about the different influence or action of the reagents used for this process.

Reverse isotope dilution (RID) analysis with an octapole reaction system ICP-MS was used to quantify both isotopically enriched tracers after deproteinization. Spike solution of natural selenium was used for the quantification by RID.

5.1.1. ISOTOPE DILUTION

Isotope Dilution (ID) is based on the modification of the isotope composition of the element or compound to be determined in the sample by the addition of an isotopically enriched or labelled form of the same element or compound. The basic ID experiment is illustrated in Fig. 11 using black and white trouts as surrogates for isotopes. If we want to know the number of white trouts contained in the first fishpond, one solution would be to count the individual trouts. However, this procedure could be

time consuming when the number of trouts is very high. Alternatively, we can use the black trouts contained in the second fishpond of the figure. The black trouts are of the same weight and size as the white ones but can be distinguished by the different colour and, most importantly, we know the number of black trouts contained in the second fishpond. To carry out the isotope dilution experiment we would add the black trouts to the first fishpond and mix them. Providing a high enough total number of trouts in the blend, we can take a handful from the black and white trout blend and determine the ratio of white (W_t) to black (B_t) trouts obtained.

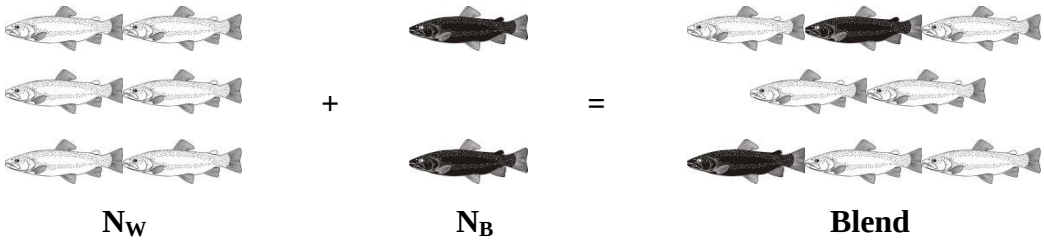

Fig. 11 A basic Isotope Dilution experiment explained with white and black trouts.

The total number of white trouts (N_W) can be obtained from the ratio of white to black trouts and the known total number of black trouts (N_B) originally contained in the small fishpond using the Eqn. 1:

$$N_W = N_B \frac{W_t}{B_t} \quad \textbf{(Eqn. 1)}$$

The strength of this procedure is that every aliquot from the blend will show the same W_t/B_t ratio (allowing for random counting variations) so any losses of trouts, once they are mixed, will not affect the final results [24].

The isotope dilution process for elemental analysis for a bi-isotopic element is shown in Fig. 12.

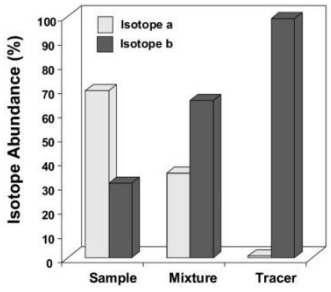

Fig. 12 Illustration of the isotope dilution principle for a bi-isotopic element [24].

5.1.1.1. REVERSE ISOTOPE DILUTION (RID)

Reverse isotope dilution, inversely to the isotope dilution analysis (IDA), uses a standard solution of natural isotope abundances as spike or tracer solution to determine the quantity of an isotopically enriched compound or sample.

RID with ICP-MS provides highly accurate and precise determinations. A major advantage of IDA (and, also, of RID) over conventional calibration procedures is that it can provide compensation for a variety of physical and chemical interferences, such as plasma fluctuations, instrument instabilities and analyte suppressions by sample matrix. This feature is of great importance for the analysis of selenium in biological samples where serious matrix effects are commonly observed.

However, the accurate determination of selenium by ICP-MS has been hampered by the presence of spectroscopic interferences. Hence, the elimination of these interferences was crucial.

5.1.2. ELIMINATION OF ARGON POLYATOMIC INTERFERENCES

Major isotopes of selenium (^{80}Se, ^{78}Se and ^{76}Se) are all subject to severe Ar_2^+ interferences. Other Se isotopes of lower abundance (^{82}Se or ^{77}Se) can also be interfered by molecular ions from halogens (BrH^+, ClO^+) present in the sample. The main polyatomic interferences, which could occur in the determination of selenium in biological samples, are listed in Table 6 [25].

Table 6 Spectral interferences on the determination of Se in biological samples by ICP-MS.

Isotope	Natural abundance (%)	Interference
^{74}Se	0.89	$^{38}Ar^{36}Ar^+$, $^{37}Cl_2^+$, $^{40}Ar^{34}S^+$
^{76}Se	9.37	$^{40}Ar^{36}Ar^+$, $^{40}Ar^{36}S^+$, $^{31}P_2{}^{14}N^+$
^{77}Se	7.63	$^{40}Ar^{36}ArH^+$, $^{38}Ar_2H^+$, $^{40}Ar^{37}Cl^+$
^{78}Se	23.77	$^{40}Ar^{38}Ar^+$, $^{31}P_2{}^{16}O^+$
^{80}Se	49.61	$^{40}Ar_2^+$, $^{79}BrH^+$
^{82}Se	8.73	$^{40}Ar_2H_2^+$, $^{12}C^{35}Cl_2^+$, $^{34}S^{16}O_3^+$, $^{81}BrH^+$

In this work, an Agilent 7500ce ICP-MS equipped with a collision/reaction cell was used to reduce or eliminate the interfering polyatomic species. The octapole was pressurized with a mixture of H_2 (reaction gas) and He (collision gas) to eliminate these interfering species by collisional dissociation and ion-molecule reactions. The collision/reaction cell can also increase the ion transmission efficiencies.

However, the formation of SeH^+ and BrH^+ ions in the octapole when using a collision/reaction cell with hydrogen as reaction gas was other effect which was taken into consideration. The SeH^+ formed could be derived from the sample matrix or from impurities of water in the cell gases [25].

Nevertheless, the complete elimination of SeH^+ and BrH^+ interferences in real samples is very difficult and it was necessary to apply mathematical corrections after the measurement of natural abundance Se and Br standards both for interference correction and mass bias correction.

5.1.3. CALCULATION PROCEDURE (INTERFERENCE CORRECTION AND MASS BIAS CORRECTION)

Five selenium isotopes (76, 77, 78, 80 and 82), two bromine isotopes (79 and 81) and the mass 83 (corresponding to $^{82}SeH^+$) were measured.

Firstly, the measured intensities of the five Se isotopes were corrected by taking into account the formation of the hydrides SeH^+ and BrH^+ using the following mathematical equations (Eqn. 2-8):

$$^{76}Se = {}^{76}I \quad \textbf{(Eqn. 2)}$$

$$^{77}Se = {}^{77}I - f_{Se}(^{76}Se) \quad \textbf{(Eqn. 3)}$$

$$^{78}Se = {}^{78}I - f_{Se}(^{77}Se) \quad \textbf{(Eqn. 4)}$$

$$^{79}Br = {}^{79}I - f_{Se}(^{78}Se) \quad \textbf{(Eqn. 5)}$$

$$^{80}Se = {}^{80}I - f_{Br}(^{79}Br) \quad \textbf{(Eqn. 6)}$$

$$^{81}Br = {}^{81}I - f_{Se}(^{80}Se) \quad \textbf{(Eqn. 7)}$$

$$^{82}Se = {}^{82}I - f_{Br}(^{81}Br) \quad \textbf{(Eqn. 8)}$$

where f_{Se} and f_{Br} are SeH^+/Se (based on the measured $^{83}I/^{82}I$ ratio in the 10 ng g^{-1} selenium standard solution) and BrH^+/Br (based on the mean of the measured $^{80}I/^{79}I$ and $^{82}I/^{81}I$ ratios in the 10 ng g^{-1} bromine standard solution) factors (f_{Se} and f_{Br} = 0 before iteration), ^{xx}I are the raw intensities measured and ^{xx}Se and ^{xx}Br are the intensities corrected for spectral interferences [26].

It is well known that heavier isotopes are transmitted more efficiently than lighter isotopes in ICP-MS. Consequently, isotope ratios measured are always biased towards the heavier isotopes. This physical effect is called mass discrimination and causes a measurable mass bias in the obtained isotope ratios that need to be corrected [27].

So, once the corrected intensities were calculated they were divided by the intensity of ^{80}Se (most abundant Se isotope) and the experimental isotope ratios were corrected for mass bias using the exponential model (Eqn. 9):

$$R_{corr} = \frac{R_{exp}}{e^{K\Delta M}} \quad \textbf{(Eqn. 9)}$$

where R_{corr} is the mass bias corrected isotope ratio; R_{exp} is the measured ratio; K is the mass bias factor (derived from the slope of the regression line of the linear relationship between the relative error $\ln(R_{exp}/R_{theo})$ in the experimental isotope ratios with respect to ^{80}Se and the mass difference between the measured isotopes from the ^{80}Se reference isotope); and, ΔM is the nominal mass difference between the considered isotopes [26]. Then, the corrected isotope abundances (A_{corr}) were calculated back using the Eqn. 10:

$$A^i_{corr} = \frac{R^i_{corr}}{\sum_{i=1}^{n} R^i_{corr}} \quad \textbf{(Eqn. 10)}$$

These corrected abundances were then introduced in the isotope dilution equation (Eqn. 11) to determine the concentration of selenium in the sample [27]:

$$C_s = C_{sp} \frac{m_{sp}}{m_s} \frac{M_s}{M_{sp}} \frac{R_m A^b_a - A^a_{sp}}{A^a_s - R_m A^b_s} \quad \textbf{(Eqn. 11)}$$

where C_s is the concentration of the element in the sample; C_{sp} is the concentration of the element in the spike; m_{sp} is the mass taken from the spike in the mixture; m_s is the mass taken from the sample in the mixture; M_s is the atomic weight of the element in the sample; M_{sp} is the atomic weight of the element in the spike; $A^a{}_{sp}$ is the isotope abundance of isotope a in the spike; $A^b{}_{sp}$ is the isotope abundance of isotope b in the

spike; A^a_s is the isotope abundance of isotope a in the sample; A^b_s is the isotope abundance of isotope b in the sample; and, R_m is the isotope ratio, measured by mass spectrometry, of isotopes a and b in the mixture (see Fig. 12).

5.1.4. WASHING SOLUTIONS USED DURING MEASUREMENTS BY ICP-MS

Analysis performed by ICP-MS using the Br standard solution showed a memory effect of bromine in the instrument. Since halogen elements (bromine in this work) are known for their severe memory effects if nebulizer-based sample introduction systems are used, it was necessary to find a rinsing solvent to eliminate this memory effect.

Bu et al. [28] studied and evaluated different rinsing solvents to remove the memory effects of the halogen elements. In their research paper, they demonstrated clearly that the memory effects of all four halogens can be eliminated effectively by washing out the ICP-MS system with 5 % (v/v) ammonium hydroxide (NH_4OH) solution.

A 5 % (v/v) ammonium hydroxide solution was prepared by diluting 5 mL of concentrated ammonium hydroxide solution from Sigma-Aldrich (Saint-Quentin Fallavier, France) to 100 mL with Milli-Q water. This solution was employed to remove the bromine of the instrument (bromine memory effect) during the analysis. The rinsing procedure, which was used in this work to wash-out the instrument between the measurement of a sample and the following one, was:

Rinse with Milli-Q water during 30 s

↓

Rinse with 5 % ammonium hydroxide solution during 90 s

↓

Rinse with 2 % nitric acid during 30 s

5.1.5. DISTRIBUTION OF SELENIUM TRACERS AFTER DEPROTEINIZATION

^{77}SeIV and ^{76}SeMet tracers were added to trout blood and plasma to determine their distribution in these samples after a deproteinization step. Also, these tracers were monitored to evaluate the loss of analytes usually observed during the deproteinization step (two different reagents for the protein precipitation were used).

The selenium concentrations of ^{77}SeIV and ^{76}SeMet tracers after the precipitation of proteins were determined by reverse isotope dilution using a natural abundance Se standard as spike. For each sample, the amount of natural Se standard added for RID was calculated using Excel spreadsheets. Selenium concentration of the tracer in the sample (C_{tracer}) was then calculated from Eqn. 12 based on that used by Tolu et al. [29].

$$C_{tracer} = \frac{(C_s A_s^{78}) - \left(\left(\frac{A_{sp}^{78}}{A_{sp}^{77}}\right) C_s A_s^{77}\right)}{\left(\frac{A_{tracer}^{78}}{A_{tracer}^{77}}\right) - \left(\frac{A_{sp}^{78}}{A_{sp}^{77}}\right)} \quad \textbf{(Eqn. 12)}$$

where C_s is the selenium concentration in the sample (obtained from Eqn. 11); $A_s^{77,\,78}$ are the abundance of ^{77}Se or ^{78}Se in the sample (determined from the monitored Se isotopes); $A_{sp}^{77,\,78}$ are the natural abundance of ^{77}Se or ^{78}Se in the natural Se spike; and $A_{tracer}^{77,\,78}$ are the abundance of ^{77}Se or ^{78}Se in the tracer (i.e., ^{77}SeIV).

Eqn. 12 was the equation used for the determination of ^{77}SeIV tracer. For ^{76}SeMet tracer, the same equation was used but instead of using isotope ^{77}Se, the selenium isotope ^{76}Se was used.

5.1.5.1. ^{77}SeIV TRACER DISTRIBUTION AFTER DEPROTEINIZATION IN TROUT BLOOD PLASMA

The distribution of the ^{77}SeIV tracer in the plasma sample after the precipitation of proteins using ACN and MAA mixture for the deproteinization is shown in Fig. 13.

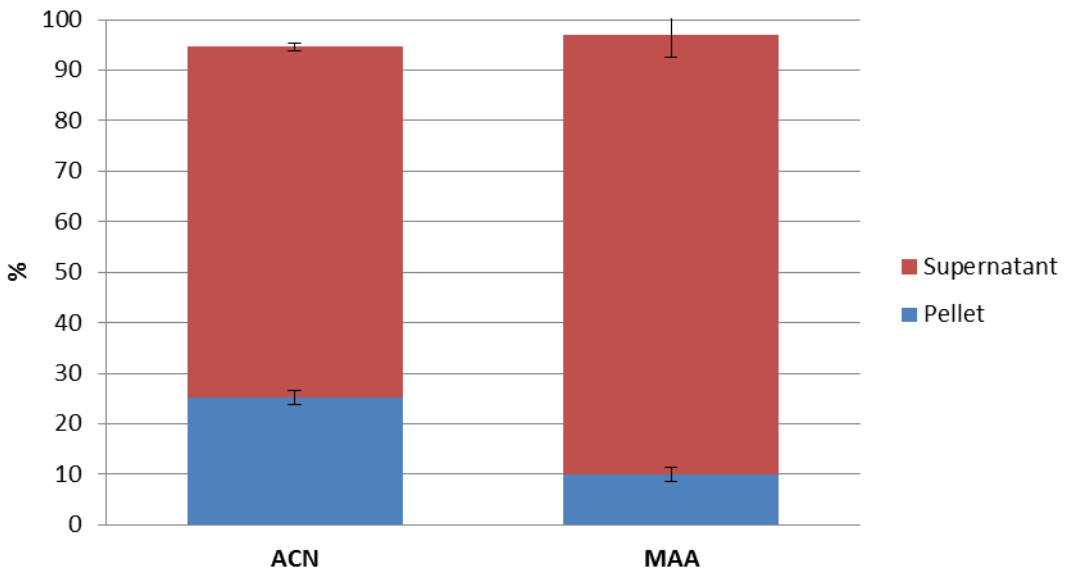

Fig. 13 ^{77}SeIV tracer distribution after deproteinization in trout blood plasma.

The distribution obtained using ACN showed that 25±1 % of ^{77}SeIV tracer (RSD = 6 %; n = 3) was in the protein precipitate (pellet), whereas that 69±1 % of tracer (RSD = 1 %; n = 3) was in the supernatant. Using ACN as deproteinization reagent, more than 95 % of tracer recovery was obtained. On the other hand, MAA mixture led to tracer being at 10±1 % in the pellet (RSD = 14 %; n = 3) and at 87±5 % in the supernatant (RSD = 5 %; n = 3). In this case, tracer recovery obtained using MAA was more than 97 %. These results showed that the losses of ^{77}SeIV tracer caused by the conventionally used deproteinization reagent (ACN) were in the order of 20 % higher than those obtained with the alternative one (MAA mixture) in trout plasma.

5.1.5.2. ^{76}SeMet TRACER DISTRIBUTION AFTER DEPROTEINIZATION IN TROUT BLOOD PLASMA

In the case of the ^{76}SeMet tracer distribution (Fig. 14), 22±2 % of tracer was in the pellet (RSD = 8 %; n = 3) and 72±3 % in the supernatant (RSD = 4 %; n = 3) using ACN; while that using MAA mixture, 9±1 % of ^{76}SeMet tracer was in the pellet (RSD = 7 %; n = 3) and 88±2 % in the supernatant (RSD = 2 %; n = 3). These results showed again that losses of tracer during the precipitation were two times more with the conventionally reagent than with MAA mixture. Tracer recoveries were higher than 95 % with ACN and 96 % with MAA.

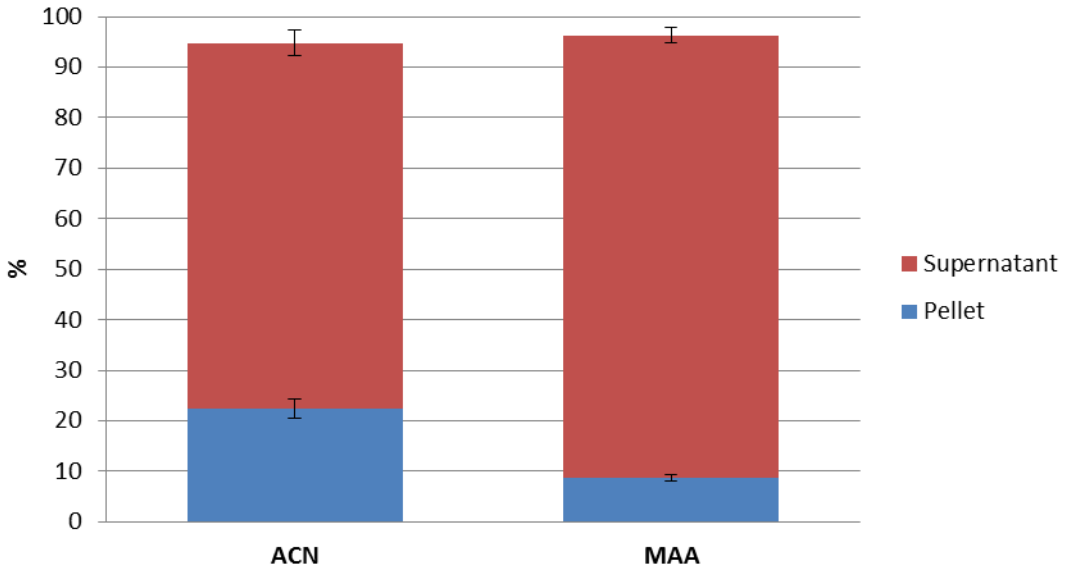

Fig. 14 ^{76}SeMet tracer distribution after deproteinization in trout blood plasma.

5.1.5.3. ^{76}SeMet TRACER DISTRIBUTION AFTER DEPROTEINIZATION IN TROUT WHOLE BLOOD

^{76}SeMet tracer was also monitored in a more complex sample, trout whole blood. The distribution of the tracer in the blood sample after the deproteinization using ACN and MAA mixture is shown in Fig. 15.

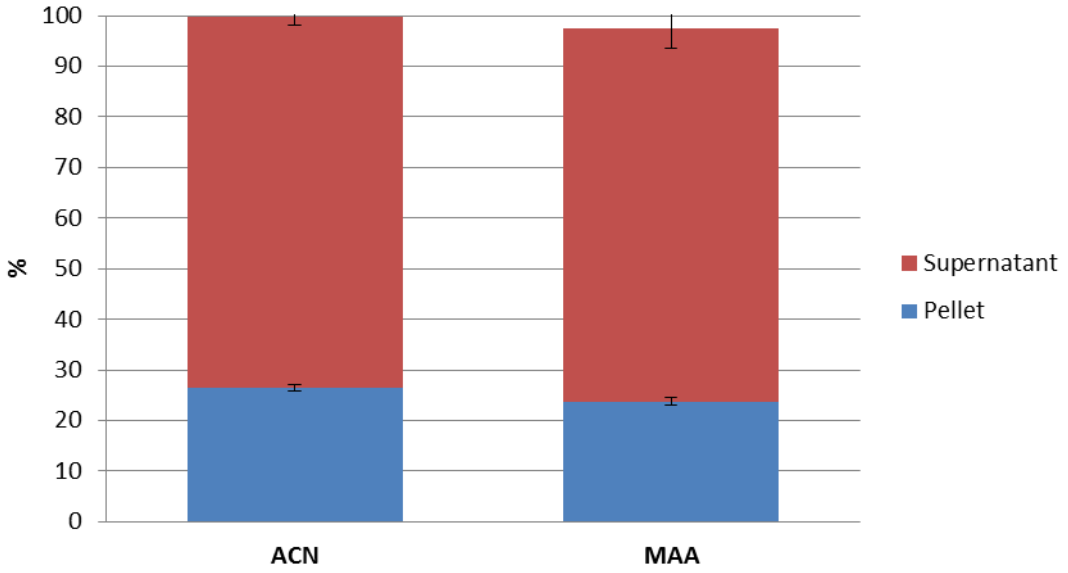

Fig. 15 ^{76}SeMet tracer distribution after deproteinization in trout whole blood.

In this case, the distribution was more or less similar both with acetonitrile as with MAA mixture. With acetonitrile, 27±1 % of tracer was in the pellet (RSD = 2 %; n = 3) and 73±2 % in the supernatant (RSD = 2 %; n = 3); and using MAA mixture, 24±1 % of ^{76}SeMet tracer was in the pellet (RSD = 3 %; n = 3) and 74±4 % in the supernatant (RSD = 5 %; n = 3). Tracer recovery was approximately 100 % using ACN and higher than 97 % with MAA. Both distributions were roughly analogous than the distribution obtained for ^{76}SeMet in blood plasma using ACN (see Fig. 14) and almost no significant differences between the use of ACN or MAA for the protein precipitation in whole blood were observed (this could be due to the presence of a greater number of proteins in whole blood than in blood plasma). However, the losses in this sample were slightly higher with ACN than with the MAA mixture.

5.1.5.4. ^{77}SeIV TRACER DISTRIBUTION AFTER DEPROTEINIZATION IN TROUT WHOLE BLOOD

Finally, the distribution after deproteinization step of the ^{77}SeIV tracer in trout whole blood was also obtained (Fig. 16) and the two deproteinization reagents were compared in this sample with the tracer.

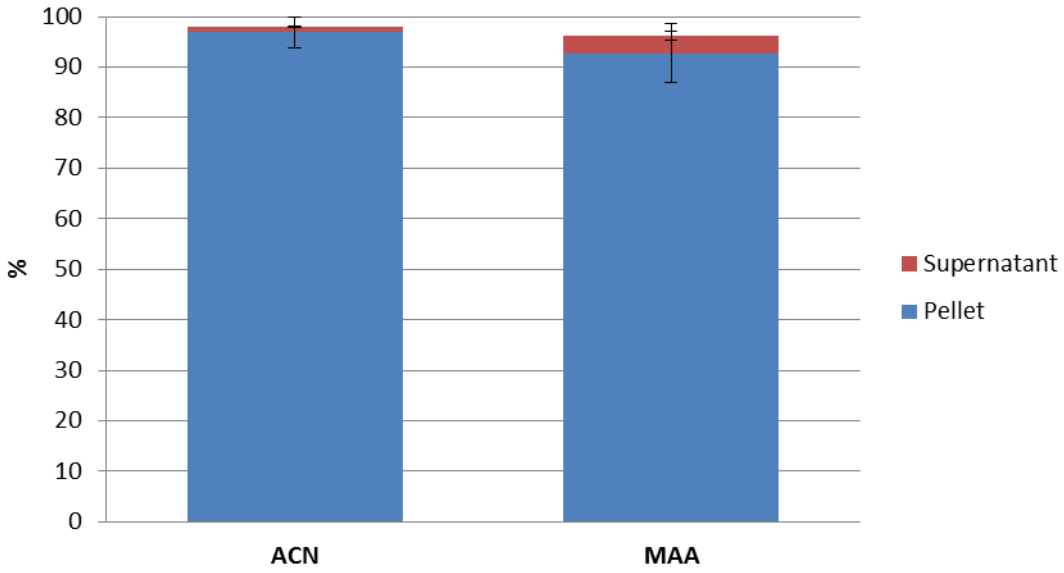

Fig. 16 ^{77}SeIV tracer distribution after deproteinization in trout whole blood.

Unlike all previously described cases, most of ^{77}SeIV tracer in whole blood was lost during the protein precipitation. The distribution obtained using ACN showed that

97±3 % of tracer (RSD = 3 %; n = 3) was in the protein precipitate (pellet), whereas that only 1±1 % of tracer (RSD = 12 %; n = 3) was in the supernatant. MAA mixture led to tracer ^{77}SeIV being at 93±6 % in the pellet (RSD = 6 %; n = 3) and at 3±1 % in the supernatant (RSD = 23 %; n = 3). Tracer recoveries were higher than 98 % with ACN and 96 % with MAA. Although most of the tracer was lost in the deproteinization both with ACN as with MAA, the losses were slightly higher with ACN than with the MAA mixture.

These large losses of ^{77}SeIV in the whole blood could be due to weak interactions of the tracer with proteins or to the undesired co-precipitation of the tracer during the precipitation of proteins of the blood. To try to explain these losses, trout whole blood and blood plasma with added Se-enriched tracers were compared by size exclusion chromatography (SEC) with ultraviolet (UV) and ICP-MS detection.

5.2. COMPARISON OF THE WHOLE BLOOD AND BLOOD PLASMA WITH THE ADDED ENRICHED TRACERS BY SEC-UV-ICP-MS

As mentioned above, Se-enriched tracers were added to the two samples and, then, they were analysed by SEC-UV-ICP-MS without any deproteinization step to try to assess if tracers could interact with proteins.

First of all, the Superdex 75 column was calibrated with a mixture of bovine albumin (67 kDa), myoglobin (17 kDa) and insulin (5.8 kDa).

The first sample which was analysed was blood plasma with ^{77}SeIV tracer. To know where the tracer in the sample was and if it interacted with proteins, ^{77}Se and ^{78}Se was monitored by SEC-ICP-MS. The chromatogram of ^{77}Se included signal of ^{77}SeIV tracer and, also, ^{77}Se signal of the selenium compounds of the blood plasma. On the other hand, the chromatogram of ^{78}Se only included the selenium compounds of the blood plasma because ^{77}SeIV tracer was almost all ^{77}Se. Thus, theoric natural ^{77}Se chromatogram was performed using the signals of ^{78}Se and multiplying these signals by

^{77}Se natural abundance/^{78}Se natural abundance ratio (0.0763/0.2377). Thereby, tracer location was found comparing ^{77}Se signals with the theoretical ^{77}Se chromatogram. In this case, there was not any interaction between tracer and proteins (this interaction should appear between 12-17 min) because ^{77}Se and theoretical ^{77}Se were overlapped until 25 min (see Fig. 17). The big peak at 26 min was free ^{77}SeIV tracer (chromatogram of ^{77}SeIV tracer standard was obtained by SEC-ICP-MS and showed only one peak at this time corresponding to ^{77}SeIV). Therefore, ^{77}SeIV tracer was totally free in the blood plasma.

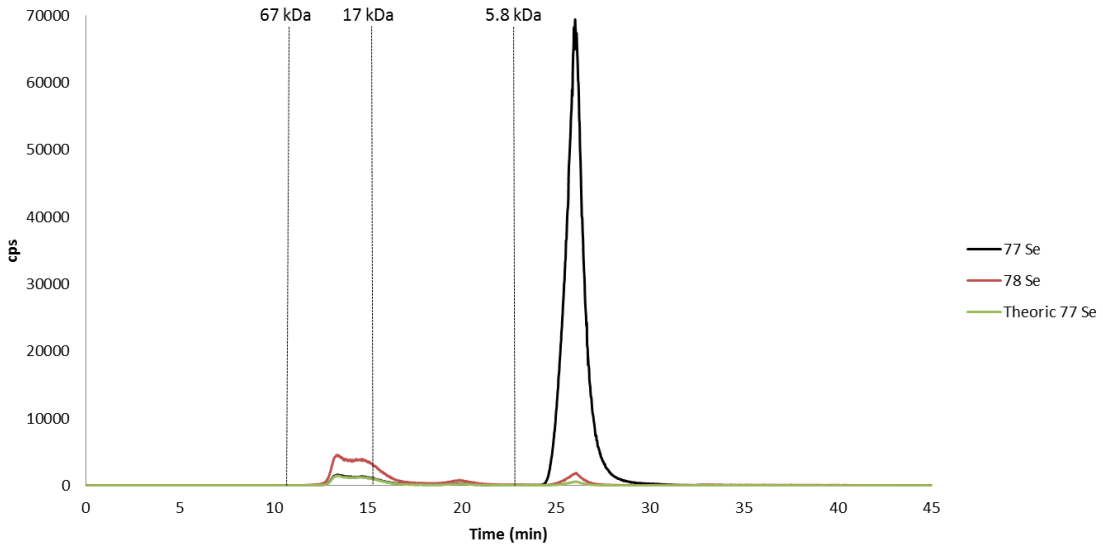

Fig. 17 SEC-ICP-MS chromatograms of blood plasma with ^{77}SeIV tracer.

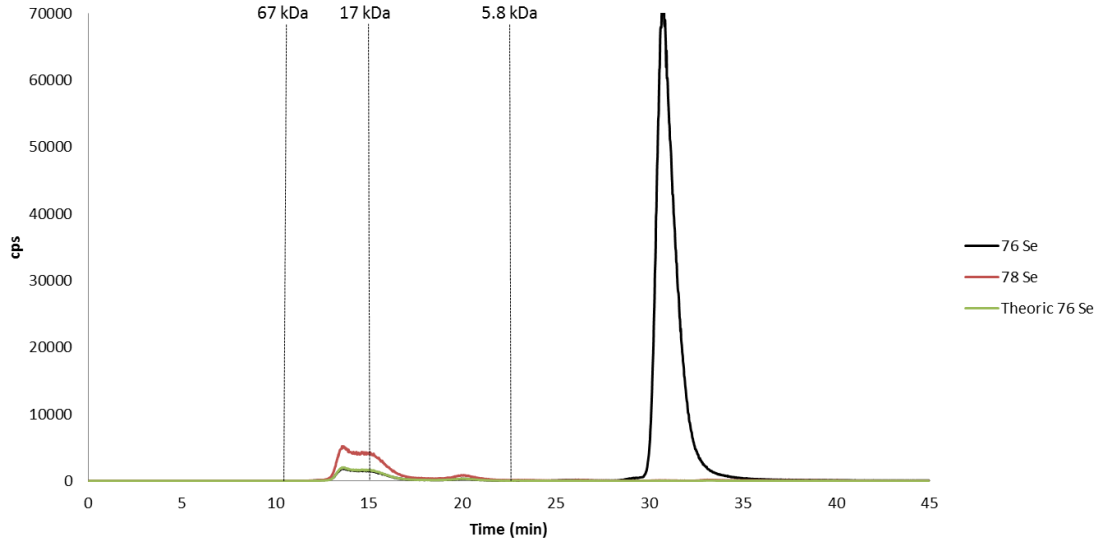

Fig. 18 SEC-ICP-MS chromatograms of blood plasma with ^{76}SeMet tracer.

Same results were obtained for blood plasma with ^{76}SeMet tracer as can be seen in Fig. 18. Therefore, ^{76}SeMet tracer was free in blood plasma and no interaction with proteins was observed. In this case, theoric natural ^{76}Se chromatogram was performed using the signals of ^{78}Se and multiplying these signals by ^{76}Se natural abundance/^{78}Se natural abundance ratio (0.0937/0.2377) and the chromatogram of ^{76}SeMet standard obtained by SEC-ICP-MS demonstrated that the peak at 31 min corresponding to free ^{76}SeMet.

With these results it seems that no interaction occurred between tracers and proteins; the losses of tracers observed in the Fig. 13 and 14 could be due to that a portion of the free tracers co-precipitated during the deproteinization step.

On the other hand, in the case of whole blood with ^{76}SeMet tracer (Fig. 19), chromatograms showed similar results (i.e., tracer was free and no interaction with proteins of the whole blood was observed) as the results obtained for the sample of blood plasma.

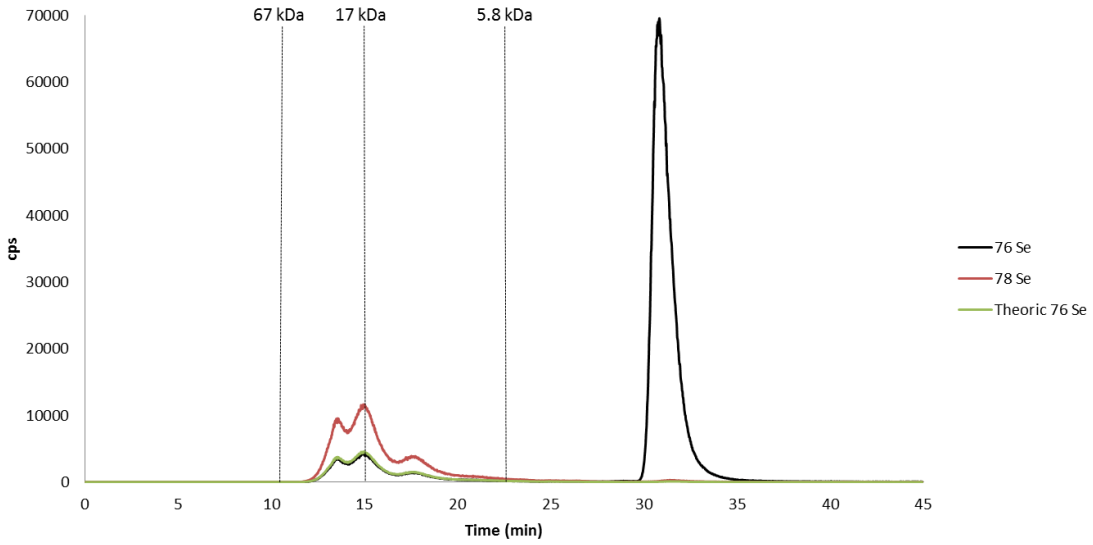

Fig. 19 SEC-ICP-MS chromatograms of whole blood with ^{76}SeMet tracer.

Regarding the difference in the losses of the ^{76}SeMet tracer observed between Fig. 14 and 15 when MAA was used as deproteinization agent (the losses with ACN were similar as can be seen in both figures), there was more losses in whole blood than in blood plasma. This could be due to the fact that higher amount of proteins is present

in whole blood than in blood plasma (see protein profiles in Fig. 20). Consequently, this could lead to a greater co-precipitation of the free tracer in whole blood having a larger amount of proteins that precipitated; since the more protein, the more co-precipitation could take place.

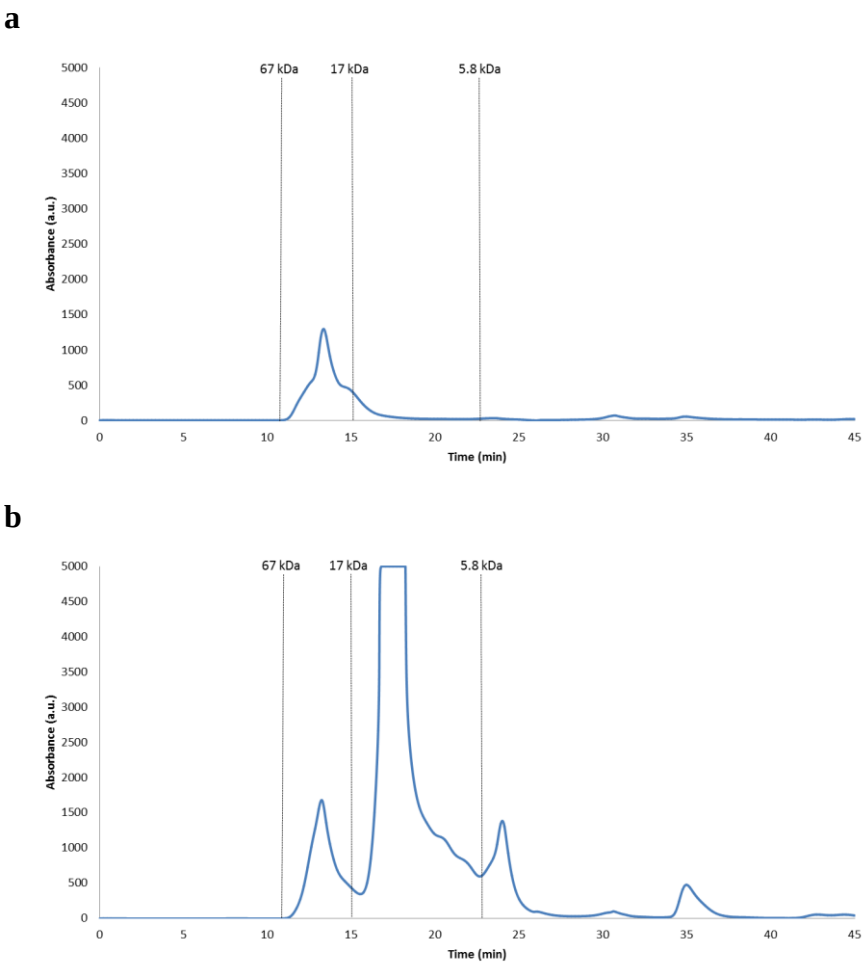

Fig. 20 SEC-UV protein profiles at 254 nm.
(a) Protein profile of trout blood plasma. **(b)** Protein profile of trout whole blood.

Finally, SEC-ICP-MS chromatograms of whole blood with ^{77}SeIV tracer are given in Fig. 21. In Fig. 16 it could be seen that almost all tracer is lost during the precipitation step. Comparing the ^{77}Se chromatogram with the theoretical one, four peaks of the ^{77}SeIV tracer could be differentiated from the natural ^{77}Se of the whole blood. One peak appeared roughly between 12 min, two small peaks appeared at 17 and 23 min, and the last one appeared at 26 min (this was the free ^{77}SeIV tracer). Unlike the previous three cases, the first three peaks of the ^{77}Se chromatogram of the Fig. 21 evidenced that ^{77}SeIV tracer was linked with the proteins of the whole blood.

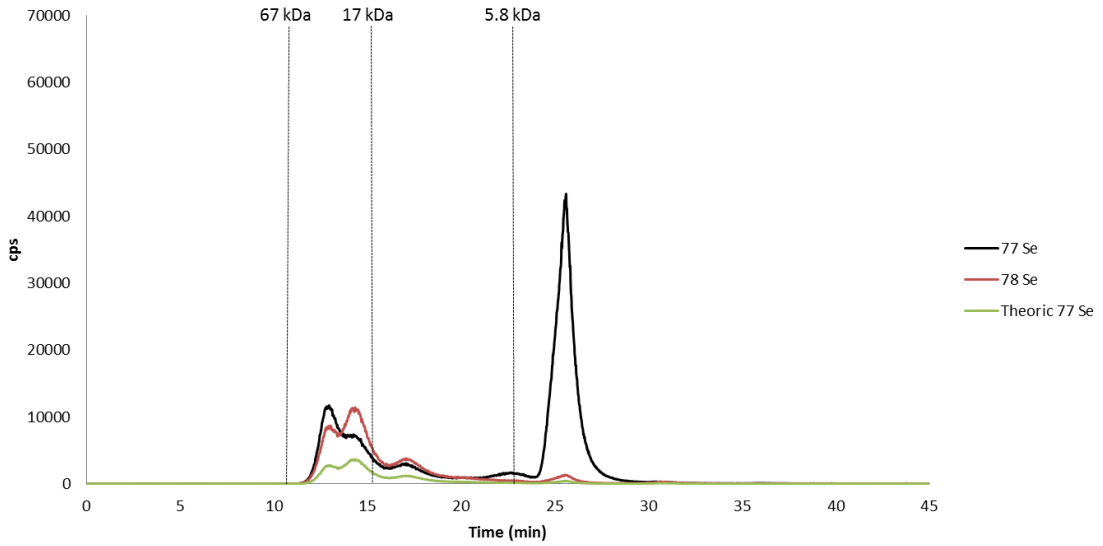

Fig. 21 SEC-ICP-MS chromatograms of whole blood with ^{77}SeIV tracer.

These results explained the much higher losses observed in Fig. 16, inasmuch as part of the tracer precipitated due to weak interactions of ^{77}SeIV with proteins and the other part of tracer co-precipitated during the deproteinization step as free ^{77}SeIV.

At last, the different interactions with proteins observed between ^{77}SeIV and ^{76}SeMet tracers in whole blood could be due to that SeIV is a smaller metabolite than SeMet. SeIV is also anionic species contrary to SeMet that should be broadly neutral, at blood pH, it should be distributed between SeO_3^{2-} and $HSeO_3^-$, and, therefore, ^{77}SeIV could be able to more easily interact with blood proteins and it could be associated with these proteins better than ^{76}SeMet.

5.3. QUALITATIVE ANALYSIS OF SELENIUM METABOLITES IN TROUT TISSUES (LIVER, KIDNEY AND SPLEEN) AFTER DEPROTEINIZATION BY MEANS OF HPLC-ICP-MS

Qualitative analysis of selenometabolites in trout tissues were carried out by performing the deproteinization step before analysis by HPLC-ICP-MS. To do the

analysis homogenous with the bibliography, the conventional reagent for the deproteinization (acetonitrile) was used.

Trout liver, kidney and spleen were chosen for the analysis because they are the organs with higher amount of Se species in most animals. ACN was used for the precipitation of proteins to simplify the tissue matrices and analyse the low molecular weight compounds of selenium (Se-metabolites).

Firstly, selenite (SeIV; t_R = 4.4 min), trimethylselenonium ion (TMSe; t_R = 1.3 min), methaneseleninic acid (MeSeOOH; t_R = 2.0 min), Se-methyl-N-acetylglucosamine (SeGlu; t_R = 7.8 min), Se-methyl-N-acetylgalactosamine (SeGal; t_R = 6.9 min) and selenomethionine (SeMet; t_R = 2.7 min) standards were analysed by HPLC-ICP-MS on Hypercarb column to obtain their chromatographic profiles (Fig. 22). The retention time (t_R) of peaks of the six standards available for this experiment were used later to identify some of these selenium metabolites in the tissue samples. The complete chromatograms of the Se standards and the enlarged chromatographic profiles of these Se compounds (0-13 min), because all species appeared before 13 minutes and after this time the profile was flat, were showed in Fig. 22.

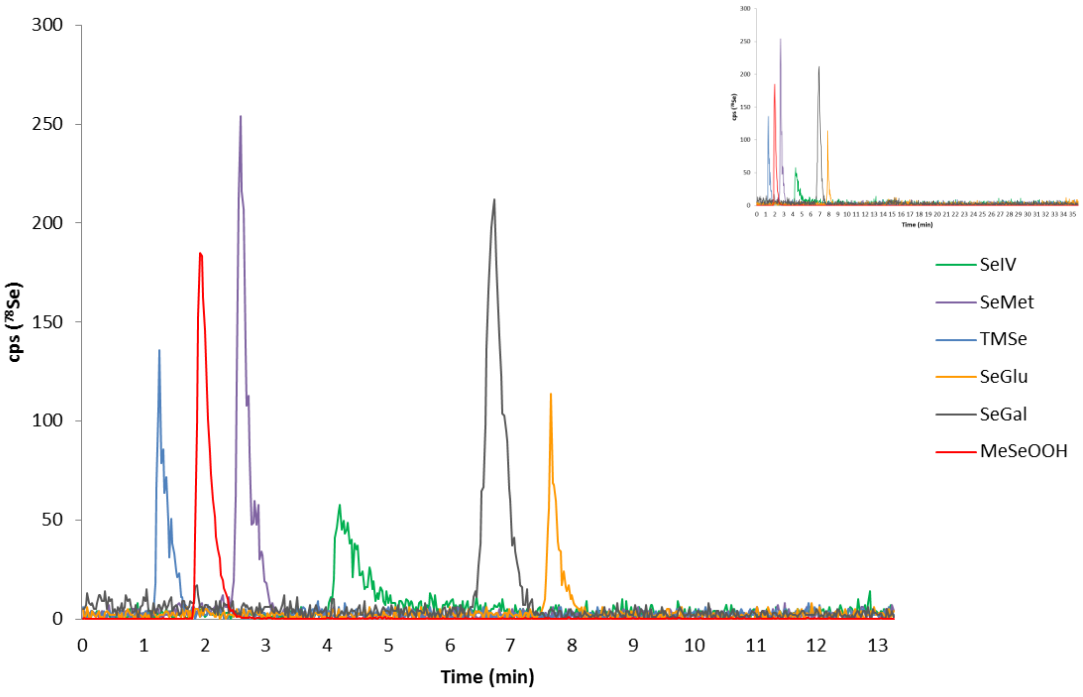

Fig. 22 Chromatographic profiles of the Se-standards obtained by HPLC-ICP-MS on Hypercarb column.

In the qualitative analysis of trout liver, as can be seen in Fig. 23, only three selenometabolites were found. They were attributed by retention time matching: MeSeOOH (t_R = 1.9 min), SeMet (t_R = 2.7 min) and SeGlu (t_R = 7.8 min). Besides, other unknown peaks were observed in the chromatogram.

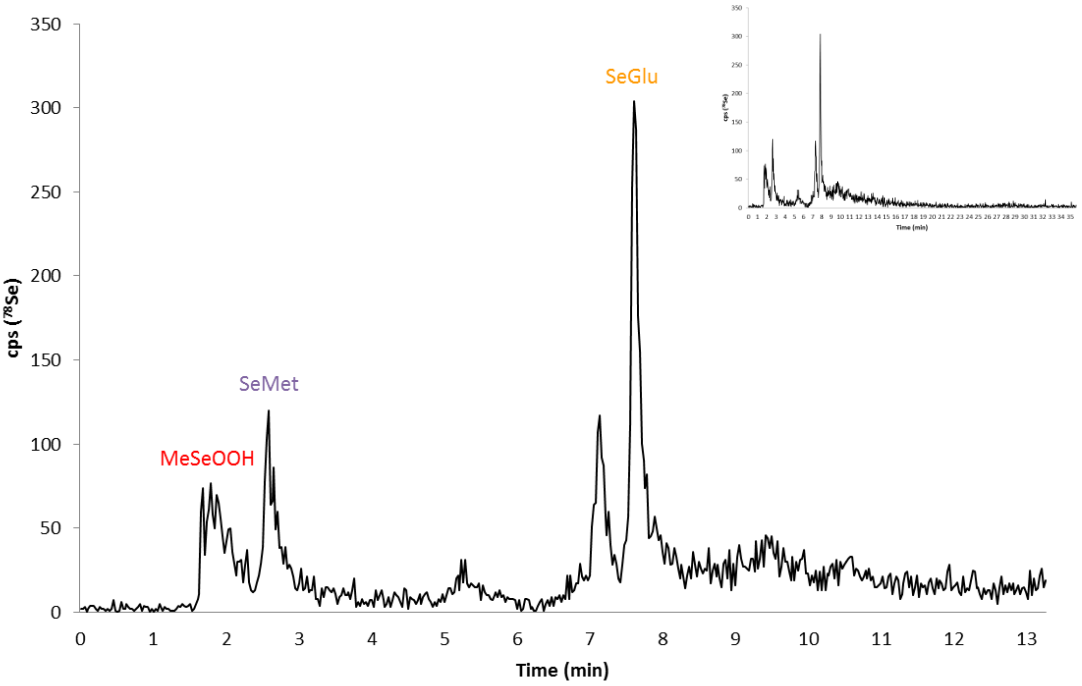

Fig. 23 Chromatographic profile of trout liver obtained by HPLC-ICP-MS on Hypercarb column.

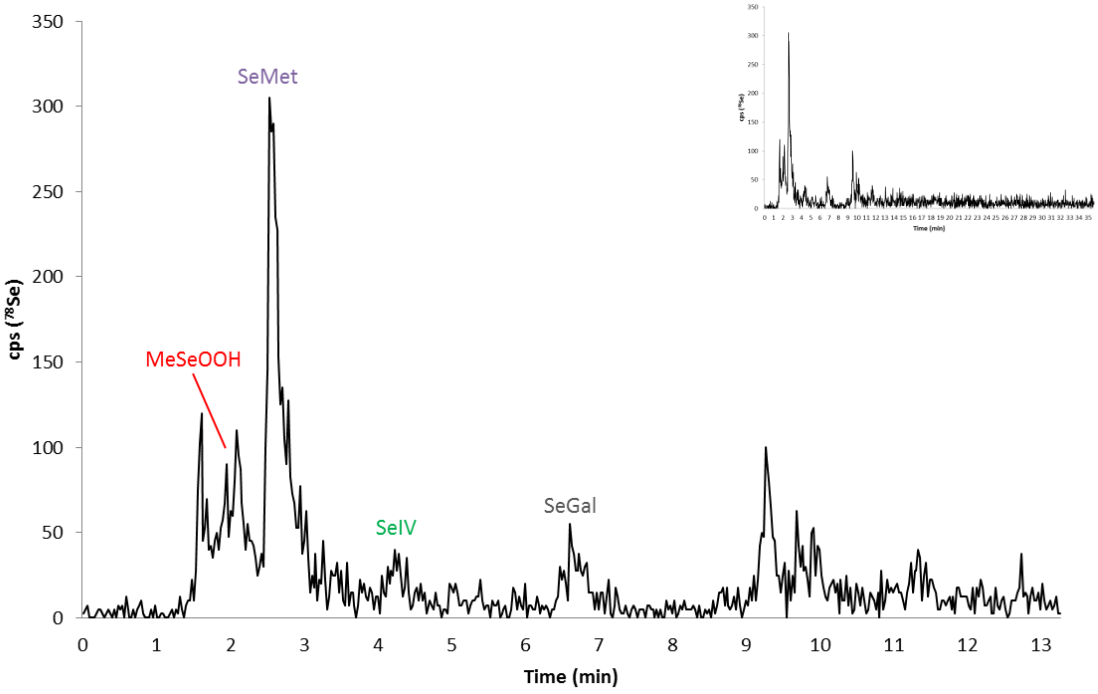

Fig. 24 Chromatographic profile of trout kidney obtained by HPLC-ICP-MS on Hypercarb column.

The Fig. 24 showed the chromatographic profile obtained with Hypercarb column for trout kidney. MeSeOOH (t_R = 2.0 min), SeMet (t_R = 2.7 min), SeIV (t_R = 4.4 min) and SeGal (t_R = 6.8 min) were identified comparing the peaks of this chromatogram with the peaks obtained for the Se-standards. However, other unknown peaks were observed as in the trout liver.

Finally, trout spleen was also analysed. In this case, only two peaks were observed in the chromatographic profile obtained (Fig. 25). One of them was identified, it was SeMet (t_R = 2.7 min). The other peak was not identified because it did not correspond to Se standards retention times. However, this highest peak could be selenoneine (see its structure in Fig. 26). 2-selenyl-Nα,Nα,Nα-trimethyl-L-histidine (selenoneine) is a novel selenium-containing compound which has been isolated by Yamashita and Yamashita as the major form organic selenium in the blood and tissues of tuna [30]. This selenium compound has strong antioxidant capacity.

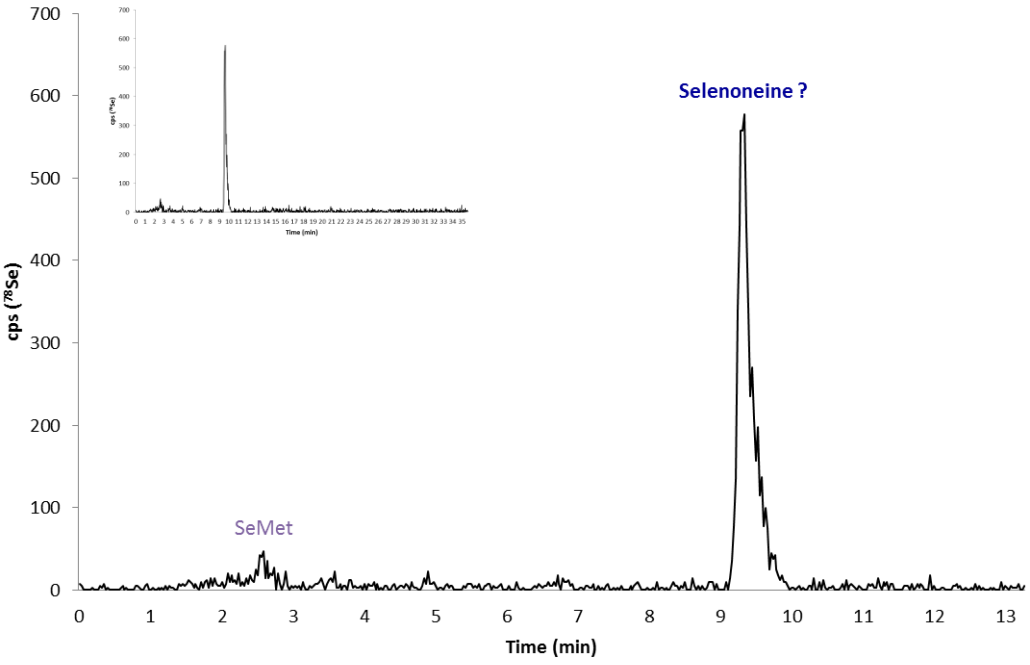

Fig. 25 Chromatographic profile of trout spleen obtained by HPLC-ICP-MS on Hypercarb column.

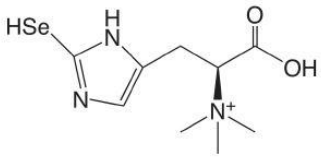

Fig. 26 Chemical structure of selenoneine (2-selenyl-Nα,Nα,Nα-trimethyl-*L*-histidine).

This hypothesis is based on other work, which is under progress in the lab, where Se-containing compound at the same retention time is observed for blood tuna sample where selenoneine is the major Se compound [30]. However, it was difficult to confirm if the peak was selenoneine due to the fact that literature on selenoneine is rather scarce and there is no selenoneine standard available. Therefore, it is complicated to say anything about this novel selenium-containing compound.

One way to know if the compound is selenoneine could be using molecular mass spectrometry (e.g., performing an analysis of the compound, which was supposedly selenoneine, by molecular MS using orbitrap as mass analyzer).

6. CONCLUSIONS

In this work was presented the optimization of sample preparation for the analysis of selenium metabolites in trout. This optimization was performed in order to minimize loss of metabolites during the deproteinization step. It was carried out using two isotopically selenium enriched tracers for the further determination of their distributions after the protein precipitation in trout whole blood and trout blood plasma by means of RID and ICP-MS. Two deproteinization solvents were compared for both samples. The obtained results showed that the losses of the tracers caused by the conventionally used deproteinization reagent (ACN) were two times more than with the alternative one (MAA mixture) in trout plasma; whereas that the losses in the trout blood were slightly higher with ACN than with MAA mixture, however interactions between the tracer and proteins and co-precipitation processes were observed for this whole blood sample by using SEC-UV-ICP-MS. In brief, this optimization of the sample preparation has allowed to reduce significantly the losses of target molecules in some of the samples, which is critical for the investigations of minor metabolites with low concentrations.

On the other hand, qualitative analysis of selenium metabolites in trout tissues after deproteinization with ACN was performed by HPLC-ICP-MS with good results. It is noteworthy that the novel selenium-containing compound, selenoneine, could have been detected in trout spleen. As future perspectives, the further analysis using the MAA mixture for the deproteinization and the use of molecular mass spectrometry could improve the experiments and consequently make easier the identification of the metabolites.

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