Universitat Autònoma de Barcelona

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Summary

Selenium is an essential micronutrient for humans, as it constitutes the enzymatic active centre of several proteins, performing antioxidant, anti-carcinogenic and anti-viral activities. It also reduces the risk of cardiovascular diseases, neurological conditions, type-2 diabetes and even decreases overall mortality. However, 500-1000 million of people worldwide suffer selenium deficiency. Their dietary selenium intakes are not sufficient to maintain adequate blood levels and maximize enzymatic activity.

Selenium intake is required in bioavailable organic forms, which are produced by plants that transform the soil inorganic selenium. Thus, selenium deficiency is caused by its low levels in certain soils of agricultural lands. Accordingly, the most effective approach to counteract selenium deficiency is the biofortification of crops with Se-rich fertilizers. Wheat is the most consumed cereal worldwide and, despite being a non Se accumulator crop, is able to tolerate and accumulate over 100 mg Se per kg of dry weight. Therefore, wheat has been chosen in the present work as a suitable candidate for Se biofortification, in order to study its potential as a functional food.

On the other hand, selenium speciation is a fundamental aspect, as different selenium species have different nutritional properties such as bioavailability and toxicity, e.g, organic selenium, in the form of selenoamino acids, is less toxic than inorganic selenium and thus it is desired in an enriched functional food.

Although plants are able to uptake and transform the Se inorganic species to selenoamino acids through their metabolic pathways, plant growth conditions and selenium application methodology define the extent of selenium uptake, metabolization and distribution through the different plant organs.

The inorganic selenium anions mostly present in soils, selenite and selenate, have different behaviour in plant metabolism: Selenite is readily reduced in wheat roots, and thus, it accumulates preferentially in underground tissues; on the other hand, selenate is highly mobile through plant xylem, and its translocation is faster than its reduction, and therefore it accumulates preferentially in shoots.

In case of excessive selenium application, the specific tissue accumulation may result in plant stress and Se-induced toxicity, which leads to decreased plant biomass production and reduced grain yield. The concentration resulting in significant impairment of the plant development also depends on the selenium species. Specifically, 10 µM selenite resulted in a 55% decrease in grain dry weight, while 10 µM selenate did not cause significant reductions. However, 25 µM selenite caused a 86% decrease of grain weight, whereas the same concentration of selenate caused severe toxicity and plants were unable to produce grain.

On the other hand, the mixture of 10 µM of both selenium species, as 5 µM selenite and 5 µM selenate, did not cause significant weight reductions and 25 µM only reduced grain

weight in a 47%. Therefore, the application of both anions may contribute to balance the Se enrichment due to their separate metabolic pathways, resulting in a more equilibrated distribution through the plant tissues, which reduces phytotoxicity but achieves the same total selenium concentration in grain.

The time in which selenium was supplied to the plants was also studied to minimize the toxicity effects of selenium. It was observed that selenium uptake was a relatively fast process, with selenium accumulation being significantly high in roots and shoots 12 hours after exposure. Furthermore, the application of selenium at the plant florescence stage, instead of at a vegetative stage, decreased the Se toxicity while maintaining, in general, a stable selenium concentration in grain.

The speciation of the accumulated selenium compounds after plant metabolization in roots, shoots and grain was also studied by both conventional methodology (indirect speciation) and direct sample observation using synchrotron light based techniques.

For conventional methodology, tamdem of high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) was used after appropriate sample digestion, to quantify the five major selenium species present in wheat plants: selenite, selenate, selenomethionine, methylselenocysteine and selenocystine. The methodology was optimized, including sample pretreatment, in order to avoid the alteration of the species previous to determination, e.g. selenomethionine oxidation.

Selenite addition resulted in almost complete reduction of selenium into organic species, in particular at roots, where the induced toxicity effects produced a more oxidizing environment within the plant that resulted in a higher accumulation of organic selenium in the form of selenocystine. Oppositely, selenate showed slower metabolization and a significant accumulation of selenium in inorganic form. However, the inorganic forms were mostly accumulated in shoots and a limited amount was translocated into grains. Besides, selenate enhanced the production of organic selenium in the form of selenomethionine, which can be unspecifically incorporated into proteins. In the same manner, the mixture of selenite and selenate produced an intermediate behaviour, with the formation of both selenomethionine and selenocystine.

Speciation was also studied by direct observation methods via X-Ray absorption Spectroscopy (XAS), using synchrotron radiation techniques. This methodology allows direct measurement of the selenium species, avoiding sample pretreatment and incomplete recoveries. The results correlate with those obtained by HPLC-ICP-MS, and additionally showed a significant accumulation of Se (0) in selenite-treated roots.

Furthermore, the combination of the potential of XAS with a small beam size in the form of μXAS, allowed tracking the selenium distribution within plant tissues, and the acquisition of spatially resolved speciation. Special insight was granted to study the distribution in grains, which showed high selenium accumulations in the germ, bran and

pigment strand, and a lower selenium concentration in the endosperm which correlate with the concentration of proteins in the different parts of the grain.

The different selenium treatments applied did not result in relevant differences in the resulting distribution of total Se uptake but affected the different selenium species accumulated in each region. In general, germ contained a higher proportion of C-Se-C amino acids, such as selenomethionine, due to its high protein content, especially in selenate-treated wheat. On the contrary, selenite treated wheat produced a significant relatively higher proportion of C-Se-Se-C (e.g, selenocystine) species in grain as indicative of the plant reaction to the higher phytotoxicity of the selenite species.

Finally, the protective effect of selenium against mercury toxicity was also studied with a simultaneous exposure of the plant to both elements. Mercury caused severe toxicity to wheat plants, resulting in impairment of the plant growth, root elongation and grain production, but the co-occurrence with selenium reduced the toxicity symptoms. Furthermore, the uptake and metabolization of both elements in the form of Se-Hg complexes was indicated from the obtained data. The root uptake of both selenium and mercury was enhanced in the presence of the two elements, and common accumulation areas were seen in the form of hotspots in roots surfaces. The translocation of mercury to shoots and grain decreased in the presence of selenium, reducing the consume risk of crop products in case of mercury pollution. Besides, the formation of the Se-Hg complex reduced selenate mobility and translocation to the shoot, but in the same manner, it also hindered selenite conversion into selenoamino acids, resulting in a Se translocation enhancement in selenite cultures. Moreover, root protein content was also affected by the selenium and mercury exposure, and the obtained infrared spectra indicate the formation of a complex in the form of protein-Se-Hg adduct.

In conclusion, selenium biofortification of wheat effectively enriched grain with sufficient selenium concentration in order to produce a functional food, however, whole-flour preparations would be advised due to a higher accumulation in grain bran and germ than in the endosperm. Selenium biofortification could be applied at florescence time, achieving similar translocation yield than at vegetative stage. In addition, the inorganic selenium species applied during biofortification are successfully metabolized into selenoamino acids, which are more bioavailable and beneficial forms for human consume.

Furthermore, the use of a mixture of inorganic selenium species reduced the selenium induced plant toxicity while maintaining grain enrichment. In addition, this strategy produced an intermediate mix of selenoaminoacids in grain, which opens the possibility for further studies in which different ratios of selenite and selenate can be used in order to define the desired combination of selenium species produced for a specific application of the enriched functional food, while ensuring the safety for human consume.

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1. Introduction and objectives

The present studies on Selenium biofortification of wheat form part of a research project of the Grup de Tècniques de Separació en Química (GTS) de la Universitat Autònoma de Barcelona that has been running during the last six years. Because of the wide readership that may not be accounted with GTS previous studies and the previous reported results on both selenium crop enrichment and selenium chemical speciation, the present thesis will be introduced from a basic perspective.

Introduction

1.1 Selenium

Properties

Selenium, with the symbol Se and the atomic number 34, is an element located in the chalcogen group, the 16th of the Periodic Table. It belongs to the period 4, block p, between the elements S and Te.

Table 1: Chemical properties of selenium [1], [2].

Standard atomic weight	78.971 (8)
Electron configuration	[Ar] $3d^{10}4s^{2}4p^{4}$
Melting point	220,5ºC (494K) (grey)
Boiling point	685ºC (958K) (grey)
Density	4,81 g/cm3
Electronegativity	2,55 Pauling scale

Selenium exists in at least eight different allotropes. The most stable is the grey "metallic" form with a hexagonal crystalline structure. There are also three red monoclinic polymorphs (α β and γ) with Se_8 rings. Additionally, there is as well amorphous red selenium and vitreous black selenium, which is the ordinary commercial form of the element. Grey Se is the most dense structure and the only one that conducts electricity [3].

Isotopes

Selenium has seven natural isotopes. Six of them occur in significant quantities, which are ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se and ^{82}Se. Besides, ^{79}Se is a trace isotope that is found in uranium ores.

Moreover, ^{82}Se is a radioactive nuclide beta$^-$ emitter with a long half-live. ^{77}Se is the only isotope with nuclear spin (1/2) and it can be used in nuclear magnetic resonance spectroscopy[3]. 23 other unstable synthetic radioisotopes have been characterized, but they quickly decay.

Table 2: Selenium isotopes and their abundance, mass and half life [1], [2], [4]. Uncertainties are given in parentheses, corresponding to the last digits.

Isotop	Abundance (%)	Mass (u)	Half life (years)
^{74}Se	0,89 (4)	73,9224766 (16)	Stable
^{76}Se	9,37 (29)	75,9192141 (16)	Stable
^{77}Se	7,63 (16)	76,9199146 (16)	Stable
^{78}Se	23,77 (28)	77,9173095 (16)	Stable
^{79}Se	trace	78,9184991 (18)	$3.27(8)\times10^5$
^{80}Se	49,61 (41)	79,9165218 (20)	Stable
^{82}Se	8,73 (22)	81,916700 (9)	$9,7\ (5)x10^{19}$

Oxidation states

There most common oxidation states of selenium are -2, +4 and +6. Selenium in -2 state is found in selenides, such as hydrogen selenide H_2Se or metal selenides such as Na_2Se, HgSe or Al_2Se_3; +4 is found in selenous acid H_2SeO_3, selenite salts such as Na_2SeO_3, selenium dioxide SeO_2 and selenium sulfide SeS_2; +6 is found in selenic acid H_2SeO_4, selenate salts such as Na_2SeO_4 and selenium trioxide SeO_3 among others.

However, other oxidation states of selenium exist: -1 in diselenides as Na_2Se_2; 0, in the allotrops previously described; +1 in compounds such as Se_2Cl_2, Se_2Br_2 and SeBr; +2 in SeF_2, $SeCl_2$, $SeBr_2$ and Se_4S_4; +3 in Se_2NBr_3 and Se_2NCl_3 and +5 in SeO_3^- and $HSeO_4^{2-}$ [2], [3], [5]–[8].

History

Selenium was discovered in 1817 by the Swedish chemists Jöns Jakob Berzelius (who also discovered silicon, thorium and cerium) and Johan Gottlieb Gahn (who is better known for the discover of manganese). They were producing sulfuric acid from copper

pyrites from Fahlun, when they observed a reddish precipitate with a characteristic horseradish smell. Berzelius analyzed this precipitate and named the new element selenium, for the greek goddess of the moon, *selene*, due to its similarities with tellurium, discovered 35 years before, and named from the latin goddess of earth, *tellus* [3].

However, the symptoms of selenium toxicity had been described a long time before. In the 13th century, Marco Polo was traveling the silk route. In the Shanxi province of China, he observed certain plants being poisonous to animals, causing their hooves to be deformed or even to drop off [9]. In the nineteenth century it was discovered, by Kurt Franke and others, that the "alkali disease" and "blind staggers" diseases of livestock in the American West and Plains States were caused by plants containing high amounts of selenium [10]. Franke, in 1936, already demonstrated that selenium was associated to the protein fraction in the plants, in a compound similar to cysteine [10]. Accordingly, selenium was classified as a toxin.

It was not until 1957, when Schwartz and Foltz probed that selenium was necessary to prevent muscular dystrophy and liver cirrhosis in rats, that selenium essentiality to animals became evident [10], [11]. Afterwards, it was discovered that selenium deficiency was the cause in several cattle illnesses and conditions, such as the "white muscle disease", cardiomyopathies, cataracts, lymphatic diathesis, ill-thrift and liver damage [9], [11].

In 1973 Rotruck et al. demonstrated that Se is part of the active centre of the glutathione peroxidase (GPx) enzyme, which reduces peroxides, protecting cells from oxidative damage [12].

And in 1976 Thressa Stadtman discovered selenocysteine, the "21st amino acid" [10], [13]. And in the 1980s it was found out that selenocysteine was encoded by the UGA codon, which also specifies termination of protein synthesis [14], [15].

The health benefits of selenium for animals and humans became well known, and countries with low selenium in soils or low bioavailability of this selenium started campaigns to increase the Se intake of the population. China added sodium selenite to table salt to combat the Keshan disease [10] and 1985, Finland decided to supplement all fertilizers with sodium selenate in order to increase the amount of selenium in agricultural products, and specially, in cereal grains [16].

Eventually, the new discoveries in the fields of selenium health benefits and selenium metabolism, together with the tragic environmental damage to wildlife in Kesterson reservoir in California in the 1980s, led to a significant increase in the interest of the scientific community in the biological implications of selenium [15].

Sources of selenium

Selenium is the 66th element in order of crustal abundance, comprising 0,05ppm of the earth's crust [3].

There are 40 different types of minerals containing selenium, and although they occur naturally, they are very rare [17]. Selenium is mainly found as an impurity in sulphide ores, associated with chalcophilic metals [3], [17]–[19]. Moreover, it is also found enriching coal (≤6500mg/kg), carbon shales (≥600mg/kg) and phosphatic rocks (≤300mg/kg) [20].

However, selenium for industrial applications is generally not obtained from minerals. Direct recovery from the ores is not economically viable due to their limited occurrence [3]. Therefore, the major source of selenium is in copper refineries. Selenium is obtained as a byproduct from the anode mud deposited during the electrolytic refining of copper. Selenium is released after a treatment with sodium carbonate or sulfuric acid [2].

Selenium can also be recovered from the sludge accumulating in sulfuric acid plants, but this method is no longer used, and from electrostatic precipitating dust collected during the processing of Cu and Pb [3].

In total, global selenium production was estimated to be 2200 tones in 2015, and some of the main producers are Japan, Germany, Belgium, Russia and Canada [21].

Biogeochemical cycle of selenium

On the other hand, apart from the anthropogenic activities, Selenium is also mobilized from the Earth's crust due to natural processes [17]. Figure 2 illustrates the biogeochemical cycle of selenium and how this element is transported from rocks to soils, to water, to biota and to the atmosphere.

Natural processes of mobilization of selenium from earth include weathering of rocks and soil leaching [15], [17]. With a high sediment–water partition coefficient (Kd), this selenium ends up in water bodies such as groundwater, rivers, lakes, estuaries and seas [15], [22]. In natural environments, the predominant forms of selenium are selenides, elemental selenium, selenites and selenates [17], [20], being the two last the most common due to the pH and redox conditions in soils and waters [23].

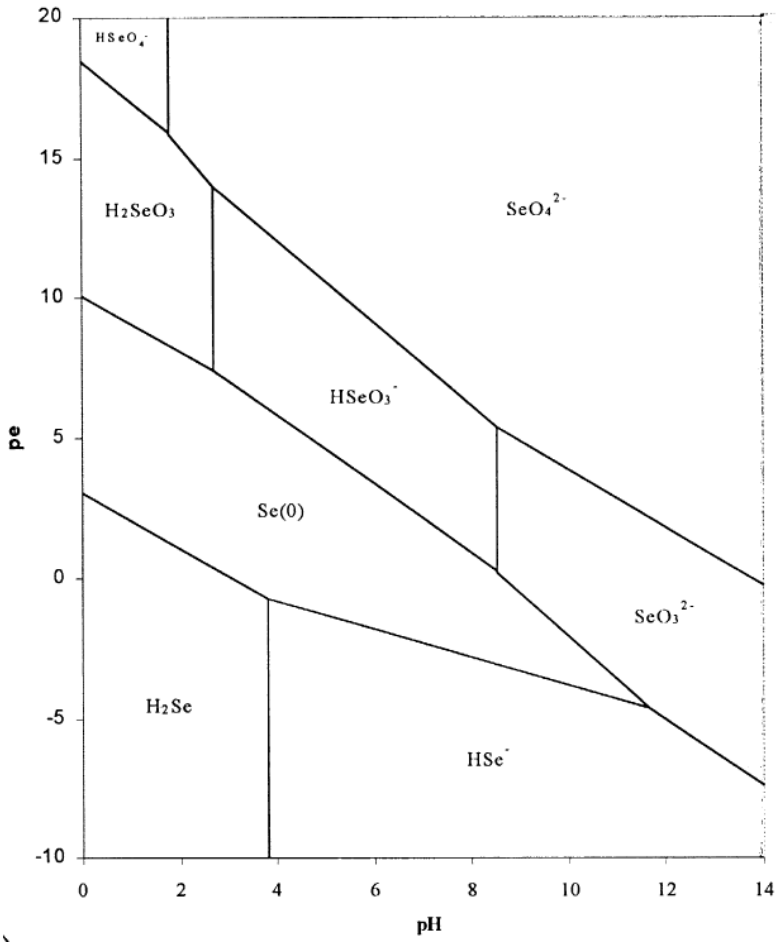

Figure 1: Selenium pE-pH equilibrium diagram (also known as Pourbaix diagram) for the system selenium-water at 25°C, 1 bar, for a selenium activity of 10^{-10} mol/l. Reproduced from [24].

Selenium is incorporated by plants from soil and water, entering the food chain. Then, selenium bioaccumulates through the food web up to apex predators. Selenium has been shown to be highly biomagnified in a study performed in an Australian lake, where a magnification factor of 1.39 per trophic level was determined, with selenium changing from 0,2 µg/g DW in microalgae to 12,9 µg/g DW in carnivorous fish [22]. Additionally, small increases in selenium concentration in water can have a big impact on biota, and specially affect the reproductive success of sensitive fish species [25].

The selenium cycle is closed by the degradation of organism, which returns selenium to the soil and sediments [15].

Selenium also reaches the atmosphere. Natural Se emissions from rich mineral areas and fossil fuel sources account for 50–65%, and anthropogenic activities for 37–40% of the emissions [15]. Selenium is released to the air from coal and oil combustion as selenium dioxide [17]. This is converted mainly to oxyanions, such as selenate [26], which can be adsorbed on dust particles and return to the soil [17].

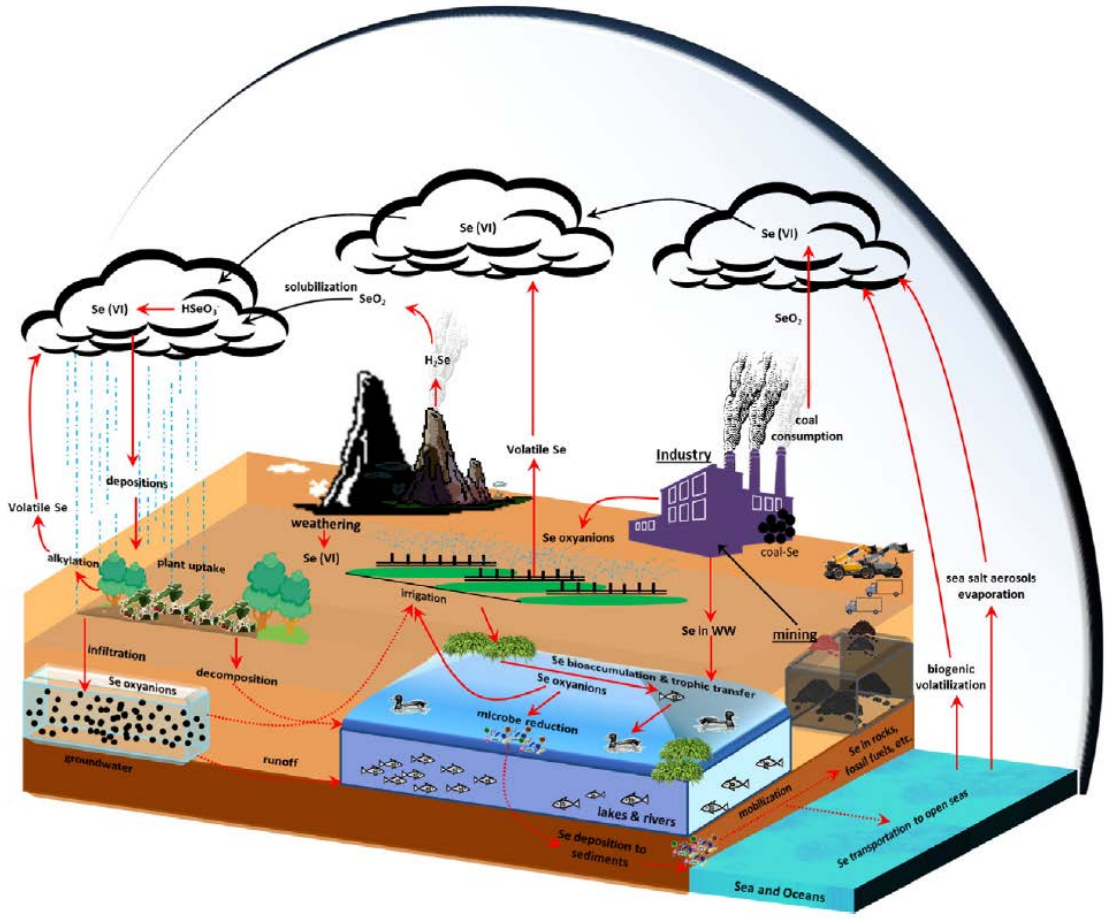

Figure 2: Biogeochemical cycle of selenium, reproduced from [15].

Uses

In 2010, the global consumption of selenium by application was estimated to be as follows: 40% electrolytic manganese production and metallurgy; 25% glass manufacturing; 10% agriculture; 10% chemicals/pigments production; 10% electronics and 5% for other applications [21].

The main metallurgic use of selenium is the production of manganese in electrolytic cells with SeO_2 in China. It is also used with bismuth as a lead substitute in free-machining brass plumbing fixtures, and in iron, lead copper, and steel alloys. In glass manufacturing, it can be used to decolorize the green tint caused by iron impurities, to produce a ruby red color for decorative purposes and to reduce the solar heat transmission through the glass. Moreover, the main selenium pigments are cadmium sulfoselenide compounds, used in ceramics, glazes, paints and plastics, giving a range of red, orange and maroon colors [21].

In agriculture, selenium is added to fertilizers. Moreover, selenium pharmaceuticals and supplements are also available commercially. These applications will be further developed in the present manuscript.

Selenium is also used on thin-film photovoltaic solar cells as copper indium gallium diselenide (CIGS) [21]. Selenium exhibits photovoltaic effect and photoconductivity. These properties enable its use in solar cells as well as in photocells and exposure meters for photographic use, rectifiers that convert alternating current (AC) to direct current (DC) and in p-type semiconductors [2].

Xerography has been another relevant application of selenium, where the photoconductive properties of selenium enabled the copying and reproducing documents. However, recently selenium is being replaced for organic compounds such as polyvinylcarbazole [2].

Lithium–selenium (Li–Se) batteries are promising energy storage systems and cadmium selenide nanoparticles are used as quantum dots. Finally, the dithiocarbamate [$Se(S_2CNEt_2)_4$] is used in the processing of natural and synthetic rubbers [3].

1.2 The importance of Selenium in health

Selenium essentiality, toxicity and deficiency

Selenium is an essential micronutrient for animals, including humans. Selenium in the human body is found substituting sulphur in amino acids, thus called selenoamino acids, which are incorporated into proteins. Specifically, selenium is incorporated in the form of selenocysteine. This amino acid functions as the enzyme active site and performs catalytic redox reactions, supporting various physiological functions [27]. Therefore, a certain amount of selenium to optimize the enzymatic functions is vital and indispensable in order to maintain a good health status.

However, the range of adequate consumption is narrow, and, as much as Se deficiency can lead to Se-related illnesses and conditions, also selenium excess can lead to toxicity.

The Recommended Dietary Allowance of Se for humans is 55μg/day [28]. However, the adequate value depends on factors such as age, gender, eating patterns, etc. Therefore, different countries may establish slightly different regulations [29], [30].

The Tolerable Upper Limit is defined as 400 μg/day by the World Health Organization [31], which is in agreement with the limit of 5 μg/Kg of body weight/day recommended by the U.S. Environmental Protection Agency (EPA) [32]. Furthermore, the 'no observed adverse effects level' (NOAEL) level has been set at 819 μg/day and the 'low observed adverse effects level' at 1540 μg/day based on studies in China [31], [33]. Over those values toxic symptoms may present. Those harmful selenium intake levels could be achieved with regular ingestion of food containing more than 1 μg Se/g [34].

However, speciation is a key factor determining the selenium toxicity, and values over those recommended can be safely ingested in certain conditions. The Inuit people of North Greenland Se intake is estimated to be 193 – 5885 μg/day, with blood concentration above 1000 μg/l. They do not show selenosis signs, probably due to the source of selenium being protein from fish and marine mammals, and thus organic selenium [31].

Oppositely, the population in other seleniferous areas around the world, such as the Hubei and Shaanxi provinces in China, Punjab in India, the great plains in USA, Venezuela and Colombia, has experienced chronic exposure to selenium [26], [31]. Specifically, cases of selenosis have been reported in Chinese provinces, where people eats a cereal-based diet with plants grown in seleniferous soils, with selenium intakes up to 6300 μg/day [35] or 4990 ± 1349 μg/day [33].

Seleniferous regions can be recognized by plants that show white chlorosis, and livestock that has cracks in the hoofs, peeling-off of horns and loss of hair, and premature abortions and deaths [20].

In humans, short-term consumption of high levels of Se may cause nausea, vomiting, and diarrhea [36]. Chronic consumption leads to selenosis in which the primary targets are the cardiovascular, gastrointestinal, neurological and hematopoietic systems [36]. Specifically, chronic exposure of subacute concentrations can lead to brittle hair and nails, hair loss, thickened and stratified nails, gastrointestinal disturbances, garlic odor from breath and skin, skin lesions, and neurological disturbances [26], [31], [36]. In extreme selenosis events, cirrhosis of the liver, pulmonary oedema, or even death can occur [37].

Oppositely, a regular consumption of food containing less than 0.1 μg Se/g will not reach the minimum Estimated Average Requirement of 45 μg/day, leading to deficiency [28][34]. Keshan, Kashin-Beck and Kwashiorkor diseases are associated with low blood Se levels.

Keshan disease is an endemic congestive cardiomyopathy with myocardial insufficiency, that principally affects children aged between 2 and 10 years, and premenopausal women [38]. It causes cardiac insufficiency, cardiac enlargement, arrhythmias, and electrocardiographic abnormalities [39].

Keshan disease occurred to population consuming only local food from deficient regions and whose selenium intake was between 7-15 μg/day [30], [31]. However, Keshan disease is not caused exclusively by selenium deficiency and is influenced by other factors such as viral infection, that leads to the heart condition, with the Coxsackie virus isolated from the patient's heart tissue [31], [38]. Anyway, a minimum of 19 μg Se/day was established by the World Health Organization (WHO) to prevent Keshan disease [30], equivalent to 0,02 mg/g in cereal crops for human consumption [31].

The Kashin-Beck disease is an osteoarthropathy found in rural areas of China, Tibet and Siberia. Kashin-Beck disease is a human rheumatoid condition that affects the joint of the four limbs and causes ankylosis, enlarged joints, shortened fingers and toes, and, in extreme cases, dwarfism [9], [40]. It occurs as a consequence of oxidative damage to cartilage and bone cells, with a decreased antioxidant defence, and an inhibition of bone remodelling by mycotoxins [31]. Although it is associated with severe Se deficiency, low iodine status and presence of fulvic acids or mycotoxins in foods are also a significant factor [31].

Kwashiorkor is a severe protein malnutrition in a low-protein diet, commonly seen in malnourished children. It is characterized by oedemas, enlarged liver, dermatosis and irritability. It is associated with low Se levels. Furthermore, higher selenium status also helps preventing the development of congestive heart failure (CHF) in children recovering from Kwashiorkor [41].

Nevertheless, these conditions and cases of severe deficiency in humans are rare and limited to specific areas in the world. However, situations of moderate deficiency are much more common, and selenium deficiency is associated with other more usual pathologies such as muscular dystrophy, reproductive disorders, cardiovascular sclerosis, dental caries, necrosis of liver, kidney and heart, digestive alterations, rheumatic diseases and many types of cancer [36], [38].

In addition, selenium deficiency can also affect livestock with reproductive impairment, growth depression, ill-thrift, and white-muscle disease, a myopathy affecting heart and skeletal muscle [9], [42].

Benefits of optimal Se levels in humans

Maintaining an adequate selenium level can highly benefit human health. Selenium intake at optimal or supranutritional doses for those with moderate shortages can not only prevent deficiency related conditions but also enhance health status, with immunostimulating effect [42] reduced cancer risk [32], reduced mortality [43] and high antioxidant capability, delaying aging and degenerative diseases [38].

- **Selenoproteins**

Selenium performs its activity through the selenium containing proteins, as the active site of the enzymes. In these proteins, selenium is specifically incorporated in the form of selenocysteine via the UGA codon [14]. Selenocysteine is analogous to cysteine, with a Se atom replacing S. However, the chemical differences between the two justify the significance of selenium in metabolism. Selenium is more polarizable, which leads to weaker bonds, more electrophilicity and nuclophilicity and faster reactivity [10]. Another relevant diference is the change in the pKa. Selenocysteine has a pKa of 5,24,

versus cysteine having a pKa of 8,25 [10]. Therefore, at physiological pH selenols are completely ionized to selenolates, while thiols are practically not ionized [10]. Consequently, this results in approximately 2 orders of magnitude higher reactivity [10] and 100 times higher catalytic efficiency [44] for selenoamino acids as the active sites of enzymes.

A total of 100 selenoprotein families have been identified [45], of which 25 are found in humans [46]. Only the function of some of the so far known selenoproteins is known (see table 3). However, their functions are clearly differentiated, with some of them being present only in specific tissues or organs [47]. Moreover, the expression of the diverse selenoproteins has different priority in the body, and accordingly different biological significance. In cases of selenium shortage some are preferential over others: brain endocrine and reproductive organs are high in the hierarchy, with the 18kD-selenoprotein being preferentially synthesized. Oppositely, liver, muscle and heart organs, blood plasma and blood cells are the first to be depleted, together with the glutathione peroxidase enzymes [47].

Table 3: Known selenoproteins and their biological functions. Data from [42]–[45], [47]–[50]

Selenoprotein	**Function**
Glutathione peroxidases (GPx1, GPx2, GPx3, GPx4, GPx6)	Antioxidant: Reduction of hydrogen peroxides and hydroperoxides to prevent oxidative damage to biomolecules and cells, maintaining membrane integrity. Recovery of the cells after oxidative stress, reduction of retroviral virulence, protection against cancer and neurodegenerative diseases. Comprises 10-30% of total plasma selenium.
Thioredoxin reductases	Oxidoreductases: maintain a reduced environment in cells with redox homeostasis. Cell signaling. Reduction of nucleotides during DNA synthesis, regulating gene expression.
Iodothyronine deiodinases	Production and regulation of the active thyroid hormone. Activation and inactivation of the hormone.
Selenoprotein H	DNA-binding protein. Transcription and regulation of gene expression.
Selenoprotein I	Phospholipid biosynthesis
Selenoprotein K	Unclear. Located in the endoplasmic reticulum (ER). Antioxidant.
Selenoprotein M	Thiol-disulfide oxidoreductases. Protein folding in ER.
Selenoprotein N	Early muscle formation and calcium mobilization from ER.

Selenoprotein O	Unclear.
Selenoprotein P	Selenium transporter. It contains 40-50% of the total selenium in plasma in 10 SeCys residues. Relevant to brain and testes functioning. Protection of endothellial cells. Protection from oxidative damage. Heavy metal complexation and detoxification. Prevention of liver necrosis.
Selenoprotein R	Methionine sulfoxide reductase. Protein repair. Antioxidant.
Selenoprotein S	Removal of misfolded proteins from ER, and protection of ER of oxidative damage and stress-induced apoptosis. Involved in inflammatory response, regulation of inflammatory cytokines.
Selenoprotein T	Calcium mobilization from ER.
Selenoprotein V	Unclear. Restricted to testes, potential role in male reproduction.
Selenoprotein W	Muscle function and growth. Skeletal and cardiac muscle metabolism. Protects myoblasts from oxidative stress. Antioxidant. Intracellular transporter.
Selenophosphate synthetase	Biosynthesis of selenophosphate as intermediate in selenocysteine synthesis. Necessary for all selenoprotein synthesis.
Selenoprotein 15kD	Antioxidant. Located in prostate epithelial cells. Protects secretory cells against carcinoma. Important form in the prostate gland. Role in unfolded protein response.
Selenoprotein 34kD	Antioxidant. DNA-bound in spermatids and sperm nuclei. Protects developing sperm.
Selenoprotein 18kD	Unclear. Mitochondrial selenoprotein
Sperm mitochondrial capsule	Protects developing sperm cells and helps stabilizing and mobilizing mature sperm.

- **Antioxidant**

One of the most predominant and well studied role of the selenium enzymes is its antioxidant activity [32], by the glutathione peroxisases family. They reduce the damage caused by reactive oxygen species (ROS). For example, harmful hydroperoxides (ROOH) react with two glutathione molecules (GSH) to form hydroxides (ROH), diglutathione (GSSG) and water, catalyzed by the GPx enzymes [44]. They are part of a complex antioxidant system in the organism, together with other compounds such as Vitamin E, Vitamin C, carotenoids and catalase, which eliminates

the free radicals and the peroxides, hydroperoxides, superoxide anions and hydroxyl radicals [38]. By preventing the damage that these compounds cause to cell membranes, they maintain the proper functioning of the organ, slow the aging process and delay the manifestation of degenerative diseases, reduce the risk of tumours, diminish the occurrence of atherosclerosis and hypertension and prevent immune dysfunction, which are all conditions associated with the presence of free radicals in the organism [38].

Accordingly, selenium deficiency would derive in an impairment in the GPx enzyme activity, inducing higher oxidative stress and cellular damage. The tissues more vulnerable to oxidative stress are heart, liver, diaphragm and striate muscle, that have high metabolic activity [38]. Thus, selenium is especially significant for those organs. A decrease in Se-related antioxidant capability is also associated with hemolysis of erythrocytes and hepatic necrosis of the liver [38]. Effects to the liver from selenium deficiency can be similar to those from excessive consumption of alcohol [38].

Moreover, increased oxidative stress also leads to increase inflammation as it enhances the production of inflammatory prostaglandins and leukotrienes [42]. Therefore, selenium deficiency is also related to rheumatoid arthritis, pancreatitis and asthma conditions, being selenium supplementation proven to reduce the pain and the severity of the illness [42]. Specifically, regarding acute necrotising pancreatitis, selenium intravenous administration resulted in zero deaths from an otherwise usually lethal illness in untreated patients [51], [52]. Furthermore, higher selenium status also reduces the risk of systemic inflammatory response syndrome and sepsis in patients [43].

- **Mortality**

High selenium levels in blood are correlated with low mortality in the population, both in all causes of mortality and in cancer mortality [43]. In a study performed in the US, serum selenium levels of 130-135 µg/L showed the lowest mortality [53]. Below 120 µg/L higher mortality was observed, in agreement with other studies [54], [55], but also levels over 150 µg/L resulted in higher mortality [43].

Also, in agreement with those values, 122 µg/L in plasma or serum is the value in which selenium supplementation produces a change in cancer risk and accordingly cancer mortality. With lower basal levels, an increase of selenium through supplementation decreased the incidence of tumors, but with basal levels over that value, supplementation did not result in an improvement [43], [56]. Consequently, selenium can reduce mortality, and specifically it is highly significant in mortality derived from cardiovascular diseases and cancer mortality, and up to one third of the cancer mortality could be prevented with adequate selenium intakes [11].

- **Cardiovascular diseases**

Low selenium status has been associated with increased risk of cardiovascular diseases as coronary heart disease[57], acute myocardial infarction, atherosclerosis, congestive heart failure, cardiomyopathy, arterial hypertension, chronic heart disease, ischemic cardiomyopathy, coronary atherosclerosis, etc. [38].

The main effects of selenium are: the prevention of the formation of lipid hydroperoxides, which may attack the vascular endothelium and which inhibits the synthesis of vasodilatory prostacyclin; the reduction of the accumulation of oxidized low-density lipoproteins in the artery wall and the decrease in the synthesis of thromboxane, which causes platelet aggregation and vasoconstriction [42].

Furthermore, selenium supplementation in elderly people decreased total serum cholesterol and non-HDL cholesterol, and increased HDL cholesterol, consequentially decreasing the risk for cardiovascular diseases [58]. The initial selenium status of the population before the supplementation is decisive to determine the potential benefit: with low Se concentrations, cardiovascular mortality was reduced, but for patients with already optimal Se and optimal selenoprotein activity, no effect was detected [43].

- **Neurological conditions**

Two neurologic diseases are associated with low Se and high oxidative stress: multiple sclerosis and Alzheimer's disease [38]. Selenium and other trace elements have been shown to be lower in patients with Alzheimer's disease, who also have lower GPx activity [59], [60], and selenium also acts as an antagonist of tellurium, which has been hypothesized to cause damage in mitochondria that leads to the neurodegenerative disease [61].

In the same manner, low selenium levels have also been found in patients with Down's syndrome [62], [63].

Furthermore, selenium is also related with depression. Several studies have demonstrated low selenium status to correlate with depressed moods [64] and increase the risk for major depressive disorder [65]. It has been seen that Se deficiency alters the turnover rate of some neurotransmitters [42] and interacts with the dopaminergic and noradrenergic systems [66]. Moreover, several organoselenium compounds have been shown to have antidepressant-like effects [66].

Other studies have attempted to relate selenium blood levels with mood status with inconsistent results. Some studies have shown low selenium to have a negative mood influence, leading to more anxiety, confusion, hostility and tiredness [42], but other studies did not show a positive effect of selenium supplementation in mood scores [67].

- **Diabetes**

Diabetes mellitus is related to the alteration in the homeostasis of certain elements such as selenium [38]. Specifically, the incidence of the type-2 diabetes has been shown to be higher in people with low Se status [68]. However, conflicting results are found in clinical studies: while some report correlations between high plasma selenium and reduced diabetes prevalence, other reveal a significantly increased risk of type-2 diabetes with selenium supplementation [43]. There are several mechanisms in which selenium is affecting diabetes that explain this variability. Diabetes has associated a systemic inflammatory response that will reduce selenium levels in plasma. Moreover, Insulin resistance can be triggered by oxidative stress and ameliorated by antioxidant treatment. Therefore, patients with high selenium status and thus high antioxidant GPx activity were protected from the development of insulin resistance [43].

However, the increased risk of type-2 diabetes with supplementation is due to the effect of selenium in insulin signaling. Thus, the binding of insulin to its receptor is accompanied by a release of hydrogen peroxide. But a higher activity of the GPx1 and other selenoproteins will remove the hydrogen peroxide and interfere with the signaling. Therefore, higher selenium concentration in blood or overexpression of GPx1 could lead to insulin resistance, hyperglycaemia, hyperinsulinaemia and obesity [43].

- **Cancer**

Several studies have found a clear relationship between higher selenium dietary intakes and lower cancer incidence and mortality [42], in epidemiologic studies, animal experiments and clinical intervention trials [69]. Specifically selenium has been shown to be effective for: cancers of large intestine, rectum, prostate, breast, ovary, lung and leukemia [70]; cancers of the lung, breast, rectum, bladder, oesophagus, and corpus uteri [71]; lung cancer [72]; hepatocellular carcinoma (HCC) [73]; prostate cancer [74]. However, other studies have not shown a positive effect of selenium supplementation in cancer prevention [75], such as in prostate cancer [76] and bladder cancer [77].

Some of the literature reviews on selenium effect in cancer have failed to consider the specific aspects of the diverse studies. The chosen model and the concentration of selenium are factors as decisive as the basal level of selenium before the intervention and the chemical form of selenium used in the study, in order to determine the effectiveness of selenium as an antineoplastic agent [32], [42], [78].

A very well-known selenium study is the Nutritional Prevention of Cancer Trial (NPC) which was the first double-blind, placebo-controlled clinical intervention in western population [42]. It proved that selenium supplementation in patients decreased the total cancer mortality by 50%, the total cancer incidence, by 37% and the incidences of lung, colorectal, and prostate cancers by 46%, 58% and 63% respectively, even if it did

not had an effect in basal cell or squamous cell skin cancer incidences [56]. However, a follow up in the also well-known SELECT trial, did not shown any significant improvement in prostate cancer incidence [79]. Yet, there were crucial differences between the two investigations: selenomethionine was used instead of selenized yeast; selenium was given in combination with vitamin E, and the population was only men with an already high Se intake and high basal level of selenium [80], [81]. Selenomethionine is the main component of selenized yeast; nevertheless, a 30-40% of the total selenium is in other forms, such as methylselenocysteine, which is more effective in chemoprevention [43], [82].

Selenium exerts an effect in cancer prevention through its antioxidant properties, its ability to inhibit cell growth and to induce tumour cell apoptosis and its enhancement of the immune system [11], [83]. It inhibits both the initiation and post-initiation stages of carcinogenesis, through inhibition of phase I enzymes (which convert chemical carcinogens to reactive adducts that attack DNA) and induction of phase II enzymes (detoxifying enzymes) [83].

Additionally, selenium can be also used for anti-tumour therapeutics to promote carcinogenic cell death [84]. Its success is based in the pro-oxidant effect at high concentrations of selenium [84]. Selenium selectively affects cancer cells preferentially than healthy cells. Tumour cells tend to have a more reducing environment that favours selenium uptake [80]. Moreover, selenium acts as a pro-oxidant at lower doses in malignant cells than in benign cells, making the former more sensitive to Se cytotoxicity and Se supplementation [78], [85]. Additionally selenium affects angiogenesis, leading to a decrease in microvascular density, reduces tumour metastases and inhibits cell growth [78], [80], [85].

The main step in the mechanism of action that leads to the death of cancer cells is the production of the methylselenol metabolite (CH3SeH) [86], [87]. Due to the low pKa value of selenol (pKa $\sim$5.8) the Se is almost fully ionized under physiological conditions, and is highly reactive [88]. Thus, this selenolate anion (RSe−) catalytically generates superoxide radical (O2·−) by oxidising reduced glutathione (GSH) and other thiols (RSH), which causes oxidative stress to the cell, inducing cell-cycle arrest and apoptosis [86].

The effectiveness of several selenium species against mammary tumours has been studied: data shows that Se-methylselenocysteine is the most effective selenocompound, followed by selenite, and selenomethionine and selenocystine are the least effective of the major species of Se in foodstuffs [86], [89]. Selenobetaine is as effective as Se-methylselenocysteine, however, it is not found in aliments [89].

Selenomethionine generates methylselenol from the enzyme methionase, and Se-methylselenocysteine from beta-lyase enzymes [86], [87]. Selenocystine is easily reduced to selenocysteine, releasing the selenolate anion form [84]. Selenite also

generates easily selenolate anion and superoxide metabolites [86]. Oppositely, selenate is not as effective producing methylselenol, and would need to be metabolized first [86], [88]. Additionally selenite causes necroptosis, necrosis and paraptosis cell death, apart of the apoptosis mechanism caused by organic selenium species [78].

Methylseleninic acid, which is not naturally present in foods, produces methylselenol without the need of enzymes, being more effective than Se-methylselenocysteine as a chemopreventing agent in the situations of low abundance of beta-lyases [89]. Other synthetic compounds are also effective against tumours, such as selenocyanates as 1,4-phenylenebis(methylene)selenocyanate (p-XSC) [84], [90].

In general, inorganic forms cause a higher genotoxic stress and systemic toxicity, leaving a smaller therapeutic window for cancer treatment [84]. However, organic Se compounds have significant anti-tumour activity and prevent metastasis, with fewer side effects and lower systemic toxicity [84].

- **Immune function**

Selenium has significant implications in the body immune function and thyroid function. The thyroid gland has the highest selenium concentration of all tissues, and a family of selenoproteins, the iodothyronine deiodinases, are responsible for the production of the active thyroid hormone from its inactive precursor [43].

Moreover, selenium is also implicated in the activity of the T cell and lymphocytes. Selenium supplementation enhanced their activity and lead to a rise in the proliferative reaction against antigens with an increase in cell-mediated response to immunity [68]

Therefore, selenium supplementation has been shown to be effective against Hashimoto's thyroiditis, the most common form of autoimmune thyroid disease, against Graves' disease, an autoimmune hyper-thyroidism, as well as, against the development of post-partum thyroid disease and permanent hypothyroidism in pregnant women with autoimmune thyroiditis [43].

- **Antiviral effects and HIV**

Selenium also has a role in the inhibition of viral expression and in delaying the progression of AIDS in HIV-positive patients [27].

Selenium deficiency, together with low GPx1 activity, has been linked to the occurrence, progression and virulence of several viruses. It has been suggested that the lack of selenium can accelerate viral replication by reducing the immune response

of the host or by increasing the oxidative damage to the RNA genome, which augments the opportunities for virus mutations to occur [91].

For this reason maintaining adequate Se levels in order to optimize the antioxidant activity of GPx1 is important to avoid viral mutation to more virulent forms [31], [43].

Moreover, supplementation can enhance antiviral effects in humans [31]. Supplementation is recommended in cases of infection, since it is noticed that retroviruses, such as HIV and Coxsackie B3, and viruses, such as *Molluscum contagiosum*, can deplete the host of selenium, by incorporating it into their seleno-proteins for protection [31].

Specifically regarding HIV, selenium deficiency has been shown to correlate with decreased survival in HIV-infected patients, whereas supplementation of HIV-positive patients has been shown to lead to decreased viral load and hospital admissions [43].

- **Fertility and reproduction**

Selenium plays a role in reproduction since it is required for testosterone biosynthesis, and for the formation and development of spermatozoa [42]. Glutathione peroxidase (GPx4), located in the mitochondria of the sperm tail[43], protects developing sperm cells from oxidative damage, and later in mature spermatozoa, it cross-links to become a structural protein surrounding the flagellum, which ensures integrity, stability and motility [42]. Moreover, sperm nuclei selenoprotein is necessary for sperm maturation and selenoprotein-P is required for Se supply to the testes [31]

The analysis of sperm samples demonstrated that selenium deficiency in men resulted in infertility, as well as morphological variations and lower feasibility and mobility of the sperm [68].

Oppositely, selenium supplementation positively improved concentration of spermatozoa in sub-fertile men, sperm motility and reproductive success [42], [68].

On the other hand, excess of selenium intake (about 300 μg per day) has been shown to decrease sperm motility [92].

Regarding woman, selenium deficiency could lead to higher miscarriage risk, as has also been observed for cattle, with selenium being essential for the protection of biological membranes and DNA [42]. In addition, higher oxidative damage with lower selenium levels can also result in preeclampsia during pregnancy [11]. Furthermore, selenium can cross the placental barrier and enter the fetus, protecting the embryo development from lipid peroxidation, and it is as well transmitted from the mother's blood into her milk [11], [27].

- **Other health benefits**

Furthermore, Selenium is also a contributing factor to other pathophysiological conditions, including neuromuscular disorders and inflammation, and participates in muscle metabolism, with selenium deficiency causing muscular dystrophy [11], [27].

Moreover, it protects the skin from ultraviolet radiation, and can counteract asthma [11].

Finally, selenium has a protective effect against heavy metal toxicity. It affects the metabolism of the heavy metals by forming insoluble metal selenides that are excreted from the body, and therefore, reduce the poisoning toxic effects and symptoms [11], [93]–[97].

1.3 Selenium supplementation and functional foods

The need for selenium supplementation

Despite all the aforementioned benefits, available data suggest that 500-1000 million of people may be Se deficient [39], which accounts for approximately a 15% of the total world population with insufficient Se levels in their blood [68] (further data in references [39], [42]).

Measuring selenium concentration in serum or plasma through several countries has shown that, in general, the selenium level of the population was not sufficient to maximize the activity of the glutathione peroxidase selenoenzyme (GPx) at 89–114 μg/L [42], or to reach the concentration of 130–150 μg/L, which has been associated with minimal mortality and reduced risk of cancer, non-melanoma skin cancer, and type-2 diabetes [42][43][98]. In addition, the Se plasma levels encountered are also significantly lower in patients suffering of cancer, cardiovascular diseases and diabetes than in healthy controls [38].

Dietary intake is the source of selenium for humans. Accordingly, Se deficiency is due to an insufficient selenium consumption. Human intakes can vary widely throughout the world. Levels are found between 3 and 6,690 μg/day [34], although the average usually falls in the range of 20–300 μg/day [34][39], with mean values of 40 μg per day in Europe [43] and 98 μg per day in the USA [34]. Reviews of mean intake per country can be found in references [31], [38], [39], [68] and references therein.

The selenium content in foodstuffs, both plant and animal products, mainly depends on the content of selenium in the soil. Therefore, the diversity in selenium intakes between regions is caused by soil Se levels that differ greatly between them. Continental crust and, accordingly, agricultural lands have uneven distributions of essential micronutrients, such as selenium [99].

Selenium levels in soils can range between less than 0,05 mg/kg to higher than 5mg/kg [68]. In addition to the endemic causes, man-made activities, such as agricultural overexplotation, can also lead to depletion of micronutrients [100].

Therefore, a lack of nutrients in agricultural soils is common. Low selenium levels in soils result consequently in a shortage through the food chain [100]. Consequently, humans have an insufficient intake of selenium through diet, which results in an incorrect expression of proteins and an increased risk of several health conditions.

Dietary selenium sources

In order to counteract selenium deficiency, selenium intakes should be increased in population with low selenium status.

Despite the content of the soil being the main factor in determining the content of selenium in edibles, some foodstuffs are naturally richer in selenium than others. In general, foods that are rich in proteins tend to have higher concentration of selenium [68]. For this reason, meat, eggs, fish, and seafood can all accumulate high concentrations of selenium, in spite of the fluctuations between animal species and origin. Mushrooms also tend to have a high content of selenium as well as sulfur [36], [101], [102]. In contrast, fruits and vegetables have some of the lowest selenium content, due to their lower protein content [68], [103]. However, some vegetables are able accumulate high concentrations of selenium in high Se media, such as broccoli, garlic, onion and cabbage [35], [104]–[107]. Cereals can also contain an important amount of selenium, since grains in particular are especially rich in protein [108], [109]. Finally some nuts are also rich in proteins, such as such as pistachios, walnuts and Brazil nuts [36]. Specifically, Brazil nuts are very high in S-containing amino acids, and are the foodstuff with the highest selenium content, with one single nut being sufficient daily to exceed the Recommended Dietary Value [34], [101], [110]–[112].

Table 4: Range of selenium content reported in selected foodstuffs coming from several countries, as analyzed or reviewed by [34], [39], [43], [68], [101], [103], [113], [114]

Food	**Selenium concentration (µg Se/Kg)**
fruits	0,023-60
vegetables	0,52-140
tubers	0,3-230
mushrooms	3,3-1340
milk and diary	1-430
eggs	20-1500
red meats	8,2-1000
poultry meats	10-3020
organ meats	400-1543
fish and seafood	20-1480
cereals	4-10000
nuts	2-38000

However, not all foodstuffs are consumed in the same amount by the population. For example, Brazil nuts, despite being the richest selenium source, are not commonly consumed [34]. Accordingly, the aliment with biggest content is not necessarily the one that provides a bigger amount of selenium to the body. In Japan, the main contribution to the selenium intake comes from fish and seafood, which represents a 60% [115]. In UK, the majority of the selenium intake comes from both cereal based foodstuffs and meats, each representing a 26% of the selenium consumption, whereas in US cereals are the main source with a 37% of the contribution [43]. In Russia the main Se supply derives from cereals, with a 50% contribution, while meat provides a 20% [116]. In China, meat contribution to total selenium intake is significantly higher, accounting for approximately a 67%, while in Saudi Arabia, cereals and legumes predominate, with a 30% and a 25% respectively [68].

Methods for increasing selenium intake

The consumption of a bigger proportion of naturally rich selenium foods is one possible approach in order to increase selenium status. However, changing the diets of deficient population can be difficult due to culture and traditions [116].

For this reason, a better approach is to increase the amount of selenium in the food already available and of usual consumption in deficient areas.

Different methods are possible in order to increase the nutritional food quality: the addition of nutrients (including minerals and vitamins) in processed food, which is called fortification, or the addition of micronutrients to plants during their cultivation to increase the content in the edible part of the plant, which is known as biofortification [36]. This could be done with the application of selenium to soil or plants with Se-rich fertilizers [68], [117]. In a similar way, it is also possible to increase the selenium content in animal feed, in order to produce selenium-enriched animal-based foodstuffs, such as meat [118], [119], eggs [116], [120] and milk [121], [122].

Other approaches can be considered such as the addition of Se to water supplies [123], or the intake of nutritional supplements, such as multi-vitamin preparates (generally containing inorganic Se) [34], synthetic selenomethionine [124]or Se-enriched yeast [125]–[127].

However, although selenium supplements are widely available in western countries, only health-conscious individuals tend to consume this type of products [123]. Moreover, discontinuation of the supplementation results in a return to a selenium deficient status, as seen in the Mianning County, where the discontinuation of the fortification of local salt with selenite led to the reappearance of the Keshan disease [128].

Besides, fortification, which has already been attempted in table salt, rice, wheat flour and biscuits, has been shown to have some drawbacks: a limited stability of the additives, the possible nutrient lose in cooking, the modification of food quality or flavour or the requirement for an industrial infrastructure to perform it [129], [130].

Oppositely, the addition of selenium in fertilizers for agronomic biofortification has proved to lead to excellent results. The nation-wide official decision of adding selenium to all fertilizers in Finland successfully changed the selenium status of the population from deficient to optimal. Its use since 1984, nowadays at a level of 10 mg Se/kg fertilizer, has achieved an increase of 10-13 fold the content of cereals, and 2 fold the blood serum Se level [9], [131], [132].

For all these reasons, crop enrichment with Se-containing fertilizers provides best short-term and long-term solution, which, additionally, can be coupled with long term genetic improvement through plant breeding [133], [134].

Functional foods

The idea to use food in order to enhance health benefits already started in the early 1980s in Japan, where, later, the concept of functional food or FOSHU (foods for specified health use) was established [135].

A functional food is defined as a food that has a demonstrated beneficial effect to one or several target functions in the body, beyond the traditional nutritional effect, in a way that improves the state of well-being, health, and physical and psychological performances, or which reduces the risk of a disease [136].

Therefore, functional foods must be natural foodstuffs, fresh or processed, which are already part of the diet, not supplements in the form of pills of capsules [135], [137]. They can be prepared by several approaches: eliminating a component known to cause a negative effect, increasing the concentration of a beneficial component which is naturally present or adding a component that is not normally found in that food, replacing a component for another, or increasing the bioavailability or stability of a desired component [136].

Therefore, selenium-enriched foods are considered functional foods due to the health benefits of this element for human health.

1.4. Selenium speciation

The importance of selenium speciation

However, is not only the amount of selenium, but which chemical species of selenium are consumed, what eventually determines the final Se status and the related health benefits. Different selenium species have different bioavailability, toxicity and are processed in a different manner once ingested [39], [42].

For example, despite fish and seafood having a relatively high selenium content, it appears that the Se they contain is less bioavailable that the one coming from other dietary sources, since it is found in a molecular form which is not readily available to be used for selenoprotein synthesis in human metabolism [113], [138]. Oppositely, selenium content in vegetables is smaller, but it is highly bioavailable. Normally, a 85-100% of the selenium from vegetables is bioavailable, in comparison with the 20-50% (but usually lower than 25%) selenium from seafood [38]. Meat products have even lower bioavailability, approximately a 15%, and diary products the lowest, with a 2-7% range [38]. Selenium from Brazil nuts is also highly bioavailable [30].

Selenium species

The difference it is mostly due to the different selenium species having different activity in the body and being metabolized in a different manner.

The most important selenium species that occur naturally, and that are relevant for the present work are found in Figure 3. Other synthetic selenium species are gaining more significance due to their use as antineoplasic agents, but are out of the scope of this work and have not been included, but can be found in other reviews [84].

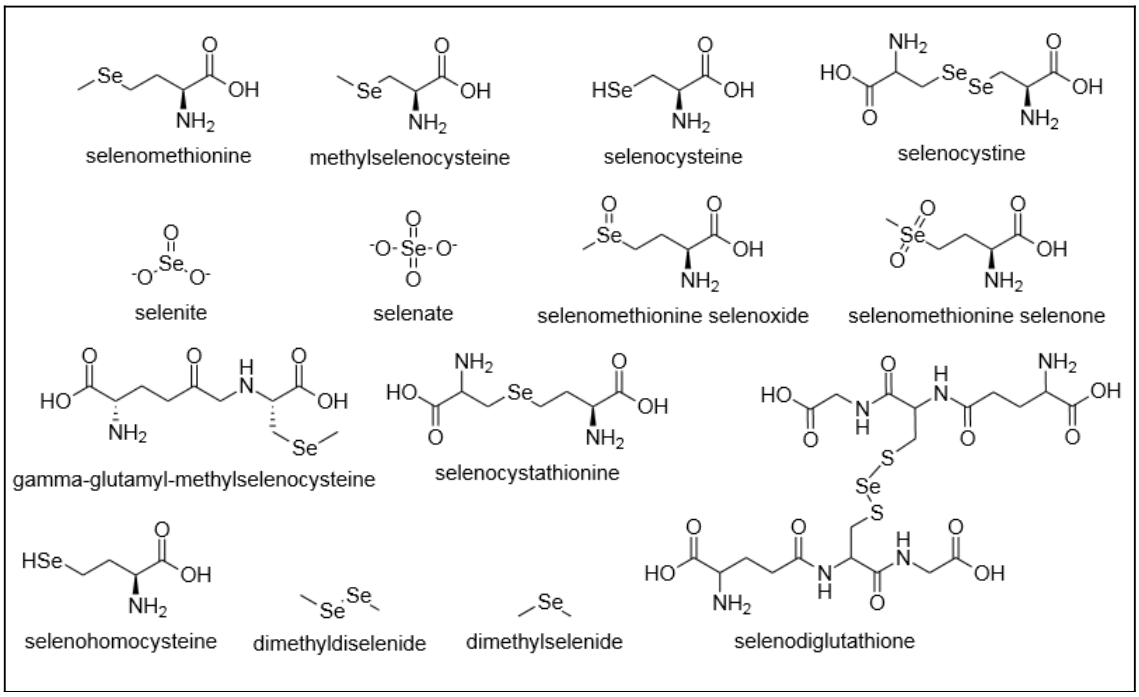

Figure 3: Main selenium species, relevant to the present work.

Those species are present in different amounts depending on the food source. In plants, selenate and selenomethionine are predominant in non-accumulating plants, while γ-glutamyl-MeSeCys is the main form in selenium hyperaccumulator plants [49]. In fish selenite and selenate are the prevalent species [49]. Finally, in cattle meat and other animal-derived foodstuffs, selenomethionine and selenocysteine are the main forms [49], [133]. However, their relative abundance in animals depends on the selenium form in the feed: inorganic selenium intake results in meat rich in SeCys, while a diet based on Se-rich plants generate meat with an elevated content of SeMet [113].

Bioaccessibility, bioavailability and bioactivity concepts

Three different terms can be used in order to describe the fraction of the ingested compound that is absorbed by the organism and effectively metabolized into active forms, as summarized by Thiry et.al. [12]:

The bioaccessibility defines the capacity of the element to be absorbed through the intestinal mucosa, and thus being retained by the body instead of being excreted. The bioaccessibility can be measured by in vitro gastro-intestinal digestion.

The bioavailability defines the capacity of an element to be absorbed and to reach the systemic circulation, where it can be distributed to organs and tissues. Bioavailability should be estimated by analyzing Se level in blood and in body tissues.

Finally, the bioactivity refers to the capacity of the element to be absorbed, distributed and converted into biologically active metabolites. For example, selenium bioactivity is estimated by measuring the activity of certain selenoproteins.

In general, the effectiveness of the selenium supplementation of the population is measured by both the bioavailability and the bioactivity. The changes of selenium concentration in plasma, erythrocyte, and whole-blood as well as the changes of activity of plasma selenoprotein P, and plasma, platelet, and whole-blood glutathione peroxidase activity can be used as biomarkers for the selenium status. Oppositely, there is not enough evidence to use the concentration of selenium in urine, hair or toenails, the plasma triiodothyroxine:thyroxine ratio, plasma thyroxine, plasma total homocysteine, or erythrocyte and muscle glutathione peroxidase activity as biomarkers [48]. Nowadays, it seems that selenoprotein P is the most promising biomarker, because it contains approximately half of the selenium in plasma, and thus is more sensitive than other selenoproteins to changes in the selenium status [49], although the GPx activity is the most commonly measured parameter through the literature.

Bioavailability and bioactivity of the selenium species

Different species of selenium are metabolized in different ways, thus resulting in different degrees of bioavailability and bioactivity.

Regarding bioaccessibility and bioavailability, organic selenium seems to be better than inorganic in order to increase selenium status. More than 90% of selenomethionine and selenocysteine is absorbed by the body and effectively increases blood Se levels [49], [133]**.** On the other hand, selenate is almost all absorbed, but its bioavailability is low. It is not well retained in the body and a big fraction of it is excreted in urine. Oppositely, only around 50% of selenite is absorbed, but its better retained than selenate [49]**.**

Regarding bioactivity, also organic selenium seems to be more adequate to increase not only blood selenium levels but also GPx activity. A selenium rich diet with organic selenium, in the form of selenomethionine-containing yeast, was more effective in increasing selenium blood concentration and GPx enzymatic activity than sodium selenite, in beef [139] and turkey [140]. Moreover, similar results were found in blood Se concentration and GPx activity between selenium-rich yeast and selenium enriched mushrooms in beef supplementation, with Se-enriched mushrooms being a significantly cheaper source of selenium than yeast [141].

Other studies confirm those observations. Tests in rats showed that the GPx activity did not increase with a selenite supplementation (98% of activity, compared with 100% of GPx activity in the control groups). Selenate supplementation effectively increased the activity (127%) as well as a diet of enriched beef meat (127-139%), which contained a significant fraction of organic selenium [142].

Furthermore, it has to be considered that other nutrients present in the diet can affect the fraction of the selenium that is bioavailable, such as minerals, fiber, phytate and pectin, but further studies are required [38]. Moreover, positive synergisms in health benefits between selenium and vitamin E and beta-carotene should also be considered [123].

All these reasons support the better suitability of a selenium rich diet with biofortified products, containing organic species selenium, over inorganic supplements or even Se-rich yeast, with biofortified foodstuffs being not only cheaper to produce but also more effective at increasing selenium status and enzymatic activity, and thus obtaining higher health benefits at lower selenium concentrations.

Selenium metabolism in humans

As previously discussed, selenium is very important in the human body, as it conforms the active site of at least 25 proteins. That selenium is exclusively in the form of selenocysteine, which is considered the 21st aminoacid, and which is the only selenoamino acid that can be synthetized by the human body [143].

Interestingly, the SeCys intaken from the diet cannot be directly incorporated into proteins in a specific way, but needs to be metabolized first [12]. In the same manner, all the dietary selenium species also need to be transformed in order to be biologically active, with most of the reactions occurring mainly in the liver [12].

Selenocysteine is synthetized in human metabolism from selenophosphate ($H_2SePO_3^-$ or $HSePO_3^{2-}$) [144]. The selenophosphate reacts with phosphoseryl-tRNA$^{[Ser]Sec}$ to form selenocysteyl-tRNA[^{Ser}]^{Sec}, which is encoded by UGA codons to insert SeCys into the amino acid chain of the proteins [113].

That selenophosphate is synthetized from hydrogen selenide (HSe^-) and ATP, catalysed by selenophosphate synthetase [144].

Hydrogen selenide is the main intermediate in the metabolization of selenium, with all the selenium species converging into this form [145].

Regarding inorganic selenium, selenate is reduced to selenite in order to be metabolized [12]. Selenite is reduced by glutathione (GSH) to selenodiglutathione (GSSeSG), and then selenodiglutathione is reduced to glutathione selenenylsulfide (GSSeH), also called selenopersulfide, by NADPH and glutathione reductase. Finally, GSSeH is reduced to hydrogen selenide, with the thioredoxin reductase (TrxR),

thioredoxin (Trx) and glutaredoxin (Grx) enzymes also playing a role in the whole process [82]. Due to the high levels of GSH in cells, this metabolization process occurs rapidly [144].

Regarding organic selenium, selenocysteine can directly produce hydrogen selenide from the reaction with selenocysteine β-lyases [144]. Selenocystine needs to be reduced to selenocysteine by GSH prior to yielding hydrogen selenide [144]. MeSeCys is also reduced by selenocysteine β-lyases, but the product of the cleavage is methylselenolate ($MeSe^-$) [144], which can later be demethylated into hydrogen selenide [145]. Despite MeSeCys being relatively nontoxic to cells, it is considered one of the most promising antineoplastic agents, due to its ability to generate $MeSe^-$, the most active selenium compound against cancer cells [82], [86]. In the same manner than MeSeCys, γ-glutamyl-MeSeCys and synthetic selenium compounds such as selenobetaine, methylseleninic acid and methylselenocyanate, also yield $MeSe^-$ [12], [83]. Finally, selenomethionine, one of the most important dietary sources of selenium, can be metabolized in two manners: it can be transformed into SeCys, by the trans-selenation pathway (equivalent to the trans-sulphuration pathway) [12], with selenohomocysteine (SeHCys) and selenocystathionine (SeCysta) as intermediates [144]; besides, SeMet can also be unspecifically incorporated into proteins, because the methionine-tRNA is not able to discriminate between Met and SeMet [12]. Thus, selenomethionine is retained in the proteins, but it is not in a biologically active form [42], and is stored until the proteins are degraded, when then it can be recycled [144]. Accordingly, SeMet is highly bioavailable and rapidly increases the apparent Se status, leading to bigger Se concentrations in tissues [69], but it is less bioactive than other selenium species.

Due to the fact that all selenium species converge into hydrogen selenide, this species can be referred as a selenide pool [49]. If there is an excess of selenium, no all the selenide is used for selenoprotein synthesis, but it can also be excreted as selenosugars in urine, dimethylselenide (DMSe), in the breath, and trimethylselenonium ($TMSe^+$) in the urine [113], [144].

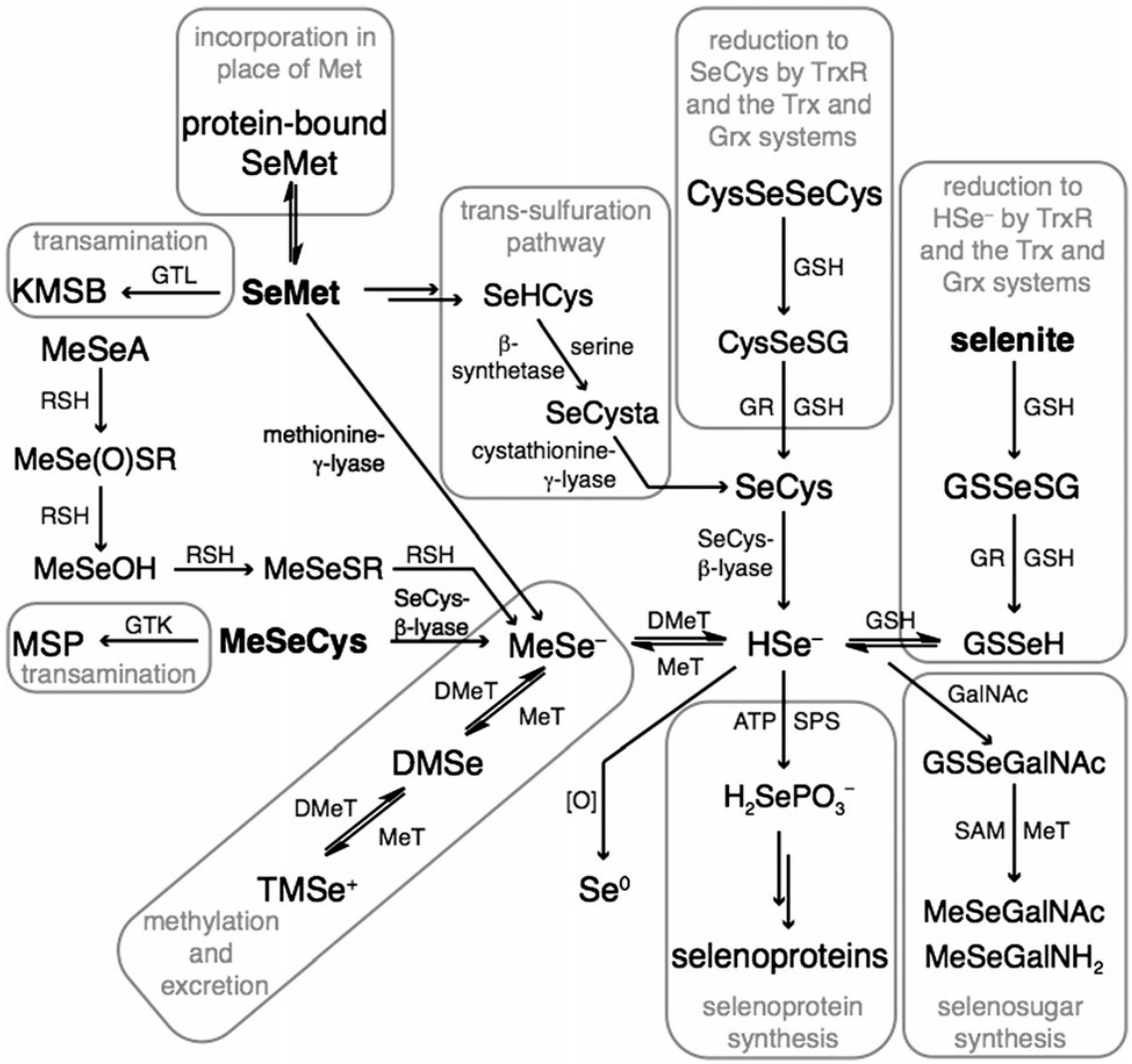

Figure 4: Selenium metabolism in humans, reproduced from Weekley et. al. [144].

1.5 Plants and selenium

Crop biofortification with selenium

As previously discussed, organic selenium species, such as selenoamino acids, are more effective at increasing selenium blood concentration and enzymatic activity than inorganic counterparts, such as selenite and selenate [42]. For this reason, a biofortified diet is preferred over direct supplementation. Both plant and animal biofortification are possible options, with vegetables as well as mammals containing mostly selenium in organic forms.

However, in order to perform the biofortification of the plants and the animals, organic or inorganic forms can be used as the starting material. The use of inorganic selenium salts is substantially inexpensive compared to the use of pure selenoamino

acids, due to a much more simple synthesis procedure. Besides, the preparation of selenium enriched yeast is also relatively expensive [141].

Accordingly, farmers would prefer the use of inorganic selenium for biofortification, for both cattle feed and crops.

However, inorganic selenium has a extremely low bioaccessibility for rumiants, since it is reduced to insoluble selenide in the rumen, and therefore it is not absorbed in the intestinal tract [141]. Besides, inorganic selenium species can be more toxic than their organic counterparts for mammals at the same concentration [113].

Oppositely, plants can efficiently intake inorganic species and transform them into selenoamino acids, being able to tolerate and accumulate relatively high selenium concentrations without hampering the crop yield.

For these reasons, plant biofortification with inorganic selenium species is the most promising strategy in order to increase selenium status of the population in deficient areas. Consequently, this is the approach selected in the present work, with specifically wheat plants being investigated.

Plants and selenium

Plants require 14 mineral elements: N, P, S, K, Mg, Ca, Fe, Mn, Zn, Cu, B, Mo, Cl, Ni, but chlorine and nickel are essential only for some plant species. Other elements are also beneficial for plants, such as Na, Si, Co, I, V [146]. Accordingly, plants are essential sources of mineral nutrients for humans.

However, selenium is not essential in land plants, despite being essential to many other organisms such as archaea, bacteria, protozoan, animals and green algae [147].

Accordingly, selenium has no specific role in plant metabolism [133]. Even though plants are able to tolerate and thrive under limited amounts of selenium, elevated concentration may result in stress and toxicity effects.

Besides, the fortification of crops with one element, in this case selenium, can modify plant metabolization of other compounds and also be detrimental to production yield. Therefore, the optimization of the enrichment methodology is needed in order to enhance selenium content without hampering crop production or plant nutrient content.

Plant accumulation capacity, tolerance and selenium toxicity

Distinct plant species are able to tolerate selenium at different magnitudes, mostly depending on how they metabolize this element and their adaptation to the environment.

Depending to the amount of Selenium that plants are able to accumulate in their cells, they are classified in three groups: hyperaccumulators, which store 1000–15000 mg Se/kg of dry weight (DW), secondary accumulators with 100–1000 mg Se/kg DW, and non-accumulators with less than 100 mg Se/Kg of DW [37], [148].

Cereals are considered non-accumulators, with average selenium content of 0.01-0.55 mg/kg fresh weight [133]. Specifically, wheat has been reported to have a selenium content up to 5.3 mg/kg fresh weight in non-enriched conditions [133] and up to 30 mg/kg in high-selenium areas of the USA [43], [89], which makes it a suitable candidate for biofortification.

Plant exposure to selenium concentrations exceeding those values can lead to toxicity symptoms. In crops, excess of selenium for extended periods is generally identified by a visible white chlorosis [20], as well as withering and necrosis of leaves [149]. Moreover, selenium can also inhibit root and shoot development, mostly in the more sensitive young plant seedlings [150], [151], resulting in biomass reduction [152]. In fact, the inhibition of the primary root elongation is considered the most characteristic sign [150]. In addition, it can also decrease germination and seed weight [148].

There are several mechanisms through which selenium toxicity occurs. The main reason for selenium phytotoxic effects is the malformation of proteins [150]. This is due to the interference of Se in the S metabolism [153] where S containing amino acids (methionine and cysteine) are non-specifically replaced by the selenium analogues [37], which modifies the structure and function of the proteins [150], [154]. Furthermore, selenium also triggers to production of reactive oxygen species (ROS), such as hydrogen peroxide and superoxide, which increase oxidative stress [150]. Specifically, superoxide is generated from the reduction of selenite by glutathione and can impair the photosynthetic activity [155]. In addition, selenium also affects the metabolization of reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, which results in reduced growth and root size [156]. Regarding the photosynthesis processes, selenium can be an intrusion in the cellular energy production pathway, where it can either enlarge the activity of the photosynthetic system [157] or reduce the levels of photosynthetic pigments, especially chlorophylls [152], [158]. Moreover, selenium also interfere in the nutritional state of the plants, affecting the content of elements such as S, P, Ca and Zn [150], reducing sugar, protein and antioxidant content [152]. Finally, selenium also affects the hormonal homeostasis, leading to an imbalance of several phytohormones involved in the growth regulation or the stress response of the plant [150].

For this reason, long term use of uncontrolled amount of selenium in crops can lead to excessive applications and phytotoxic effects on plants and, in extreme cases, to a threatening release of selenium to the ecosystem and the environment that can lead to contamination [152].

On the other hand, the addition of small amounts of selenium, much lower than the tolerance limit, can be beneficial for the plant. Selenium can promote plant growth, stimulating seed germination and delaying senescence, therefore increasing yield. Besides, selenium can act as an antioxidant, protecting the pant from the stress induced from cold weather, drought, and exposure to excess of salinity or heavy metals, and maintaining plant homeostasis. It can also enhance the plant respiratory potential, facilitating the flow of electrons during photosynthesis and increasing the chlorophyll biosynthesis [159]. On the other hand, it can also serve as a mechanism of plant protection against herbivores, including vertebrates and invertebrates, due to an effect of deterrence from the odoriferous forms of volatile Se, and against pathogens [148], [160]–[162].

For this reason, the optimization of the biofortification process, and specifically, of the amount of selenium used, is essential, and also requires a complete understanding of the uptake, metabolization and accumulation mechanisms occurring in the plant.

Plant accumulation regarding selenium speciation

Furthermore, distinct plant species are able to accumulate selenium up to different thresholds due to the use of different metabolic pathways, which results in the accumulation of diverse Se metabolites.

Hyperaccumulators tend to accumulate selenium in methylated forms of the amino acids, mostly MeSeCys, and to vaporize it as dimethyldiselenide (DMDSe). They translocate selenium to shoots via xylem transport and from leaves to reproductive organs via phloem transport, thus storing selenium mainly in young leaves and reproductive organs, within epidermal vacuole. In contrast, secondary accumulators and non-accumulators usually store inorganic Se (selenite and selenate) sequestering it in leaves in the vascular tissues and the vacuoles of mesophyll cells, and additionally, they volatilize it as dimethylselenide (DMSe) [37], [148], [163].

For this reason, some hyperaccumulating plants such as *Astragalus bisulcatus* and *Stanleya pinnata* and enriched hyperaccumulating vegetables such as garlic (*Allium sativum*), onion (*Allium cepa*), leek (*Allium ampeloprasum*) and broccoli (*Brassica oleracea*), store Se predominantly as MeSeCys [148], [164]. On the other hand, non-hyperaccumulating plants such as *Arabidopsis thaliana* and *Brassica juncea* contained Se in the form of selenite [165].

In contrast, cereals such as wheat, barley and rye store selenium predominantly as SeMet [164].

Additionally, some secondary accumulators have been found to contain prevalently other less common selenium species. *Stanleya albescens* (Brassicaceae) accumulates

selenium as selenocystathionine [166] and *Cardamine hupingshanesis* (Brassicaceae) accumulates up to 99% of its total Se as selenocystine [164].

Moreover, selenocystine has also been previously reported to be present in small concentrations in other plants, such as different varieties of selenate fortified wheat, rye and barley [167] and chicory (*Cichorium intybus L.*), lamb's lettuce (*Valerianella locusta L.*) and parsley (*Petroselinum crispum Mill*.) [168].

Other selenocompounds identified in plants include selenohomocysteine, γ-glutamyl-selenocystathionine, γ-glutamyl-methylselenocysteine, selenomethionine selenoxide, selenocysteineselenic acid, Se-proponylselenocysteine selenoxide, selenosinigrin and different selenopeptides [89].

The fact that hyperaccumulators store Se as MeSeCys contributes to their Se tolerance, since this species of selenium does not cause oxidative stress (in contrast to selenate and selenite), and also it does not get unspecifically incorporated into proteins (as SeCys and SeMet), and thus it can be accumulated without resulting in severe toxicity [163].

Selenium metabolism in plants

Plants are able to take up both inorganic and organic selenium via active membrane transporters, with roots being the main organ for nutrient uptake. Leaf surface can also uptake selenium, and some crop experiments have focused in foliar application [149], [169], but soil application has proved more effective at increasing Se content in the grains of cereals [117].

The different selenium species follow different assimilation pathways, as can be seen in figure 5 [170]. Selenate is taken up actively by roots via sulfate transporters in the root plasma membrane [37][171]. Thus, there is a competition with sulfate [172], with high S concentrations suppressing selenate uptake and low amounts enhancing it [163]. Selenite uptake is driven by other processes, since there is little inhibition of selenite uptake with high sulfate concentrations [170] and there is no evidence that it is mediated by membrane transporters [151]. Oppositely, selenite has been assigned to the phosphate transport pathway, since excess or starvation of P effectively modifies the Se uptake [173][174], and to aquaporins involved in silicon transport [175]. Organic species of selenium are known to be taken up at higher rates than inorganic compounds, but the specific mechanisms are not yet elucidated, despite amino acid transporters being involved [163], [176]. Moreover, besides the presence of competing S and P, the rate of selenium uptake also depends on the concentration of Se available to the plant in the soil solution and rhizosphere conditions, such as pH and redox potential, since Se ions are taken up across the plasma membrane into the roots epidermal cells through an electrochemical gradient [11], [177].

After the Se species have been taken up by the root cells, they are transported intracellularly from the apoplast into the symplast [177].

The first step in the selenium metabolization is the reduction of selenate to selenite via the ATP-sulfurylase, APS-reductase and gluthathione-reductase enzymes [11]. It is known that sulfate reduction in plants is performed exclusively in plastids, for this reason it is considered that selenate metabolization takes place mainly inside chloroplasts [133], [151], [177], [178]. The selenite formed is, in general, readily reduced to organic compounds, but some plants are able to oxidize small amounts of selenite back to selenate [177].

The reduction of selenite into organic selenium requires the formation of selenide as an intermediate, which can take place by enzymatic (with sulfite reductase) and non-enzymatic (with glutathione) processes [163]. In fact, the selenide will be found in the form of HSe^-, due to the pKa of this compound [24], or as a glutathione-conjugated selenide ($GS\text{-}Se^-$) [11], [151]. Then, selenide, which is not an stable intermediate, is rapidly converted into organic species, in a pathway that is parallel to that of sulfur [34], [178], [179].

Selenide is transformed exclusively into selenocysteine (SeCys), via the coupling with O-acetylserine and catalyzed by the cysteine synthase enzyme [163]. Then SeCys is further metabolized, forming the rest of organic compounds encountered in plants.

SeCys is transformed into SeMet through a several step process, with selenocysthationine and selenohomocysteine as intermediates [163]. The final step to convert selenohomocysteine to selenomethionine is performed by the methionine synthase, which is localized mainly in the cytosol, which indicates that the final steps in the metabolization and mutilation of selenium take place outside the chloroplasts, in the cytoplasm [177], [180].

On the other hand, SeCys can also be methylated into MeSeCys, through the action of the enzyme SeCys methyltransferase [133], [163].

The current knowledge on Se metabolism does not fully explain the mechanism of SeCystine (SeCyst) synthesis in higher plants [164], however, it will most probably, be derived from a further oxidation of selenocysteine, since this species is almost fully ionized to a selenolate at neutral pH, and thus, highly reactive [10].

Furthermore, SeMet and MeSeCys can be further metabolized into the methylated species DMSe and DMDSe, which are volatile [163].

Finally, both SeCys and SeMet can be incorporated into proteins, by specific and non-specific mecanisms: SeCys can be specifically incorporated into proteins through the action of the cysteinyl-tRNA synthetase, as well as SeMet through the methionyl t-RNA synthetase [151]. However, the mechanism for specific incorporation via the UGA codon, occurring in mammals, has not been found in yeast or land plants yet [181],

only in algae such as Chlamydomonas reinhardtii [182], [183]. Non-specifically, SeCys and SeMet can be incorporated in the place of methionine and cysteine, with Se randomly substituting a S atom [46]. Besides, selenium can also bind to proteins, with Se binding covalently to two adjacent cysteine residues in a R-S-Se(II)-S-R-type complex [147].

As previously discussed, these alterations of the proteins are a major cause of selenium toxicity, and therefore, the synthesis of non-protein amino acids such as MeSeCys and SeCyst, allows the accumulation of higher selenium concentrations in plant tissues [113], [133], [177].

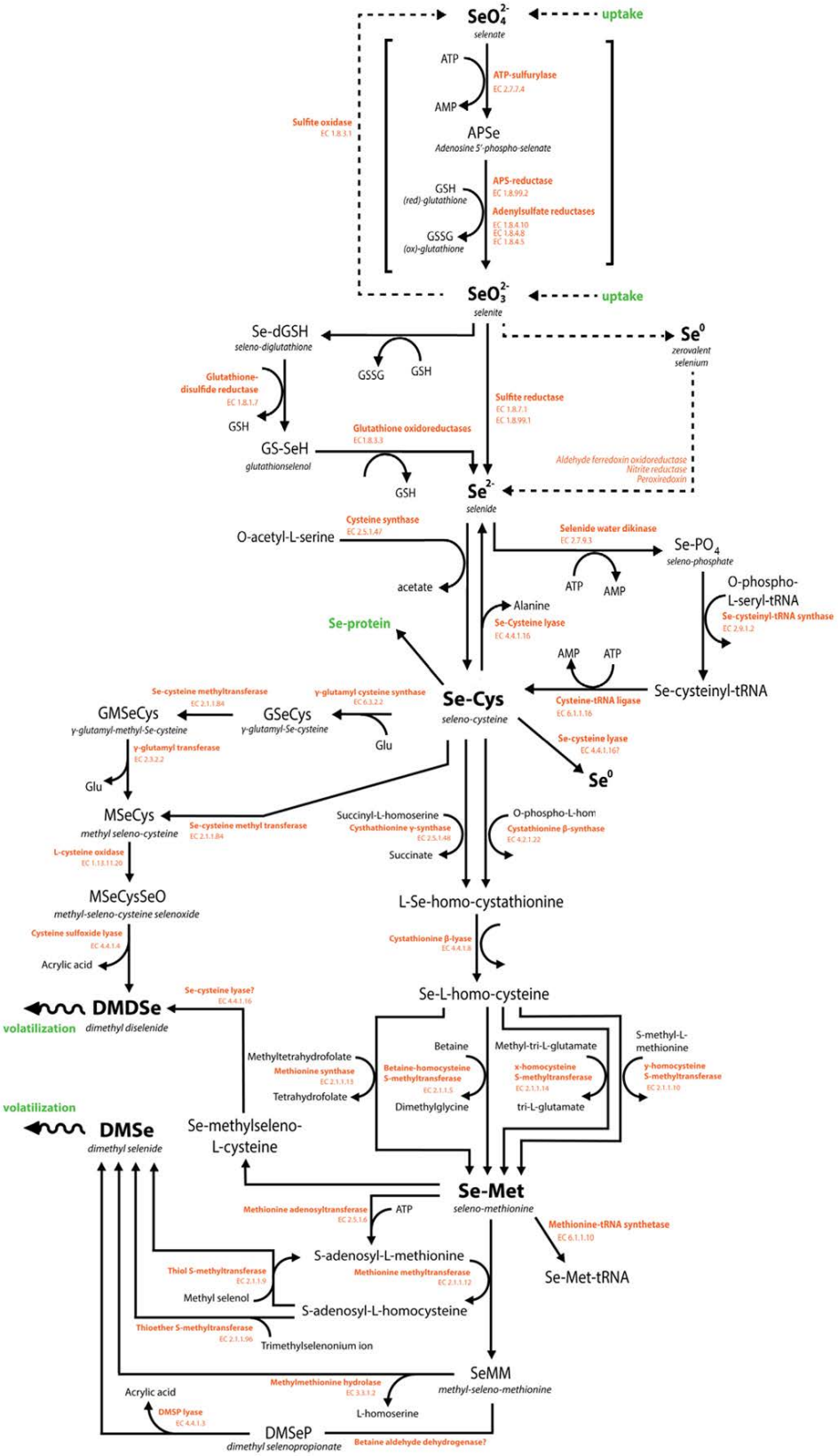

Figure 5: Plant metabolic cycle, adapted from [163]*: Diagram indicating the selenium compounds formed during plant uptake and metabolism. The enzymes and their Enzyme Commission (EC) numbers are indicated in orange at the corresponding reactions.*

1.6 Selenium and wheat

Selection of wheat for the biofortification study

Wheat plants were selected as the most adequate crop to be biofortified in order to produce a selenium enriched functional food that can effectively increase selenium status in the population.

As previously discussed, crop enrichment with Se-containing fertilizers has been proved to be successful and more adequate than other options such as the introduction of a new type of naturally Se-rich food in the diets of the population, the promotion of the consumption of nutritional supplements or the fortification of already processed foodstuffs.

Wheat was chosen among other kinds of crops because it fulfils the required characteristics of an ideal foodstuff to be selected for enrichment, as summarized by Fisinin et. al. [116]: It is already part of the population traditional food, is consumed regularly and in moderate amounts by the majority of the population, it is an affordable foodstuff, is able to supply a meaningful amount of the desired nutrient, and could be additionally complemented with other nutrient enrichment.

As previously discussed, cereals are already the most important selenium source for the whole population from the countries in Europe, America and Asia that have been studied, with meat also being a significant source of this element. In addition, despite cereals being non-accumulators of selenium, they can still store relatively high Se amounts in their tissues, without significant decreases of yield, when grown in a Se-rich environment, in contrast to other plants such as legumes [106].

Furthermore, in general, cereals and starchy crops (such as wheat, rice, maize, legumes, sweet potato, sorghum and millet) dominate diets worldwide, and constitute a staple and affordable food even for impoverished and malnourished populations, which are more vulnerable to nutrient deficiencies [184].

Precisely, the starchy endosperm of cereals (the part remaining after grain processing), provides the majority of the calories in human intake, specifically, rice provides a 23% of the global calories, wheat a 17% and maize a 10% [129].

Moreover, cereals can additionally be enriched simultaneously with more than one nutrient, in areas where populations suffers from multiple micronutrient deficiencies, as in some African countries, where there is a high incidence of Ca (54% of the continental population), Zn (40%), Se (28%), I (19%) and Fe (5%) deficiencies [184] or China, with Zn (50%), Fe (24%) and Se (20%) deficiencies [134]. Previous experiments have studied simultaneous biofortification with selenium, zinc, iron, calcium, magnesium, copper, folate, carotenoids or vitamin C, among others [129], [185]–[187].

Specifically, wheat has been chosen among the other cereals for several reasons:

Wheat is one of the cereals most produced and consumed worldwide. During the 20th century wheat was the most produced crop, and by 2010, the global production of grain was 651 MT, but consumption and demand are still rising [188]. Wheat cultivations covers more land than any other crop, with 240 million hectares (ha) worldwide (see Figure 6) [189]. Despite its production in weight ranking third after corn and rice, its gross world trade is bigger that the combination of all the rest of crops, since it is the most common grain for human consumption [189].

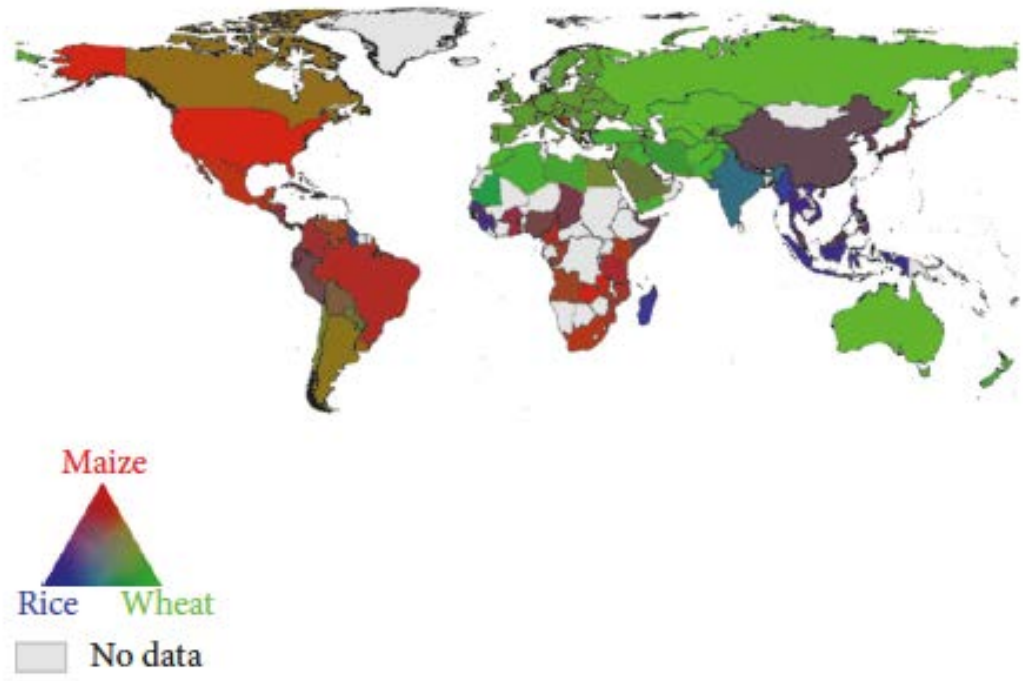

Figure 6: Map showing the most important grain consumed per country, including wheat, rice and corn [189].

Besides, wheat already contains the essential mineral micro and macronutrients for the human body such as calcium, phosphorus, magnesium, sodium, potassium, iron, zinc, the trace elements copper, chromium, manganese, molybdenum, selenium, and iodine and even some non-essential or semi-essential elements as fluoride and boron [190], [191]. But further biofortification in cases of deficiency to increase the content of one or several of these elements is feasible.

Moreover, wheat is a major food staple because it is a highly adaptable plant that can grow in many regions, and the wheat grains can be easily stored for long periods and easily transformed into flour that can be further transformed into a big variety of products [189], such as bread, pasta, noodles, tortillas, pizzas, cookies, cakes, couscous

and even beer [188]. Therefore, this big variety of forms and preparations allows it to be part of the diet of the majority of the cultures.

Finally, wheat has a couple of significant advantages over rice (which is also a staple food worldwide and of special significance in Asian countries [152]): Wheat has been shown to accumulate higher selenium content than rice [180], and to preserve a bigger Se fraction after the milling and cooking processing [184].

Wheat

Wheat was domesticated approximately 10 000 years ago in the Fertile Crescent region. This achievement was essential for the humankind transition from nomadic to sedimentary lifestyle. Later, the fact that wheat grain could be stored for long periods, enabled the rise and growth of cities and empires in Babylon, Assyria and Egypt, as well as the development of western civilization, ensuring the survival in cold European winters [188].

Initially, the earliest cultivated forms were diploid (genome AA) and tetraploid (genome AABB), but selection, breeding and hybridization over time lead to a hexaploid form (genome AABBDD). Nowadays, about 95% of wheat is hexaploid, with only a 5% being tetraploid (durum wheat) [188].

Specifically, the specie *Triticum aestivum*, also known as common bread or bread wheat, is the most extensively cultivated wheat species worldwide, and thus the one chosen for the present study. The data from the scientific classification of this species is found in Table 5.

Wheat is a highly adaptable crop. Therefore, it can grow in many different regions around the world, being found between latitudes of 30^{o}N to 60^{o}N and 27^{o}S to 40^{o}S, practically from the artic to the equator, in elevations from sea level up to 4000 meters in the Tibet, and in every soil condition. It can also withstand all kinds of weather, tolerating average temperatures from 3C to 32C, and dry and humid weathers with precipitation ranges from 250 to 1750mm [189], [192].

Table 5: Classification of wheat [193].

Kingdom	Plantae
Division	Magnoliophyta
Class	Liliopsida
Order	Poales
Family	Poaceae
Subfamily	Pooideae
Tribe	Triticeae
Genus	Triticum
Species	*Triticum aestivum*

State of the Art of selenium biofortification of wheat

The literature regarding wheat biofortification with selenium is large, however, there are some limitations to it that the present work aims to fulfil.

In the first place, a big amount of selenium research has only focused in the total selenium content in wheat grain or wheat-based products (such as flour or bread).

This has been the case in most of the studies that had aimed at assessing native state of selenium in a specific country, in order to assess the need for selenium biofortification, as for example in the investigations carried out in widespread places such as in UK [172], [194], Australia [195], Argentina [196], Spain [197], Libya [198], Greece [199], Egypt [200], Portugal [114], Ireland [201] and Korea [202].

On the other hand, the defect of only assessing total selenium content has also occurred in biofortification experiments, which have only quantified the concentration reached in grain after selenium application, in wheat cultivations performed in Australia [203], [204], New Zealand [205], [206], UK [207] and Slovakia [208]. It is also the same case for the nation-wide application of selenate in all fertilizers that has been implemented in Finland, where reports have mainly focused in total content achievements [9], [131], [132].

However, and as previously discussed, determining the selenium speciation is of paramount important in order to assess the bioavailability, bioactivity, metabolization, toxicity and consequent health benefits of consuming a selenium-biofortified functional food.

A fair amount of research has indeed focused on selenium speciation in wheat grain, flour or bread. The majority of the studies have followed a methodology based in the extraction of the selenium species from the sample matrix, the separation of the species by chromatographic techniques and their quantification with a mass

spectrometry detector, being the high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) the most applied technique for Se speciation.

However, even if different separation mechanisms have been used, such as anion exchange [167], [174], [209], [210], cation exchange, reverse phase [130], size exclusion [211], [212] or the parallel application of several of them [100], [213], with a wide variety of methodologies, columns and mobile phases, only partial Se speciation has been achieved in wheat.

The most important selenium species present in wheat are the amino acids SeMet, MeSeCys and SeCyst and the inorganic forms selenite and selenate. The majority of the studies have only been able to quantify some of them, due to the concentrations being close to the limit of detection and the low stability of the species after the pretreatment for extraction.

Accordingly, several authors have just reported selenomethionine to be the most abundant species in wheat grain, with values within 45-100%, but generally over a 70%, of the total selenium regardless of the selenium source (native or supplemented), the amount of selenium used for the biofortification, the chemical form (selenite or selenate), and the application method (foliar or soil application) [100], [167], [204], [209], [213]. Selenate is also commonly quantified, with literature generally reporting values below 5% [100]. Very few works have succeeded in quantifying these five more important species [209], and even less have focused in other minor selenium compounds present in wheat such as selenosugars [212].

In addition, also a small number of works in wheat selenium speciation have used complementary techniques, such as electrospray ionization-tandem mass spectrometry (ESI-MS-MS) in order to confirm the identification of the structure of the eluting compounds [210], [212].

On the other hand, to a lesser extent, gas chromatography mass spectrometry (GC-MS) has also been used for Se speciation in wheat flour [214], [215], but only SeMet was able to be quantified with this methodology.

The main drawback of these chromatographic techniques for speciation is that they are indirect methodologies that require several pretreatment steps in order to extract and solubilize the Se species, what leads to, not only, the reactivity of these species and therefore changes in their structure (such as the oxidation of selenomethionine in the wheat extracts), but also to incomplete recoveries, where species that have not been solubilized or extracted adequately (such as metallic selenium or selenopeptides) are not taken into account by the present methodologies.

Oppositely, direct speciation techniques, such as X-Ray absorption spectroscopy (XAS) using synchrotron radiation, can measure selenium speciation in the sample, in solid form and without the need of extraction and pretreatment steps.

Moreover, this technique can also be complemented with X-ray fluorescence mapping (XRF) and a microfocused beam, in order to perform fine and localized analysis of selenium speciation within selected points in a sample. Therefore, this combination is a powerful methodology that not only provides speciation quantification but also spatial distribution information of the element and of their species.

A limited number of studies have used this technique to analyse Se speciation in wheat samples due to a limited accessibility of beamtime in synchrotron facilities. The have focused in wheat roots and leaves [216] and in roots, stems, leaves and grain [217].

However, the same technique has been used to study selenium speciation in other plants, such as rice [216], [218], [219], garlic [220], broccoli [170], lentils [221], cowpea (*Vigna unguiculata*) [222], Indian mustard (Brassica juncea) [217], [223], *Astragalus bisulcatus* [161], [224] and *Stanleya pinnata* [166], [224].

Objectives

Considering the state of the art and the lack of a scientific strategy for selenium enrichment, the aim of the present work is to develop and characterize a methodology for selenium biofortification in wheat plants. This main goal will be performed by developing the following tasks:

i. The first objective is to investigate the different behavior of the two main inorganic selenium anions, selenite and selenate, in the plant metabolism. The differences in the mechanisms of uptake, metabolization, translocation, distribution and accumulation, as well as their intrinsic phytotoxicity and effect on plant growth, grain production and yield have not been previously studied in depth, and thus they will be addressed in this thesis. Besides, in order to obtain additional information on the pathways of the two species, cultures will be also performed with a 1:1 mixture of the two, which can benefit from the possible synergisms of these species.
ii. Furthermore, the influence of low and high selenium concentrations and the time in which selenium is supplied to the plant on the final enrichment will be considered, in order to optimize the biofortification strategy to maximize selenium concentration in grain while minimizing the toxic effects on plant development and grain yield.
iii. A following objective is to characterize the transformation of the selenium species through the plant metabolism. The form in which selenium is present on the grain needs to be precisely determined, since selenium consumption is recommended in the form of organic compounds such as selenoamino acids, which are less toxic than inorganic forms and more bioactive and effective to increase blood selenium levels and enzymatic activity. Therefore, the determination of the species in which selenium accumulates will ensure the safety and the related health benefits of the consumption of a functional food produced with the obtained wheat flour.
iv. The succeeding objective is to determine the species spatial distribution and accumulation in roots, shoots and grain, and to characterize, more precisely, the location of the selenium compounds in the different parts within a wheat grain. Common flour preparation techniques use only the grain endosperm, removing the bran and germ in the process. Therefore, it is necessary to characterize the location of the selenoamino acids in the different grain parts, in order to ensure effective enrichment profit.
v. Finally, the protective effects of selenium against heavy metals, specifically mercury, will be exploited in order to assess the consequences of selenium biofortification of crops grown in contaminated lands, to assess the benefit of selenium addition in potential polluted soils. Therefore, the interactions between the two elements will be studied to obtain further information on the mechanism of mercury detoxification with selenium, while evaluating the minimization of mercury accumulation in edible tissues.

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2. Dynamics of selenium uptake and effect on wheat plants

Introduction

Biofortification of wheat aims to maximize the content of beneficial selenium species in wheat grain, without hampering plant development and without risk of safety concerns for the environment or human consumption of the enriched wheat. Therefore, it is necessary to obtain a detailed characterization of the mechanisms of selenium uptake, metabolization, translocation and distribution, as well as to determine the effect of the enrichment on the plant nutrient status and production yield.

Accordingly, simulating the conditions encountered in field culture in lab-scale studies, would promote applicability in large-scale wheat crop enrichment. The biofortification of wheat with selenium is influenced by several factors such as the Se species used, the application methodology, the concentration applied, the timing and the particular soil and environmental conditions [1]. Otherwise, hydroponic cultures in growth chambers allow controlling the plants environmental conditions and the nutrient supply in order to avoid the influence of soil and weather characteristics over selenium behavior.

Consequently, this permits to study the effects of selenium concentration, speciation and application timing on the physiology of wheat plants, in order to develop an optimum biofortification strategy.

Selection of selenium species for biofortification

In natural environments, such as soils and water bodies, inorganic forms of selenium are dominant [2]. Specifically, selenate and selenite are the prevailing forms due to the pH and redox conditions of soils [3]. Selenate dominates in alkaline and well-oxidized soils, whereas selenite is common in acidic and neutral soils [4], [5].

Besides, the two species have different solubility in soils. Selenite is immobilized by adsorption on metal oxides surfaces (iron oxides/hydroxides and aluminum oxides) or soil organic matter [6], [7]. Selenate is less absorbed and more soluble in the rhizosphere solution, and consequently, more bioavailable for plant uptake [3].

Despite the fact that selenite is more common in arable lands [8]–[10], due to the big variability of agricultural soils worldwide, both species can be encountered. Moreover, selenite and selenate can be easily oxidized and reduced respectively, and therefore, both species will coexist in a big range of pH and Eh soil conditions [11].

In addition to the effect of the soil characteristics, selenium speciation is also influenced by the environmental and climatological conditions, which are subjected to

seasonal variability, such as the amount of rainfall, water leaching, organic matter content, bacterial activity, and even the preceding crop and the application of fertilizers [11]–[13].

Therefore, the study of the biofortification of wheat with a single selenium form may fail to properly assess the complexity of speciation in natural conditions. For this reason, the effect of selenite and selenate on wheat plants needs to be considered individually, and also in combination.

Previous studies have shown that plants have different uptake mechanisms for selenite and selenate [14], which results in unequal distribution and accumulation through plant tissues, and even different speciation [15]–[17]. However, the interactions between selenite and selenate when both are available have been marginally studied [5], [11] and require further investigation .

Hydroponic culture

Several methodologies exist in order to perform crop cultures and biofortification, with several technologies available to increase the amount of selected nutrients in the edible parts of the plant, such as seed treatment (seed dressing or seed soaking), granular or prilled fertilizers, and liquid fertilizers, which can either be applied to the soil or to the plant leaves (foliar treatment), and a either at sowing (planting) or at later plant developing stages [1], [13], [18], [19].

Previous studies have shown that selenium application to growing plants, i.e. at a seedling, tillering or flowering stages, was more effective than seed treatments or application at sowing stage. Moreover, liquid fertilizers were also more effective than granular or prills [1], [13].

Regarding the option of foliar application, the scientific community is divided. Many authors have favored foliar application as the most effective and economic methodology for biofortification [15], [19], [20], whereas other studies have demonstrated soil application to be more effective and with a simpler logistic than foliar application [15], [21].

In fact, roots are the main organ for nutrient uptake in plants. Accordingly, it has been shown that total recovery of the applied Se was only about 7% for foliar applications but of 20-35% for soil applications [18], [22]. For these reasons, the present study focuses in the selenium uptake through roots.

In addition, the need to obtain basic knowledge on the effects of selenium in the selected conditions, motivated the selection of a hydroponic culture methodology, thus growing the plants in nutrient solution instead of soil, and in a growth chamber with controlled environment.

Hydroponic culture has been used since the mid 18th century, and nowadays, it is still a widely-used, well-accepted methodology that enables a precise control of the elemental environment surrounding the root and the physical and chemical parameters [23], [24]. It is a fundamental approach in order to study the physiological effects of a deficiency or excess of a particular nutrient or stress-inducing agent, avoiding the intricate influence of complex soil systems and the seasonal variability coming from changing weather conditions [25].

It suppresses absorption processes of selenium and nutrients occurring in the metal oxides surfaces and organic matter of soils, which will decrease the bioavailability for plant uptake [6], [7]. Moreover, since selenite is more absorbed in soils than selenate, understanding and comparing the behavior of both selenium oxyanions in wheat physiology is only feasible if those absorption processes are avoided [3].

Optimization of selenium species concentration for biofortification

The selenium concentration for biofortification process needs to be optimized in order to maximize selenium content in the grain to be employed for producing a Se-enriched functional food. However, an excessive selenium application to the crop will cause toxic symptoms to the plants, resulting in stressed plants with reduced biomass and diminished grain production.

In previous wheat biofortification experiments, selenium application has been reported as the amount of selenium in the fertilizer, using between 6-16 mg Se/kg of fertilizer in Finland's nation-wide application [26], [27], or as the amount of selenium per crop size, with values between 1 and 600 g Se/ha [1], [13], [18], [19], [28]–[30].

However, in order to accurately quantify selenium uptake by plants, the amount of selenium per the amount of substrate, such as kg of soil, should be taken into account. This approach has also been reported in a wheat biofortification study, which used from 0,05 to 0,2 mg Se/kg of soil, which is roughly equivalent to 150-600 g Se/ha [31].

These values resemble the Se concentrations naturally found in soils. In general, native selenium levels can range between 0,05 mg Se/kg of soil in deficient areas to 5mg/kg or higher in seleniferous regions [26], [32].

Accordingly, values comprised in that range should be relatively adequate. If an approximate soil density of 1,5g/cm^3 is considered [31], those values are equivalent to 0,075 - 7,5 mg Se/L or 0,95 - 95 µmol Se/L.

Hydroponic cultures have been enriched with a variety of selenium levels, also depending on the plant to be fortified: 40 mg Se/L for sunflowers, 20 mg Se/L for radish [33], 3,7 mg Se/L for *Brassica juncea* [34], 1-4 mg Se/L for alfalfa, soy and lentils [35], 2-60 µM for lettuce [36]and 2-80 µM for cucumber [37], among others.

In a previous study, wheat seedlings were treated with concentration of selenium in the range 1-100 µM for 5 days in hydroponic cultures. It was seen that 100 µM Se caused visible toxicity symptoms, with a 50% decrease in root elongation. Oppositely, 10 µM Se did not show any significant effect on root elongation or on root and shoot weights; parameters that started to be affected at Se additions equal or higher than 50 µM [11]. In agreement, another short term study applied 10 µM of selenium during 24h to wheat seedlings, and no effects were reported on wheat growth parameters [5].

Therefore, 10 µM Se was selected as the main selenium concentration applied to wheat plants in the present study. Besides, a concentration of 25 µM Se was also selected in order to study both the effects of a high concentration on the maximization of selenium content and the appearance of toxicity symptoms.

Plant growth stages and selenium application time

Plants have different responses to herbicide application depending on the growth stage in which it is administered, with wheat seedlings being more sensitive and at risk of developing toxicity symptoms than plants at a tillering stage [38].

The same is true for selenium biofortification: young plant seedlings are more sensitive to high selenium concentrations than mature plants, resulting in inhibited root and shoot development and thus smaller biomass production [39], [40].

Therefore, since Se application to growing plants is more effective for enrichment purposes and less challenging for seedling development, five days of germination plus two weeks of preculture conditions were allowed in this study before selenium supply.

Different Se application times were also considered, in order to maximize selenium content in the edible part, in this case the grains, while maintaining production yield and minimizing both plant toxicity and the economic cost of selenium supplementation.

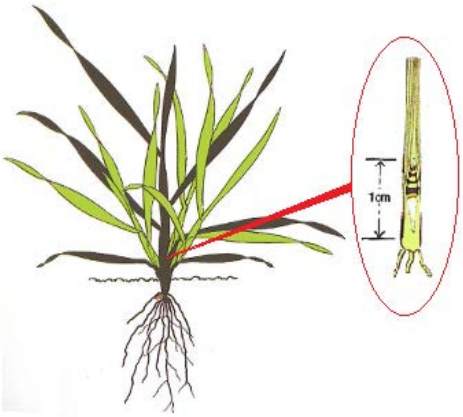
Plant development stages are detailed in figure 1 and table 1. In the present study two different Se applications were performed: one at the stage 13 (named vegetative stage) when plants have at least 3 leaves unfolded and another at stage 53 (named florescence stage) when approximately there is ¼ of the ear emerged. Once the Se supply was started, it was continued until plant harvest, and constant levels were ensured by weekly renewal of the solution.

Table 1: Wheat main growth stages. Adapted from [41]*. Green marks the stages in which selenium application has begun in the present work.*

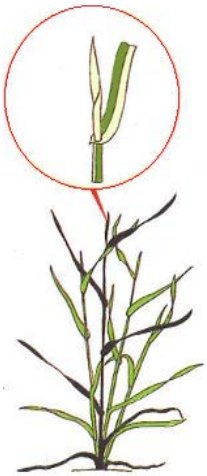
Stage	Process
1	Seed imbibition
5	Radicle emergence
7	Coleoptile emergence
10	Seedling vegetative growth
13	3 leaves unfolded
21	Tillering start: main shoot and tiller formation
30	Ear at 1cm and start stem elongation
31	Start node formation
37	Flag leave visible
40	Booting start
45	Boots swollen
49	First awns visible
51	Inflorescence start: first spikelet emerging
53	1/4 inflorescence emerged
55	1/2 inflorescence emerged
59	Emergence of inflorescence complete
61	Beginning of anthesis, flowering
69	Anthesis complete
71	Kernel development: water ripe stage
73	Kernel development: start of milk stage
85	Kernel development: soft dough
87	Kernel development: hard dough
90	Kernel start ripening
93	Kernel loosening
94	Senescence: straw dead and collapsing

Stage 12: 2 leaves

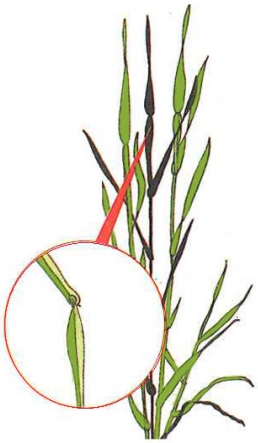
Stage 13-21: 3 leaves, 1 tiller



Stage 30: Ear at 1 cm



Stage 37: Flag leave visible



Stage 45: Boots swollen

Stage 55: ½ Ear emerged

Stage 61: Flowering, anthesis

Stage 90: Ripening, kernel hard

Figure 1: Representation of the most relevant growth stages of wheat. Reproduced from [41].

Objectives

The main objective of this chapter is to elucidate the processes of selenium uptake and distribution in wheat plants. In order to complete this main goal, the following detailed objectives will be accomplished:

- ✓ To determine the amount of selenium take up by roots and its translocation from roots to shoots in wheat seedlings during the first hours of exposure to selenium.
- ✓ To resolve the distribution of selenium in the different tissues in mature plants and to quantify the amount that successfully reaches the grain.
- ✓ To elucidate the distinct behavior of the different selenium species (selenite, selenate, and mixture of the two forms) on wheat Se metabolism and distribution.
- ✓ To measure the effect of the selenium application to plant development and biomass production.
- ✓ To optimize the Se concentration, speciation and exposure time in order to maximize enrichment while minimizing Se-induced plant stress.
- ✓ To investigate the influence of selenium application on the uptake and distribution of other elements.

Materials and Methods

Wheat culture

Plants of common wheat (*Triticum aestivum* cv. Pinzón purchased from Semillas Fitó S.A.) were grown in hydroponic culture.

Seeds were germinated in moistened filter paper at room temperature for 5 days. Then, seedlings were transferred to opaque plastic containers filled with continuously aerated ½ strength Hoagland's nutrient solution (see Table 2) buffered with MES (2-(N-morpholino)ethanesulfonic acid, $C_6H1_3NO_4S$) to maintain a stable pH of 6,0. Plants were precultured for at least two weeks before exposure to selenium. The solution was renewed weekly in order to maintain constant levels of water, nutrients and selenium.

The culture was carried out in a controlled-environment growth chamber with 18-24^{o}C temperature, 60–70% humidity and 320 µE m^{-2} s^{-1} light intensity with a short photoperiod of 8h light/16h darkness during vegetal growth and a long photoperiod of 12-16h light/8-12h darkness for flowering and grain production.

The uniformity of the solution was ensured by monitorization of the elemental concentrations by inductively coupled plasma-mass spectrometry (ICP-MS), and the stability of the species by high-performance liquid chromatography with Inductively

coupled plasma Mass spectrometry (HPLC-ICP-MS), with a PRP-x100 anionic exchange column, with the conditions detailed in chapter 3.

Table 2: Composition of the modified Hoagland ½ nutritive solution.

KNO_3	3.0mM
$Ca(NO_3)_2.4H_2O$	2.0mM
KH_2PO_4	1.0mM
$MgSO_4.7H_2O$	0.5mM
H_3BO_3	3µM
$MnCl_2$	2µM
$ZnSO_4.7H_2O$	2µM
$CuSO_4.5H_2O$	1µM
$(NH_4)_6Mo_7O_{24}.4H_2O$	0,1µM
Fe(Na)EDTA	60µM
$C_6H_{13}NO_4S$	2mM

Selenium treatments:

Selenium was supplied at a concentration of 10 or 25 µM, in the form of sodium selenite, sodium selenate and a 1:1 mixture of the two salts. A control treatment without selenium, was also included in all cases for comparison.

Table 3: Selenium treatments used in the present work and concentrations used of each species.

Treatment	10µM Concentration	25µM Concentration
Control	-	-
Selenite	10µM Se as Na_2SeO_3	25µM Se as Na_2SeO_3
Selenate	10µM Se as Na_2SeO_4	25µM Se as Na_2SeO_4
Mixture	5µM Se as Na_2SeO_3 +5µM Se as Na_2SeO_4	12,5µM Se as Na_2SeO_3 +12,5µM Se as Na_2SeO_4

The different cultures performed were classified according to the duration of the Se treatment before plant harvest (from few hours or days, with plant recollection at a vegetative stage, to few months, with plant recollection at senescence stage) and the time in which selenium was applied to the plants (in the early vegetative stage or at the florescence stage).

In the first experiment, plants were exposed to 10 µM selenium for up to 10 days during the vegetative stage. Plants were collected at several times in order to monitor the kinetics of the uptake and translocation of selenium: after 12 hours, 1, 2, 4, 6, 8 and 10 days of exposure. Two different cultures were performed, each comprising 6 plants per Se treatment (control, selenite, selenate, mixture), therefore, resulting in a total of 12 plants exposed to each condition.

In the second experiment, plants were exposed to 10 µM Se at the vegetative stage and Se supply was kept constant during the whole plant cycle, until plants reached senescence and mature grains were obtained. Two different cultures were performed in two different years with the same conditions, each comprising 4 plants per treatment, therefore resulting in a total of 8 plants for each treatment.

In the third experiment, plants were exposed to either 10 µM or 25 µM Se, applied either at the vegetative stage (veg) or at the florescence stage (flor), and maintained until harvest at the senescence stage. This resulted in a total of 13 conditions: control and selenite, selenate and mixture applied in 4 different manners each (10 µM veg, 10µM flor, 25µM veg, 25µM flor).

Sample collection:

After treatment, plants were harvested: Roots where washed with ice-cold $CaCl_2$ solution to remove the elements from the root apoplast, and then rinsed with deionized water.

Afterwards, the plants were cut and divided into roots, shoots and spikes if present. The different parts were weighted and stored at -20ºC until further processing.

Plant material was dried in an oven at 45ºC during four days until stable weight. Air drying was chosen over lyophilization, since for X-ray absorption spectroscopy (XAS) investigations the later method could potentially modify chemical speciation in redox-sensitive elements such as Se [42].

Plant biomass (shoots and roots) was calculated by its dry weight, and the effect of Se on plant development was also tracked by means of stems and grains yield. The significance of the results was assessed by means of an ANOVA statistical analysis with 95% confidence, with the software TIBCO Statistica (StatSoft).

Total elemental analysis:

An acid digestion was performed in closed HP500 PFA vessels in a microwave digestion system (Mars 5, CEM, USA) under EPA 3052. Briefly, 50 mg of plant material was placed in the digestor tube with 4ml of HNO3:H2O2 (3:1), and a gradient of

temperature and pressure was applied up to 180 ºC and 1.9 atm for 45 minutes. Samples were stored at 4 ºC until analysis [35], [43].

The plant digestates were adequately diluted and the contents of ^{24}Mg, ^{31}P, ^{55}Mn, ^{56}Fe, ^{64}Zn, ^{65}Cu, ^{78}Se, ^{98}Mo were measured by Induced Coupled Plasma Mass Spectrometry, ICP-MS, (X Series 2, Thermo Elemental, UK). Commercial standard solutions of 1000 mg L−1 of the elements were used for the calibration procedure. ^{45}Sc, ^{69}Ga, ^{89}Y and ^{115}In were monitored as internal standards.

The macronutrients S, Ca and K were measured by Induced Coupled Plasma atomic emission spectroscopy (ICP-AES), also referred to as Induced Coupled Plasma optical emission spectrometry (ICP-OES). These analyses were performed by the external analytical service at "Scientific and Technological Center of the University of Barcelona".

A certificate reference material (CRM), SELM-1, consisting of a selenium-enriched yeast (NRC, Canada), was used to validate the procedure of sample digestion and selenium determination. The determined Se concentration 1993±39 mg/kg (mean±SD with n=8) agrees with the certified value of 2031±70 mg/ kg [44], [45].

Results and Discussion

PART I. Evolution of selenium concentration and plant status with plant age: Early stages of plant development

Kinetic study of selenium uptake in the plant early stages of development: Plant development

Two cultures were performed at different times to study the kinetic effect of the selenium uptake by wheat plants, at 12 hours, 1 day and 2, 4, 6, 8 and 10 days. Each culture contained 6 different plants for each selenium condition: control, selenite 10µM, selenite 10µM and a mixture of 10µM of both species (5 µM selenite+5 µM selenate). Results are presented in figure 2 for roots and figure 3 for shoots.

It was observed that the plants collected at 12 hours of Se exposure presented significant differences ($p<0,05$) in the dry matter of both roots and shoots between the two cultures, but after 24 h of Se exposure until the end of the kinetic study (10 days), no more significant differences in the dry weight between them could be seen.

It can be possible that the variability at 12 hours was due to plant homeostasis alteration caused by the supply of selenium, which can cause severe differences to the more sensitive young seedlings. After more hours of Se exposure, nutrients and hormone levels stabilize, and plants continue the development of biomass with converging values.

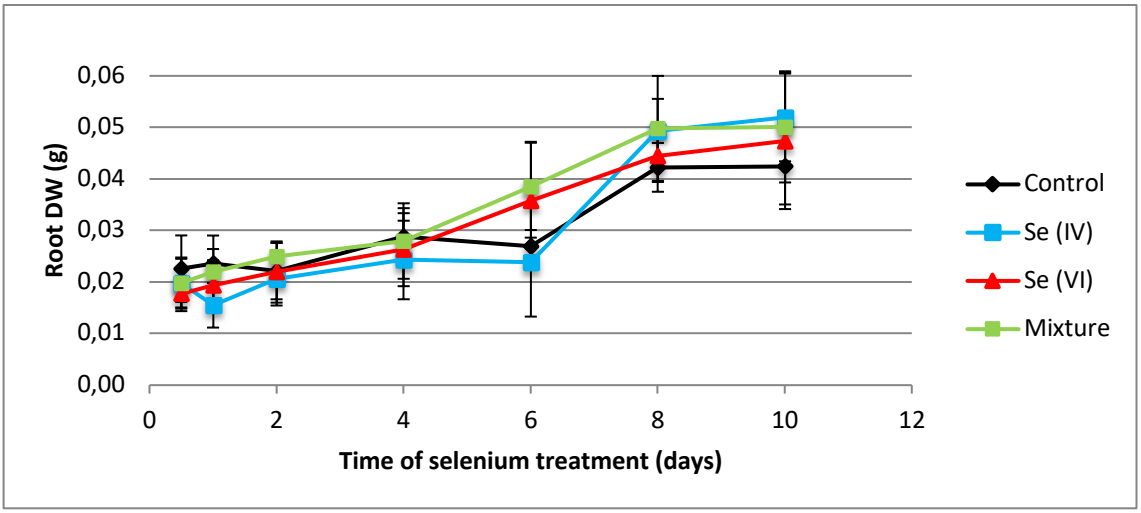


Figure 2: Root dry weight (g) of wheat plants collected after 12h, 1, 2, 4, 6, 8 and 10 day of Se exposure: control (black), 10µM of Selenite (blue), 10µM of Selenate (red) and 10µM of a mixture of both selenite and selenate (green) Results are expressed as means± SD (n=12).

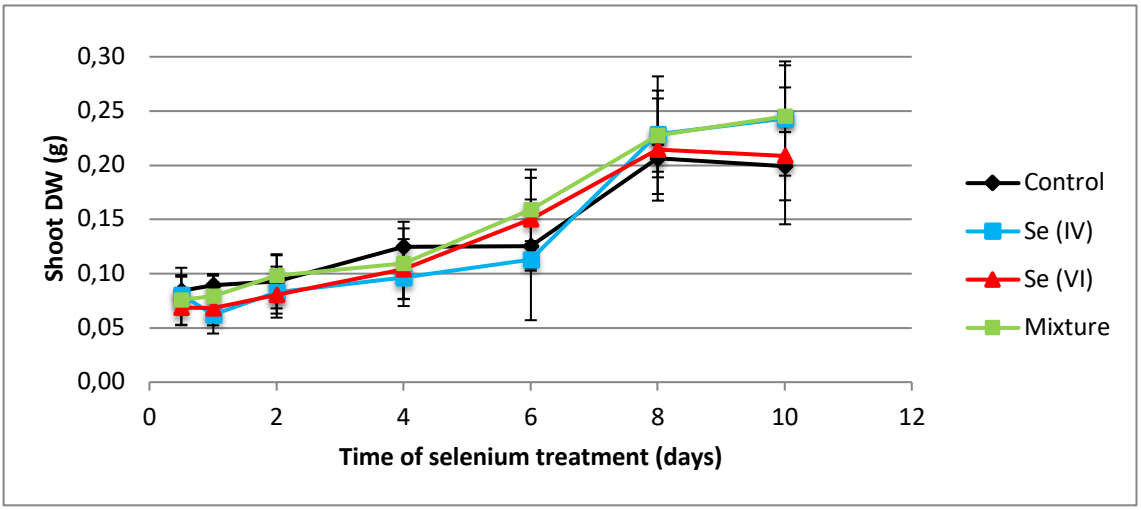


Figure 3: Shoot dry weight (g) of wheat plants collected after 12h, 1, 2, 4, 6, 8 and 10 days of Se exposure: control (black), 10µM of Selenite (blue), 10µM of Selenate (red) and 10µM of mixture of both selenite and selenate (green) Results are expressed as means± SD (n=12).

No significant effects of selenium application to plant weight were detected for any of the selenium treatments. In conclusion, 10µM Se for 10 days did not cause any observable toxic effect to the plant, as root and shoot weights were not different than control treatment. This is in agreement with previous studies in wheat seedlings [5], [11]. However, also no significant beneficial effect to enhance plant growth was observed as opposed to the previous reported data [11], [25].

Finally, significant differences in root and shoot weight, respect to the initial weight, were detected after 8 days for control, 8 days for selenite plants, 6 days for selenate and 6 days for mixture plants. Accordingly, Se experiments with wheat seedlings should be longer than 8 days in order to have sufficient plant growth that could enable the possibility to observe existing differences.

Kinetic study of selenium uptake in the plant early stages of development: Selenium uptake and distribution

Selenium concentration in roots and shoots of wheat seedlings were analyzed at each time, for each treatment, and can be seen in figures 4 and 5 respectively.

It can be observed how, after 12 hours of selenium exposure in any form, the selenium level in roots is already significantly different than the one in control plants. Therefore, root uptake can be considered a relatively fast process that started immediately after selenium is supplied.

Even Se exposure times as small as 30 min result in a significant uptake by roots of rice [46] and cowpea [47], and, in previous studies, the effect of selenium in wheat seedlings was measured on only 2 days of treatments [5].

Besides, selenium uptake was constant during the studied time, and accordingly the concentration in roots steadily increases, without reaching a plateau.

As seen in figure 4, all the Se species follow the same kinetic pattern, with parallel evolutions in the selenium content. It has been reported that selenite and selenate uptake by wheat roots can be described with a kinetic equation that follows a Michaelis-Menten model [5], [46]. This enzymatic model has been established to describe general ion transport into root cells at low external ion concentration (<1mM) [48] and has been confirmed with several other nutrients [49], even if there were changes in the dynamics of nutrient uptake in the different growth stages of plants [50].

Furthermore, at all the exposure times studied, selenite was accumulating in roots at a significantly higher concentration than selenate, with the mixture of the two species resulting in Se concentrations between them and with no significant differences to the individual species.

However, this should not be interpreted as a smaller rate of Se uptake by selenate treated plants, since the translocation to aerial tissues is also a relevant factor.

The Se translocation to shoots presented an even more differentiated behavior between the three selenium treatments.

Selenate treated plants showed the highest Se accumulation in shoots. Moreover, it was significantly higher than mixture treated plants, which had an intermediate Se accumulation, and finally selenite plants had the lowest Se content in shoots.

For selenate and mixture treatments, translocation was a relatively fast process and the Se concentration in shoots was already significantly higher than in controls after 12 hours of Se exposure.

However, for selenite enrichment, the concentration in shoots when all the treatments were considered, was not significantly different to control plants even after 10 days of exposure. If the statistical analysis was performed individually for the selenite condition, the selenium concentration increased significantly only after 2 days of treatment. Therefore, the translocation of selenium from roots to shoots in selenite applications can be considered a slow process.

The translocation factor (TF), which is defined as a the ratio between the selenium concentration in shoot and the one in roots, can serve to evaluate the transfer potential [36], [40], [46].

Accordingly, the translocation factor, as can be seen in figure 6, confirms a different behavior for the two selenium species, with selenate translocation being highly favored at all times, with TF values well above 1, while selenite translocation is not favorable, with TF values close to 0.

The reasons for these differences between the two species will be further discussed in the next section but could be summarized as an easy transfer of selenate from the root epidermis to the xylem, and thus a fast translocation to shoots, versus a quick reduction of selenite to less mobile organic Se compounds.

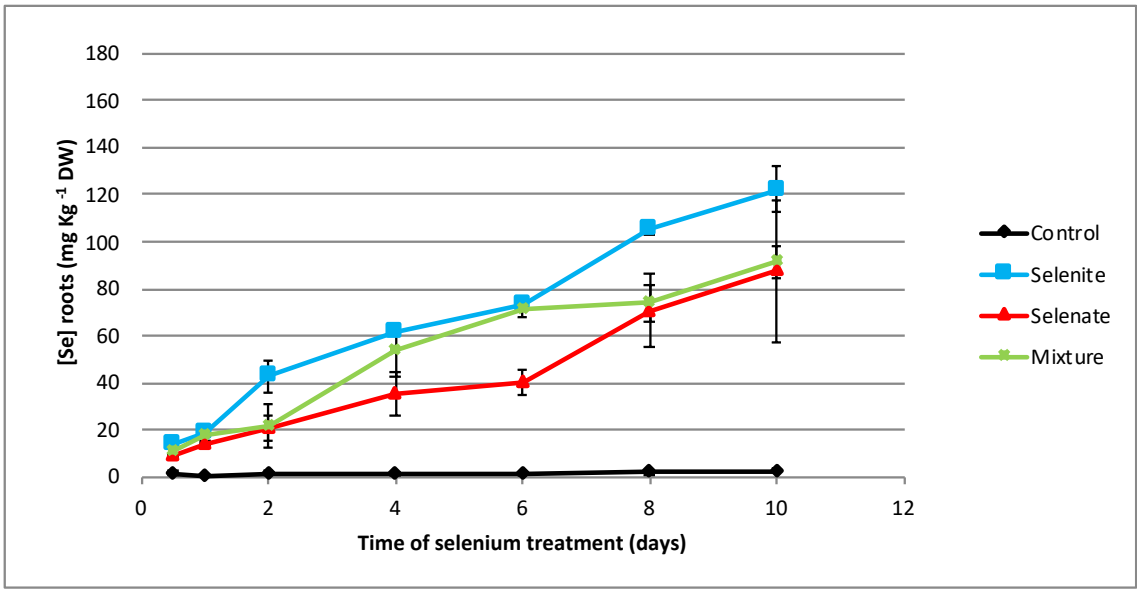


Figure 4: Changes in selenium concentration in roots of wheat plants harvested after 12h, 1, 2, 4, 6, 8 and 10 days, after no treatment (black), 10µM of Se as selenite (blue),

10µM of Se as selenate (red) and 10µM of Se as a mixture of both selenite and selenate (green). Results are represented as mean±SD (n=12).

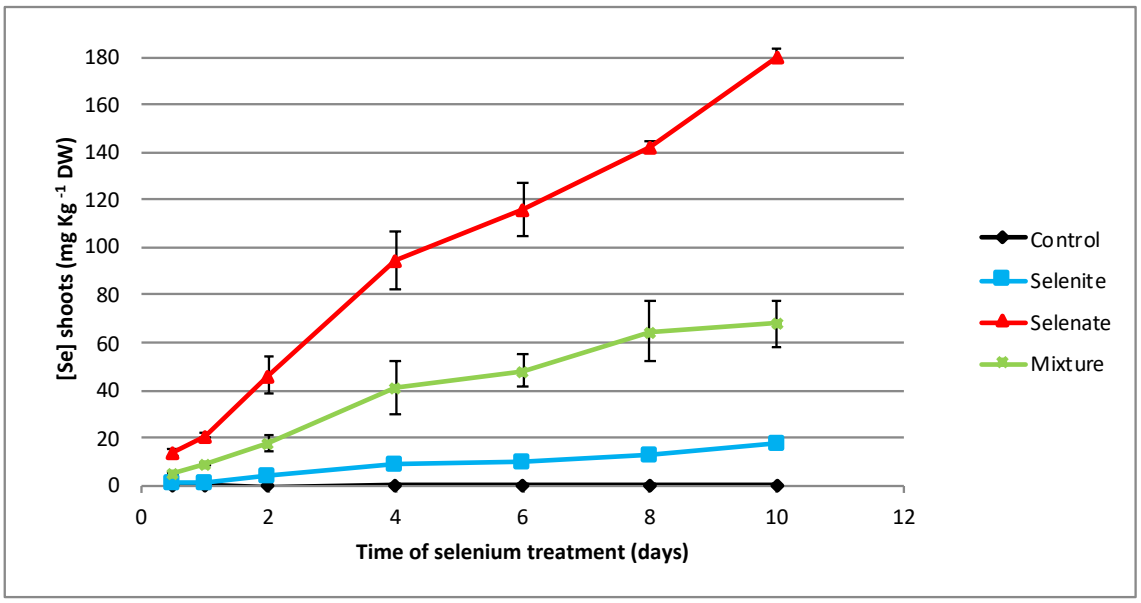


Figure 5: Changes in selenium concentration in shoots of wheat plants harvested after 12h, 1, 2, 4, 6, 8 and 10 days, after no treatment (black), 10µM of Se as selenite (blue), 10µM of Se as selenate (red) and 10µM of Se as a mixture of both selenite and selenate (green). Results are represented as mean±SD (n=12).

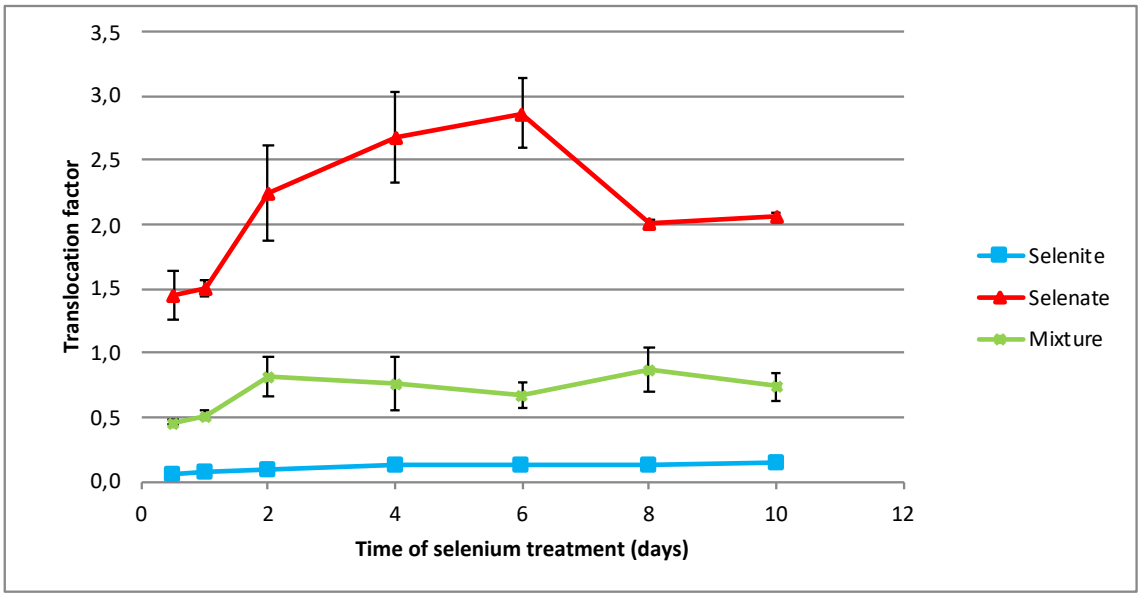


Figure 6: Selenium translocation factor (concentration in shoots/concentration in shoots) at 12h, 1, 2, 4, 6, 8 and 10 days of 10µM Se exposure as selenite (blue), 10µM of Se as selenate (red) and 10µM of Se as a mixture of both selenite and selenate (green). Results as mean±SD.

Kinetic study of selenium uptake in the plant early stages of development: Effect on nutrient uptake

The effects of selenium over the uptake, accumulation and translocation of mineral nutrients was also studied during these 10 days of Se exposure.

Selenium induced significant changes in the sulfur levels in wheat seedlings, whereas no significant effect was seen on the other macronutrients (P, K, Ca, Mg), as can be seen in figure 7, and neither on micronutrients (Fe, Mn, Cu, Zn), figure 8, in the selected timespan for either roots or shoots.

Regarding sulfur, selenium addition did not change significantly the accumulation in roots. However, shoot S concentration in plants treated with selenate started to be significantly higher than controls after 2 days of exposure. Mixture treated plants had also an enhanced S accumulation after 4 days of exposure, even if there was a punctual descent at 8 days. Oppositely, S concentration in selenite treated shoots was not significantly different to controls at any time.

As will be further discussed, selenate is taken up by root cells by sulfate transporters present in the membranes [4], [51]. Moreover, due to the similarities between the two molecules, selenate and sulfate have parallel metabolic pathways. The increase of selenate concentration in the media, enhances the transporter activity, promoting a faster uptake and translocation through xylem, for both compounds, into shoot organs where they can be safely stored [52].

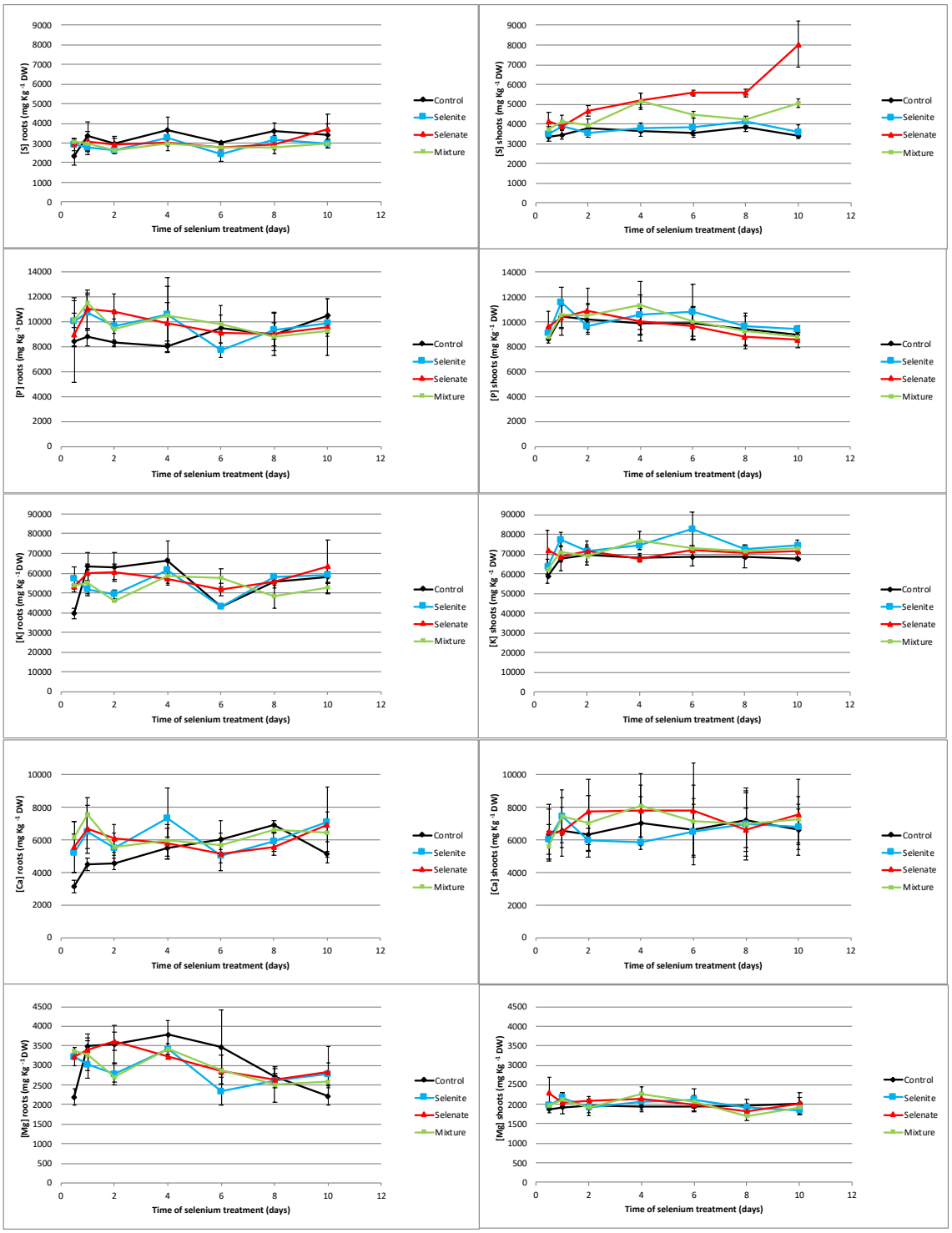


Figure 7: Macronutrient (S, P, K, Ca and Mg) concentration in roots and shoots (mg/Kg DW), after 12h, 1, 2, 4, 6, 8 and 10 days of Se exposure, represented as mean±SD (n=12).

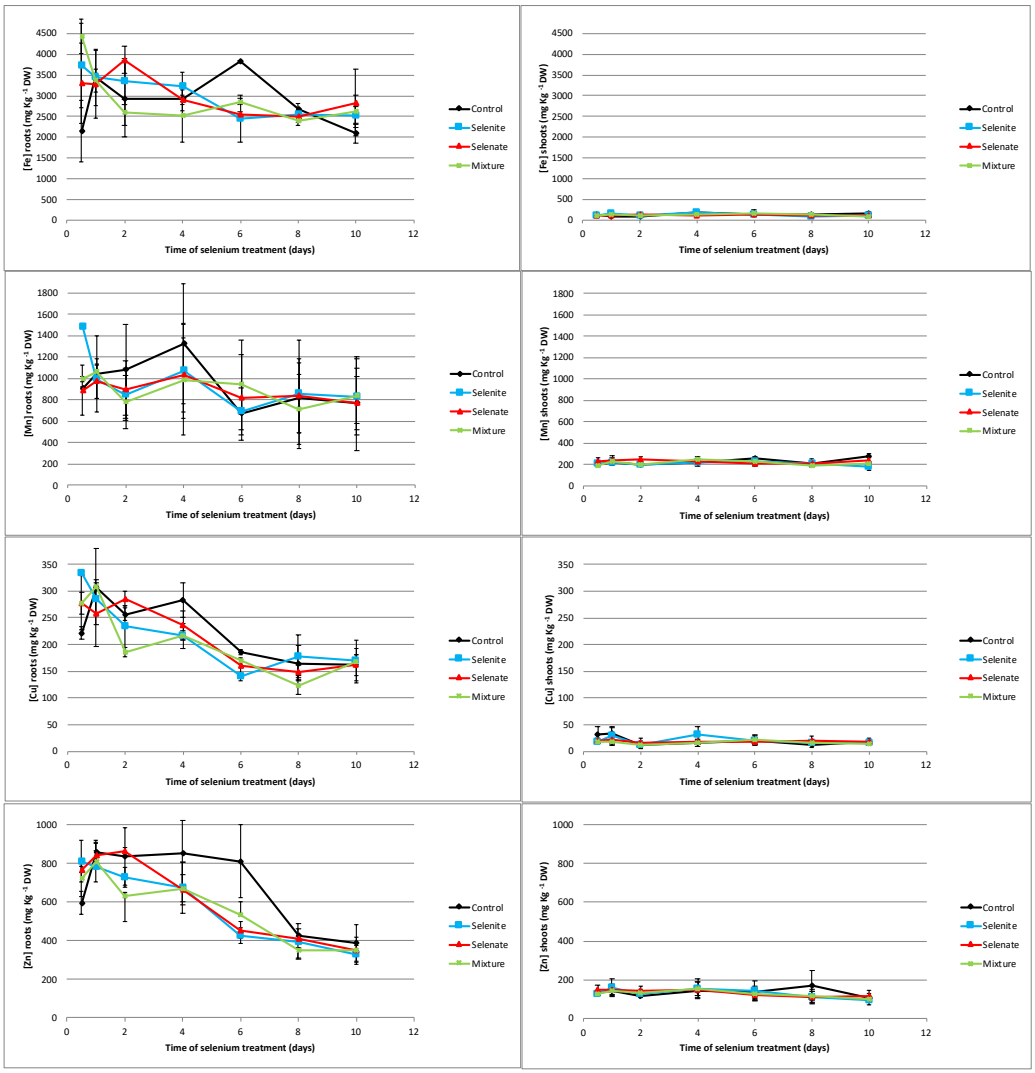


Figure 8: Micronutrient (Fe, Mn, Cu and Zn) concentration in roots and shoots (mg/Kg DW), after 12h, 1, 2, 4, 6, 8 and 10 days of Se exposure, represented as mean±SD (n=12).

PART II. Evolution of selenium concentration and plant status with plant age: Mature plants

Selenium effect on wheat plants at grain maturity stage: Plant development

Two experiments were performed, in two different years, in order to determine the concentration and distribution of selenium in plants that have completed their life cycle and have reached a senescence stage.

These experiments allowed the study of the selenium translocation and accumulation in ripe grain, as well as the assessment of whether the selenium biofortification affects the yield or the nutrient content of the produced grain.

In order to study the selenium physiological effect on wheat development and yield, a comparison of plant root, shoot and grain weight, as well as the total number of stems,

spikes and grains produced, the average number and weight of grains in each ear and the average individual weight of a single grain was determined.

The two cultures performed in different years did not present significant differences, therefore, the results were averaged to a total of 8 plants per each treatment.

The results in figure 9 showed a significant low plant biomass with reduced weights of shoots and grain of plants treated with selenite, compared with control plants and with the other two Se treatments. Selenate and mixture treatments did not result in any statistically significant (ANOVA, 95% confidence) change compared to control plants, for any of the parameters considered.

Furthermore, the selenite treatment did not alter the number of stems produced compared to control plants, but the number of stems that were able to generate a spike, the grains produced and even the average weight of a single grain were significantly reduced (Figure 10). The single grain weight was even significantly lower than the values determined for the other two Se treatments.

Therefore, selenite plants produced less grain weight per ear, even if the number of grains generated was not significantly affected. Consequently, the selected Se species used is relevant for wheat biofortification.

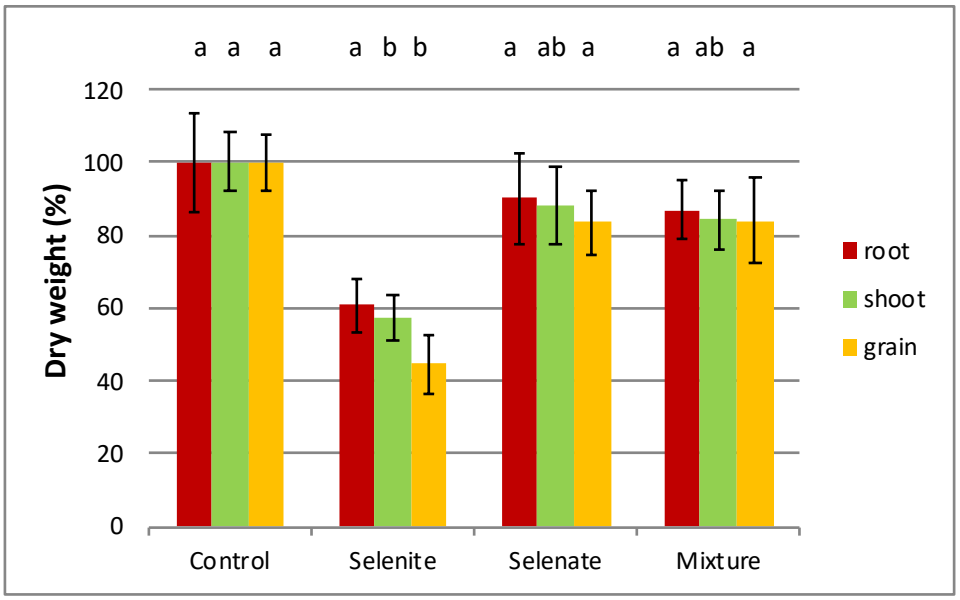


Figure 9: Root, shoot and grain weight after selenium treatment as a percentage of that of non-exposed plants, represented as mean±standard error (n=8). Letters indicates significance (p<0.05) between different treatments.

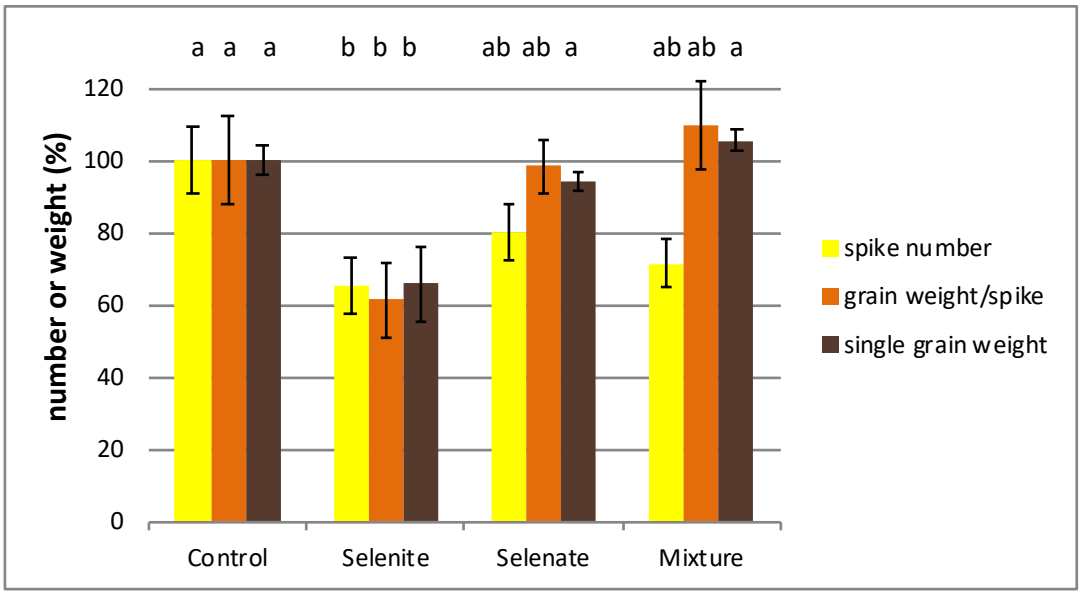


Figure 10: Spike number, grain weight per spike and average weight of a single grain after selenium treatment as a percentage of that of non-exposed plants, represented as mean±standard error (n=8). Letters indicates significance ($p<0.05$) between different treatments.

In the presence of selenite, the observed decrease in the measured parameters is understood as a Se-toxicity response in the plant. For non-accumulators such as cereals, over 100 mgSe/Kg of DW exert a toxic effect on the plant [4], that may come from an interference of Se in the S metabolism [36], which leads to a malformation of proteins because of the replacement of amino acids for the selenium analogues [4], an increase in oxidative stress [4][40], or an intrusion in the cellular energy production pathway, among other causes.

This behavior is in agreement with previous published data, where the impact of selenium on plant development was evident at lower concentrations for selenite in non-accumulators such as wheat [11], lettuce [36] and cucumber [37]. However, opposite results have been reported for Se hyperaccumulators such as pak choi (*Brassica chinensis L.*) [53].

This difference in the toxicity effects of the two inorganic species is due to differentiated processes in the uptake and metabolization mechanisms occurring in the plant, which leads to significant differences on wheat plant accumulation and distribution of selenium, as will be further discussed in the next section.

Selenium effect on wheat plants at grain maturity stage: Selenium uptake and distribution

The selenium concentration in the different wheat organs and grain, shown in figure 11, revealed that the distribution of Se in wheat is not homogeneous through the different plant parts (roots, shoots and grain) and that this Se distribution depends on the form of selenium present in the substrate.

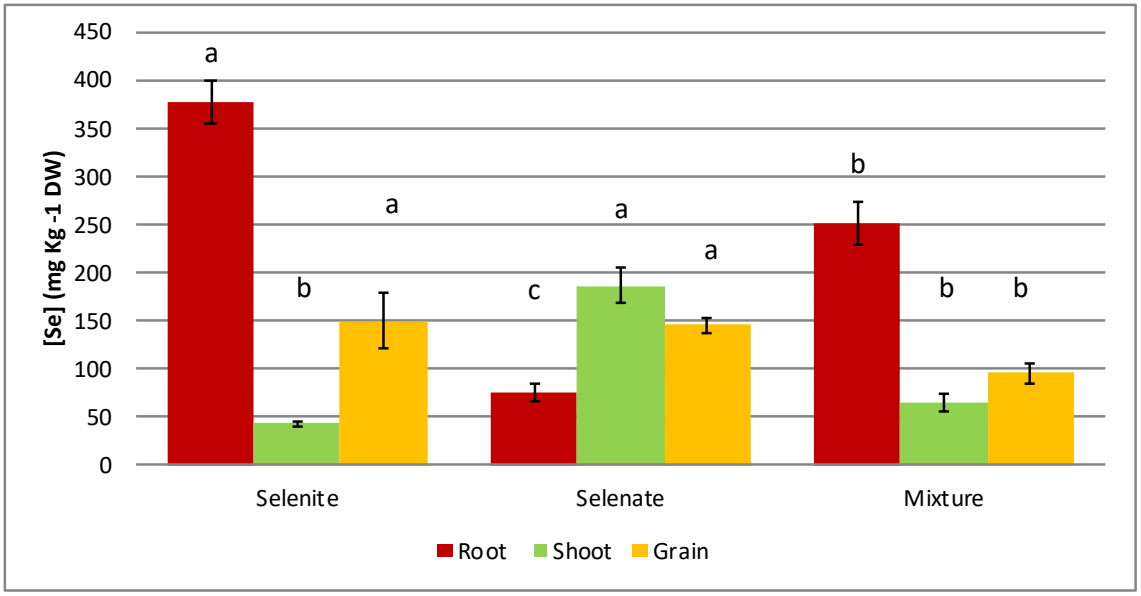


Figure 11: Selenium concentration in root, shoot and grain (mg Se/Kg DW), represented as mean±SD (n=8). Letters indicates significance ($p<0.05$) between treatments.

The current study has shown that the selenium concentration in the plant, considered as a whole, reaches significantly higher accumulation levels in selenite treated plants than in plants treated with selenate or with both species together.

This result agrees with previous reported data of higher selenite uptake by wheat plants than selenate, when they are available at the same concentration [5], but is opposed to previous data in Indian mustard (*Brassica juncea*) where the accumulated selenium was ten times higher for selenate than selenite [54]. These divergences can be attributed to the different metabolism of non accumulators and hyperaccumulator plants, with the later having, additionally, mechanisms for fast selenium volatilization, particularly for selenite treated plants, and a significant faster selenate uptake than wheat plants [54].

In selenite treated plants the selenium concentration was mostly located in roots, which contain a 66% of the total Se, and very little was translocated to shoots. But once this selenium was in the shoots, only an 8% was accumulated in stems and leaves, and a 26% was transported into grain.

In contrast, in selenate treatments, only an approximate 18% of Se accumulated in roots, whereas 46% accumulated in shoots, and a 35% translocated to grains.

Coherently, the mixture enrichment had an intermediate behavior. Approximately 61% of the selenium was found in the roots, and 15% was found in the shoots, and 23% of the selenium reached the grain.

The translocation factors, which are shown in figure 12, were lower than 1 for selenite and mixture treatments, and higher than 1 in the selenate exposure. Therefore, the translocation to shoots was favored over root accumulation only with the selenate treatment.

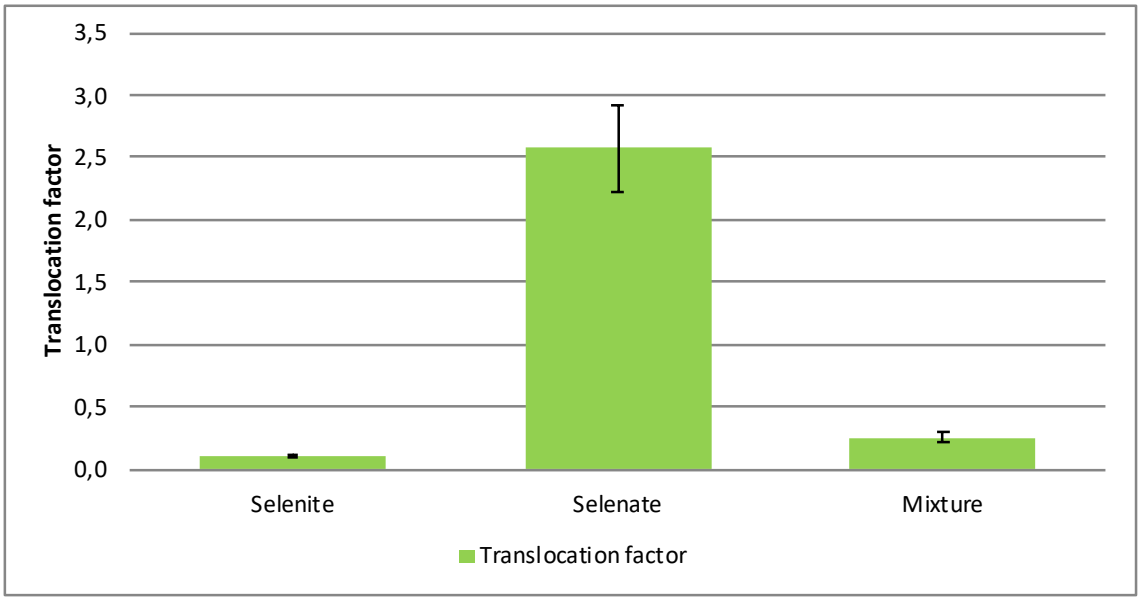


Figure 12: Selenium translocation factor (shoot Se concentration/root Se concentration) for each treatment, as mean±SD.

The Se concentrations in roots were statistically different between the three treatments, with selenite being accumulated at higher amounts in this organ. Shoots contained a significantly higher concentration of selenium in plants enriched with selenate rather than selenite and mixture. However, despite the big difference in the translocation from root to shoot between selenite and selenate, the final selenium concentration in grain was not statistically different in the two treatments.

Only the mixture treatment resulted in a significantly lower selenium amount in grains, with a 95 ± 11 mg Se/Kg DW, instead of the 149 ± 29 mg Se/Kg DW and 145 ± 8 mg Se/Kg DW in selenite and selenate grains, respectively. This can be attributed to a separate distribution pathway for the two species, which did not result in an additive effect, thus hindering the final translocation.

The reason for the observed diverse behaviors is the separate pathway mechanisms for selenite and selenate assimilation in roots [14], which are the main organ for

nutrient uptake. Selenate is taken up actively by roots via sulfate transporters in the root plasma membrane [4], [51], competing with sulfate [55]. Selenite uptake is driven by other processes, since there is little inhibition of selenite uptake with high sulfate concentrations [14] and there is no evidence that it is mediated by membrane transporters [56]. Oppositely, selenite has been assigned to the phosphate transport pathway, since excess or starvation of P effectively modifies the Se uptake [5], [57], and to aquaporins involved in silicon transport [58].

Furthermore, the translocation of selenium compounds from root to shoot also depends on the chemical species of selenium in the media, and, in addition, is also affected by the plant species, its development stage and the external physiological stress [59].

It is well known that selenate is more easily transported to shoots than selenite or organic selenium [37], [54], [56]. This occurs due to several reasons: firstly, selenite reduction to organic forms is quicker [56], while selenate reduction to selenite is slower and is considered the limiting step of Se metabolism [14]. Thus selenite will be easily converted to organic forms, while selenate will remain relatively stable. Secondly, selenate is easily transferred from the root epidermal cells to the plant xylem, with selenate-treated plants showing higher Se concentration in xylem exudates than selenite treatments [60]. It is through the plant xylem that plants transport water and nutrients from roots to shoots (with phloem contributing in the transport from shoots to reproductive organs) [61], [62], with this translocation depending on the rate of xylem loading as well as plant transpiration [4], [63]. Finally, mobility through xylem depends on the diffusion coefficient of the species in solution, with selenate diffusion coefficient being 2 to 3 orders of magnitude bigger than that of selenite in a variety of media and conditions, and diffusion coefficients of organic Se species being somewhat in between [64], [65].

Consequently, the translocation capacity in plants has been summarized as selenate >> selenoamino acids > selenite [14], which has also been demonstrated for wheat plants in the present experiment.

The quick reduction of selenite and its low translocation capacity results in a high Se accumulation of 377 ± 23 mg Se/Kg DW in selenite-treated roots, is well above the toxicity threshold of 100 mgSe/Kg of DW for non-accumulator plants. Those high levels can interfere in plant homeostasis and cause Se-induced stress, which triggers the production of reactive oxygen species (ROS) and generates phytotoxicity, which justifies the decrease of plant development and crop yield observed in figures 3 and 4.

Selenium effect on wheat plants at grain maturity stage: Effect on nutrient uptake

Regarding the effect of the selenium treatment on the nutrient status of wheat plants, it has been seen that it depends on the form of selenium used, which can be seen in figure 13 for wheat macronutrients, and figure 14 for micronutrients.

Selenium metabolism is parallel to sulfur metabolism, and selenate uses sulfate transporters to be taken up. For this reason, an effect of the selenium application on sulfur concentration on wheat plants was foreseen.

Sulfur accumulation was significantly lower in selenate treated roots than in control plants. Oppositely, selenite and mixture treatments did not show significant differences in roots. On the other hand, selenate treated shoots presented higher accumulation of sulfur than controls, with, again, selenite and mixture treatments showing no effect. In grains none of the treatments resulted in significantly different sulfur content than controls, despite presenting differences between them.

Therefore, the presence of bioavailable selenate in the solution decreased the sulfate uptake by roots, since they competed for the assimilation by sulfate transporters. However, once the selenate and the sulfate had entered the plant xylem, the simultaneous occurrence had a synergic effect, and the translocation to shoots of both molecules is enhanced, leading to significantly higher Se and S accumulation in shoots of selenate treated plants. However, then this Se and S increased content in shoots dod not result in higher phloem translocation to grains, since the content was not higher in selenate grains.

In the same manner, selenium, mainly as selenite, can also use phosphate transporters to be uptaken, and thus, changes in P content could be expected to be similar to S.

It was shown that selenate biofortification greatly enhanced the phosphorus accumulation in roots, and selenite and mixed treatments decreased root P content. Oppositely, regarding shoots, selenite treated plants showed significantly higher P accumulation than selenate plants, which was proportional to final grain P concentration, with selenite treated plants having significantly higher P than selenate and mixture treatments.

Thus, selenite had a similar behavior towards phosphate than selenate with sulfate. Selenite tended to reduce the phosphate content in roots, due to competition in the transport pathways, but then the translocation of P was enhanced, in this case to both shoots and grain.

On the other hand, a significant increase in the P content of selenate-treated roots was observed. Although the selenite competitive inhibition of phosphate has been previously reported, selenate enhancement of P uptake has not been previously observed to our knowledge [5], [66], [67]. However, due to the high variability in the P content of selenate treated plants, this effect needs to be further studied.

Regarding other macronutrients, potassium content in roots was not affected by selenium application. However, selenite significantly increases shoot and grain K concentration, whereas selenate and mixture treatments have no effect.

Calcium content in roots was significantly higher in selenate treatment. Shoot Ca content was not affected by selenium, and grain Ca was significantly enhanced with selenite application.

Magnesium uptake was parallel to that of calcium in selenium biofortification. Selenate application led to significantly higher Mg accumulation in roots, and selenite to a higher content in grains compared to the other selenium conditions, with the Mg content in leaves and stems not being affected in any case.

Concerning micronutrients, which can be seen in figure 14, Iron uptake was also not significantly modified by the introduction of selenium in wheat metabolism.

Manganese showed a significantly high concentration in selenate treated roots but shoots and grain content were not modified with any selenium treatment.

Copper accumulation was significantly higher in selenite treated roots than in the selenate treatment, although this enhancement was not observed in the aerial parts of the plants, with shoots and grains showing equal Cu levels.

Regarding molybdenum, roots and grain concentrations were not modified by selenium, but Mo in shoots treated with a mixture of both Se species was significantly lower than selenate treated plants.

Finally, zinc accumulation was significantly enhanced in roots biofortified with selenate, but its concentration was not modified in shoots or grains under any Se condition, in the same manner than Mn.

To sum up, selenite treatment enhanced the amount of Cu accumulated in roots, while decreasing P concentration. Also, it increased P and K in shoots and P, K, Ca and Mg in grains. Selenate increased several folds the nutrient uptake and accumulation in root, with high S, P, Ca, Mg, Mn and Zn in this organ, while it decreased shoot S. Finally, the mix of the two species lead to a decrease in root P and shoot Mo.

In general, the mix of the two selenium species did not cause such a significant effect over nutrient content than the application of one species individually. Therefore, the use of the two selenium species simultaneously affects less the plant health status and normal nutrient homeostasis. This is because selenite and selenate use different pathway processes in the first steps of plant metabolism, with selenite being converted into organic species in roots and selenate being translocated to shoots, and therefore their accumulation in each tissue is smaller and lead to less effect in the normal functioning of that organ.

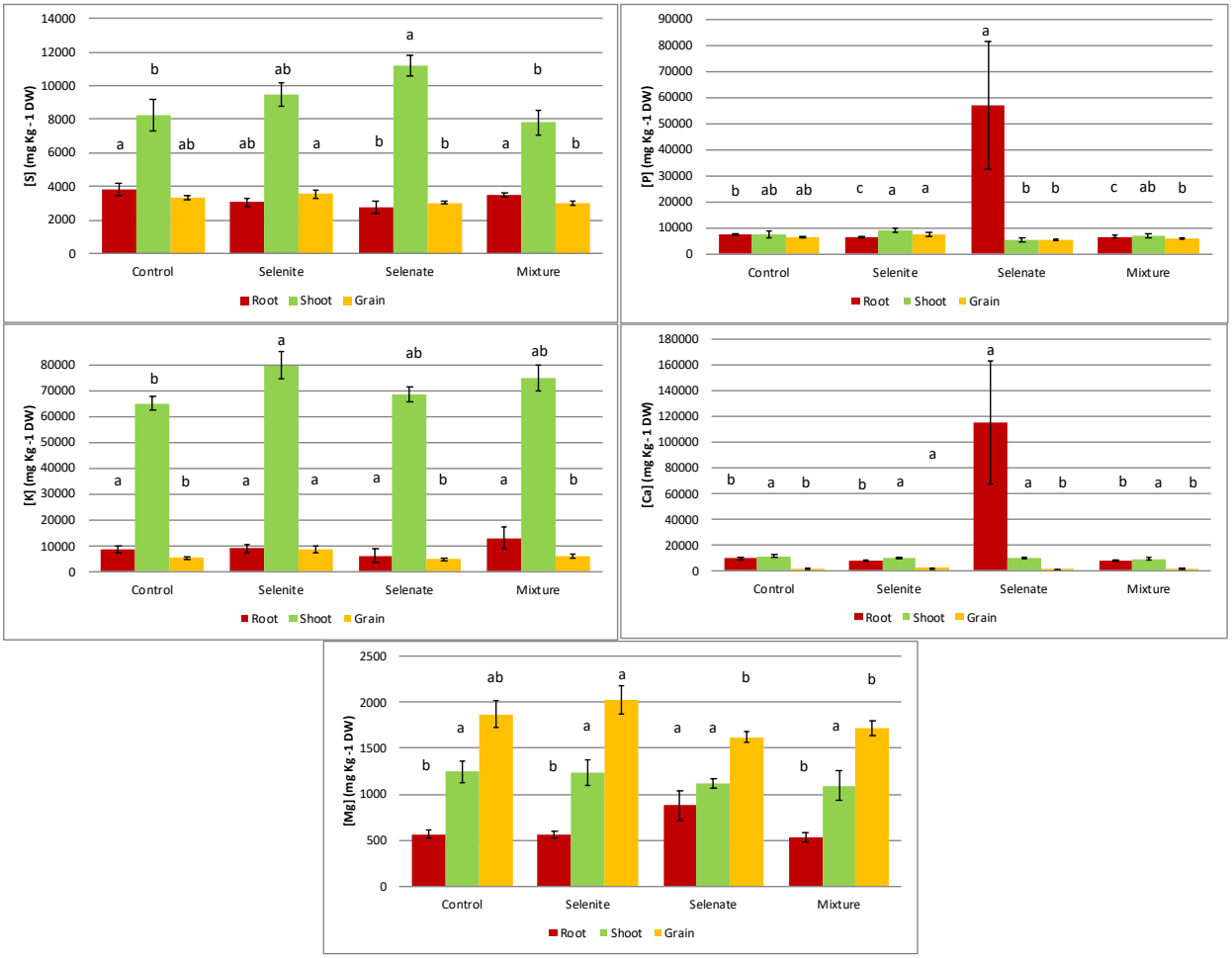


Figure 13: Macronutrient (S, P, K, Ca and Mg) concentration in roots, shoots and grain (mg/Kg DW), represented as mean±SD (n=8). Letters indicate significant differences ($p<0.05$) between treatments.

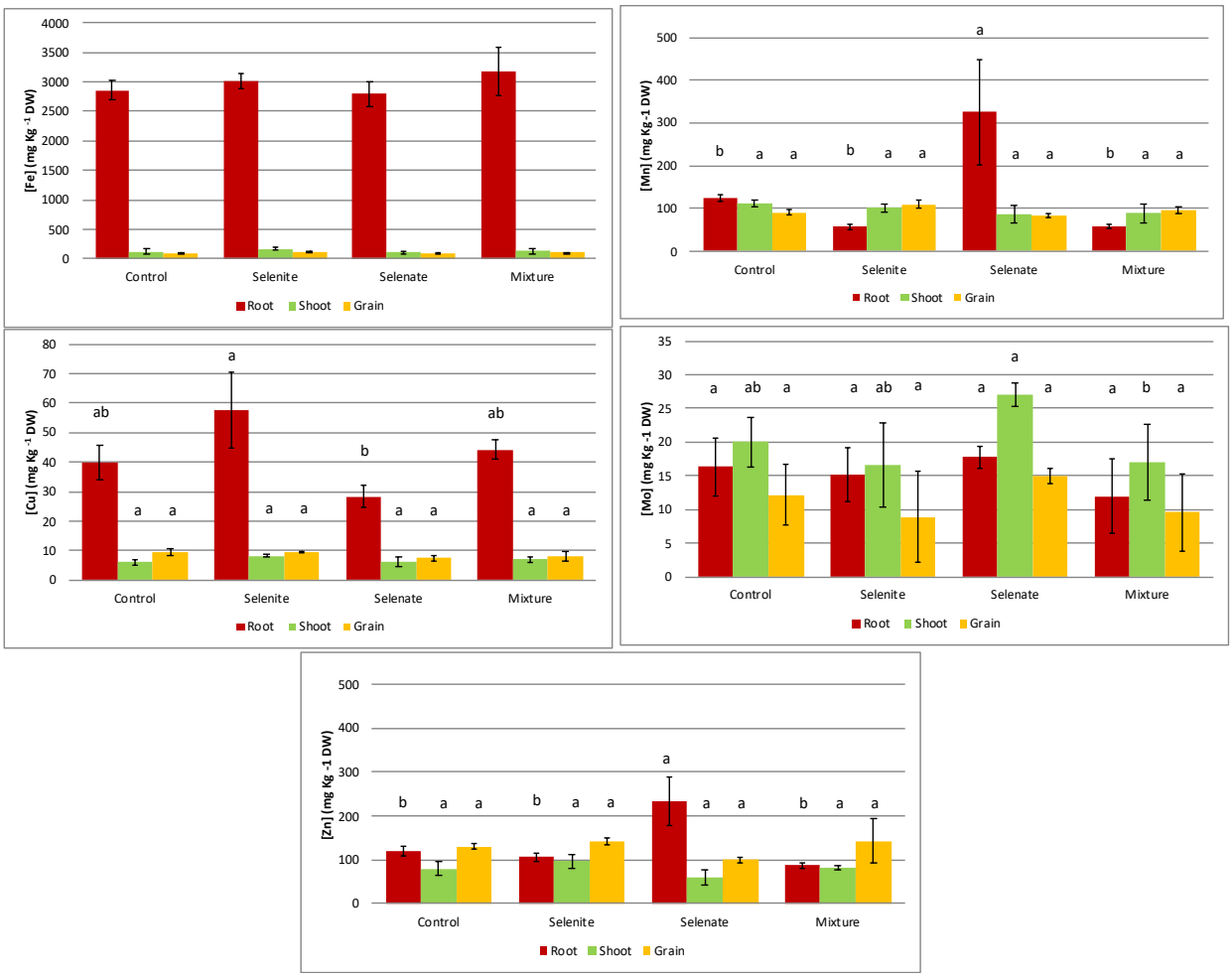


Figure 14: Micronutrient (Fe, Mn, Cu, Mo and Zn) concentration in roots, shoots and grain (mg/Kg DW), represented as mean±SD (n=8). Letters indicate significant differences (p<0.05) between treatments, in the cases where significant differences have been found.

PART III. Influence of Se concentration and time of Se application in the biofortification

Effect of Se concentration and time of Se application: Plant development

A new experiment was set up in order to find the optimum parameters to maximize wheat selenium biofortification without hampering the plant health status. The effects of selenium concentration and application time were considered, adding either 10 µM or 25µM Se at two different plant stages; vegetative and florescence.

Figures 15, 16 and 17, show the effect of the selenium application in the selected conditions for roots, shoots and grains, respectively. Furthermore, visual comparison

of the plants and an example of a representative spike can also be found in figures 18 and 19 respectively.

The plants exposed to 10 µM of selenium at vegetative stage had the same response (with no statistical difference, ANOVA, 95% confidence) than the previously discussed results: none of the selenium treatments caused a significant reduction in the root dry weight, and selenate and mixture treatments had no effect on the shoot dry weight either, but selenite significantly decreased the dry weight of shoots and grains, therefore confirming the good reproducibility of the results between different cultures in different seasons.

When the application of 10 µM Se at vegetative stage was compared to the application of 10 µM at florescence time, no effect on roots was observed for any selenium species. On the other hand, the selenite effect of shoots observed at a vegetative stage did not occurred when the application was performed at florescence. Regarding grain, the selenite-induced toxicity observed for vegetative stage was slightly reduced at florescence, but, in contrast, selenate and mixture treatments both resulted in toxicity and generated grain with significantly smaller weight.

Besides, the increment of selenium concentration from 10 µM to 25 µM at the vegetative stage showed a significant decrease in root weight for selenate, whereas selenite and mixture treated roots had no significant effect. Regarding shoots, all three conditions resulted in significantly lower shoot biomass compared to control plants, and there was a relevant decrease in the weight of selenate shoots with the Se concentration increment to 25 µM. This effect can be easily observed in the pictures of the cultures in figure 18: it can be seen qualitatively how plants treated with 25 µM at the vegetative stage reached senescence much earlier than the plants growth with other conditions, and how the biomass of stems and leaves is effectively smaller. Moreover, the same effect was transmitted to grain. The three selenium conditions affected the capacity of grain generation significantly. In particular, as can be seen in figure 19, wheat treated with selenate at 25 µM resulted in deformed spikes that were not able to produce any grain. Besides, selenite at 25 µM also resulted in a considerably smaller production of grain.

Oppositely, the application of 25 µM selenium at florescence stage did not hinder root growth, but it could even enhance it. Even though the dry weight of the roots was not significantly different than that of the control plants, the roots treated with 25 µM of selenate at florescence were significantly bigger than the roots with 25 µM of selenate at the vegetative stage. In the same manner, the roots of selenite plants treated with 25 µM at a florescence stage were also bigger than the roots of selenite with 10 µM at the vegetative stage. Moreover, 25 µM of selenium at florescence stage did not cause a significant decrease in shoot weight, for any of the forms of selenium used. In figure 18 it can be appreciated qualitatively how the effect of shoots is smaller than with the 25 µM at vegetative stage. Regarding grain, the application of selenium at florescence

also served to avoid the hampering in grain formation. Thus, none of the selenium conditions resulted in a grain weight decrease respect to the control. This was especially evident for mixture treated plants, for which 25 µM at florescence even lead to more grain weight than 10 µM at florescence.

In conclusion, the application of selenium at a concentration of 25 µM to young sensitive wheat plants at a vegetative stage, and during an extended period of time, resulted in enhanced toxicity, which inhibited plant development and biomass production. However, it is relevant that selenate species was the most toxic to the plant at this concentration, which even resulted in deformed ears and totally impeded grain formation, whereas 10 µM selenite caused more phytotoxicity to the plant than 10 µM selenate. The reason for this effect will be further discussed in the following section.

Furthermore, the exposure to selenium at the florescence stage, and thus during a much shorter time, leads to less effects on the plant, avoiding part of the toxic symptoms and resulting in a minimum effect on plant development, than exposure of young seedlings at the vegetative stage. Moreover, since at florescence stage is when the grain gets filled with nutrients, the application of Se at this moment may be sufficient to produce an equivalent enrichment, as will be discussed in the following section.

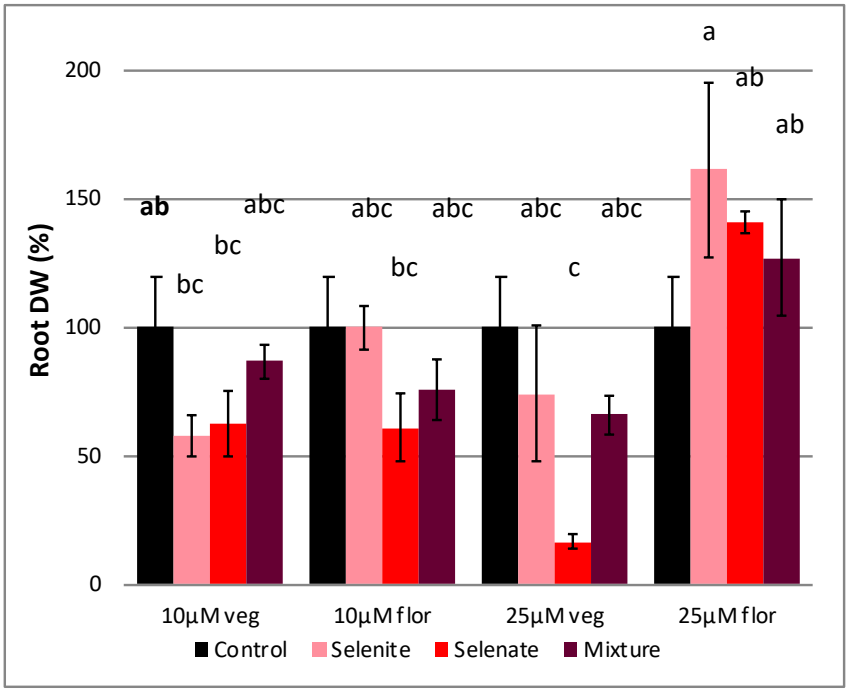


Figure 15: Root weight after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as a percentage of that of non-exposed plants, represented as mean±SE (n=4). Letters indicates significance (p<0.05) between different treatments. Control (black) is repeated for every condition for easier comparison, but its letters are only indicated in the first column in bold.*

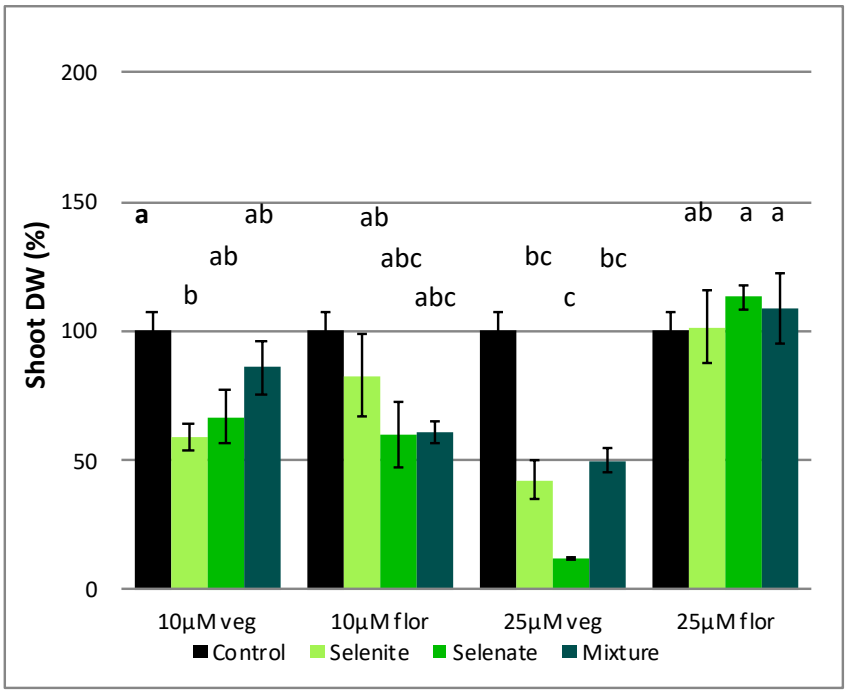


Figure 16: Shoot weight after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as a percentage of that of non-exposed plants, represented as mean± SE (n=4). Letters indicates significance (p<0.05) between different treatments. Control (black) is repeated for every condition for easier comparison, but its letters are only indicated in the first column in bold.*

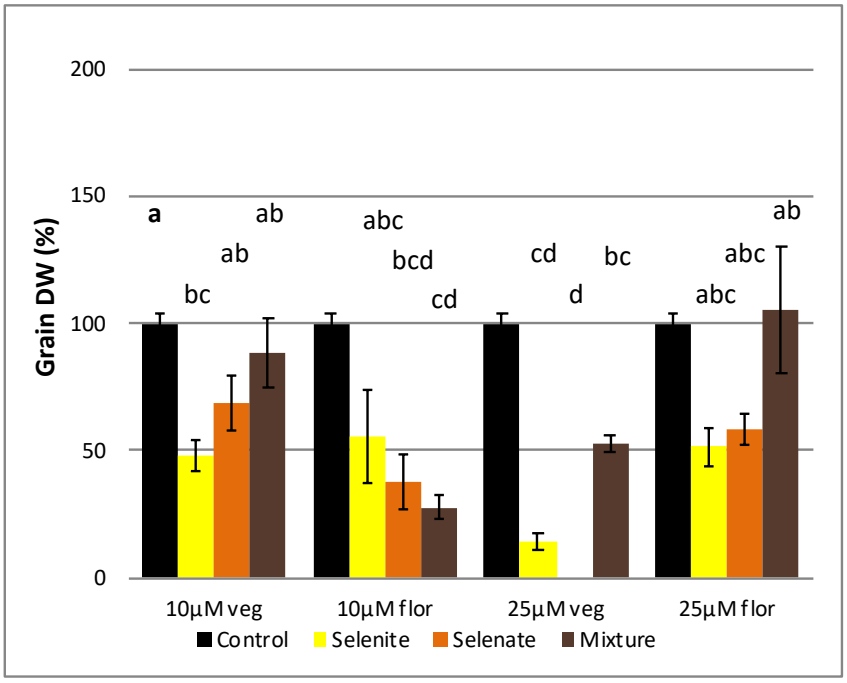


Figure 17: Grain weight after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as a percentage of that of non-exposed plants, represented as mean± SE (n=4). Letters indicates significance (p<0.05) between different treatments. Control (black) is repeated for every condition for easier comparison, but its letters are only indicated in the first column in bold.*

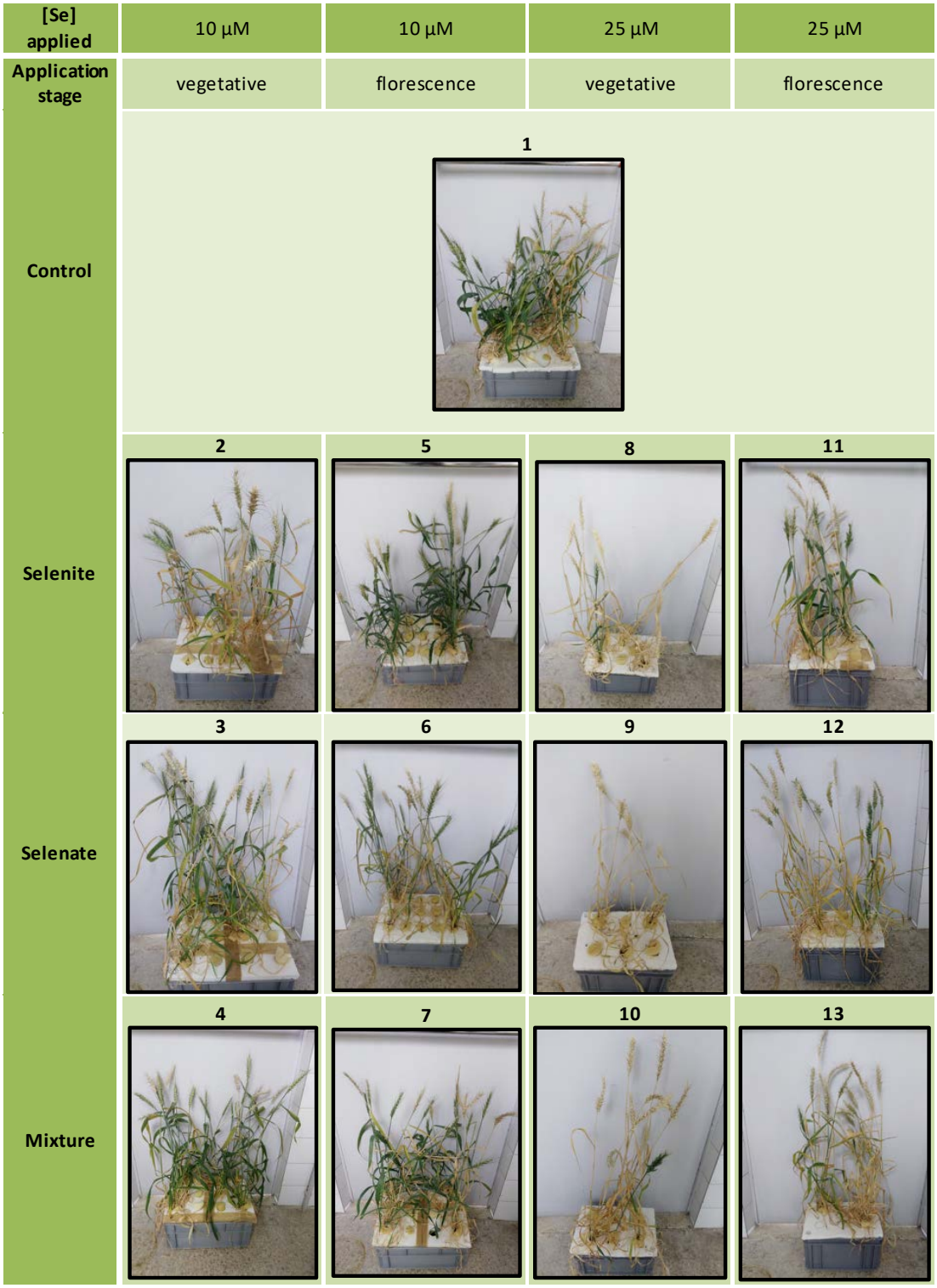


Figure 18: Pictures of wheat plants after selenium treatment, taken the same day, which allow a visual comparison of the effect of the different Se conditions on the plant development.

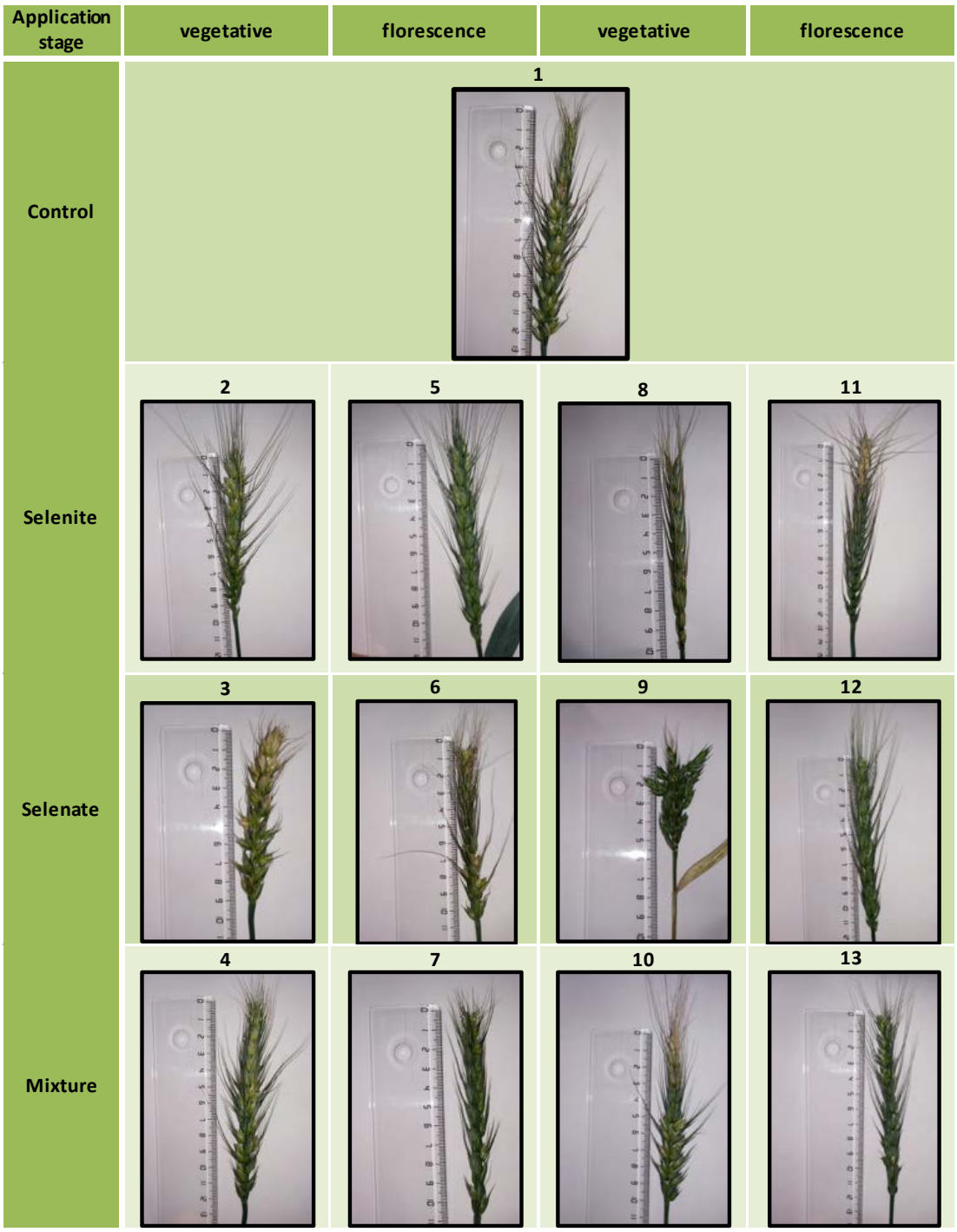


Figure 19: Pictures of a developing spike, representative of the ears in each condition, taken the same day, which allow a visual comparison of the effect of the different Se conditions on their size and shape.

Effect of Se concentration and time of Se application: Selenium uptake and distribution

In this experiment, all the conditions assayed resulted in a significant increase in the levels of Se in wheat roots, shoots and grain compared to control plants, as can be seen in figures 20, 21 and 22. However, plants exposed to 25 µM selenate at vegetative stage, did not produce any grain, and therefore, could not be reported in figure 22. Moreover, the translocation factor from root to shoot is shown in figure 23, and the effect of the increase in concentration from 10 µM to 25 µM in figure 24.

As in the previous experiment, the supplementation of 10 µM selenite at vegetative stage caused the highest accumulation in roots, followed by the mixture and finally selenate. Accordingly, selenite-exposed roots had a significantly higher concentration than selenate roots, but neither of them was different from the mixture. Regarding shoots, similarly to the previous experiment, selenate treatment resulted in a significantly higher Se accumulation than selenite and mixture. Selenate treatment resulted also in a significantly higher Se concentration in grain than the mixture of the two species, with selenite not being significantly different to neither of them.

The application of 10 µM selenium at the florescence stage resulted in similar accumulations than the early application at vegetative stage. Thus, the final concentration in roots, shoots and grain was not significantly different between the two application times, for each of the conditions.

Conversely, an increment of the selenium concentration to 25 µM resulted in higher Se accumulation. Addition of 25 µM Se as selenite or mixture in the vegetative stage resulted in a significant increment of selenium concentration in roots whereas the addition of 25 µM selenate did not result in a significant increase. Regarding shoots, selenate and mixture treatments caused a significant increase in Se levels in stems and leaves, whereas the effect of selenite was not significant. In addition, selenite and mixture treatments increased the Se translocation to grain, leading to high concentrations. In contrast, selenate treated plants could not produce grain.

A similar effect occurred for the application of 25 µM Se at the florescence stage. Selenite and mixture exposed roots significantly increased Se concentration respect to the 10 µM Se supply at florescence, but not in the selenate treated roots. Oppositely, shoots accumulated a significative amount of selenium only in the selenate treatment, whereas addition of 10 µM of both selenium species increased the selenium levels of the grains, although the mixture resulted in a smaller amount compared to the 25 µM Se at the vegetative stage.

More specifically, a 2,5-fold increase of selenium concentration from 10 µM to 25 µM Se, increased root Se concentration above 2,5-fold in all cases, as can be seen in figure 24, and in general, this increment was enhanced with longer exposure times.

This amplification of the concentration was distributed in a different manner depending on the selenium species and on the application time, showing a different physiological response in the plant.

For the applications at the vegetative stage, the increment of 2,5 fold of selenite resulted in an enhancement of the Se concentration in roots of 4,0 fold, although the shoots and grains did not increase proportionally, with an increase of only 2,3 folds. Instead, the addition of selenate resulted in a higher enhancement of selenium concentration in the shoot, with a 3,1 fold increase, than in the roots, with a 2,7 fold increase (grain was not formed for selenate 25 µM so it cannot be compared). Finally, the mixture treatment led to a more equally distributed increment: 3,1 fold in roots, 3,6 in shoots and 3,4 in grain.

In contrast, the increment from 10 µM to 25 µM Se at the florescence stage did not result in an equal enhancement of the plant selenium concentration, with an increase of selenium concentration by less than 2,5 fold in most of the tissues. In this case, selenite caused an increment of 2,1 fold in grains, 1,6 in roots and a 1,7 in shoots. For selenate, the increment of Se in shoots was similar to the one at vegetative stage, with a 3,2 fold increase, but roots did not accumulate more selenium being the ratio 1,0. Besides, in this condition there was grain formation. Finally, for the mixture of the two species, the increments were reduced for all tissues, with only a 2,5 for roots, a 2,2 for shoots and a 1,9 for grain.

This highlights the different behaviors that the different selenium species, concentration and time have on selenium accumulation in plants.

For selenite treatments, in all the conditions the selenium was mostly accumulated in roots, due to a fast reduction of this species and a slow translocation to above ground tissues, as previously discussed (part II). Accordingly, this high root accumulation led to plant stress and phytotoxicity, hampering wheat development and yield.

However, when the selenite application took place at florescence stage, the accumulation of selenium in roots was less relevant although the increment of Se in grain was similar. This is because in this stage the kernel formation and nutrient filling was starting, which favored a faster mobilization of selenite from roots to aerial parts.

In contrast, selenate triggered a high Se accumulation of selenium in shoots due to a fast translocation of this species. Once in the above-ground plant tissues, some amount of selenate can accumulate innocuously in the vacuoles of the mesophyll cells of leaves [67]–[69]. Therefore, the plant can tolerate up to a certain amount and thus the plant biomass production is not significantly affected with 10 µM of selenate. However, with 25 µM of selenate, the selenium concentration in shoots reaches 871± 151 mg Se/Kg DW. The plant is not able to safely store such a high Se concentration, and thus it results in phytotoxicity that hampers plant development and inhibits the production of grain.

On the other hand, similarly to selenite, the application of selenate at the florescence stage resulted as well in an enhanced increment of shoot Se levels, while roots did not accumulate more selenium, which confirms that a faster translocation to aerial parts was taking place in the process of grain ripening.

However, for mixture treatments, due to the different assimilation pathways and preferential organs of accumulation of each species, this effect was more distributed between all the tissues and thus being less evident.

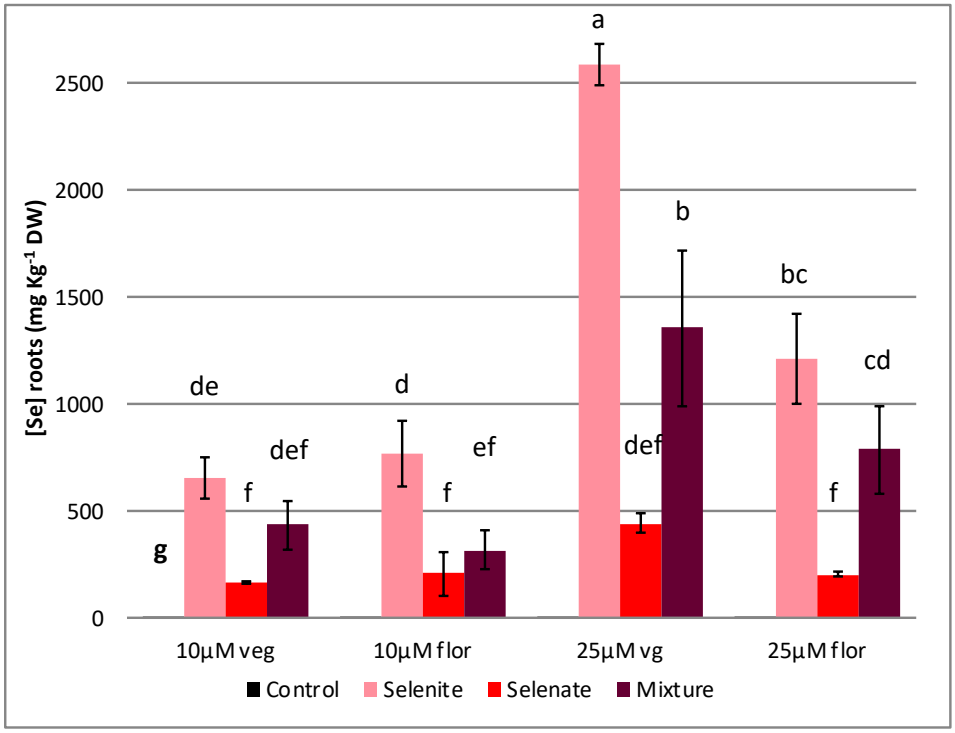


Figure 20: Selenium concentration in roots after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as mg Se/Kg DW, represented as mean±SD (n=4). Letters indicates significance (p<0.05) between different treatments.*

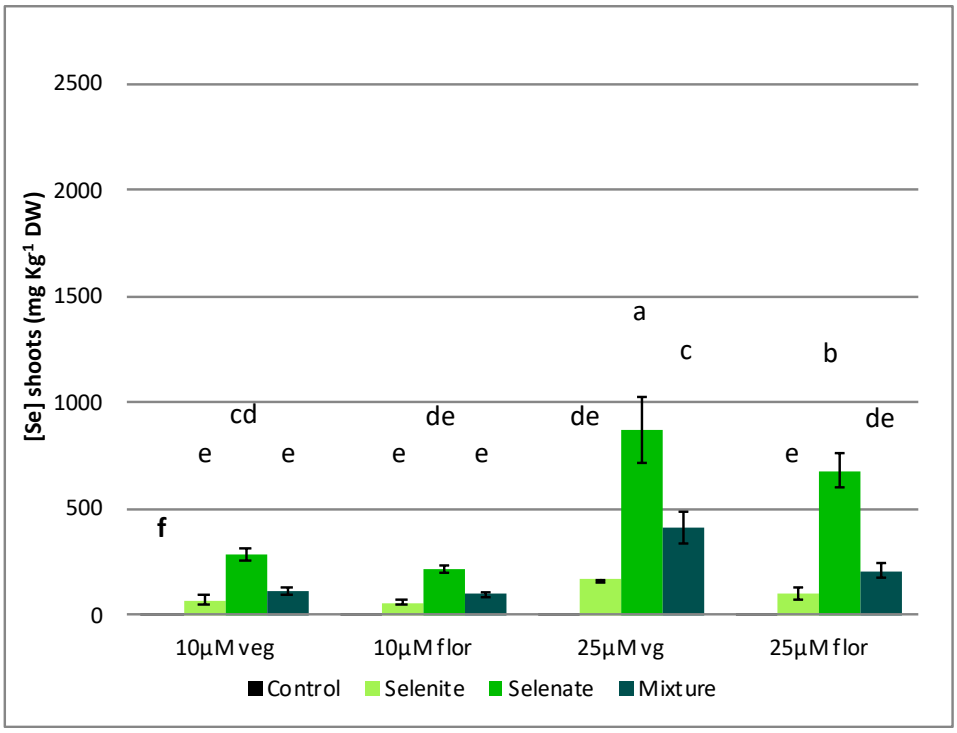


Figure 21: Selenium concentration in shoots after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as mg Se/Kg DW, represented as mean±SD (n=4). Letters indicates significance (p<0.05) between different treatments.*

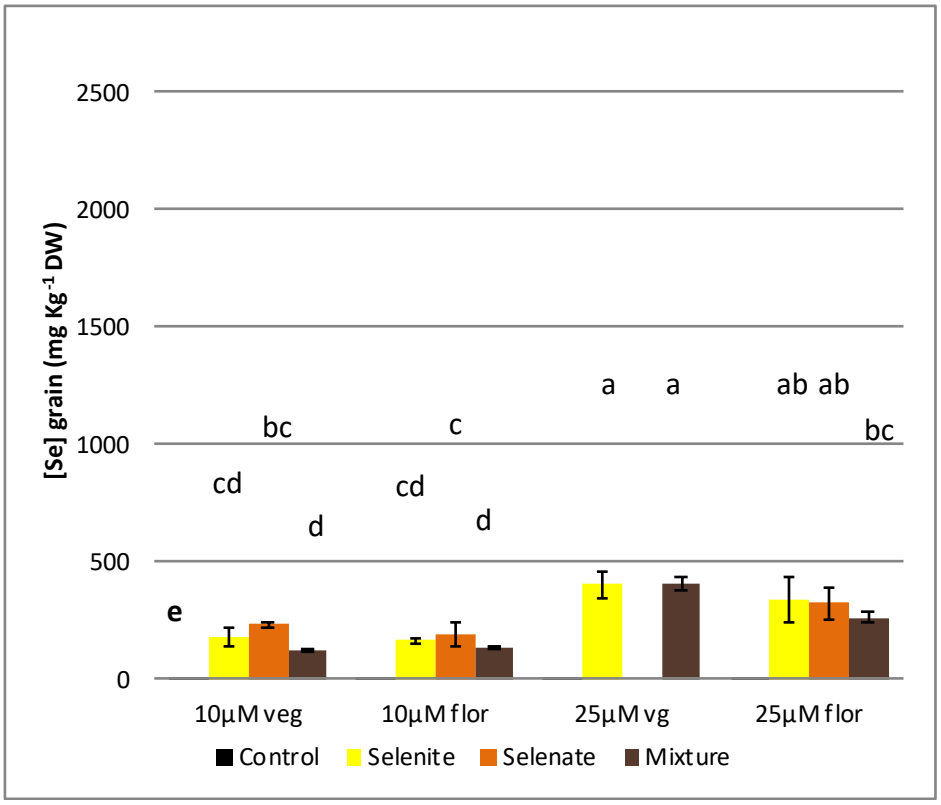


Figure 22: Selenium concentration in grain after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as mg Se/Kg DW, represented as mean±SD (n=4). Letters indicates significance (p<0.05) between different treatments.*

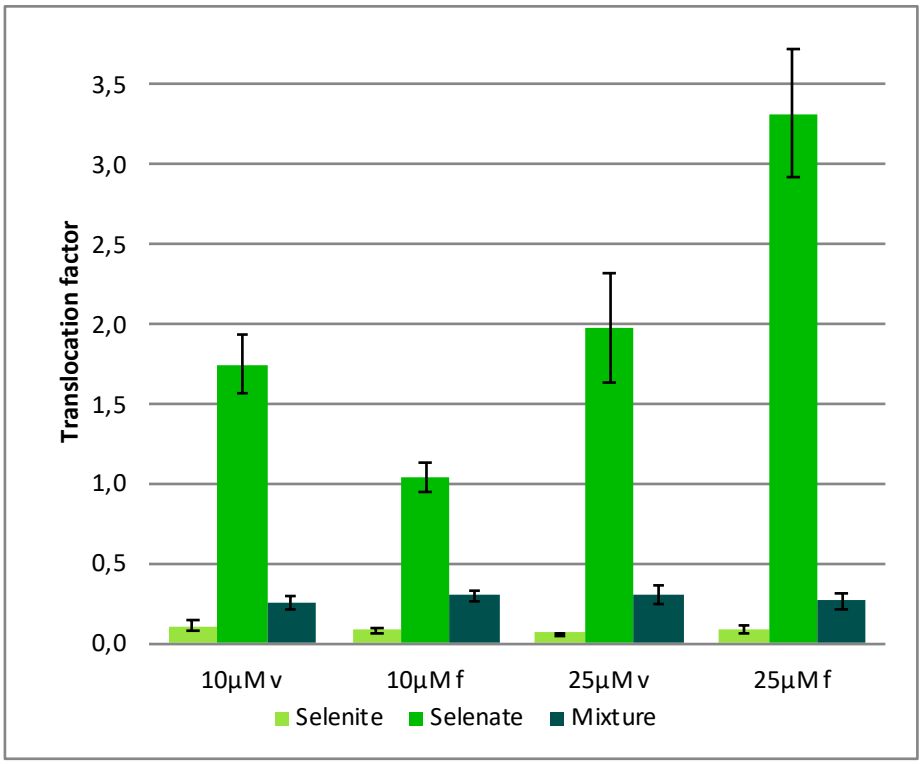


Figure 23: Selenium translocation factor (shoot Se concentration/root Se concentration) for each treatment, as mean±SD.

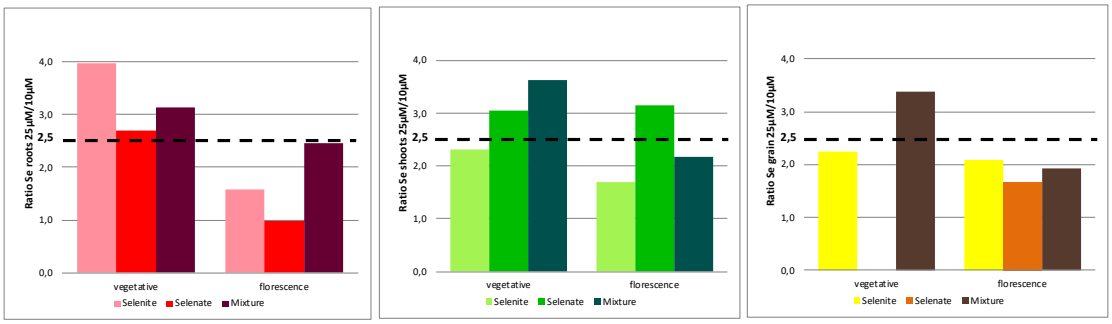


Figure 24: Ratio of selenium concentration between selenium applications of 25 µM over 10 µM, in roots, shoots and grain, as an indication of the increment of the selenium in tissues with the increment of 2, 5 fold (dash line) of the selenium applied.

Effect of Se concentration and time of Se application: Effect on nutrient uptake

Selenium addition caused a significant change in nutrient uptake and accumulation in roots, shoots and grain, depending on the species and concentration of selenium applied in the biofortification, as well as on the time of application.

The influence of Se in nutrients did not follow a general trend, but had a separate behavior for each element, as can be observed in figures 25 and 26:

Sulfur concentration in roots did not change after 10 µM Se exposure, for any of the Se species. However, at concentrations of 25 µM Se, supplied at the vegetative and florescence stages, both selenite and mixture treatments resulted in a significant decrease of sulfur levels in roots. Regarding sulfur accumulation in shoots, selenate treatment caused a significant increase in all the conditions considered. Oppositely, selenite did not cause any modification in S levels, while the mixture treatment only led to an enhanced S accumulation in plants treated with 25 µM Se at the vegetative stage. None of the conditions assayed changed significantly the amountof S content in grains.

Phosphorous concentration decreased with 10 µM Se exposure at the vegetative stage. However, the addition of 25 µM selenate at the florescence stage significantly increased P in roots, and a similar behavior was seen with the addition of the mixture of both Se species. Oppositely, P decreased significantly in the shoots of plants treated with 25 µM of selenate both at the vegetative and florescence stage. Similarly, P concentration decreased significantly in the grains treated with 25 µM of selenate at florescence time, showing an interconnected behavior, in which this condition is affecting the roots, the shoots and also the grain concentration.

Potassium concentration in roots significantly decreased with selenite application at 10 µM at vegetative stage but did not change with any Se application at the florescence stage. Regardless of its speciation, a selenium concentration of 25 µM significantly decreased K concentration in roots at vegetative stage, whereas a significant effect was noticed only upon selenate and mixture treatment in florescence stage. K concentration in shoots was not significantly modified by Se application with respect to the control. However, K concentration in grains was significantly higher for selenite application at 10 µM at vegetative stage.

Calcium accumulation was enhanced significantly in roots upon addition of 25 µM selenate and mixture at florescence stage. In all the other treatments, Ca in roots did not significantly change. In shoots, Ca concentration slightly increased upon selenium addition, regardless of the speciation, but the amount in grain did not significantly change in any case.

Magnesium concentration in roots significantly increased for 10 µM selenite application at the florescence stage, and significantly decreased with 25 µM selenate at the vegetative stage. On the other hand, magnesium on shoots and grain was not affected by selenium treatment.

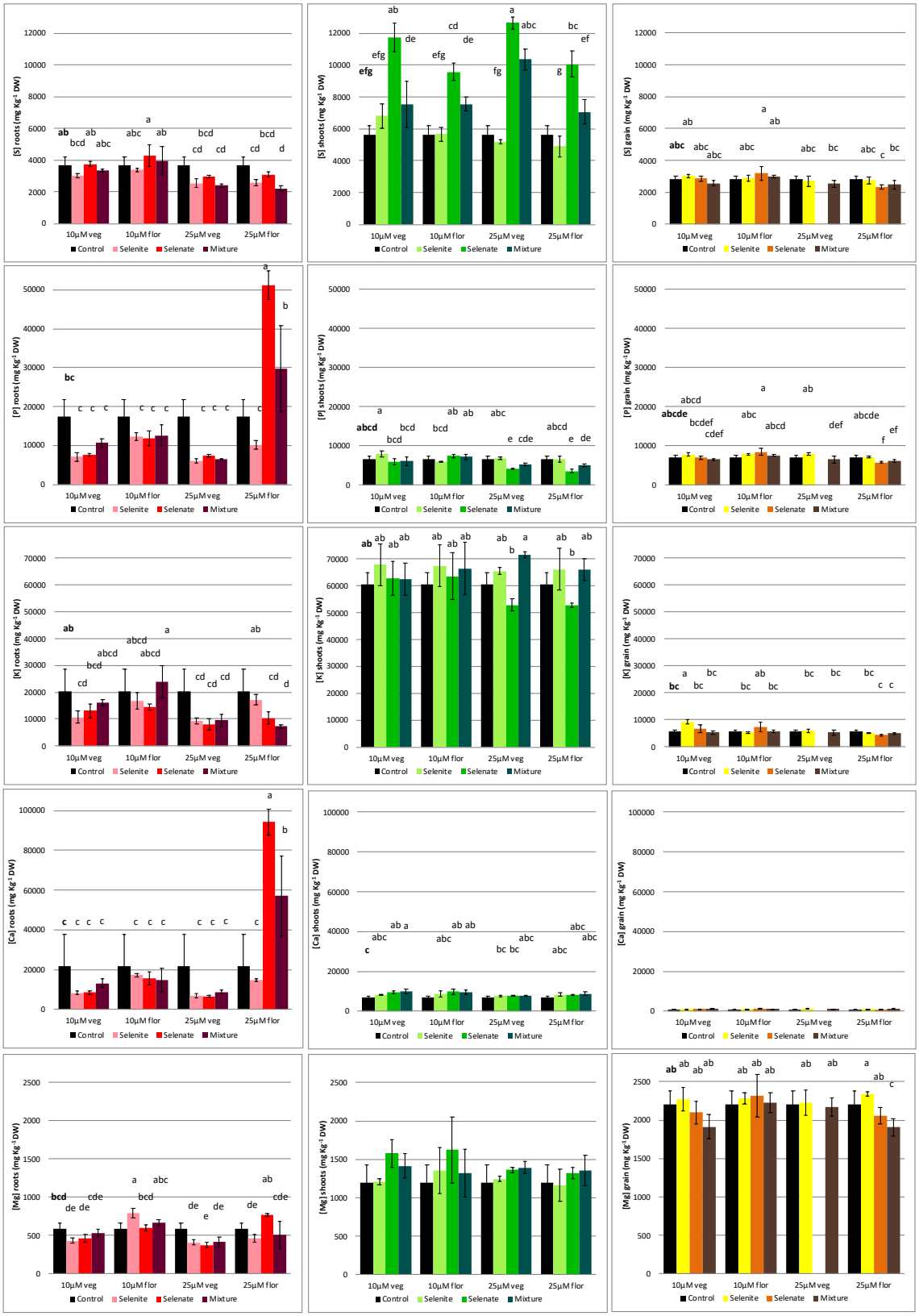


Figure 25: Macronutrient (S, P, K, Ca and Mg) concentration in roots, shoots and grain after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as mg Se/Kg DW, represented as mean±SD (n=4). Letters indicates significance (p<0.05) between different treatments, in case they exist.*

Iron accumulation in roots tended to increase with selenium treatment, especially upon application of 25 µM selenate during vegetative stage. However, any selenium application did not change significantly the iron concentration in shoots and grains respect to that of the control plants.

Manganese in roots increased significantly with the application of 25 µM selenate and mixture at florescence stage but did not change in other conditions. Mn concentration in shoots increased significantly upon addition of 25 µM selenate at vegetative stage, while Mn concentration in grains did not change with respect to control plants.

Copper accumulation in roots increased upon application of 10 µM Se at vegetative stage, regardless of the species, but significantly increased at florescence stage only upon addition of selenite. At 25 µM Se, only the addition of selenite at vegetative stage increased Cu concentrations. Besides, the selenite enhancement of Cu accumulation was significantly higher for 10 µM than for 25 µM. Oppositely, selenium application did not produce any significant change in Cu levels in shoots and grain compared to control plants.

Molybdenum concentration in roots increased with Se application, particularly in the form of selenate. Application of 25 µM selenate at vegetative stage resulted in the highest increase. Mo in shoots increased significantly with selenate treatment, regardless of the concentrations and application times, with 25 µM at vegetative stage resulting in the most significant increment. Mixture treatment at 25 µM at vegetative stage also enhanced Mo accumulation in shoots. In contrast, Mo concentration in grains did not change in any condition.

Zinc concentration in roots increased significantly with application of 10 µM selenite or 25 µM selenate at florescence stage. Zinc accumulation in shoots was enhanced by 25 µM of selenate or mixture treatments applied at the vegetative stage. Zinc concentration in grains was not changed by any of the selected conditions.

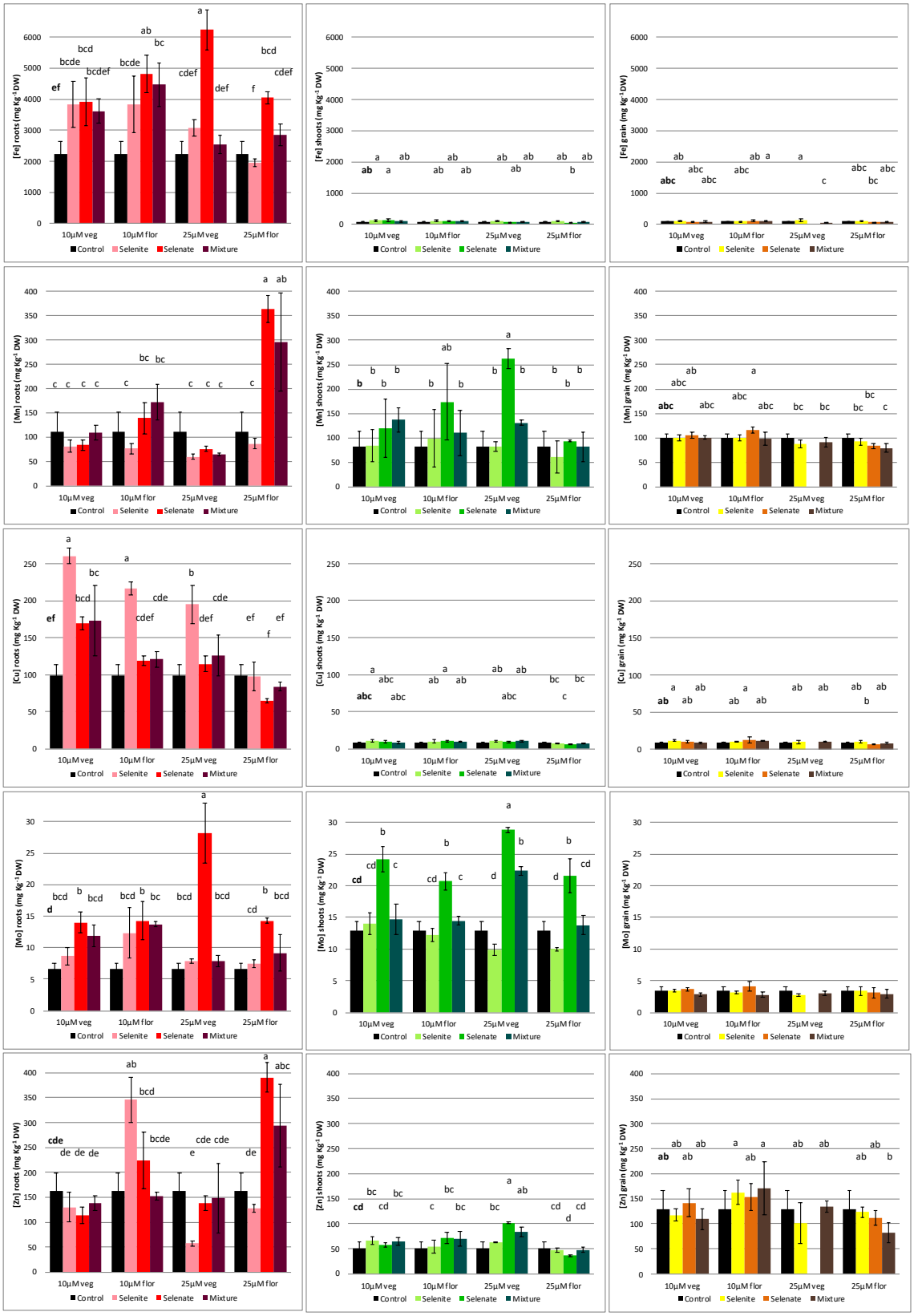


Figure 26: Micronutrient (Fe, Mn, Cu, Mo and Zn) concentration in roots, shoots and grain after selenium treatment (10 and 25 µM, at vegetative or florescence stages) *as mg Se/Kg DW, represented as mean±SD (n=4). Letters indicates significance (p<0.05) between different treatments, in case they exist.*

Although no general trends can be established, selenate produced the strongest effect on nutrient content, causing a relevant enhancement of the concentration of S, Mo and Mn in shoots and P, Fe, Ca and Mg in roots, among others.

Selenium species are taken up, translocated and metabolized within the plant by the sulfur and phosphorus transporters and metabolic pathways. Therefore, the biofortification with selenium was expected to modify the content of these two elements.

However, no known metabolic pathways are shared between selenium and the other nutrients. Therefore, the reason for the modification of their content in the plant may not be due to active processes, but due to indirect interactions, induced toxicity and alteration of the plant homeostasis.

Interestingly, the effects of selenium exposure to the nutrient content in grains were less evident than in other tissues of the plant i.e. roots and shoots. This is due to the translocation of nutrients to grain being the principal process during the stages of kernel ripening in the plant senescence. Therefore, mobilization of nutrients from other plant organs to the grains will be enhanced even in cases of shortage.

Consequently, this result is of a special relevance for the process of Se biofortification of wheat, which has been shown to not modify the nutritional value of the produced grain.

Conclusions

The present work investigated whether selenium biofortification affects wheat yield and the nutrient composition of the produced grain. Those two parameters are of paramount importance to establish an economically-viable biofortification strategy to produce an enriched grain that will contribute to enhance health status of the population.

Moreover, the determination of the selenium distribution in plant tissues (roots, shoot and grain) is of a particular importance in order to understand the process of uptake, translocation and metabolization of selenium.

Grain production is the pivotal factor of wheat crop culture, since is the edible part for humans. It has been shown that selenium biofortification of wheat can effectively increase selenium content in grains, regardless of the selenium species, concentration or the plant stage at Se application.

However, the application of 25 µM selenate at vegetative stage resulted in selenium accumulation in wheat shoots above their toxicity threshold, hampering grain formation. Furthermore, selenite application, at the same concentration and time, also

resulted in excessive selenium accumulation in roots and induced toxicity, which resulted in a significantly reduced grain weight.

On the other hand, the application of a mixture of the two inorganic selenium salts generally led to a more distributed selenium concentration in wheat tissues, with accumulation favored in both root and shoots. Coherently, the accumulation in an individual tissue was lowered, thus reducing the resulting toxicity, and allowing the formation of grain with less affectation.

Furthermore, the application of selenium at a florescence stage, and thus for a shorter period of time, produced a minimum effect on plant development and yield, whereas the application at vegetative stage caused phytotoxicity in the sensitive wheat seedlings. However, both applications resulted in similar accumulations in roots, shoots and grain. Therefore, Se application directly at florescence was equally effective than at vegetative stage. Selenium metabolization and translocation are fast processes and thus, the Se addition when the process of grain nutrient filling and ripening takes place is sufficient to achieve successful grain Se enrichment.

Selenium application also caused an alteration of the nutrient content in roots and shoots. However, this effect was less relevant in the grains. Consequently, the biofortification of selenium of wheat plants can be performed without hampering the nutritional value obtained in the grain, and thus of a flour-based foodstuff.

Finally, the selenium accumulation in other plant tissues apart of the grain, such as in shoots, can also serve for other applications than flour-based products, thus minimizing the economic cost of the selenium biofortification and reusing the otherwise biomass waste. For example, selenium enriched wheat straw can serve as cattle feed [70], [71], or can be used as the selenium source for the culture of other Se-enriched foodstuffs, such as se-enriched mushrooms [72].

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3. Conventional speciation

Introduction

The analysis of selenium speciation, including inorganic selenium forms and organic selenium such as seleno-amino acids, is commonly done using a methodology as high-performance liquid chromatography (HPLC) coupled with element-specific detection, such as ICP-MS [1].

Three steps are needed in this conventional speciation analysis: species extraction from the sample matrix, species separation and species detection and quantification [2].

Selenium species are found in a biological matrix, therefore, the first step in the present methodology is an extraction into an aqueous solution, previous to the instrumental determination.

Because the species have to be removed from the sample matrix prior to their determination, the methodology can be considered as “indirect speciation”.

Water soluble fraction of inorganic and low molecular weight organic selenium compounds can be extracted by mild extraction protocols, including water-methanol and low concentrated acids [3]. However, these mild processes generally provide extraction efficiencies of 50% of the total selenium [3]. The use of enzymes or chemicals in order to break the peptide bonds of the proteins, and release the protein-bound amino acids, generally increase extraction efficiency to around 80% [1], [3].

In enzymatic digestions to analyze selenium speciation in biological matrices, proteases such as protease XIV and proteinase K are commonly used, as well as a protease-lipase mixture [4]. Alternatively, methanesulfonic acid provides high extraction efficiencies and cyanogen bromide yields volatile species for gas chromatography analysis [4]. However, the enzyme protease XIV from *S. griseus* is considered the most effective enzymatic extraction procedure [4].

Additionally, the extraction procedure selected should release the species with minimum modifications to its chemical structure. The soft conditions required for enzymatic reactions, 37ºC and pH 7,5, lower the risk of species transformation to a certain extent [5].

On the other hand, and regarding species separation, both ion exchange chromatography and ion-pair reversed-phase chromatography can be employed for this purpose, due to the high polarity of most selenium compounds and the zwitterionic nature of the amino acids [1]. In solution they are found in dipolar form, with both cationic and anionic ends. That facilitates their separation with chromatographic methods.

However, previous experiments by reversed-phase (RP) HPLC-ICP-MS showed that several Se-compounds were not adequately separated under the commonly employed conditions, with most of selenium eluting in the void volume of the C8 and C18 columns [6]. Oppositely, strong anion-exchange (SAX), employed in the tandem HPLC-ICP-MS, has demonstrated to be more adequate for highly polar selenium compounds, and allowing better recoveries of Se from enzymatic extracts [6].

The Hamilton PRP-X100 anion exchange column is robust and stable, with a Poly(styrene-divinylbenzene) with trimethyl-ammonium exchange sites, which has been highly employed in literature for selenium speciation [7], [8].

The pH and the concentration of the mobile phase are the main factors determining the retention of the different selenium compounds in the stationary phase.

Finally, ICP-MS is the preferred detector for trace element analysis due to its high selectivity, sensitivity and precision, even in in complex biological mixtures [9], [10]. However, there are some difficulties in the analysis of selenium speciation: the low ionization of selenium in argon plasma (around 33%) due to its high first ionization energy (9,75 eV) [9] and the polyatomic interferences such as $ArAr^+$ or $CaAr^+$ [10]. The most abundant isotope ^{80}Se has a high interference from $^{40}Ar^{40}Ar^+$ dimer. The use of Collision Cell technologies with H_2/He mixtures can reduce polyatomic interferences, but they can give rise to other induced interferences, such as hydride formation [9]. For all these reason, ^{78}Se is the recommended isotope to monitor, with the best abundance vs. interferences ratios, and detection limits as low as 14-30 ng/L can be achieved under improved HPLC-ICP-MS conditions [9].

Objectives

The main objective of this work is to quantify the selenium speciation in different parts of wheat plants.

In order to achieve this objective, the following tasks will be accomplished:

- ✓ To optimize the chromatographic methodology for selenium speciation in plants in order to achieve optimum separation of selenium standards.
- ✓ To determine the stability of selenium species with the chosen method.
- ✓ To test different reducing agents to minimize seleno-amino acid oxidation.
- ✓ To validate the methodology with the use of a selenium certificate material.
- ✓ To measure the extraction efficiency of selenium with the selected methodology in roots, shoots and grain of wheat.
- ✓ To Compare the achieved speciation in mature plants enriched with either selenite, selenate or a mixture of the two species

Materials and Methods

Sample preparation:

Plants of common wheat (*Triticum aestivum* cv. Pinzón purchased from Semillas Fitó S.A.) were grown in hydroponic culture, in the conditions detailed in chapter 2.

After germination and two weeks of preculture, at the vegetative stage plants were exposed to selenium treatments: Control, 10 µM Na_2SeO_3, 10 µM Na_2SeO_4 and a mixture of 5 µM Na_2SeO_3 and 5 µM Na_2SeO_4. The nutrient solution containing the treatment was renewed weekly.

At harvest, roots where washed with ice-cold $CaCl_2$ solution and rinsed with deionized water. Afterwards, plant material separated into roots, shoots and grain.

Samples were dried in an oven at 45^oC during four days until stable weight. Then, plant material was ground with an automatic mortar, and stored in a dry atmosphere until analysis.

Sample pretreatment:

Selenium species were extracted by means of an enzymatic digestion, using protease XIV in order to break the long protein chains: 50 mg of plant material were treated with 10 mg of protease and 5 ml of $NH_4H_2PO_4$. The solution of ammonium dihydrogen phosphate was previously degassed by sonication and nitrogen bubbling in order to remove the dissolved oxygen. The flask was sealed and placed in an incubator for 16 hours at 37^oC, in darkness and with continuous stirring.

Immediately after the 16 hours the samples were cooled down with an ice bath and filtered. They were maintained at 4^oC and analyzed within the day.

On the other hand, sample enzymatic digestion was also performed in the presence of reducing agents. 2-mercaptoethanol, dithiotreitol and tris(2-carboxylethyl)phosphine were separately tested by adding them in the incubation mixture.

In addition dithiotreitol reduction was combined with a derivatization with iodoacetamide.

Sample analysis:

Selenium speciation was analyzed with the tamdem of high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS).

A PRP-X100 (Hamilton) strong anion exchange column, 250x4.1 mm, with a stationary phase of 10µm particle diameter, was used for analysis at 20ºC of 100 µl of sample, injected with a HPLC Spectra system (pump P4000 and autosampler AS3000, Thermo Thermo Fisher Scientific)

The chosen mobile phase was ammonium citrate + 2% MeOH at pH 5,0. A gradient elution was performed, changing the mobile phase concentration and flux as reported in table 1.

The detection of ^{78}Se elution with time was done by ICP-MS (X-series 2, Thermo Fisher Scientific). ^{115}In was used as an internal standard, and peak identification and quantification was performed by external calibration and spikes with commercial standards of sodium selenite, sodium selenate, selenomethionine, methyl-seleno-cysteine hydrochloride and selenocystine.

Additionally, these standards were digested in the same manner as samples in order to determine the stability of the species.

A standard for selenomethionine oxide has been prepared by selenomethionine oxidation with 30 µL of H_2O_2. Although precautions were taken to ensure selenomethionine oxidation is minimized, the formation of this species needs to be considered on the selenium analysis by HPLC-ICP-MS.

PART I: Method optimization

Results and discussion

The quantification of the selenium speciation in the parts of wheat plants required a preliminary optimization of the procedure for the species extraction and determination, which will be detailed in the following sections.

Optimization of the mobile phase

10mM ammonium Citrate at pH 5,0 is a commonly used mobile phase for the separation of selenium compounds with a PRP-X100 anion exchange column. A 2% of methanol is added to improve sensitivity and enhance signal response [7], [9].

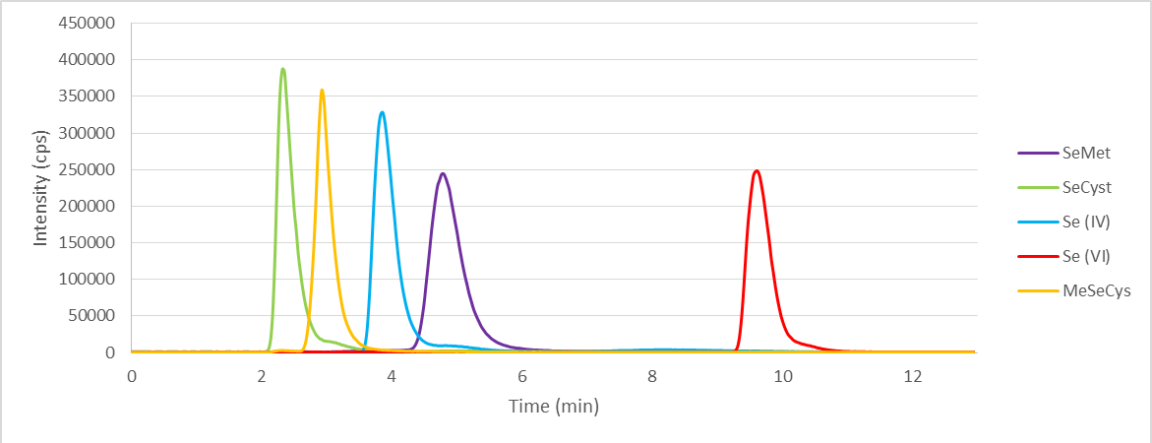

Figure 1: Chromatogram with the retention times of the standard species, analyzed individualy, using the optimized gradient method of table 1.

The elution order was shown to be, in the conditions used, the following: Selenocystine, methylselenocysteine, selenite, selenomethionine and selenate.

<u>Influence of pH:</u>

Different variations in pH and concentration of the mobile phase were tested in order to optimize the separation of a mixture of 5 commercially available selenium standard compounds from the widely reported isocratic gradient with 10mM ammonium citrate at pH5,0 with 2% of MeOH.

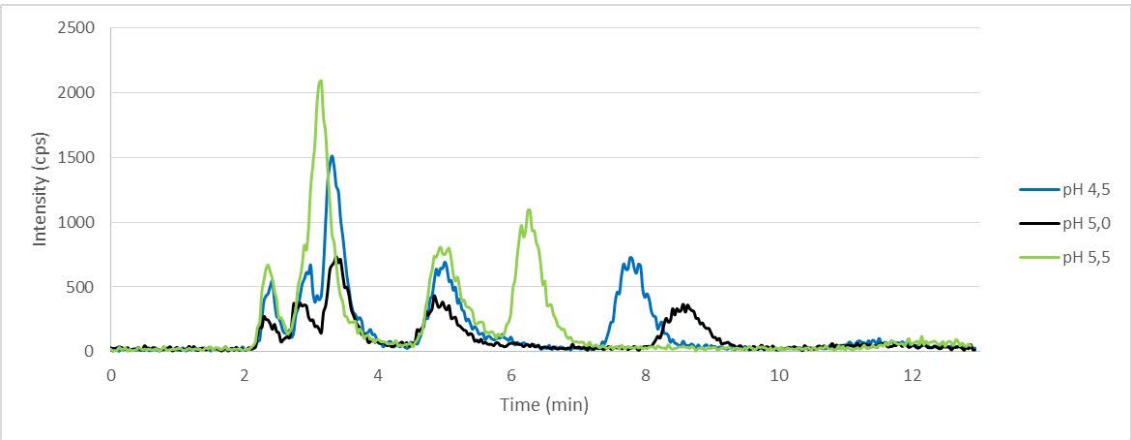

Figure 2: Chromatogram depicting the elution of a mixture of the 5 standards at three different pH values (4,5, 5,0 and 5,5) with a mobile phase of 10mM of ammonium citrate.

The use of pH 4,5 and pH 5,5 did not improve the separation of the 5 species, but oppositely, they showed bigger peak overlapping than at pH of 5,0, which is the most common mobile phase used in literature. The reason for this was a modification of the retention times of, mainly, the inorganic selenium species, due to their diverse pKa values.

The pKa values for Selenite are 2,70 and 8,54 and for selenate -2,01 and 1,8 [11]. Accordingly, in the mobile phase at pH 5,0 these species will be encountered as hydrogen selenite $HSeO_3^-$ and fully deprotonated selenate, SeO_4^{2-}. Thus, selenite elutes earlier in the chromatogram than selenate, the latter having higher charge.

The seleno amino acids have pKa values of 2,1-2,6 in the carboxylic group and 8,9 in the amino group [12]–[14]. Due to their zwitterionic nature, the amino acids in the mobile phase at pH 5,0 will be encountered both positively and negatively charged. Their isoelectric point, where charges are in equilibrium, is the average between the two pKa, approximately at pH 5,6. Selenocysteine, the reduced form of selenocystine, would have a selenol group with a pKa of 5,24 [15].

In addition, important interactions between the selenium atom in the amino acid and the stationary phase are occurring. Consequently, selenocystine and selenomethionine selenoxide (the oxidized form of selenomethionine) that have the selenium atom hindered from the anion exchange, are less retained and coelute close to the chromatographic void volume [16], [17].

Because of the proximity of the isoelectric point of the amino acids to the pH of the mobile phase, modifications in the chosen pH have a limited effect on the retention times of the amino acids. Oppositely, the two inorganic forms are the most affected due to pH changes.

From this point on, in the rest of the study a pH of 5,0 was always used for the mobile phase.

Influence of concentration:

Regarding the concentration of ammonium citrate in the mobile phase, isocratic elution with 5mM and 10mM were tested, in order to improve the resolution of the species in the chromatogram.

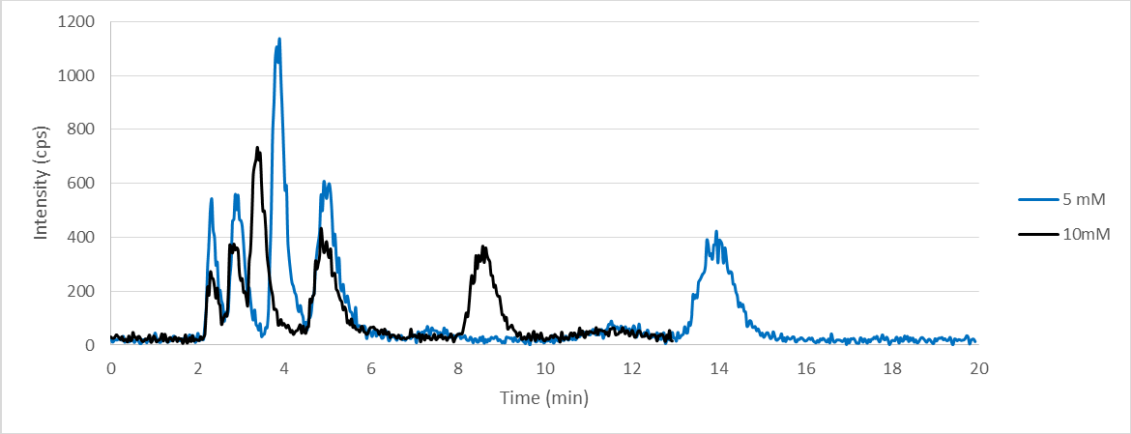

Figure 3: Chromatogram depicting the elution of a mixture of the 5 standards at 5mM and 10mM concentration of ammonium citrate with 2% of methanol.

Results showed that lower concentrations resulted in longer retention times and better peak separation, and higher concentrations in shorter retention times and peak overlapping. The 5mM mobile phase resolved better the peaks of SeCyst, MeSeCys, SeMet and Selenite than the usually employed 10mM mobile phase. But, despite the improved elucidation of these species, Selenate eluted after 13-15 min which would significantly increase the analysis time.

For this reason, a gradient elution was optimized with a progression from 5mM to 15mM ammonium citrate, as found in Table 1. The resulting chromatogram for the mixture of the 5 selenium compounds is found in Figure 4, matching the retention times of the compounds injected individually that can be seen in Figure 1.

Table 1: Method of elution used with a gradient from mobile phase A (5mM of ammonium citrate at pH5,0 with 2% of MeOH) and mobile phase B (15mM of ammonium citrate at pH5,0 with 2% of MeOH), with a flow gradient from 1,0 to 1,2 ml/min.

Time (min)	MP A: 5mM (%)	MP B: 15mM (%)	Flow (ml/min)
0	100	0	1
4,5	100	0	1
5,5	0	100	1
6,5	0	100	1.2
10	0	100	1.2
11	100	0	1.2
12	100	0	1

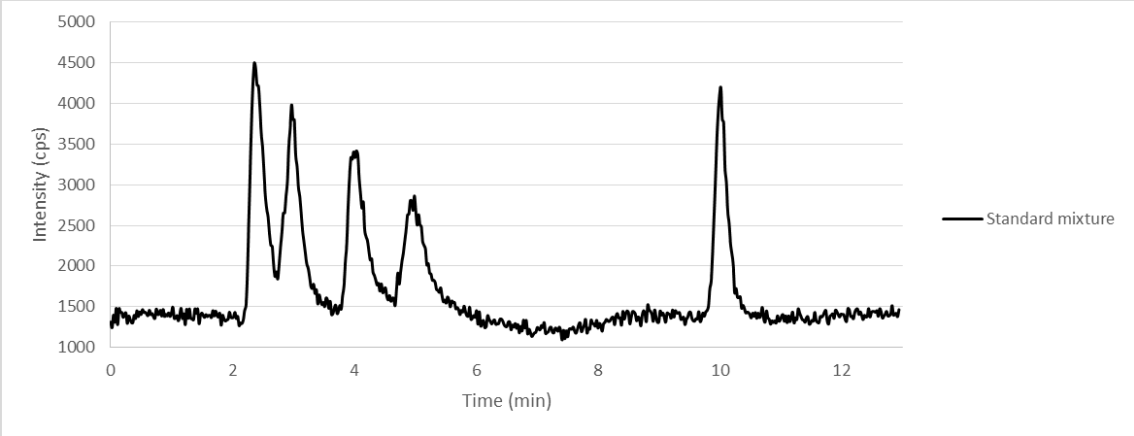

Figure 4: Chromatogram of a mixture of the 5 selenium standards at a concentration of 5 µg Se/L each, with the elution method of table 1.

The 5 selenium compounds eluted well resolved and good separation was achieved in less than 12 minutes. After this time, two minutes of stabilization time with 100% of 5mM mobile phase were granted before the start of a subsequent sample, giving a total analysis time of 14 minutes per sample.

Sensitivity for the detection of selenium species

Selenium species were determined by means of external calibration of the undigested standards.

After a preliminary study of the samples, it was determined that SeCyst, MeSeCys and Selenite were present at lower concentrations than SeMet and Selenate in the samples.

For this reason, calibration curves were prepared from 5 µg/L to 250 µg/L of Se for SeCyst and MeSeCys, from 5 µg/L to 500 µg/L of Se for selenite and from 5 µg/L to 1200 µg/L of Se for SeMet and selenate.

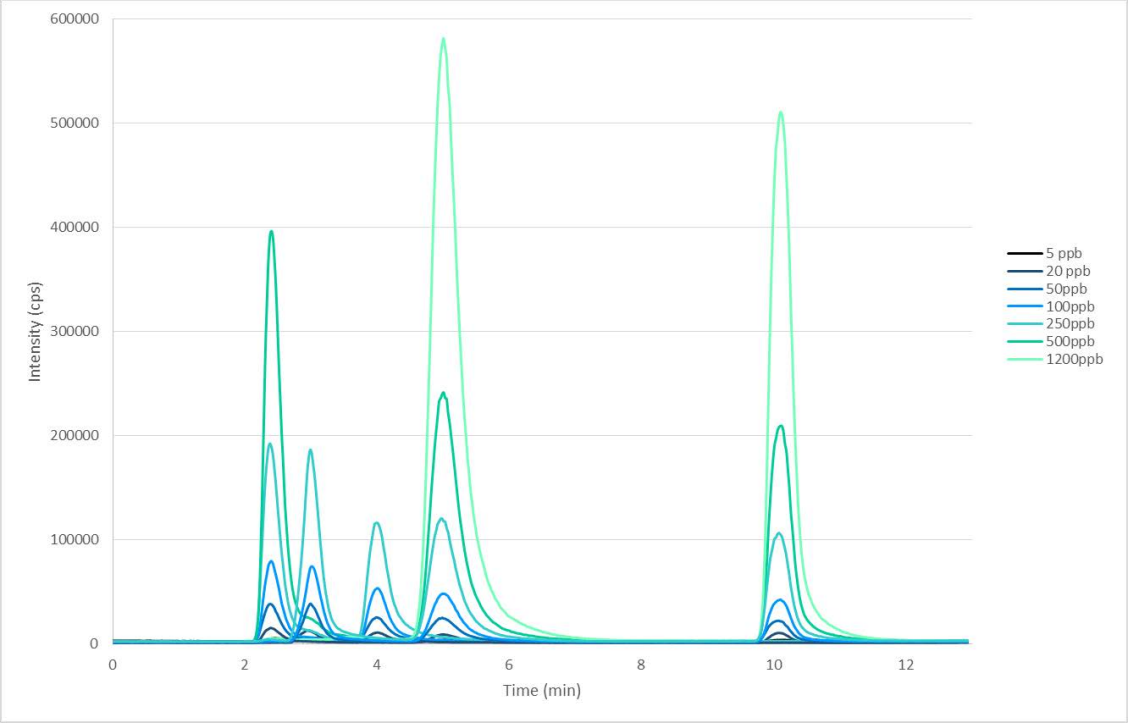

Figure 5: Calibration chromatograms using mixtures of related standard compounds.

Furthermore, slightly different sensitivities were found for each of the selenium species. It is known that species that elute earlier in the chromatogram result in narrower peaks and therefore, higher sensitivities [18]. Moreover, it is also possible that the ionization of selenium in the plasma varies to a certain extent depending on the nature of the species i.e. organic or inorganic selenium.

Repeatability of the analysis in the same day was excellent, and the standards remained stable up to 12 hours. However, for longer periods, storage of the selenium standard solutions in the fridge at 4ºC is strongly recommended to avoid further degradation or oxidation of the species.

Limits of quantification (LOQ) were calculated as $LOQ = \frac{10s_y}{b}$, where S_y is the error of the interpolation in the regression line and b is the slope [19]. In this case, the limit of quantification is the lowest concentration of the selenium species in the extract that can be quantified unequivocally. This value is in agreement with the calculated signal to noise ratio from several repetitions of the 5ppb standards in Figure 4. All standards give an approximate S/N of 10:1 [19].

Table 2: Signal to noise ratio and limits of quantifications for repeated measurements of standards of 5 µg/L of Se.

	S/N of 5ppb	LOQ with s_y (µg Se/L)
SeCyst	12,9	14,4
MeSeCys	12,1	11,2
Se (IV)	8,5	11,6
SeMet	6,6	22,7
Se (VI)	9,5	16,5

Evaluation of the extraction methodology with the use of a certificate reference material

SELM-1 is a certificate reference material (CRM) of a yeast enriched with selenium, which content and speciation of selenium are certified [20], [21].

It has a reported total Se concentration of 2031 ± 70 mg Se/kg in the form of selenomethionine. SELM-1 was analyzed by means of an acid digestion in order to determine the total selenium. The result obtained was not statistically different to the certified value.

Consequently, the obtained value was used to calculate the extraction efficiency for the Selenium compounds after the enzymatic digestion of the sample. The extraction efficiency is defined as the difference in percentage between the element concentration in the extracts and the total content in the sample [1].

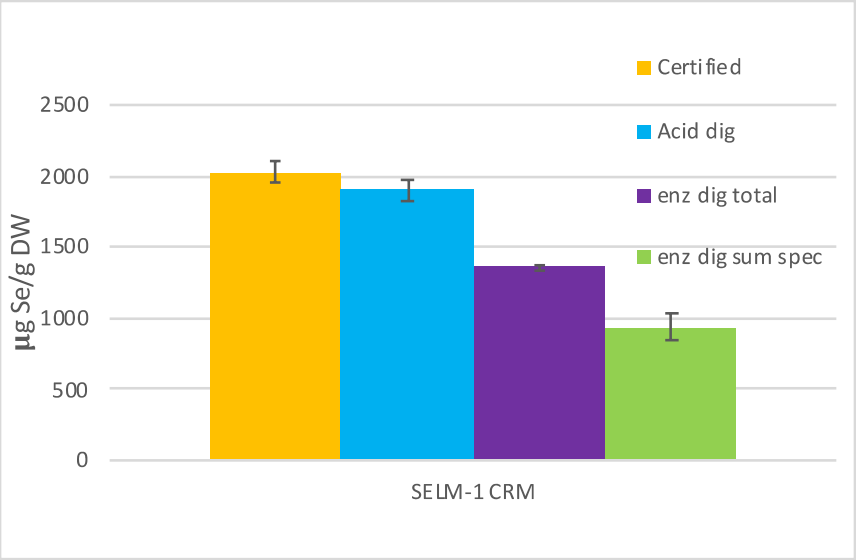

Figure 6: Selenium in selenium enriched yeast SELM-1 certified reference material. Certified value (yellow), total selenium after acid digestion by ICP-MS (blue) with n=8, total selenium after enzymatic digestion by ICP-MS (purple) with n=4, sum of all selenium species after the chromatography by HPLC-ICP-MS (green). Results shown as mean±SD.

The employed methodology achieved an extraction efficiency from SELM-1 of 71,3 ± 0,9%. This result is not far from the general 80% extraction efficiency for enzymatic extractions, previously discussed. Nonetheless, extraction efficiency for selenomethionine in yeast have been reported specifically in other works, with values of typical extraction recoveries of 70-90% [2]. In the same way, it has been reported that extraction procedures may fail to extract all the selenium from the yeast sample, and a 3–11% can remain in the solid residue after proteolysis [4]. However, three consecutive proteolitic digestions of the selenized yeast were able to achieve extraction yields up to 90–98% of selenium [4].

On the other hand, chromatographic recovery is defined as the difference in percentage between the amount quantified after the chromatography and the concentration present in the extract.

The analyzed CRM sample provided a 73 ± 3% chromatographic recovery.

Apart from the loose of recovery from incomplete releases from the solid phase to the aqueous phase, two other factors can explain the observed decrease in recoveries with HPLC-ICP-MS. Incomplete digestion may cause the solubilizing of peptides instead of amino acids and additionally, selenomethionine can interact unspecifically with other sample components and elute as a continuum throughout the chromatogram [4].

Previous studies have reported a 75±4% recovery of selenium in the proteolytic extract of selenized yeast after quantification with HPLC-ICP-MS [4]. However, the collection

and analysis of total selenium in the column effluent provided a recovery of 103±4%, indicating that the selenium in the sample is eluting from the column but approximately a 25% of it does not produce a well-defined peak [4], due to the reasons previously discussed. By combination of various chromatographic techniques, this study also demonstrated that a 15% of the undetected selenium is in the form of unidentified peptides that have failed to achieve complete digestion [4], with some of these peptides being identified as selenium compounds containing glutathione, selenogluthathione and selenocysteine [6].

Verification of the methodology and stability of Selenium species in a certificate reference material.

The reference material SELM-1 was enzymatically digested and analyzed in order to verify the methodology. Figure 7 shows an example of the related chromatogram obtained. The results from the quantification of the areas of the peaks with the calibration curve provided the results in table 3.

A main peak of selenomethionine was observed, accounting for a 91±3% of the total selenium species recovered in the sample.

Other minority peaks were also detected, coming from either an oxidation of selenomethionine or an incomplete digestion. The most important of those peaks is the one appearing at a retention time of 2-3 minutes, which accounts for a 4±2% of the selenium in the sample.

A similar chromatogram was obtained when the standard of pure selenomethionine was left at room temperature for 6 days, when an oxidation of around 15±13% was detected (with n=4).

This peak matches the void volume of the column, where it also coelutes selenocystine [16], [17], making it impossible to accurately quantify these two species in the cases were both species are present in the samples.

Further analyses were performed with selenomethionine standard in order to identify the peak. Forced oxidation of selenomethionine was performed with 30 µL of H_2O_2 to completely oxidize this compound, as seen in Figure 8. It produced a species with a peak maximum at 2,35 min, matching the smaller peak of Figure 7.

Identification of the structure was previously done in literature, where it was reported that the oxidation with hydrogen peroxide yielded selenomethionine selenoxide [22], [23]. Further oxidation can also be achieved with 1000 µL of H_2O_2 and 6 days of reaction time, which yields as well selenomethionine selenone [1], which also elutes before selenomethionine, as can be seen in Figure 9.

Thorough care is required in order to prevent oxidation of selenomethionine, and thus avoid errors in quantification. The buffer used in the enzymatic extraction was

previously degassed with sonication and saturated with nitrogen using a bubbling nitrogen flow in order to remove the oxygen dissolved in the solution. After sample preparation, the flask was sealed to avoid further contact with oxygen, and the enzymatic extraction was performed in darkness for 16h at 37ºC. Afterwards, samples were placed in an ice bath in order to prevent further reactions and were maintained at a temperature of 4ºC until analysis in the same day.

Tests performed with the SELM-1 extracts showed than even after 2h at room temperature the area of the peak of selenomethione selenoxide started to increase, but it remained stable at 4ºC for one day.

Table 3: Quantification of selenium species in the reference material SELM-1 as mean±SD with n=4

	Concentration (µg Se/g DW)	Percentage (%)
SeCyst	41±24	4±2
MeSeCys	9±4	1,0±0,5
Se (IV)	13±7	1,3±0,7
SeMet	849±71	90±3
Se (VI)	8±4	0,9±0,4
sum of unknown	19±12	1,9±1,1

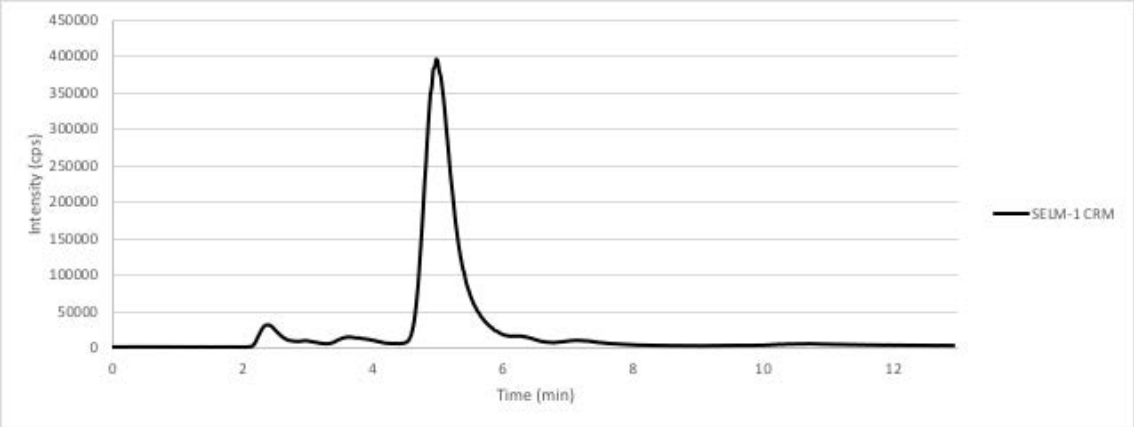

Figure 7: Chromatogram of the reference material SELM-1 using the gradient method in table 2.

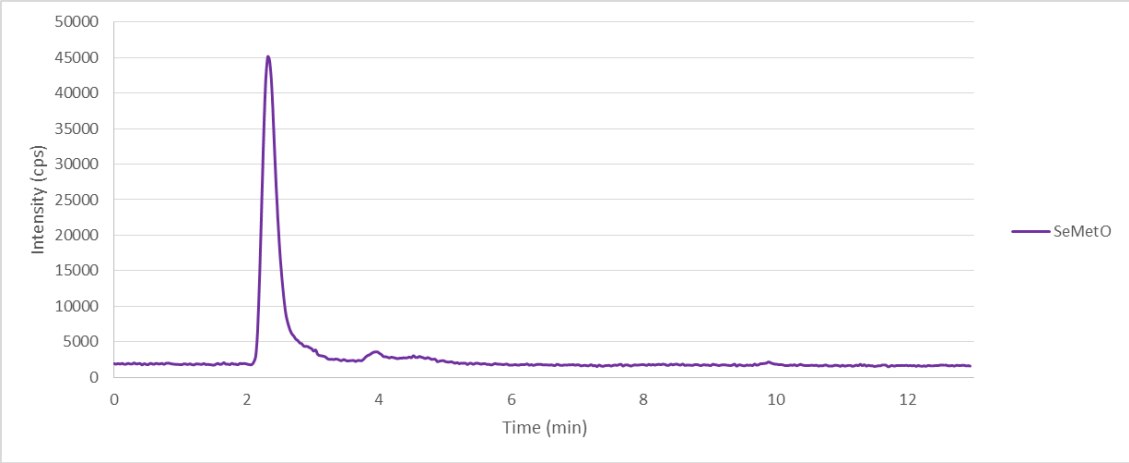

Figure 8: Chromatogram selenomethionine selenoxide, prepared with commercial selenomethionine and 30 µL of hydrogen peroxide.

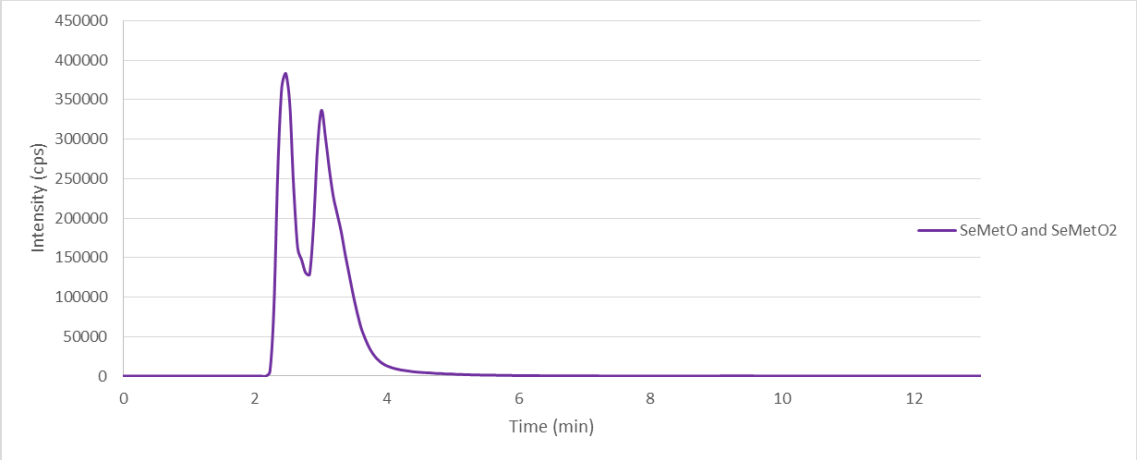

Figure 9: Chromatogram showing selenomethionine selenoxide and selenomethionine selenone, prepared with commercial selenomethionine and 1000 µL of hydrogen peroxide, after 6 days of reaction time.

Use of reducing agents in order to minimize oxidation in speciation analysis

The studies performed in the SELM-1 CRM proved oxidation of selenomethionine a possible source of inaccuracies in the determination of the speciation [17], [24]. The same phenomenon is observed as well in the preliminary investigations performed on wheat samples.

For this reason, the usage of a reducing agent was proposed as a method to prevent oxidation and preserve the integrity of the selenium species.

Three different reducing agents were tested: Dithiothreitol (DTT), 2-mercaptoethanol (BME) and tris(2-carboxyethyl)phosphine (TCEP). Moreover, they were added to the

sample before the enzymatic extraction in order to prevent oxidation during all the procedure. Results will be detailed in the following sections.

Use of 2-mercaptoethanol on the determination process of selenium compounds

2-mercaptoethanol, also known as β-mercaptoethanol (BME) is a known reducing agent with a thiol group.

As a reducing agent, it has been used to prevent oxidation of selenium species such as selenomethionine, in order to maintain its stability and ensure unambiguous quantification [4], [17], [25].

However, disulfides and diselenides are reduced as well to their thiol and selenol forms [26], following the reaction in Figure 10.

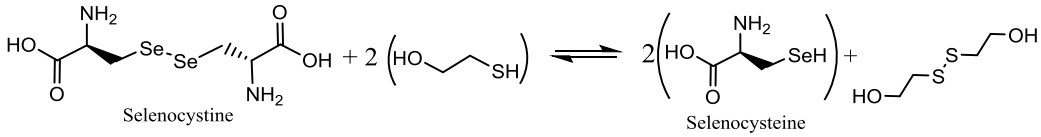

Figure 10: Reduction reaction of selenocystine with 2-mercaptoethanol

The result of the enzymatic digestion of the standards of the selenium compounds with BME is shown in Figure 11.

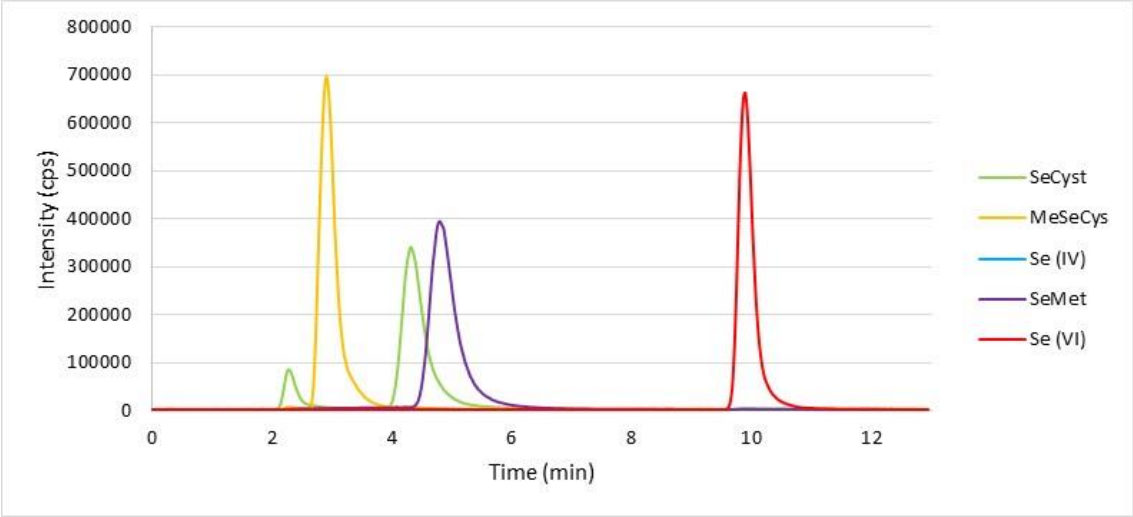

Figure 11: Chromatograms depicting the retention times of the standard compounds, injected individually, after enzymatic extraction with BME.

2-mercaptoethanol has a reduction potential of -0,207 V at pH 7,0 [27], which is comparable with that of other thiols such as glutathione of -0,205 V at pH 7,0 [27]

(although other authors have reported -0,16 V as the reduction potential of glutathione [28]). In contrast, redox potentials of the peptides are calculated to be the following: a SeCys-SeCys peptide showed a redox potential of -0,381 V, a SeCys-Cys peptide -0,326 V, and Cys-Cys peptide -0,180 V [27]. Accordingly, diselenides are theoretically more stable against reduction than disulfides and mixed Se-S peptides under ambient conditions [27], [29]. However, the stronger acidity and nucleophilicity of selenols, makes the selenol/diselenide exchange reactions occur 10^7 times faster than thiol/disulfide exchange reactions [30].

For these reasons, the reduction of selenocystine with BME is only partial, as reported for other monothiols such as glutathione [27]. The extent in which the reaction takes place depends on the amount of BME used. However, an excess of BME did not achieve complete reaction, even with molar ratios from 4:1 BME:Se to 25000:1 BME:Se. In the same manner, the use of very little BME, such as 0,5:1 BME:Se molar ratio did not suppress the formation of the reaction product, and proved to be not sufficient to prevent SeMet oxidation.

The second peak in the SeCyst chromatogram could be the reduced form selenocysteine (SeCys). Due to the pKa of SeCys of 5,24 [15], at pH 5,0 (pH of the mobile phase), this species will be in equilibrium between protonated and deprotonated forms. Additionally, since selenocysteine is highly reactive and the oxidation of BME is reversible, further reactions can occur between selenocysteine and BME, forming a SeCys-BME complex. The same effect has been seen with thiolate-disulfide exchanges [31].

SeMet, MeSeCys and Se (VI) did not show any change of retention time or any byproduct after treatment with BME.

Oppositely, selenite standard after digestion with BME did not appear in the chromatogram of Figure 11. Most probably, selenite is reduced with BME. At the working pH, selenite has the following reduction potential:

$HSeO_3^- + 5H^+ + 4e^- \rightarrow Se\ (s) + 3H_2O$ 0,780 V [11]

Further reduction of elemental selenium:

$Se\ (s) + H^+ + 2e^- \rightarrow HSe^-$ -0.227 V [11]

Therefore, reduction of selenite to elemental selenium with BME is very favorable. Further reduction to selenide could occur but is not favored according to their redox potential values.

In agreement, the chromatographic analyses of samples treated with BME resulted in a gradually but marked increase of pressure in the system, that could be due to the accumulation of little amounts of Se (0) in the precolumn. The replacement of the precolumn returned pressure values to the initial, and so, the use of a precolumn was essential to protect the column, and maintain the system.

The analyses of wheat samples are shown in Figures 12 and 13. The product of the reaction of selenocystine resulted in a peak eluting very close to selenomethionine, and thus not well resolved, making it difficult to quantificate properly the peaks. Mathematical approaches could be proposed as a way to quantify the two overlapping peaks, such as the fit of the curve of the chromatographic peak to a function such as a modified Gaussian function [32], or a multivariate calibration with artificial neural networks [33], [34]. Oppositely, MeSeCys, is very well resolved and can be adequately quantified, as well as selenate. Additionally, an increase of the area of SeMet with the use of BME is also observed in Figure 13, which is an indication of the hindrance of its reduction by the use of BME.

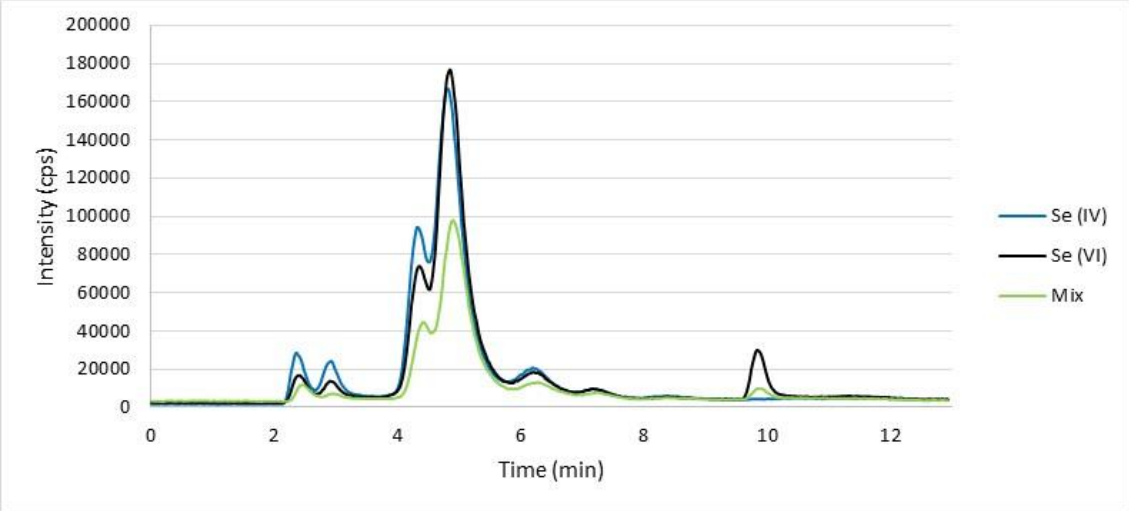

Figure 12: Chromatogram depicting wheat grain samples enriched with selenite, selenate and mixture of both species. The enzymatic extraction was performed in presence of BME. SeCyst show produced a second peak at a retention time of 4-5 min.

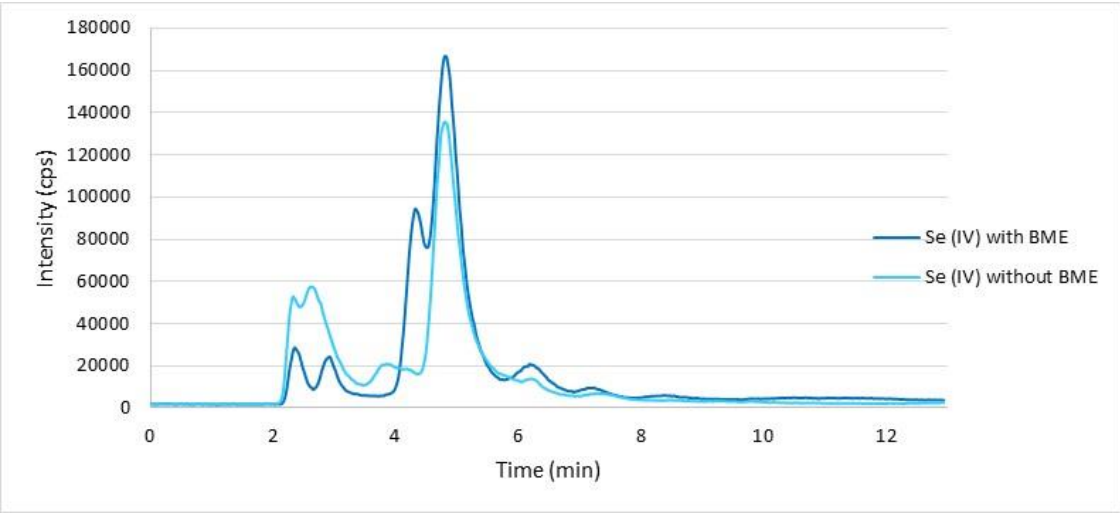

Figure 13: Chromatogram of a selenite-enriched wheat grain sample digested with and without the presence of 2-mercaptoethanol. The differences in the result depending on the two treatments are clear.

Use of Dithiothreitol on the determination process of selenium compounds

Dithiothreitol is a reducing agent that contains two thiol groups. Its oxidized form contains a disulfide bond between the two thiols, forming a ring [27]. DTT is easily oxidized in solution, but this reaction is reversible [35].

DTT is a strong reducing agent with a reported reduction potential of -0,33 V [28], [35], [36], although also -0,323 V at pH 7,0 and -0,366 V at pH 8,1 have been determined [27].

As seen in Figure 15, and in agreement with previously reported data, dithiothreitol successfully prevents the oxidation of selenomethionine [17].

However, DTT also reduces Se-Se, Se-S and S-S bonds [35], [37], thus leading to the formation of thiols and selenols. In contrast to monothiols such as BME, dithiol compounds such as DDT are able to quantitatively reduce diselenides, as long as the dithiol is in excess [27].

Selenols are unstable and reactive. Therefore, selenocystine, which is no longer protected by the Se-Se bond, can be oxidized to selenoxide and further react through a syn-b-elimination of selenenic acid [18]. A derivatization reaction with iodoacetamide (IAM) is usually combined with DTT reduction in order to protect the formed selenocysteine [18]. This protocol has been applied in selenium enzymatic extractions to a variety of biological samples [37]–[39].

The reaction occurring is seen in Figure 14. The product obtained is carboxyamidomethyl-selenocysteine (CAM-SeCys), which is chemically stable in acidic and neutral media [40]. The reaction yield for the derivatization was more than 99%, with no residual SeCyst and no by-products [24].

However, this derivatization procedure can lead to the appearance of other by-products, since an excess of IAM can react with selenomethionine and yield carboxymethylated selenomethionine [18]. The best solution to avoid the formation of this by-product is the elimination of the excess IAM by further addition of DTT [37], as has been used in the present work, although the complete derivatization of SeMet has also been proposed [18].

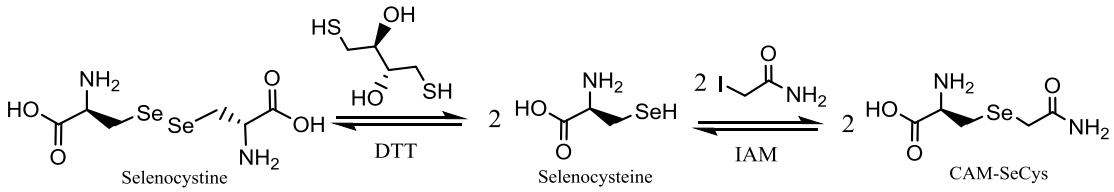

Figure 14: Reduction and derivatization reactions of selenocystine with dithiotreitol and iodoacetamide.

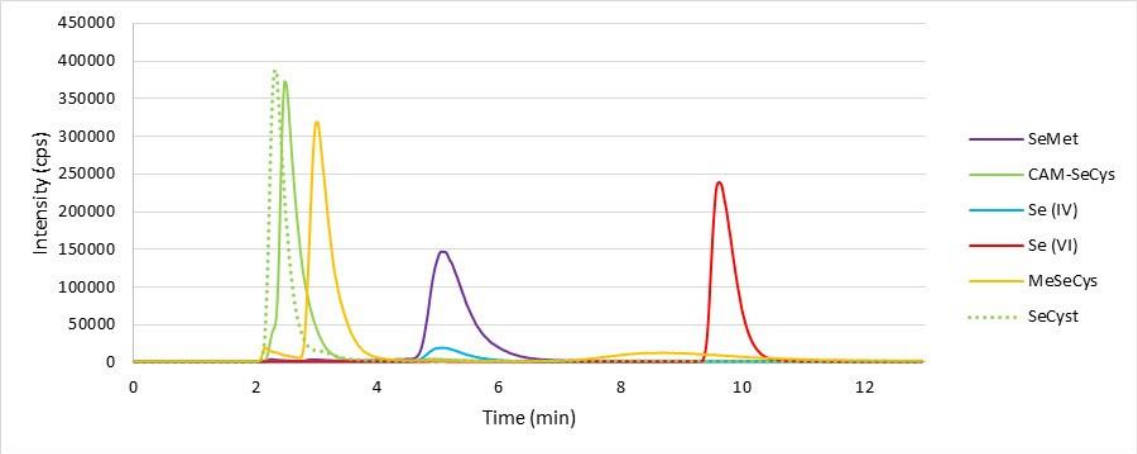

Figure 15: Chromatogram depicting retention times of the standard compounds, injected individually, after enzymatic extraction with derivatization with DTT and IAM. Underivatized selenocystine (dotted line) included for comparison of retention time.

Selenomethionine and selenate show no by-product formation or modification of their retention time with the methodology employed. Recoveries of selenite are very poor, since this species can be easily reduced with DTT, with the appearance of a new species at a different retention time. This new species could be a selenide compound after reduction or a selenite complex. MeSeCys shows the appearance of a small amount of two by-products: one elutes in the void volume and the second does not give a defined narrow peak.

On the other hand, SeCystine has successfully formed CAM-SeCys with no detectable byproducts. CAM-SeCys elutes at a slightly higher retention times than unreacted SeCyst, because CAM-SeCys is slightly more polar and so it is more retained in the column. This shift in retention time of CAM-SeCys with PRP-X100 column has already been reported in previous works [24].

However, this shift of retention time for CAM-SeCys results in a hindrance to successfully resolve this species from MeSeCys in real samples as seen in Figure 16.

Consequently, this approach was deemed unsuccessful.

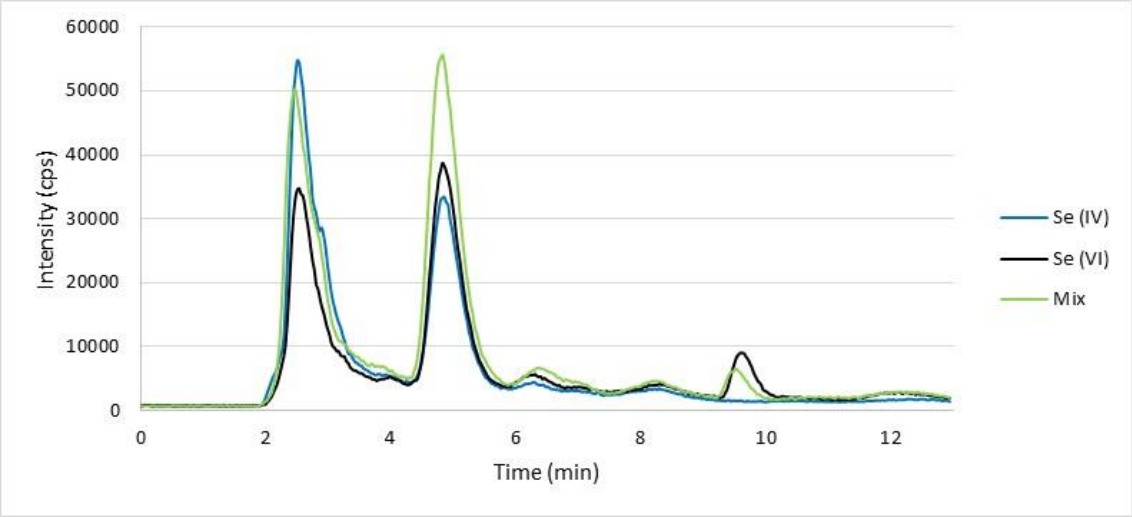

Figure 16: Chromatogram depicting wheat grain samples enriched with selenite, selenate and mixture of both species. The enzymatic extraction was performed in presence of DTT and IAM, with SeCyst being derivatized into CAM-SeCys.

Use of TCEP on the determination process of selenium compounds

The next approach was to use a reducing agent with a different functional group than a thiol. Tris(2-carboxyethyl)phosphine was selected as it is a common substitute for dithiothreitol [41].

TCEP is known to be an excellent disulfide reducing agent, and consequently, it is a suitable diselenide reducing agent. Its reduction potential is -0,29V [28], [36].

The mechanism of action for the reduction of the disulfides with TCEP is well known. The first step is the formation of a thiophosphonium salt between the disulfide and the phosphine, this being the limiting step. A hydrolysis that release the thiol and forms phosphine oxide occurs rapidly after [41] . A similar mechanism is expected in diselenides, where TCEP will be also be oxidized to Tris(2-carboxyethyl)phosphine oxide (TCEP=O). Moreover, the oxidation of phosphines can be considered practically irreversible, due to the strength of the oxygen-phosphorus bond [31], [35].

TCEP is often preferred over DTT and BME because it is highly stable in solution, can work in a wide range of pH, is odorless and is unreactive towards other functional groups [35], [42].

Furthermore, the reactivity of TCEP for the reduction of peptides containing disulfides has been shown to be more than 10 fold bigger than that of DTT at pH 7.5 [41].

The active nucleophilic form of both compounds occurs when they are deprotonated. Accordingly, their differences in pKa explain their differences in reactivity. The pKa of DDT is 9.2, and the pKa of TCEP is 7.6 (with the pKa of the protons in the carboxylic

groups being 4.7, 5.9, and 6.6) [35], [41]. Therefore, at pH 7.5, only approximately 2% of the DTT is in active form while almost half the TCEP is in active form [41].

However, the steric hindrance of the carboxylic groups over the phosphine decreases the reactivity of TCEP with disulfides contained in proteins, almost 100 fold [41]. Oppositely, DTT has lower steric hindrance, what allows higher reactivity. Notwithstanding, the effect of the protease enzyme in the enzymatic digestion allows the rupture of the protein in smaller peptides. Therefore, TCEP will exert its diselenide reducing effect after the selenium containing peptides are extracted during the digestion.

The optimization of the methodology for the use of TCEP in the enzymatic extraction with the SELM-1 CRM showed that a ratio of 5:1 mols of TCEP for each mol of selenium was not enough to successfully prevent oxidation of selenomethionine, since, at the working pH, not all the TCEP was reactive. Ratios ≥10:1 were required in order to prevent oxidation. A slight improvement was seen with 50:1 instead of 10:1, therefore this molar ratio was selected for the rest of the experiments. However, proportions over 50:1 were not advisable, because it was observed that SeCyst reacts with TCEP and the peak intensity decreasesconsiderably, and at 200:1 TCEP:Se the peak area was severely reduced (see figure 18).

Extraction efficiency of SELM-1 in the precence of TCEP was 70,0±0,9%. Therefore, the extraction efficiency did not significantly change respect to the extraction without TCEP.

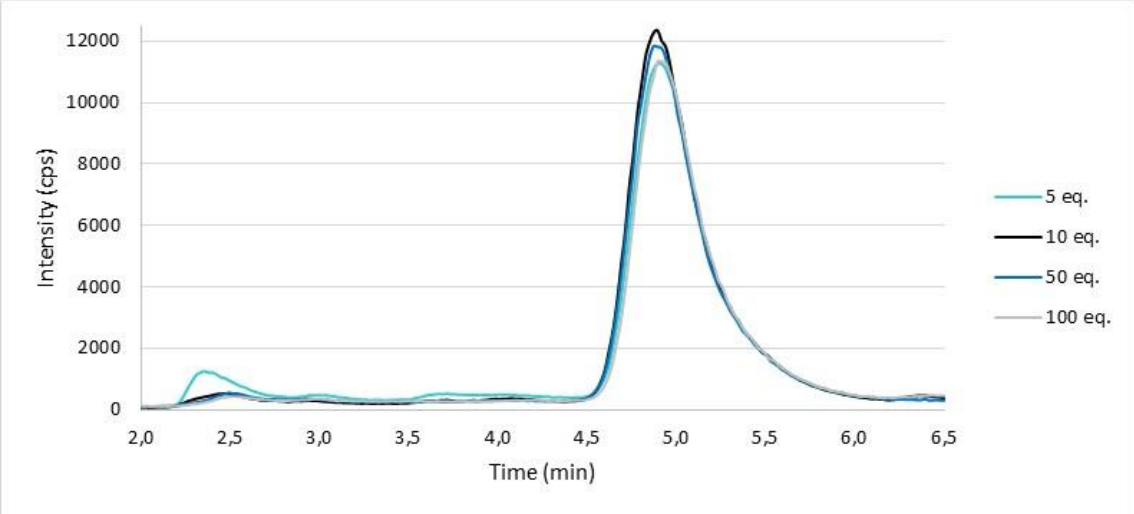

Figure 17: Section of the chromatograms of the enzymatic digestions of the SELM-1 with 5:1, 10:1, 50:1 and 100:1 TCEP:Se molar ratios.

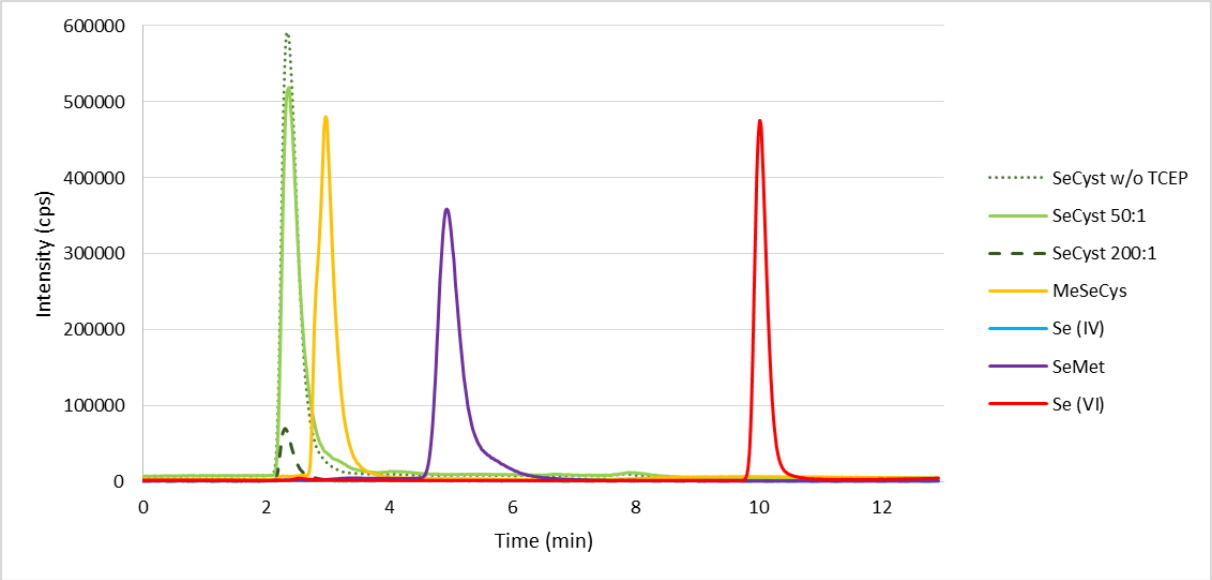

Figure 18: Chromatograms depicting the retention times of the standard compounds, injected individually, after enzymatic extraction with TCEP. SeCyst is shown to react with TCEP and thus, it is represented in different proportions: without TCEP (pointed line), with 50:1 TCEP:Se (solid line) and 200:1 TCEP:Se (dashed line).

This protocol applied to the standard compounds showed that there was no modification in the retention times of the peaks of MeSeCys, SeMet and Se(VI) or formed by products. Selenite was totally reduced to either elemental selenium or selenide, but the formed compounds did not result in a chromatographic peak.

Selenocystine peak intensity decreased its intensity, however, no other peak was seen in the chromatograph. Accordingly the reduction of SeCyst with TCEP generates a compound that also does not produce a detectable peak.

Excess TCEP has been shown to further react with the reduced SeCys. It can lead to the deselenenization of the amino acid to yield alanine (in anaerobic conditions) or serine (in aerobic conditions) [43], [44]. Unreacted TCEP (which was not oxidized to TCEP=O) forms TCEP=Se, and after two weeks, a red precipitate of elemental selenium is also encountered [43].

This reaction follows a radical mechanism. Temperature can enhance this process even if the reaction is performed in the dark [43]. The addition of sodium ascorbate, a radical quencher, could be a feasible solution in order to inhibite the deselenization reaction in future works [43].

Root, shoot and grain samples were analyzed with the addition of TCEP to the enzymatic extraction and the results can be seen in Figure 19.

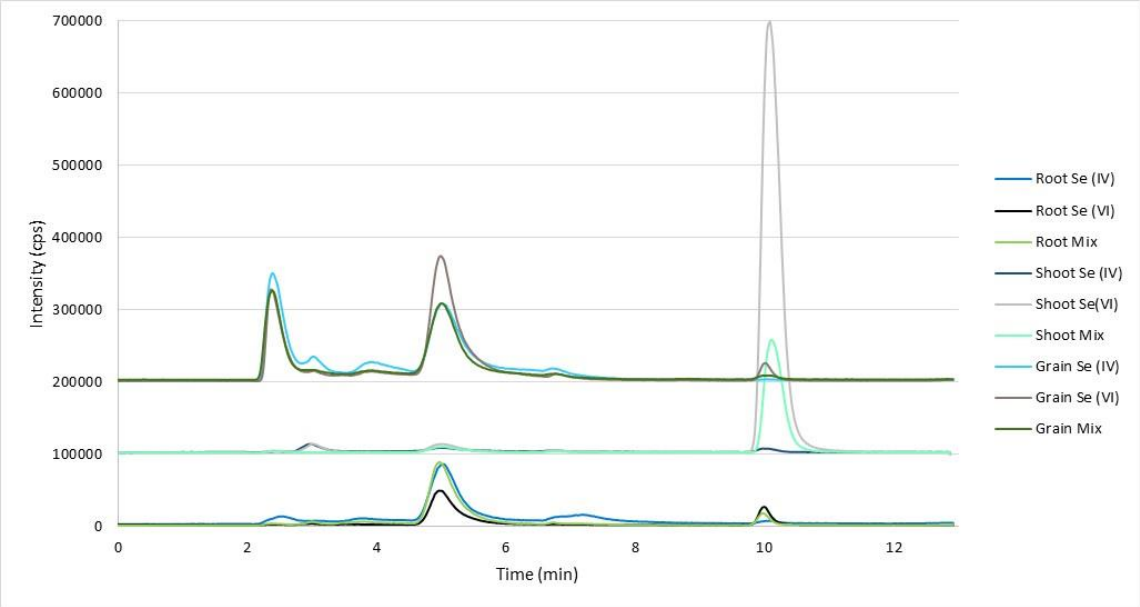

Figure 19: Chromatograms of roots, shoots and grains of wheat samples enriched with selenite, selenate and mixture of both species. The enzymatic extraction was performed in presence of TCEP.

Conclusions

Successful separation of the 5 selenium standards, which were the selenium species expected to be predominant in wheat, was achieved with an elution gradient with mobile phases with ammonium citrate 5mM and 15mM and pH of 5,0 and 2% of methanol.

The chosen methodology for the enzymatic extraction was tested with a certificate reference material. The protocol achieved extraction efficiencies of 71,3 ± 0,9%. Although this value matched those reported in previous works, in further analyses an optimization of the efficiency of the extraction could be attempted.

Speciation analysis performed on SELM-1 resulted in a major peak of selenomethionine. However, a fraction of selenomethionine was converted into selenomethionine selenoxide, which coeluted with the standard of selenocystine causing inaccuracies in the determination of these two species.

Preparation of the enzymatic extraction in order to avoid the presence of oxygen minimized the amount of oxidation occurring, and resulted in 91±3% of SeMet and only 4±2% of SeMetO in SELM-1.

Subsequent attempts to further reduce oxidation with three reducing agents, BME, DTT and TCEP, were performed. Although oxidation of selenomethionine was prevented, the reduction of selenocystine and selenite was caused.

Selenite did not appear in the chromatogram in neither of the cases, and therefore, the enzymatic extraction with reducing agents is not adequate for quantifying selenite.

Selenocystine gave different reactivity in the three case, but in none of the cases it was possible to obtain a well defined peak that allowed a proper quantification.

For this reason, enzymatic extractions for the quantification of the five selenium compounds simultaneously are better without the use of reducing agents, considering that the oxidation of selenomethionine will be lower than 6%.

However, reduction with BME and TCEP can be useful to ensure the limitation of the oxidation occurred, and confirm that the accuracy of the concentration of SeMet, SeCyst and MeSeCys in the sample.

PART II: Wheat sample analyses

Extraction efficiency for selenium species in wheat roots, shoots and grain

The described protocol used for the treatment of SELM-1 was applied to the extraction of Se species from wheat roots, shoots and grains, that were enriched with either selenite, selenate or a mixture both species, to study the speciation behavior of selenium in the different parts of the plant. The use of reducing agents, was only used as supporting information for the proper identification of certain compounds, but the main information was obtained without them.

Enzymatic extraction showed a big variability depending on the type of tissue that was analyzed, as can be seen in Figure 20. The extraction efficiency considering plant tissues, but disregarding enrichment type, was 50±11% for roots, 118±34% for shoots and 115±8% for grain.

Accordingly, extraction efficiency (purple bars in Figure 21) was significantly different depending on the part of the plant that was being analyzed. On the contrary, chromatographic recoveries (green bars in Figure 21) were not significantly different with average values of 54±20%.

The limited selenium recoveries after enzymatic extraction of plant tissue are well in agreement with other works that have reported values from 25% to 73% [2], [45]. However, wheat grain flour has been reported to give high extraction efficiencies with 93±1% and 101±2% of Se extracted in single-step and two-step extraction protocols [46], what is in agreement with our results.

On the other hand, the high standard deviations in the obtained results are a consequence of the complexity of the matrix and the diversity of selenium species in the plant tissues.

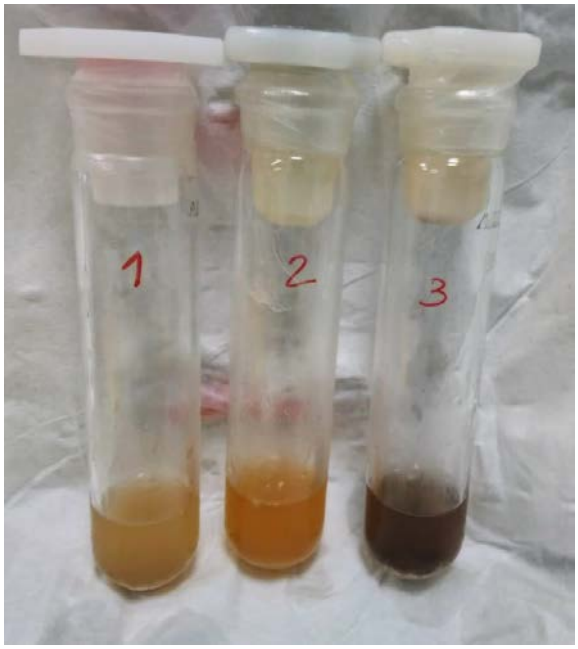

Figure 20: Result from enzymatic digestion from: 1. Root, 2. Shoot, 3. Grain

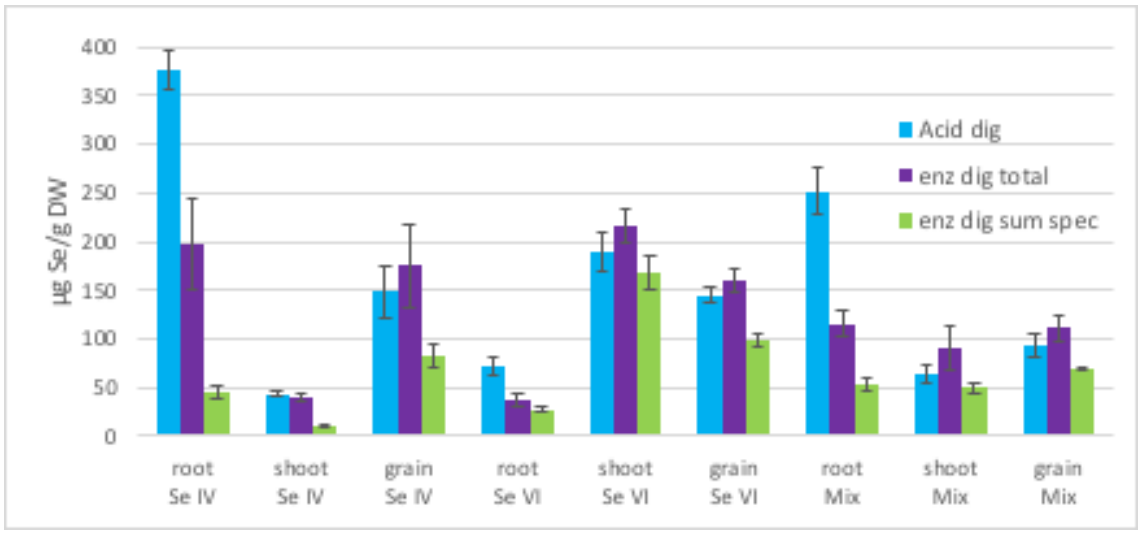

Figure 21: Selenium in wheat roots, shoots and grains, enriched with either selenite, selenate or mixture of the species. Total selenium after acid digestion by ICP-MS (blue) with n=6, total selenium after enzymatic digestion by ICP-MS (purple) with n=4, sum of all selenium species after the chromatography by HPLC-ICP-MS. Results shown as mean±SD.

As discussed with the SELM-1 yeast results, low extraction efficiencies and low recoveries are mostly a consequence mostly of incomplete digestions that releases small peptides and the presence of selenium species that are not soluble in aqueous media [4].

Shoots and grains show excellent extraction efficiencies and accordingly, selenium is mostly found in the form of species that are easily soluble and extractable by mild extractions or by breakage of the peptide bond.

Oppositely, roots show very poor extractions, with only around 50% of the species being solubilized and even less being quantified by chromatography. This is in agreement with studies performed on the roots in other plants species, where it is highlighted the need of the use of complimentary techniques in order to fully discern selenium speciation [3], [17], [19].

A SEC-ICP-MS analysis of the extracts of *Brassica juncea* roots found selenium present as both non-peptidic high molecular weight compounds (>70 kDa) and low molecular weight species (≈6,5kDa). Instead, shoots contained only low molecular weight compounds such as small proteins, peptides and Se-amino acids) [19]. Considering the recoveries obtained in roots and shoots of wheat, a similar pattern is expected.

For the unequivocal determination of the species remaining unable to be identified by peak assignation to known standards in HPLC-ICP-MS, further analysis with techniques such as LC-ESI-MS or MALDI-TOF are adequate approaches [5], [8], [17], [46]. HPLC-ICPMS/ESI-MS studies of selenite exposed roots of other plants, such as *Thunbergia alata,* were able to identify several different selenopeptides such as selenocysteinyl-2-3-dihydroxypropionyl-glutathione, seleno-diglutathione and seleno-phytochelatin [3].

These evidences from other plants supported the idea of the presence of a significant amount of selenium in roots in the form of peptides of high molecular weight that are not fully digested with the enzymatic protocol.

On the other hand, the presence in roots of selenium in forms that are not soluble in water would equally explain the low recoveries. Insoluble selenium would remain in the solid residue instead of being extracted to the aqueous phase with the current protocol.

In this concern, elemental selenium is insoluble and is a possible intermediate in the metabolization of inorganic selenium. Selenite and selenate need to be reduced before being converted into organic selenium. Elemental selenium and selenide are intermediates in this reduction.

Elemental selenium has been determined in the roots of plants such as *Astragalus bisculatus* and *Thunbergia alata* [3], [47], and accordingly, it could be present in wheat roots. An extraction with Sulfite has been proposed as a quantification method for elemental selenium in biological tissues as selenosulfate [3].

Apart of elemental selenium coming from the reduction of inorganic selenium, enzymes members of the cysteine desulfurase family or the selenocysteine lyase family can also transform selenocysteine to alanine and elemental Se. These enzymes are present in eukaryotes and bacteria [48], [49], and therefore, also microorganisms

in the media have been considered to play a role in the fabrication of elemental selenium.

Speciation in selenium enriched roots, shoots and grain tissues

Due to the incomplete chromatographic recoveries and the limited extraction efficiency for roots, quantification of selenium species in wheat tissues is only partial, and accounts only for small soluble compounds. However, they can provide a preliminary insight of the occurring selenium metabolization in wheat, the transformation from inorganic to organic selenium and its incorporation into the plant protein pool, and, last but not the least, how the Se species used for the enrichment determines the final selenium compounds produced in the plant, which can affect both the plant tolerance to selenium and the characteristics of a possible enriched functional food.

Table 4: Selenium speciation determined by HPLC-ICP-MS, in wheat roots, shoots and grain, enriched with selenite, selenate and mixture of the species. Results shown as mean±SD of the selenium determination with n=4.

	Concentration (µg Se/g DW)				
	SeCyst	MeSeCys	Se (IV)	SeMet	Se (VI)
Root Se (IV)	<LOQ	4±3	4±3	29±6	<LOQ
Root Se (VI)	<LOQ	4±4	<LOQ	11±2	6,8±0,9
Root Mix	<LOQ	21±2	<LOQ	22±6	5,2±0,9
Shoot Se (IV)	<LOQ	3,1±0,7	<LOQ	3±1	<LOQ
Shoot Se (VI)	<LOQ	3±2	<LOQ	6±1	156±21
Shoot Mix	<LOQ	<LOQ	<LOQ	3,6±0,8	41±7
Grain Se (IV)	23±15	3±2	<LOQ	53±9	<LOQ
Grain Se (VI)	15±1	1,9±0,2	<LOQ	73±7	5,7±0,6
Grain Mix	11±2	1,4±0,4	<LOQ	50±3	2,7±0,4

Speciation in roots:

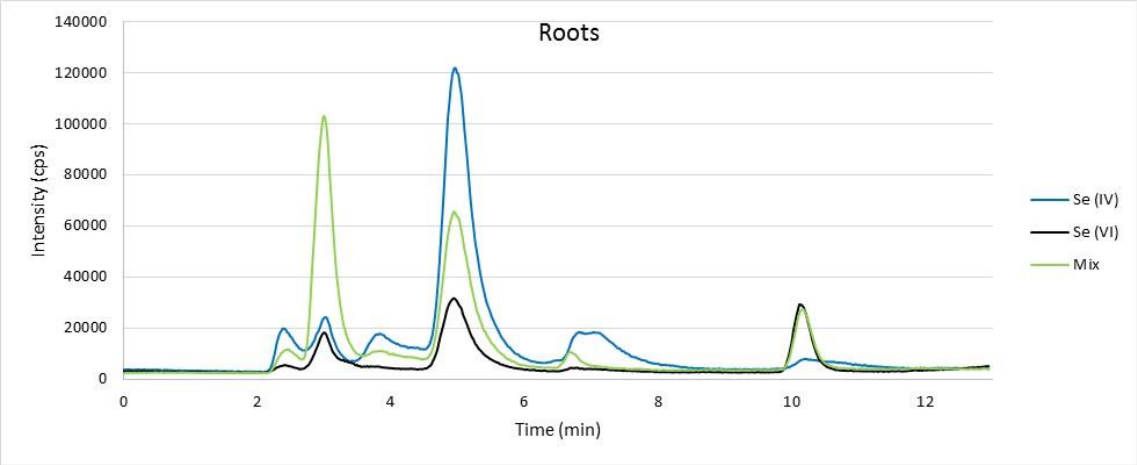

Figure 22: Chromatograms of roots of wheat samples enriched with selenite, selenate and mixture of both species.

Chromatographic results from root extract show always SeMet as the most abundant species in roots, as seen in table 4 and Figure 22.

Selenite enriched roots contain SeMet as 64±5% of the total selenium, with remaining inorganic selenium being <LOQ. However, from the chromatogram it can be seen that part of the selenite has been oxidized to selenate, indicating a relatively oxidant environment in wheat roots.

Oppositely, selenate enriched roots only contain a 43±7% of SeMet, and a 27±6% of selenium still remains in the form of selenate. However, very little selenite is detected.

Selenate needs to be reduced to selenite before being further reduced and converted into organic selenium. However, in selenate enriched roots there is still lot of selenate which has not been metabolized, indicating that selenate reduction to selenite is a slow process. On the other hand, there is practically no selenite detected, which indicates that, once selenite is formed, its reduction to organic selenium occurs fast, as seen in previous works.

In agreement with this observation for these two types of enrichment, in roots enriched by a selenium mixture, selenite has been all metabolized and there is very little remaining, but there is a 10±3% of selenate still remaining in the roots.

In addition, SeMet accounts for a 39±7%, but there is another significant selenium species that comprises the 40±7% of the total selenium. It seems coelute with the standard of MeSeCys. However, repetitions in different days of the same sample, seen in Figure 23, showed that when there is a reduction in the peak area of SeMet, there is an increase in the peak area of this compound. Therefore, what is quantified in table 4 as MeSeCys in roots, is most probably a by-product of SeMet.

The fact that this peak is not significant when the roots are digested in the presence of a reducing agent such as with TCEP, as it is seen in Figure 19, provides an indication that this peak is the product of an oxidation. Since the retention time of this peak matches that seen in Figure 9 for selenomethionine selenone, it could be preliminarily assigned to this structure.

Spiking experiments were attempted in order to confirm the identification, but the resolution in the chromatogram is not enough to obtain conclusive results. Additionally, the spike species used are not incorporated in the biological matrix in the same way as the species to be determined. Therefore, for the digestion of such complex matrix, spiking does not provide trustful results [3], [4]. LC-ESI-MS would be a more suitable method for the identification.

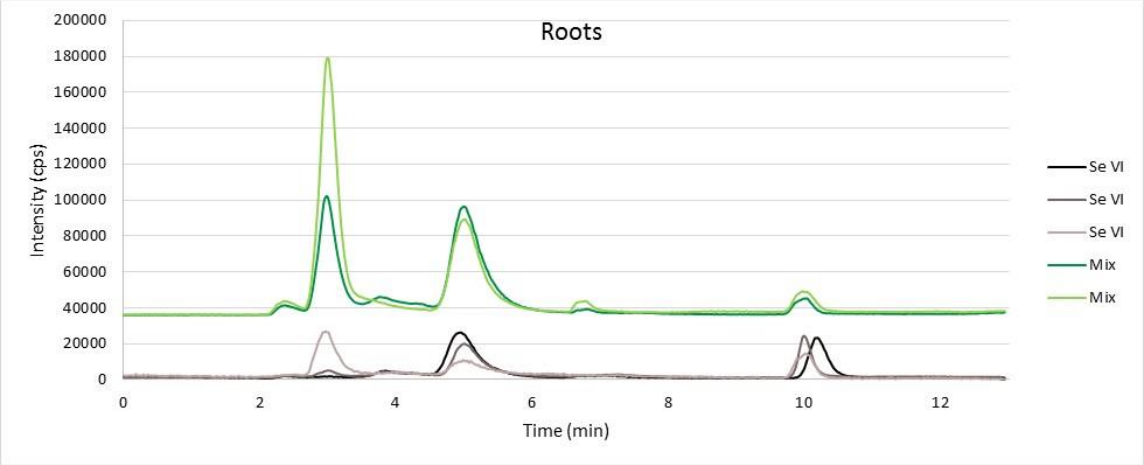

Figure 23: Chromatograms of repetition of the same root sample were a decrease in SeMet peak and an increase in the peak at rt of 3min is visible.

Speciation in shoots:

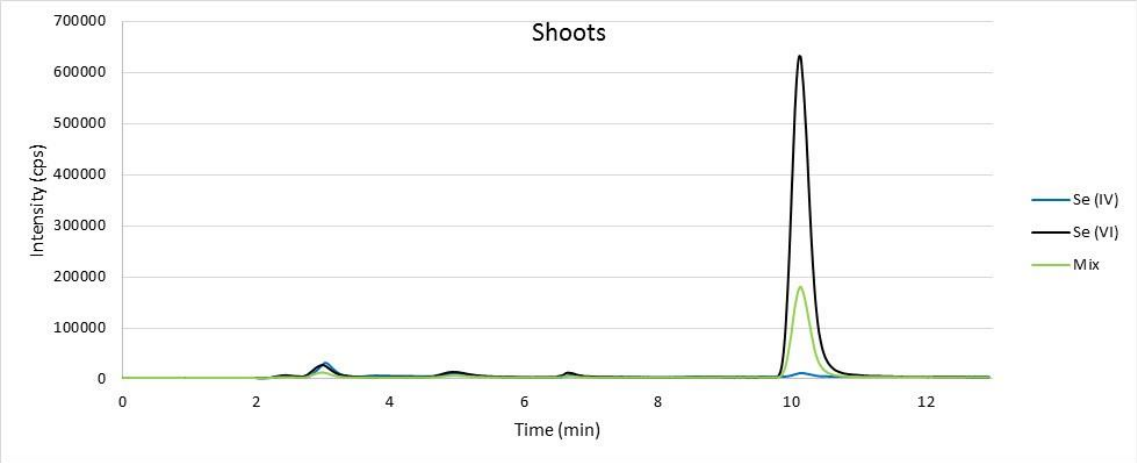

Figure 24: Chromatograms of shoots of wheat samples enriched with selenite, selenate and mixture of both species.

Chromatographic results from extracts of wheat shoots are shown in Figure 24 and table 4.

Selenite enriched shoots showed very little selenium accumulation. All species are detected but they all are below the limit of quantification.

Contrarily, selenate enriched shoots show an outstanding accumulation of selenate, with 156±21 µg Se per g of dry weight.

Enriched shoots by Selenium mixture also present a predominant accumulation of selenate, with 41±7 µg Se per g of dry weight.

This indicates that there is a preferential channel for selenate translocation from roots to shoots, occuring this translocation faster than the reduction of selenate to organic species in roots. Moreover, part of the selenate in shoots is also accumulated unchanged at high concentrations.

Other selenium species, i.e. selenite and organic selenium, are not translocated from roots to shoots in the same way, since very little accumulation is observed.

Speciation in grain:

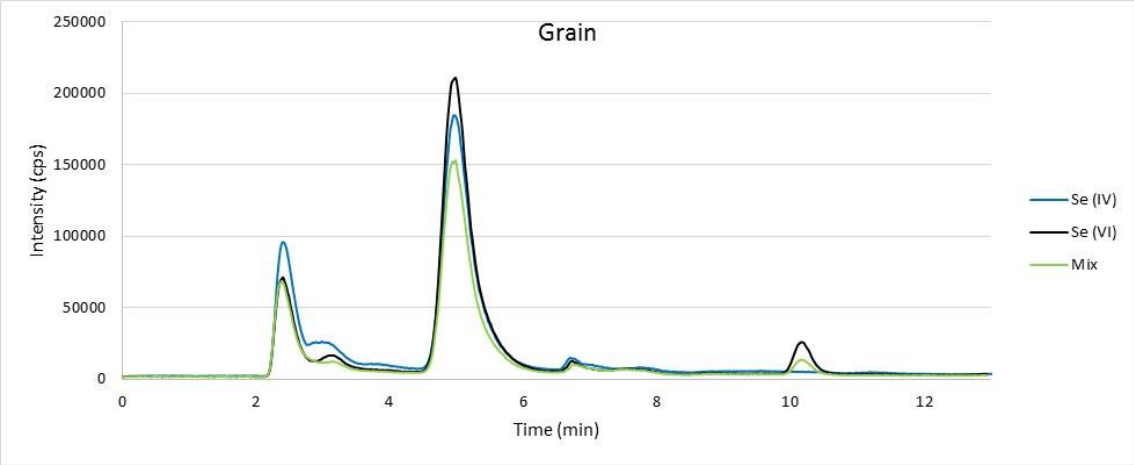

Figure 25: Chromatograms of grain of wheat samples enriched with selenite, selenate and mixture of both species.

Chromatographic results from wheat grain are seen in Figure 25 and are summarized in table 4.

For the three types of enrichment SeMet is the prevalent species, followed of a significant amount of SeCyst.

Selenite enriched grain seem to have a bigger tendency to accumulate selenium as SeCyst, with a 25±12% of SeCyst in front of a 64±15% of SeMet. Selenate enriched grain has a 15±2% of SeCyst and 73±3% of SeMet and mix enriched grain a 16±3% of SeCyst and 73±3% of SeMet.

This diversity in the production of selenoaminoacids due to different enrichments, can be explained with the difference in toxicity of each selenium specie to the plant. It was observed in chapter 2 that selenite enrichments caused bigger plant toxicity leading to smaller biomass production. This toxicity could also cause a bigger stress to the plant, resulting in the accumulation of reactive oxygen species (ROS) [50]. It is known that plants subjected to environmental stress reduce their capacity to eliminate the free radicals of oxygen produced as intermediate species in the photosynthetic metabolism. That results in the formation of superoxide and hydrogen peroxide [51]. Thus, these ROS species accumulate in the plant causing damage to the cellular structures and biomolecules, which results in inhibited growth [52]. Moreover, these ROS create an oxidative environment in the plant, which will enhance the formation of Se-Se bounds, and selenocystine, from the selenol group of selenocysteine [53].

Furthermore, the different production of selenoamino acid can also be explained by a different tolerance mechanism to selenite and selenate. It is known that different plant genus favor the formation of different organic species resulting in different tolerance to selenium. Hyperaccumulators tend to accumulate selenium in methylated forms, as

SeMet and mostly MeSeCys, and to vaporize it as dimethyldiselenide (DMDSe). Non-accumulators usually accumulate mainly inorganic Se, sequestering it in the vacuoles of mesophyll cells of leaves and volatilizing it as dimethylselenide (DMSe) [49], [54]. Accordingly, some enriched vegetables such as garlic (*Allium sativum*), onion (*Allium cepa*), leek (*Allium ampeloprasum*) and broccoli (*Brassica oleracea*), store Se predominantly as MeSeCys. In contrast, cereals such as wheat, barley and rye, that can accumulate intermediate amounts of selenium, store it predominantly as SeMet [55].

SeCys and SeMet can be non-specifically incorporated in proteins in place of methionine and cysteine, where Se randomly substitutes a S atom [56]. The increase in size, polarizability and reactivity by the substitution of a S by a Se atom [53] leads to protein misfolding, which alters protein structure and function and cellular biochemical reactions [57][58]. This nonspecific incorporation of seleno amino acids into proteins may be a major cause of the harmful effect of selenium on sensitive plants. The enhanced formation of Selenocystine and MeSeCys, which cannot be incorporated into proteins, can be a defence mechanism of the plant in order to counteract the toxicity caused by the selenite enrichment.

Furthermore, very little inorganic selenium reaches wheat grains. Selenite is hardly detected in any of the three enrichment conditions. Otherwise, selenate is present in selenate enriched grains and mixed enriched grains. For mixed enriched grains, selenate is 4,0±0,5%, however, for selenate enriched grain a 5,8±0,5% of selenium is found in the form of selenate.

Different unknown compounds have been identified in the three target tissues and at the three enrichment conditions. An approximate quantification has been made with the average sensitivity of the other selenium standards. The results show that a range between 2-15% of the total selenium present in the sample is found as unidentified compounds. However, this value is meant to provide only a qualitative idea.

These unidentified compounds would need to be elucidated by means of LC-MS in future works. However, some of the peaks seem to be a product from SeMet, as the retention times match the retention time for unknown peaks of the SELM-1 CRM. Other peaks are unique to some of the plant parts, indicating a specific presence of certain selenium compounds in determined tissues. However, all these unidentified compounds were found at concentrations below quantification limits.

Additionally, it must be taken into consideration a small error arising from the oxidation of selenomethionine. With the developed protocol, an approximate 4±2% of the SeMet in the biological tissue is oxidized during enzymatic extraction to SeMetO, which coelutes with selenocystine. Accordingly, the amount of SeMet can be slightly undervalued and the amount of SeCyst slightly overrated.

Considering all the previous information, it is observed that selenate is being less metabolized to organic Se than selenite, because much less organic Se is found in the roots and much more selenate is found in the shoots, compared to the selenite enrichement. Accordingly, it can be deduced that the translocation of selenate to the aerial parts of the plant occurs faster than its reduction, and for this reason, it accumulates unchanged in the plant, in both shoots and grain.

Despite selenate reduction being an slow reaction, it is also the first phase to the transformation of selenium. Selenate is transformed to selenite and selenite to selenide, and further on to a variety of organic species, where selenoamino acids are the predominant ones [59], [60].

Therefore, selenite has a shorter chemical pathway to the final Selenoaminoacid moiety than selenate. In contrast to what is observed with selenate enrichments, very little selenite is found in the plant. Despite a few amount of selenite is still visible in the root, none of it is found to accumulate in shoots or grain, indicating that this species is efficiently reduced. Accordingly, selenite metabolization is easier and faster that that of selenate.

This fact indicates that the transformation of selenate to selenite is the rate limiting step in selenium metabolism in plants [61], [62].

Regarding the translocation of the species from root to aerial part, the selenium species move from the root epidermal cells to the plant xylem, and from there they are distributed into the aerial parts.

Selenate diffusion coefficient is 2 to 3 orders of magnitude bigger than that of selenite in a variety of media and conditions, and diffusion coefficients of organic species such as selenomethionine, and selenocystine are somewhat in between [63][64].

Therefore selenate is much easily transported to the shoots of the plant through the xylem. In agreement, a previous study on tomato plants demonstrated that selenate enriched plants had 6 to 13 times higher selenium concentration in the xylem exudates than in the external media, and that this selenium was found mainly in the form of selenate. Oppositely, on selenite treated tomatoes the concentration in the xylem exudates were always lower than in the external solution and less than a 7% was found as selenite, being organic selenium and selenate the main components[65].

Last but not the least, despite all differences in behavior between selenite and selenate in roots and shoots, differences in grain are somewhat smaller. The three types of enrichment result in SeMet being the most abundant species.

However, enrichment made with selenate or mix, result in grain that contains a certain amount of inorganic selenium. Due to the higher toxicity of inorganic selenium

compared to organic forms [66], accumulation in grain may not be desirable in terms of producing a functional food for human consumption.

Another difference between the three enrichments is a possible divergence in the ratio of SeCyst:SeMet produced in grain. This trend will be confirmed in a further study in chapter 4. However it can have also important implications, regarding the applications of the enriched grain for functional foods. SeCyst and SeMet are metabolized differently by the human body. Furthermore, the capacity of SeCyst to form a selenol group, confers it a higher toxicity, but as well a bigger potential as an anticarcinogenic compound [66], [67].

Conclusions

The selenium species were successfully quantified in selenium enriched tissues of wheat with the methodology previously developed and optimized.

Selenite enrichment results in an almost quantitative conversion of this species into organic selenium, which is then found thorough the plant.

Oppositely selenate metabolization is the slower than its translocation, for this reason this species in found to accumulate mainly in shoots and also in grain.

Mixed enrichments result in a synergism between the two species and intermediate results are encountered. An approach with the mixture of the two inorganic species can be the indicate solution in order to be able to tune the proportion of selenium species found in grain to the desired application of the flour produced.

Some species are found in limited concentrations and could not be quantified; however, they can be also relevant to fully understand the implications of selenium to the plant and to the production of a food product. Further research in order to improve the sensitivity of the technique and lower the limit of quantification should be performed. The quantification by the method of standard addition could be a advisable in order to improve the results.

Furthermore, some peaks could not be identified as they did not match the retention times of the selenium standards. Identification of these compounds with unequivocal techniques such as LC-MS is recommended.

Last but not the least, limited extraction efficiencies and chromatographic recoveries imply that only a fraction of the selenium in the tissue is actually quantified. Amounts vary depending on the tissue studied or the enrichment applied.

For this reason, the use of a speciation technique that did not require a pretreatment step in order to extract and solubilize the species could ensure a quantification of the total selenium present in the sample, even if this is found in insoluble forms or unbroken peptides. Moreover, the elimination of the pretreatment step can also

eliminate the risk of reactivity of the species and the formation of oxidized by-products.

X-Ray absorption Spectroscopy with synchrotron light is a direct speciation technique that offers the mentioned advantages, and that will be the subject of study in chapter 4.

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4. Direct speciation with X-Ray Absorption Spectroscopy

Introduction

X-Ray Absorption Spectroscopy (XAS) is a technique that allows the study of the local structure around a selected element at an atomic and molecular scale [1].

Accordingly, XAS provides information of the chemical speciation of an element in a sample. Furthermore, its combination with X-Ray Fluorescence mapping (XRF or μXRF) brings complementary knowledge regarding the elemental distribution and the species spatial distribution [2].

This technique can be applied to all kinds of materials (crystalline or amorphous, solid, liquid or gas) and accordingly it can answer scientific questions from every discipline, including plant biology [1].

XAS measurements provide reliable speciation data that have significant advantages respect to conventional techniques such as HPLC-ICP-MS, described in Chapter 3.

XAS data is obtained by the direct application of X-ray radiation on the sample. Therefore, there is no need of sample pretreatment or species extraction before analysis, thus avoiding drawback issues such as incomplete recoveries or species modification and ensuring that all species in the sample are considered, independently of their state.

X-Ray Absorption Spectroscopy brief history:

In 1895 X-Rays were discovered by W. C. Roentgen. Years later, in 1913, Maurice De Broglie observed the first X-ray absorption spectrum of silver and bromine atoms but, due to the low energy resolution, the fine structure of the spectra was not resolved. It was not until 1920, that Hugo Fricke first observed the fine structure, thanks to the vacuum X-ray spectrometer invented by Siegbahn and Stenström in 1916. In 1931, J.D. Hanawalt observed that the chemical and physical state of the sample affected the fine structure of the obtained XAS spectra thus revealing the potential of the technique. This was confirmed when, in 1971, Sayers, Stern and Lytle achieved structural determination from the interpretation of the post-edge oscillations with Fourier analysis [1], [3].

Moreover, significant improvements to the technique occurred in the 1960s when single-crystal monochromators were used to select the desired energies by diffracting the X-rays in Bragg condition. Later on, in the 1970s, the advent of synchrotron radiation sources replaced the conventional X-ray tubes [3].

Synchrotrons:

The first synchrotron radiation was observed at the General Electric Research Laboratory (Schenectady, New York) in 1947. The idea of a synchrotron was independently proposed by Ed McMillan and Vladimir Veksler in 1945, based on previous betatron and cyclotron concepts [4], [5]

The synchrotron radiation is produced by relativistic charged particles when they are accelerated. In synchrotron radiation light sources, as the one depicted in Figure 1, electrons are produced by an electron gun and accelerated, first in the linac and then in the booster sections, to achieve the required energy. Then these relativistic electrons travelling at speeds close to the speed of light are injected into the storage ring where they can be maintained in circulation for several hours. The storage ring is composed by both straight and curved sections hosting insertion devices (wigglers or undulators) or bending magnets respectively [3]. These devices create magnetic fields that bend the path of the particle which undergoes a centripetal acceleration and emits electromagnetic radiation tangential to the trajectory [1], [6].

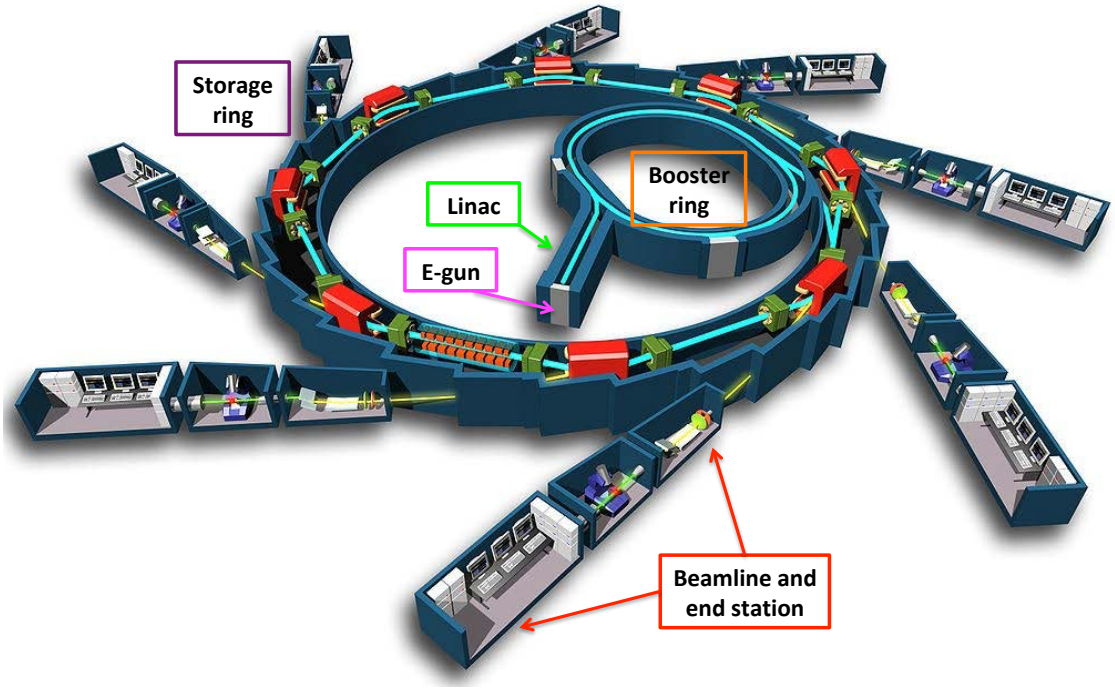

Figure 1: Conceptual layout of a synchrotron [7].

The synchrotron radiation has high flux, high intensity and high brilliance. The beam flux is defined as the total number of photons per second. The intensity is the number of photons per second per area incident on a target. And the brilliance is the number of photons per second per source area (in mm^2) per source angular divergence (in $mrad^2$) per spectral bandwidth. Therefore, a brilliant source emits many photons per second, with a small source size, and a small angular divergence [1], [4].

After exiting the ring, the synchrotron light reaches the beamlines where it can be used for a wide range of techniques. The main elements in the beamline optics are focusing devices and the monochromator. The most common type of monochromator used consists in two parallel single crystals. Monochromatic radiation is achieved by Bragg diffraction in the atomic planes. The energy of the beam is selected by the angle of incidence, thus, rotating the crystal changes the photon energy of the outgoing beam. This enables scanning the energy to collect a XAS spectrum [1], [3].

In the present study three XAS beamlines at different synchrotrons were used: CLAESS beamline at ALBA (Spain), beamlines 10-2 and 2-3 at the SSRL (USA) and BM25A beamline at the ESRF. The three synchrotrons are shown in Figure 2.

Figure 2: Views of the synchrotrons where the experiments for the present study were performed: ALBA, SSRL, ESRF.

X-Ray Absorption Spectroscopy theory:

XAS is based on the fact that, when a sample is irradiated, part of the incident radiation is absorbed and part of it is transmitted. The relationship between the incident and the transmitted radiation is the absorption coefficient μ (E).

In general, the absorption coefficient decreases as energy increases. However, sudden increases in absorption are encountered at certain energies. Those are called edges and are characteristic of the elements present in the samples. The edges occur when the incident x-ray photon has sufficient energy to excite one core electron, for example from a 1s orbital, to the unfilled states of the continuum (Figure 3) [8].

The main difference with optical spectroscopy is that, in that technique, it is one electron of the valence band that is excited, what occurs at much lower energies.

The required energy to excite one of the core electrons depends on the electron binding energies, and can be found tabulated [4]. Different edges occur when electrons from different states are excited (Figure 3). K-edges, coming from the excitation of an electron of the 1s orbital, are the most common to be measured, because, although occurring at higher energies, they have a larger absorption cross section.

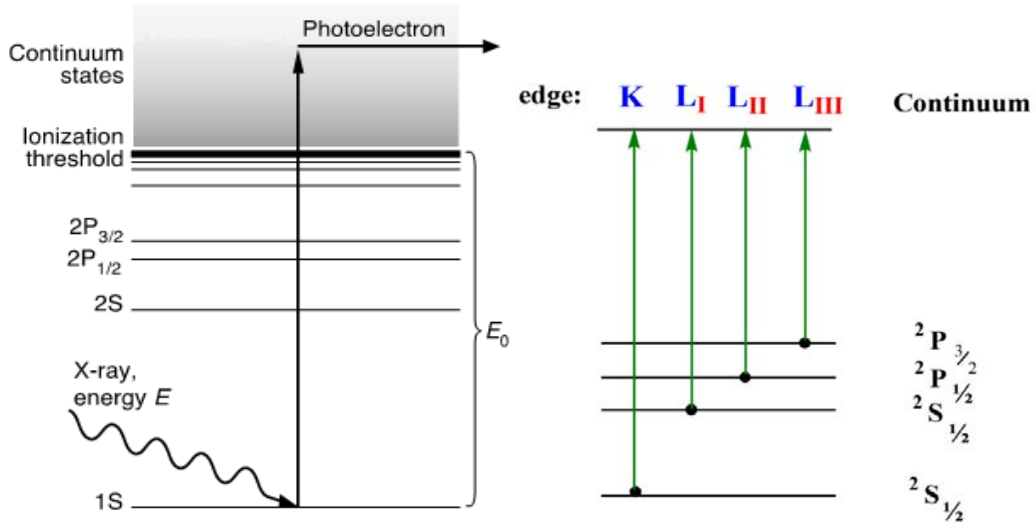

Figure 3: Electron excitation phenomenon in XAS.

If the excited atom was isolated (e.g. gas) the absorption signal after the edge would look like a flat line (Figure 4). However, since atoms are generally not isolated, in their condensed state, the propagated wave of the photo-electron generated scatters with the surrounding atoms when travelling away from the excited atom. The interference (constructive or destructive) between the outgoing wave and the backscattered waves create an oscillatory modulation of the absorption coefficient (Figure 4) [9].

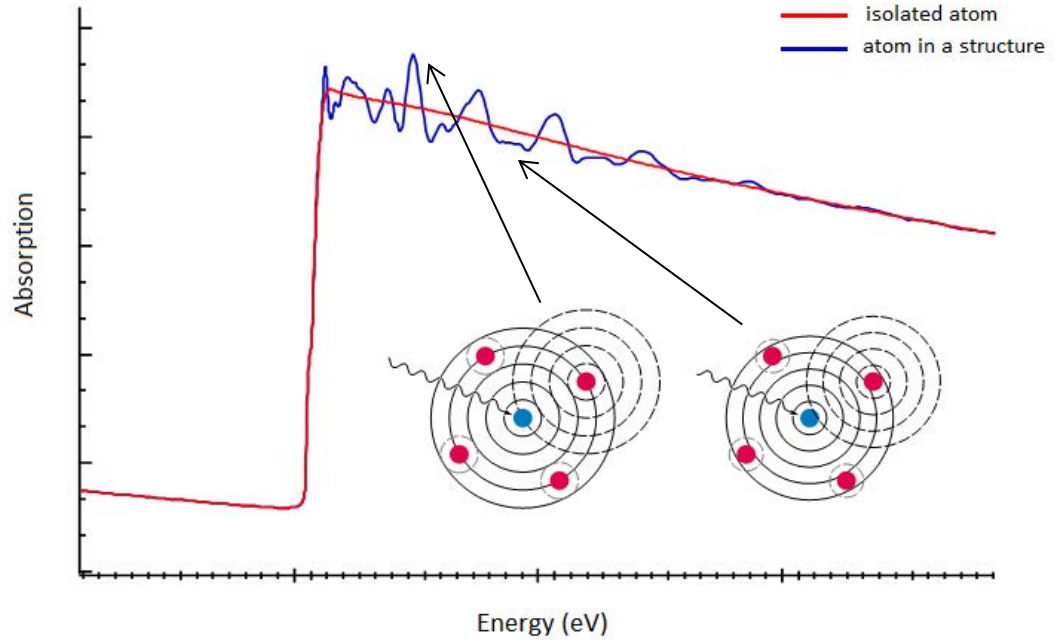

Figure 4: XAS spectra from an isolated atom and from an atom in a condensed state. The inner figure depicts the constructive and destructive interferences between the outgoing wave from the absorber atom and the backscattered waves of the surrounding atoms.

The obtained XAS spectrum contains the following parts:

- Edge: region in which the incident energy is larger than the binding energy of the electron of a chosen element and thus the electron is excited (photoelectric effect) giving rise to a sudden increase in absorption. This intense absorption is also known as white line.

- Pre-edge: region before the edge where the incident energy is not enough to excite the desired photo-electron.

- Post-edge: region after the edge where oscillations due to the scattering interactions of neighboring atoms are observed.

- XANES: X-ray Absorption Near Edge Structure. Region of the spectra that includes the pre-edge, the edge and a post-edge of 50-100 eV after the edge.

- EXAFS: Extended X-ray Absorption Fine Structure. Post-edge region above 100 eV.

- XAFS: X-ray Absorption Fine Structure. XANES+EXAFS, the entire absorption spectra. It can be used as a synonym for a XAS.

- E_0: the inflection point in the absorption edge. Its position depends on the electronic density surrounding the studied atom.

- Jump: the difference between the absorption in the pre-edge and in the post-edge. It is proportional to the number of absorbing atoms in the sample. However, during sample treatment, the jump is usually normalized to 1.

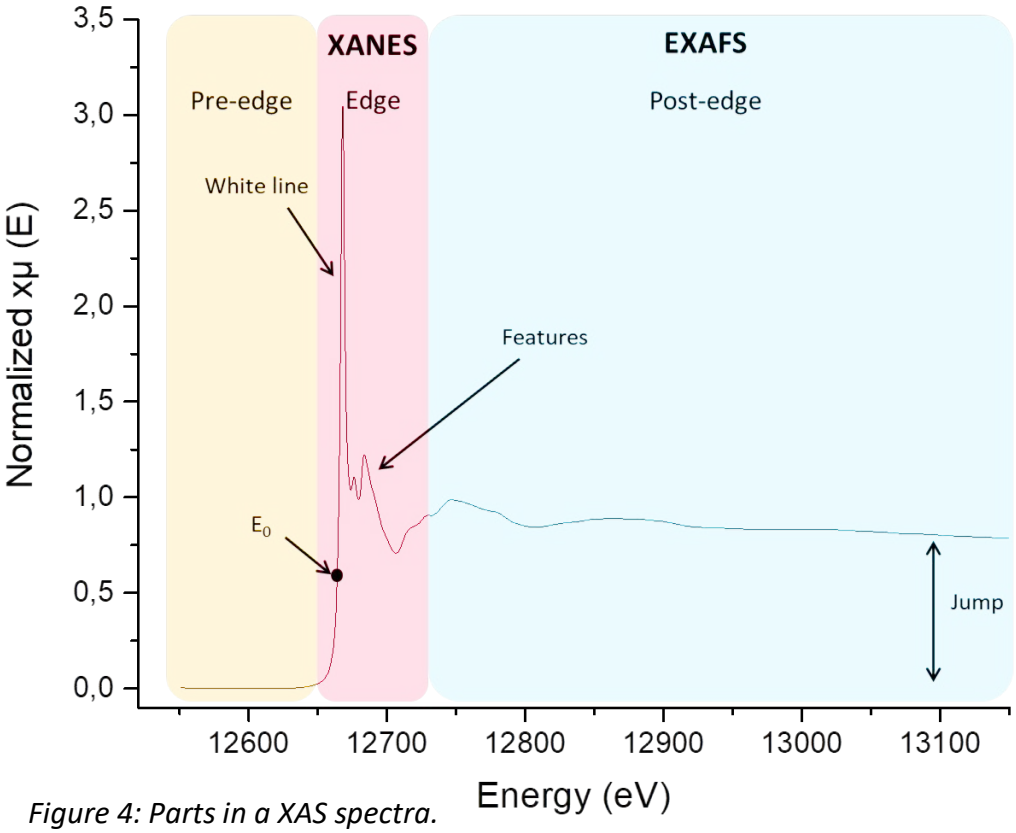

Figure 4: Parts in a XAS spectra.

The XANES region of the spectra is due to multiple scattering phenomena (Figure 5, panel a) and provides information on the local structure, the oxidation state, the coordination, the unoccupied electronic states and the spin state. The XANES area is used to obtain information of the composition of the sample. The shape of the spectra of the sample is the sum of the spectra of the individual compounds that compose the sample (Figure 6). Accordingly, XANES is said to allow a "fingerprint analysis" since the shape will be uniquely corresponding to a determined chemical state.

Therefore, for those cases that the obtained spectrum is originated by the contribution of different atomic environments of the same element, it might be possible to analyze the spectrum as the sum of each individual contribution. This requires the use spectra collected on pure standards (commercial or synthetic) to be used as references for performing a linear combination fit (LCF) analysis. The method of least-square linear combination fitting is able to identify which references are present in the sample and to quantify them. However, it is considered that the accuracy of the LCF method is around 5-10% [2].

The EXAFS region of the spectra is mostly due to single scattering from different neighboring atoms (Figure 5, panel b). The observed oscillations are extracted into a wave function, which is a combination of the interactions of the wave functions from the scattering of each individual neighbor. The EXAFS data is interpreted by means of a Fourier analysis. And the wave is fitted to a model created from crystallographic data. It provides information of bond distances, coordination numbers and static and dynamic disorder.

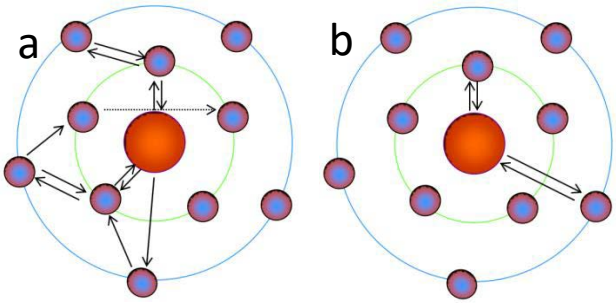

Figure 5: a) multiple scattering phenomena and b) single scattering phenomena.

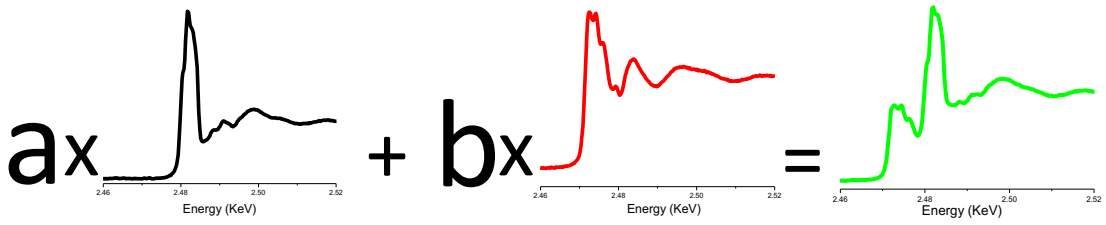

Figure 6: the spectrum of the sample (green) is the sum of the spectra of the pure reference compounds present (black and red) multiplied by a coefficient corresponding to their relative abundance.

X-Ray Absorption Spectroscopy measurements:

X-Ray absorption generally measures the linear absorption coefficient (μ) as the relationship between the incident radiation (I_0) and the transmitted radiation (I_1) with the formula:

$$\mu x = ln\frac{I_0}{I_1}$$

Where x is the sample thickness.

Accordingly, in measurements performed in transmission mode, the absorption is measured in ionization chambers located before and after the sample, in a straight line (Figure 7).

However, in order to be able to measure absorption in this manner, it is necessary that the sample is thin enough to be able to allow most of the radiation to be transmitted. Moreover, the desired element needs to be in enough concentration in order to provide sufficient absorption signal.

In addition, the sample needs to be uniform and homogeneous, therefore, they are usually prepared in the form of pressurized pellets with the sample powdered and, if necessary, diluted with a nonabsorbent matrix, such as cellulose or boron nitride.

However, in the case of having thick samples or the element of interest in low concentration, it is possible to measure the emitted fluorescence instead.

As seen in Figure 3, when the radiation is absorbed, one core electron is excited, leaving a hole. Then, one of the electrons in the higher states relaxes to occupy the empty hole, therefore emitting radiation in the form of fluorescence [1]. This fluorescence can be measured by placing the sample at 45 degrees and a detector at 90 degrees from the incident beam (see Figure 7). The absorption coefficient μ in this case is measured as:

$$\mu \approx \frac{I_F}{I_0}$$

Since fluorescence signal do not need to be transmitted, thick samples can be measured. Furthermore, fluorescence signal is more sensitive, since it detects only the photons of interest that are generated after the absorption [3]. Therefore, lower concentrated samples can be measured.

However, in general, the fluorescence signals have larger statistical noise and accordingly, a much longer acquisition time is required in order to obtain a good signal-to-noise ratio [3]. This is due to the fact that the total number of photons emitted from the element of interest and reaching the fluorescence detector can be small compared to the transmitted signal, and, additionally, photons scattered or fluorescence from other elements can also reach the detector, reducing the dynamic rage of the detector [3].

On the other hand, very concentrated samples should not be measured, due to the self-absorption effect. If the element of interest is too concentrated in the sample, it leads to an attenuation in the oscillations of the fluorescence signal [3].

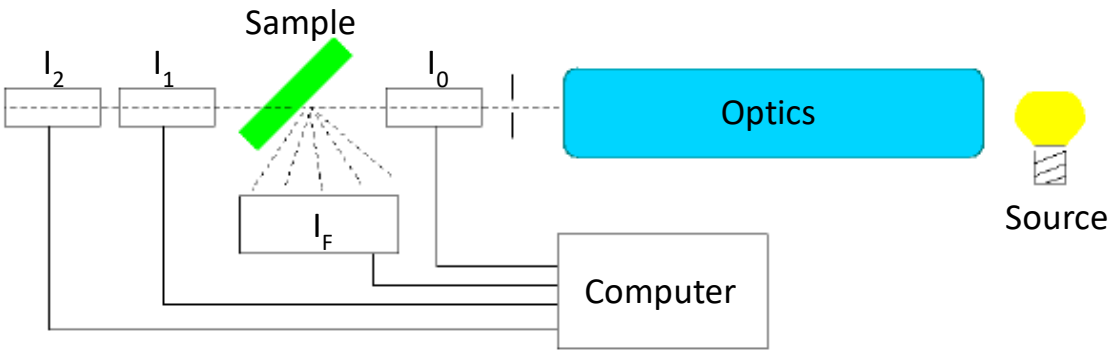

Figure 7: Parts of the experimental set-up in a XAS beamline: *source (bending magnet or insertion device), optics (mirrors and monochromators), sample, detectors (*I_0, I_1, I_2 and I_F*), amplifiers and computers* [9].

Furthermore, fluorescence radiation can also be used in order to obtain information on the localization and distribution of the elements. This approach is called X-Ray Fluorescence (XRF) mapping.

In XRF a small beam of few micrometers is used, the energy is held constant above the absorption edge and the position of the sample is scanned to create an elemental map based on the intensity of fluorescence at each position [10].

The required energy to excite the element of interest will also excite the elements with lower atomic numbers, and therefore, XRF allows the measurement of several elements at the same time, generating simultaneously various elemental maps [10].

Thus, XRF measured on inhomogeneous samples allows identifying areas where the element of interest is accumulated. Subsequently, XAS spectra can be collected at those points using the same micro-focused beam as for the XRF maps. This approach is called µXAS (micro-X-ray Absorption Spectroscopy).

Finally, the last thing to consider when measuring X-Ray Absorption Spectroscopy is the possibility of radiation damage on the sample. Radiation damage consist in the modification of the species present in the sample due to the intense flux from the beam, and it is a common effect in biological material [3]. In order to prevent radiation damage it is recommended to work at cryogenic temperatures (such as liquid nitrogen temperature, 77K, and to perform quick scans with fast acquisition times over a test sample at the beginning of the experiment, in order to see if there is any modification in the spectra such as shifts of the edge due to changes in the oxidation state or changes in the intensities of the features due to changes on the coordination geometry [3].

Objectives

The main objective in the present chapter is the analysis of the selenium species in wheat plants with a direct speciation technique, in this case X-ray absorption spectroscopy and X-Ray fluorescence. The following tasks will be accomplished:

- ✓ To characterize the transformation of inorganic selenium into organic selenium species in wheat plants.
- ✓ To determine the distribution of the species through roots, shoots and grain, depending on the Se species used for the enrichment i.e. selenite, selenate or mixtures.
- ✓ To study the accumulation regions of selenium inside wheat grains, and the correlation with other nutrients.
- ✓ To identify the different selenoamino acids present in enriched wheat grain and their distribution.

Materials and Methods

Wheat culture and treatment:

Plants of common wheat (*Triticum aestivum* cv. Pinzón purchased from Semillas Fitó S.A.) were grown in hydroponic culture, in the conditions detailed in chapter 2.

After germination and two weeks of preculture, at the vegetative stage plants were exposed to selenium treatments: Control, 10 µM Na_2SeO_3, 10 µM Na_2SeO_4 and a mixture of 5 µM Na_2SeO_3 and 5 µM Na_2SeO_4. The nutrient solution containing the treatment was renewed weekly.

At harvest, roots where washed with ice-cold $CaCl_2$ solution and rinsed with deionized water. Afterwards, plant material separated into roots, shoots and grain.

Sample preparation:

Samples were dried in an oven at 45^{o}C during four days until stable weight. Roots, shoots and grain were ground with an automatic mortar for bulk analysis. The powder was pressurized into a pellet and enclosed in kapton tape.

However, a few grains from each condition were separated for µXRF and µXAS analysis. Grains were embedded in paraffin and cut using a microtome. The precise microtome cuts were done vertically in order to expose selected regions of interest of the interior of the grain, where the main features of grain physiology were visible, mainly the germ, the bran and the endosperm, and subfeatures inside them, as the pigment strand.

Bulk XAS experiments in roots, shoots and grains:

XAS analysis in pellets of roots, shoots and grains were performed at beamline BM25A at the European Synchrotron Radiation Facility (ESRF). The beamline is equipped with a bending magnet source and a double crystal Si (1 1 1) monochromator. Measurements were performed at Se K-edge after energy calibration with metallic selenium at 12658 eV.

Samples were analyzed at low temperature, with 4 repetitions per sample in fluorescence mode, with a 13-element Si detector. The obtained spectra were normalized and merged with the Athena software [11], and analyzed by a least-square linear combination fit of reference spectra.

The references were prepared from pure selenium standards, diluted in cellulose. They were analyzed in transmission mode in gas ionization chambers, with the same beamline configuration as the sample measurements, in order to provide comparable data.

Furthermore, the reproducibility of the speciation results in grain were validated by the analysis of a new set of grain samples from different plants, but biofortified with the same selenium treatments. This analysis was performed at CLAESS beamline, ALBA synchrotron. The Se K-edge of samples was measured in fluorescence mode with a silicon drift detector, and a double crystal Si (1 1 1) monochromator was employed.

µXRF and µXAS experiments in grain:

High quality XRF maps were obtained at beamline 10-2 and 2-3 at the Stanford Synchrotron Radiation Lightsource (SSRL). Beamline 10-2 was equipped with a 30 pole, 1.45-Tesla Wiggler ID end station and a double crystal Si (1 1 1) monochromator. A beam size of 50x50µm was used, with a dwell time of 50ms/point. Maps of Se, P, S, K, Ca, Mn, Fe, Cu and Zn were obtained by selecting regions of interest in the corresponding Kα emission energies, when the sample was excited at 12677.0 eV, over the Se K-edge energy.

Further detail on specific areas of interest in grains was acquired with µXAS measurements at beamline 2-3. This beamline was equipped with a 1.3 Tesla Bend Magnet and a double crystal Si (1 1 1) monochromator, with a beam size of 2x2 µm and 20ms/point dwell time. Fluorescence data was acquired with a vortex detector. Two grain samples, considered to be the most representative, were selected and selenium speciation was measured in 6 points of interest. Data was analyzed by the software SMAK [12] and Athena.

Furthermore, a more complete analysis was performed on 3 grains at CLAESS beamline, ALBA synchrotron. CLAESS beamline is equipped with a multipole wiggler

source and measurements were performed at Se K-edge with a double crystal Si (1 1 1) monochromator. Energy calibration was performed on metallic selenium.

The sample was mapped by XRF and approximately 100 points of interest were acquired by μXAS in each sample. The measurements were performed in fluorescence mode with a silicon drift detector, with a beam size of 200x90 μm.

The acquired points were grouped in areas within the same grain tissue, i.e. germ, pigment strand, bran and endosperm (a slightly different number of areas resulted from each sample, in order to group a similar number of points to have comparable signal to noise ratio). The selection and grouping of points were performed with the software PyMCA [13]. The spectra normalization and least-squares linear combination fit analysis with selenium references was performed with the software Athena.

PART I. Distribution of selenium species by XAS

Results and Discussion

XAS study of reference selenium compounds

X-Ray Absorption Spectroscopy provides information on the local structure around a selected element. Every different local environment provides an additive contribution to the XAS spectrum.

If a sample contains one element with different environments, the resulting XAS spectra will be the sum of the spectra of the individual environments, multiplied for a coefficient corresponding to their relative abundance (see Figure 6).

For this reason, XAS is an outstanding chemical speciation technique, allowing the identification of the different species of a certain element present in the sample, as long as the spectrum of each component is measured individually as a reference.

For this reason, it is very important to obtain the spectra of the selenium species that can be contained in wheat tissues. The studies performed with HPLC-ICP-MS (see chapter 3) indicate selenomethionine, methylselenocysteine, selenocystine, selenite and selenate to be the most abundant species on wheat tissues. Moreover, elemental selenium could also be present in wheat roots, even if it was not successfully determined by HPLC-ICP-MS due to its insolubility.

These selenium species are commercially available as high purity standards. They were selected as references for the following XAS experiments and their spectra were acquired by transmission mode at the start of each experiment.

The data presented in this section were acquired in CLAESS beamline at ALBA synchrotron. However, very similar results were obtained from other synchrotron facilities.

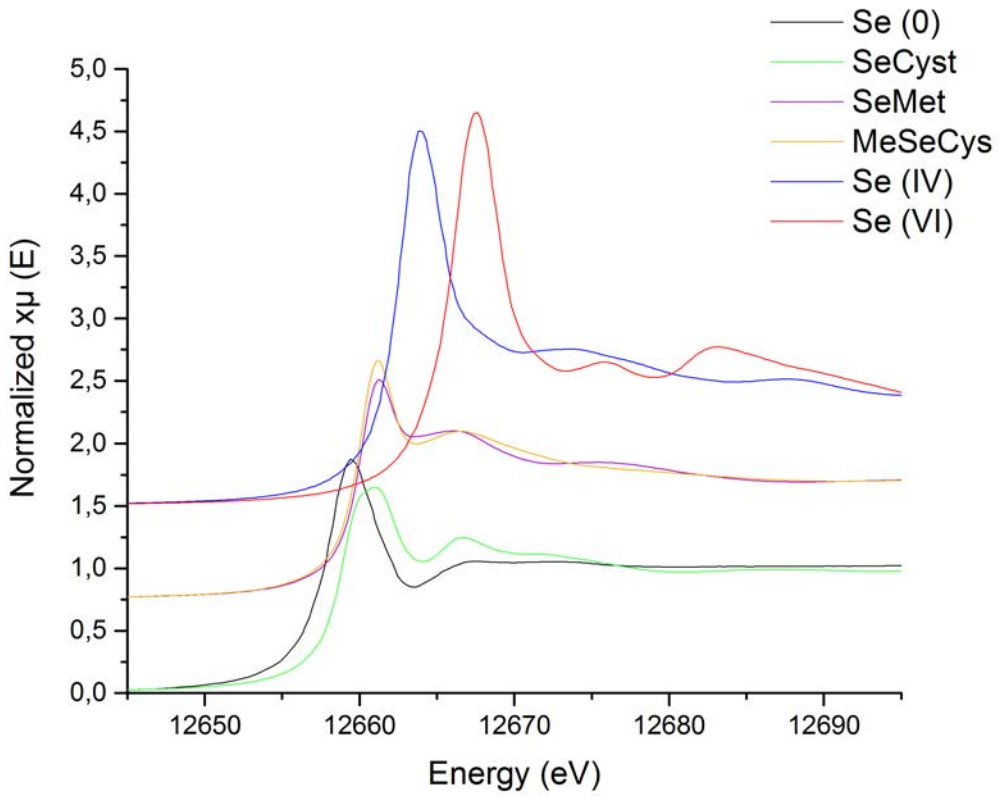

Figure 8: Normalized Se K-edge XANES spectra of the reference compounds: elemental selenium, selenocystine, methylselenocysteine, selenomethionine, sodium selenite and sodium selenate.

The analysis of the XANES region of the references showed distinctive edge position and features for the selected selenium compounds.

The edge position is characterized by E_0, the inflection point. E_0 correlates with the electron density around the selenium atom and accordingly with the oxidation state. E_0 at lower energies indicates more electrons closer to the selenium and E_0 at higher energies less electrons surrounding the selenium. E_0 for the selected selenium references can be found in table 1.

Elemental selenium has the edge at the lowest energy. Its E_0 depends on the electron binding energy of the element and can be found tabulated for the transitions of the different orbitals, being 12658 eV the required energy to remove an electron of the 1s orbital, the K-edge [4].

Organic selenium E_0 are found at intermediate energies. Selenocystine, which has a C-Se-Se-C structure, has a lower E_0 than MeSeCys and SeMet, which both contain a C-Se-C structure.

Inorganic selenium species have the edge at much higher energies because oxygen is more electronegative than selenium or carbon. In this sense, selenate has E_0 at higher energies than selenite, because of its higher oxidation state.

Regarding the selenoamino acids measured, SeMet and MeSeCys provided very similar spectra, with just a little difference in the intensity of the white line and the features (see Figure 8). This is because Selenomethionine and methylselenocysteine have the same scatterer atoms surrounding selenium (both containing CH2-Se-CH3 bonds), resulting in very similar Se XANES spectra. This fact makes their differentiation difficult with only XAS data. Moreover, this is also the case of other selenium compounds with the same structure around selenium, such as γ-glutamyl-Se-methylselenocysteine and seleno-cystathionine, which could be also present in plant samples [14], [15]. Accordingly, these species cannot be successfully elucidated and have to be determined as C-Se-C compounds. Furthermore, selenocysteine, the protonated form of methylselenocysteine, could also be present in the plant samples, and the spectra of this species is also very similar to those of MeSeCys and SeMet, even though the peak position of the major feature of Selenocysteine is shifted 0.1 eV to lower energies and it is slightly broader [16].

Otherwise, selenocystine presents a significantly broader feature due to two transitions 1s→σ* [Se-Se] and 1s →σ* [Se-C]. The edge position is found also at significally lower energies [16], around -1.3eV respect to SeMet (see table 1). Accordingly, it can be unambiguously distinguished from C-Se-C compounds.

In the same manner, selenite and selenate are also totally distinct, with edge positions at +2,6 eV for selenite and +6,2 eV for selenate respect to SeMet.

The acquired knowledge from the study of the selenium references will serve to identify these compounds contained in the different wheat tissue samples.

Radiation damage study

In order to ensure the stability of the measured species in the samples when they are irradiated by the high intensity X-ray photons from the synchrotron beam, two approaches were used: all measurements were carried out at a temperature around 77K using a liquid nitrogen cryostat and a radiation damage study was performed in one sample before starting the measurements, in order to confirm that during the time scale of the measurements there were no observable changes in the spectra. Two beamlines were used for the present study, with slightly different approaches.

At the beamline BM25A in the ESRF, measurements for radiation damage were carried out in fluorescence mode on a powdered grain sample at low temperature (77K). Several fast scans were acquired in one position, which are shown in Figure 9.

Since no changes were visible in observed in the XANES region, it is assumed that no changes are occurring to the selenium species during the measurement under these conditions. Therefore, measurements were performed with 4 repetitions in each sample.

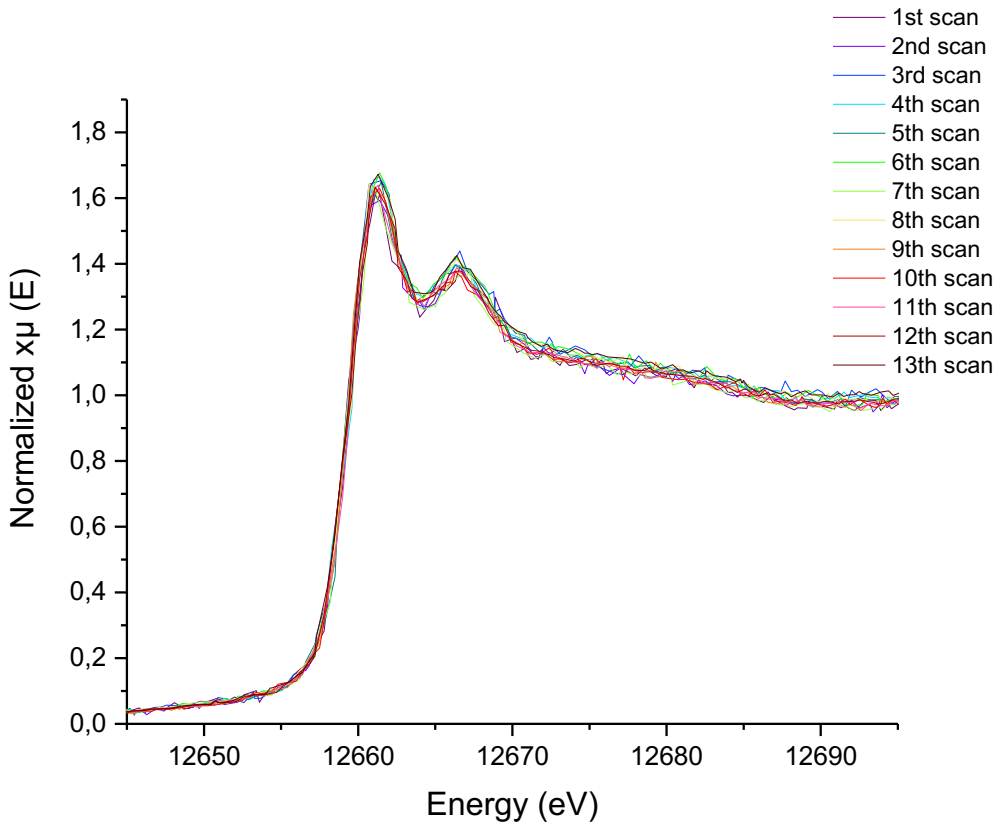

Figure 9: Example of a radiation damage study: normalized Se K-edge XANES scans measured in a grain sample, where no visible changes are seen in the electronic structure of selenium due to the beam radiation.

Since different beamlines have different settings and different intensity of the beam and photon flux, the radiation damage study was repeated at CLAESS beamline before the start of the experiment there, however, with a slightly different approach than in BM25A at the ESRF.

In CLAESS beamline, at ALBA synchrotron, measurements for radiation damage were performed also in fluorescence mode on a powdered grain sample, but in this case, at room temperature (~20^{o}C).

Ten fast scans of 2 minutes each were acquired with a focused beam of 200x90 µm in one point in the sample. After 20 minutes there was detectable radiation damage, where intensities of the features in the spectra started to change.

However, until 12 minutes of measurement there was no detectable change. Therefore, it was safe to measure the samples for at least 12 minutes. Additionally, the decrease of temperature for the actual measurements would prevent the radiation damage even further.

Therefore, the experiment was performed acquiring scans of 7 minutes each. In order to get enough statistics to achieve proper signal to noise ratio in the fluorescence, 10 repetitions of the measurement were obtained in 10 different points through the samples, which was possible thanks to the small size of the focused beam. In the data analysis these 10 repetitions were merged, obtaining an average spectrum with less noise.

Distribution of selenium species by XANES measurements in the different wheat tissues

The metabolization of the selenium species needs to be characterized in order to completely understand their different behavior between the three Se treatments. The XAS spectra acquired in BM25A at ESRF synchrotron of the selenium K-edge on wheat samples are shown in Figures 10 and 11 (they include the same spectra with a different organization in order to facilitate the comparison of the data).

The data include measurements in roots, shoots and grain of wheat plants enriched with selenite, selenate and a mixture of these two inorganic species. The main features of their spectra were compared with those of commercial selenium standards (seen in Figure 8). The position of E_0 and the existence of different features with their relative intensities were correlated with the presence of each species firstly in a qualitative manner. Secondly, a least-square linear combination fit of the references provided quantitative results as seen in table 2.

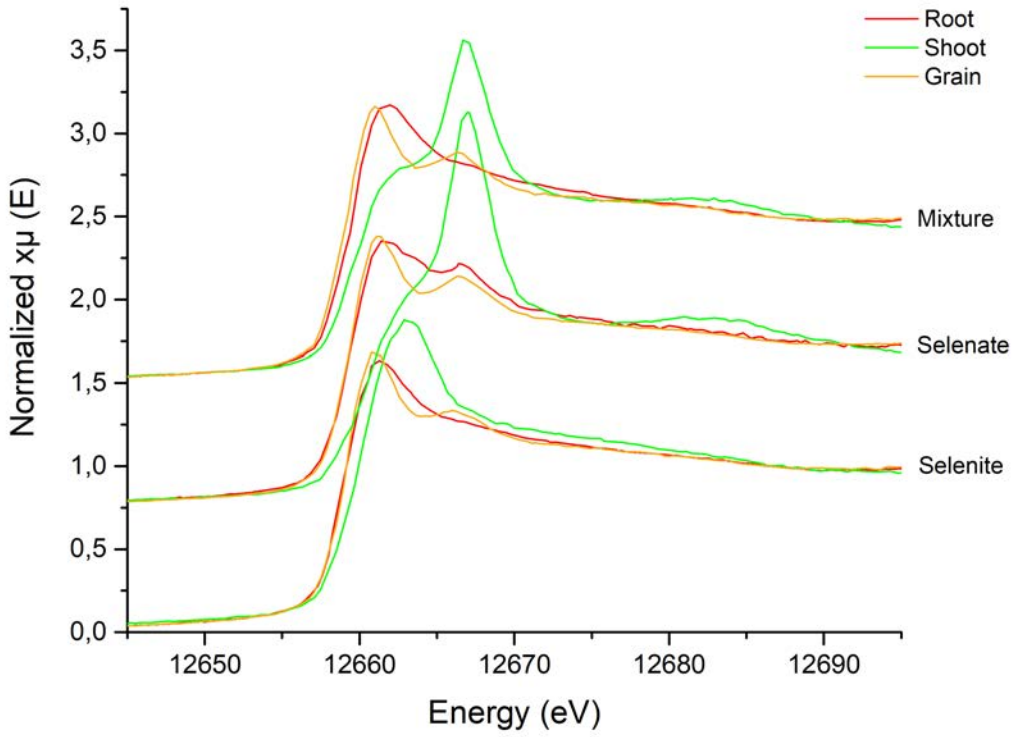

Figure 10: Normalized Se K-edge XANES spectra of roots (red), shoots (green) and grain (yellow) enriched with selenite (bottom), selenate (middle) and mixture (top) treatments, organized by type of treatment.

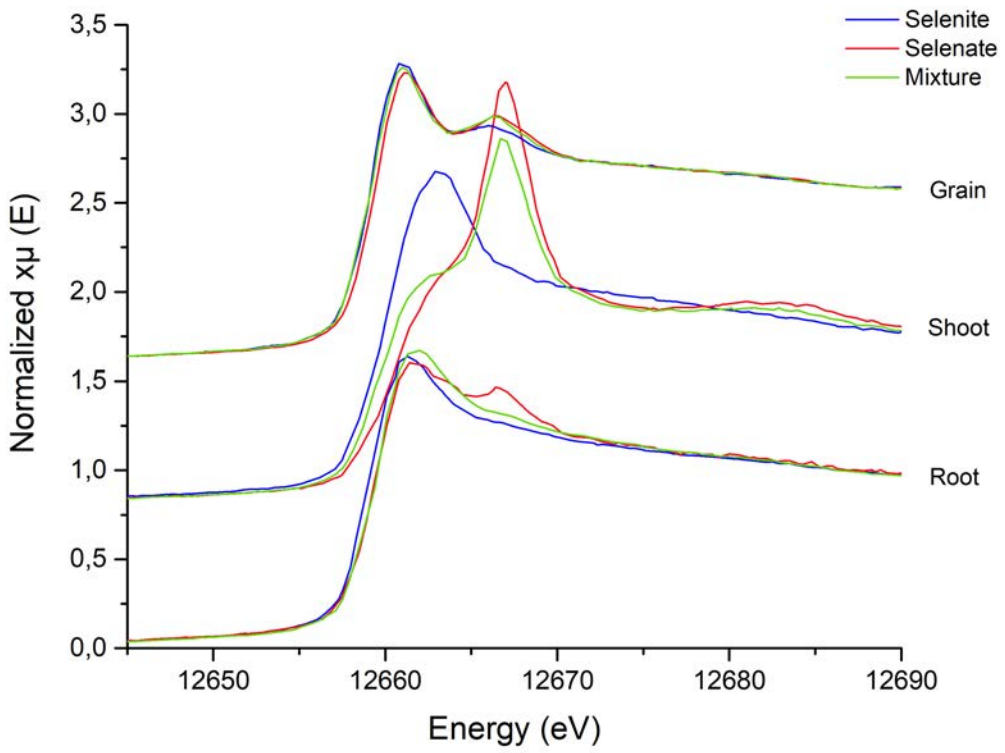

Figure 11: Normalized Se K-edge XANES spectra of roots (bottom), shoots (middle) and grain (top) enriched with selenite (blue), selenate (red) and mixture (green) treatments, organized by tissue type.

In order to perform the comparison of the spectra of the samples to those of the references, the E_0 position and the visible features were considered.

Table 1: E_0 value determined from the spectra of selenium references and samples.

Standard	E_0 (eV)	Sample	E_0 (eV)
Se (0)	12658,00	Root Se (IV)	12658,85
SeCyst	12658,53	Root Se (VI)	12659,15
MeSeCys	12659,30	Root Mix	12659,48
SeMet	12659,83	Shoot Se (IV)	12659,84
Se (IV)	12662,40	Shoot Se (VI)	12660,45
Se (VI)	12665,98	Shoot Mix	12660,35
		Grain Se (IV)	12659,17
		Grain Se (VI)	12659,35
		Grain Mix	12659,25

In samples, edge position is determined by the oxidation state and the ligands surrounding the absorbing atom and their geometry [17]. However, edges at smaller energies would indicate a higher amount of the reference compounds with lower E_0 (those more reduced or with more electrons closer to Se), while edges at higher energies represent the opposite effect.

Figures 10 and 11 display the comparison of the XANES spectra of roots, shoots and grains for the three different Se treatments. The comparison showed that the spectra profile obtained from the grains and roots was similar for all the treatments, being slightly more different for roots than for grains. Indeed, it resembled, in special for grains, the organic Se references. On the other hand, the spectra for shoots were markedly different depending on the Se-enrichment used. As it will be discussed in detail below, the case of shoots is far more complex due to the particularities of this part of the plant.

Speciation in roots:

From the spectra shown in Figures 10 and 11, it was possible to observe that in roots the main component was organic selenium.

Selenite enriched roots showed a single feature in the spectra, the white line, in an energy position characteristic of a predominance of organic species. The presence of inorganic species was not observed.

Selenate enriched roots spectra presented three features. There was a dominant organic selenium, a second shoulder which is characteristic of a small selenite presence, and the third feature, which was more marked, is characteristic of a significative selenate content.

The mixed enriched roots had only one feature, although this was wider than in the spectra of selenite roots, therefore, there were some differences in the species present.

The edge position in the three cases was characteristic of organic selenium. A comparison of the E_0 energy can provide an insight into the speciation of these organic Se species. Selenate and mixed enriched roots had slightly higher E_0 values than selenite roots. Therefore, this could correlate with more C-Se-C species in selenate and mixed roots and Se-Se bonds in selenite enriched roots.

The organic C-Se-C compounds could not be unequivocally assigned to SeMet and MeSeCys amino acids,since the spectra of roots did not show the characteristic decrease in intensity and increase in a second feature that can be seen in all the selenoamino acid references, but just a single feature. However, SeMet has this structure less marked, and therefore its presence is more probable than the one of MeSeCys (see Figure 8).

Regarding the Se-Se content in selenite enriched roots, it could be due to elemental selenium or SeCyst species. HPLC-ICP-MS results from chapter 3 did not indicate a significant SeCyst content, but, a content of elemental selenium was hypothesized due to low extraction efficiencies. The elemental selenium presence would agree with the lower E_0 values for this type of enriched samples, what was confirmed by the linear combination fit results in Table 2.

Finally, from the root analysis it could be hypothesized that the inorganic selenium taken up by the roots from the hydroponic solution was converted into organic selenium efficiently. Selenite conversion was fast, and this specie was not detected in selenite enriched roots. The resulting organic species accumulated in the roots. Moreover, some elemental selenium seemed to be present. Selenate conversion was slower, and selenate was still detected in selenate enriched roots, as well as some selenite, which is the intermediate specie in the reduction pathway. Roots enriched with the mixture did not show selenate, because its concentration in the hydroponic solution was half of that of selenate enrichment, but it seemed that some selenite may still present. These qualitative results will be confirmed by a quantitative analysis by a linear combination fit of the sample spectra with those of the standards in the following sections.

Speciation in shoots:

In wheat shoots enriched with selenite there is a predominance of organic species. However, selenate and mixed treatments showed more abundance of inorganic species in shoots.

Selenite enriched shoots showed a single feature, very broad, corresponding to the white line. The range of energy of this feature contained inside the energy of the main feature of both organic selenium and selenite species. The signals of these two species could have overlapped resulting in this unique broad feature in the sample and thus, it could indicate that both organic Se and selenite are present in substantial amounts.

Selenate supplied shoots contained a small amount of organic species and selenite, and the predominant form was selenate. This can be seen from a small first shoulder in the edge jump and a main feature in the spectra matching that of selenate.

In the mixture treatment, selenate was still a predominant component but its content was smaller than in the selenate application, resulting in a less intense feature. Higher amount of organic Se and selenite was seen in a more defined shoulder at lower energies.

These results are in agreement with a higher mobility of selenate through the wheat xylem, and a faster reduction of selenite to organic Se species.

The study of shoots provided an idea about the process of translocation and accumulation of selenium species in above-ground tissues. Since Se species are transported through the vascular system in the stem, but then they can accumulate in the leaves vacuoles, it is important to study the shoots as a whole to determine the form in which selenium is stored. However, in order to obtain a more detailed knowledge on the Se metabolization and translocation processes from roots to shoots, the first three centimetres of the stem were investigated separately in an additional experiment. The results are shown in Figure 12.

In all cases, the analysis of the speciation in the first three centimetres of the stem showed a bigger organic content than the combination of the complete stem and the leaves.

Selenite enriched stem section showed a narrower peak than the full shoot, indicating less selenite content. Selenate enriched stem section showed a much smaller amount of selenate. And the mix enriched stem section did not show selenate presence at all.

Therefore, it can be deduced that the organic selenium was synthesized in roots, and, in combination with the remaining inorganic selenium not metabolized, was transported through the plant xylem into the shoots. However, the diffusion coefficient of organic selenium is smaller than that of selenate [18], [19]. Therefore, uncomplexed inorganic selenium is highly translocated into all tissues in the aerial part of the plant, but C-Se-C compounds have low mobility through the plant xylem and are more abundant in the lower parts of the stem. This result is in agreement with previous findings in cereal plants [20].

Furthermore, the higher abundance of selenate when the complete shoot was measured than when only the lower part of the stem was analysed can also be explained by a sequestration of inorganic selenium in vacuoles, as it happens with sulfur.

It is well known that sulfate is assimilated and reduced in chloroplasts [21], [22]. However, when the concentration in the xylem is too high, the part that cannot be assimilated by chloroplasts is stored in leaf vacuoles [21]. Sulfate in vacuoles is not metabolized, it is innocuous to the plant and it is very little remobilized [21]. The same phenomena occurs with selenate [21]–[23], that use the same sulfate transporters [24].

Since results from chapter 2 demonstrated a high concentration of selenium in selenate and mixed enriched shoots, the higher content of selenate in the shoots (which include leaves) than in the stem alone could lead to the conclusion that part of this selenate is indeed sequestered in leaf vacuoles.

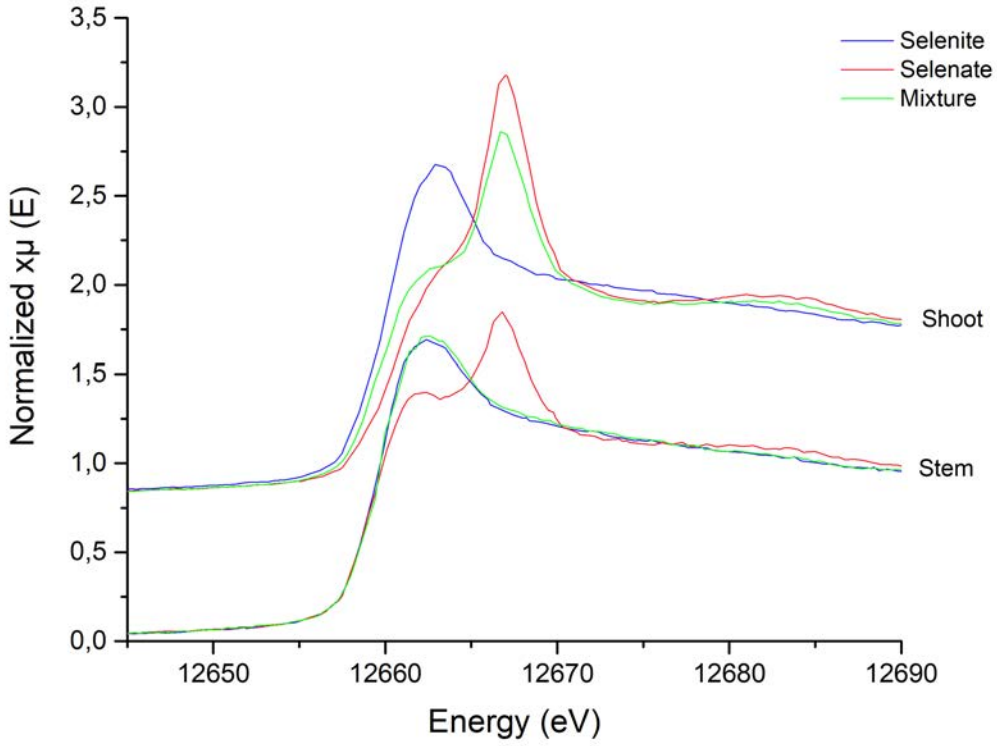

Figure 12: Normalized Se K-edge XANES spectra of shoots (top) and the first centimeters of the stem (bottom) enriched with selenite (blue), selenate (red) and mixture (green) treatments.

Speciation in grain:

In grains, organic species were the principal Se compounds, as the sample spectra resembled those of the selenoamino acid references in the edge position and the two features.

As can be seen in Figure 11, the three enrichment types provided similar spectra for the grain samples.

However, the edge position differed between the three treatments, providing an insight into the organic selenium composition of the sample.

Selenite enriched grain had the edge at the lowest energy. Mixed enriched grain had the edge at a middle energy position and selenate enriched grain at the highest energy. The edges of the three samples were found within those of the C-Se-Se-C reference (SeCyst) and the C-Se-C references (SeMet and MeSeCys), as seen in table 1. Accordingly, selenite enriched grain was expected to have the highest proportion of C-Se-Se-C respect to C-Se-C containing species, mixed enriched grain a middle proportion

and selenate enriched grain the lowest amount of C-Se-Se-C species. Quantitative results from linear combination fits will be used to confirm this qualitative observation.

Moreover, selenate and mixed enrichments showed a slightly more marked second feature than selenite enriched grain. This higher intensity could be due to both a higher content of selenate (which has the white line at that energy) and a different proportion of organic compounds that have a second feature at that energy (such as C-Se-C amino acids).

However, due to the similarities in the spectra of the C-Se-C compounds, the small differences in the second feature of grain samples could not be unequivocally assigned to a specific C-Se-C compound.

Quantitative speciation:

In order to get a more quantitative information, a linear combination fitting (LCF) analysis was performed using the Se reference standards mentioned previously. This methodology allowed a more reliable assessment than the fingerprint analysis of the chemical speciation present in the different parts of the plant.

In addition to a good visual agreement between the data and the fit, a R factor lower than 0,005 and a small value for the reduced chi square can also indicate the goodness of the fit. In general, a good fit indicates that the combination of references in the indicated proportions can be used to describe the species in the sample. The results obtained from the linear combination fit are shown in Table 2 and they agree with what was estimated by qualitative visual investigation of the spectra.

Table 2: Best fits from least-square linear combination of selenium standards to represent the spectra of the samples. R factor and reduced chi square indicate the goodness of the fit. Samples written in italics indicate results that should be considered an approximation, either because their R factor of their fit was too big (R factor>0,005) or because the visual comparison of the spectra and the fit did not match completely.

	R factor	**reduced chi-square**	**Se (IV) (%)**	**Se (VI) (%)**	**C-Se-C (%)**	**C-Se-Se-C (%)**	**Se (0) (%)**
Root Selenite	*0,00271*	*0,00072*	*n.d.*	*n.d.*	*82±6*	*n.d.*	*18±8*
Root Selenate	0,00096	0,00026	4,4±0,7	1,4±0,3	94±5	n.d.	n.d.
Root Mixture	*0,00216*	*0,00059*	*n.d.*	*n.d.*	*99±7*	*n.d.*	*1±1*
Shoot Selenite	*0,00981*	*0,00285*	*19±2*	*n.d.*	*81±14*	*n.d.*	*n.d.*
Shoot Selenate	*0,00922*	*0,00314*	*19±2*	*47±1*	*35±15*	*n.d.*	*n.d.*
Shoot Mixture	0,00466	0,00137	12±2	32,1±0,8	56±11	n.d.	n.d.
Grain Selenite	0,00267	0,00070	n.d.	n.d.	57±6	44±2	n.d.
Grain Selenate	0,00116	0,00031	n.d.	1,5±0,3	74±5	25±2	n.d.
Grain Mixture	0,00195	0,00050	n.d.	n.d.	62±6	38±2	n.d.

However, some of the samples provided fits that were considered to be unsuccessful in representing the totality of the spectra with the considered selenium references. Those samples were the ones whose fits had R factors>0,005 or that the resulting fit did not visually match totally the observed features of the spectra. Those fits should be considered only as semi-quantitative results, which can provide a better idea of the amount of the Se species than only a qualitative consideration of the spectral shape. Additionally, it is worth mentioning that the species identified at concentrations lower than 5% can be considered within the error level of the methodology and should be treated with caution.

From the linear combination fits it was confirmed that the predominant species in roots were, in every treatment, C-Se-C compounds. Moreover, the content of inorganic selenium in roots seems to be higher in selenate treated roots, even if these species are below a 5%. Additionally, it was indicated the presence of elemental selenium in selenite enriched roots, which explains the edge at lower energies observed in Figure 11, and the very low extraction efficiency of selenium with an enzymatic digestion of this sample, observed in chapter 3. Further discussion of the pathways to produce elemental selenium in plants can also be found in chapter 3.

Regarding shoots, the linear combination fit confirmed that selenite enriched shoots contained predominantly C-Se-C selenium and some unmetabolized selenite. Oppositely, selenate enriched shoots accumulated mainly selenium in the form of selenate, but some organic C-Se-C and some selenite were also found. Finally, in mixed enriched shoots organic C-Se-C was the most abundant form but there was also an important presence of selenate and less amount of selenite.

Finally, the linear combination resulted in very good fits for grains. The three enrichments resulted in grains containing both C-Se-C species and C-Se-Se-C species.

However, grains enriched with selenite had a predominance of C-Se-Se-C, which corresponds to selenocystine, grains enriched with selenate a bigger amount of C-Se-C selenoamino acids, and mixed enriched grains intermediate amounts. These proportions matched the trend observed for the E_0 position of grain samples: selenite < mixture < selenate

As discussed previously in chapter 3, this diversity in the production of organic amino acids, depending on the enrichment strategy used, is due to the different behavior of each species in the wheat plant.

Selenite and selenate are taken up by wheat roots by different transport pathways. Selenite reduction into elemental selenium and organic selenium is faster, but these species have low diffusion coefficient. Therefore, they accumulate in the roots in high concentrations, as seen in chapter 2, and cause plant stress and toxicity, resulting in smaller biomass production and the accumulation of reactive oxygen species (ROS). These ROS species create an oxidative environment in the whole plant tissues that

later result in an enhanced oxidation of selenocysteine residues to selenocystine in the grain [25], [26], [27]. Oppositely, selenate reduction is slower than its translocation to shoots. Therefore, selenate is preferentially translocated to above-ground tissues, where a considerable amount accumulates in the vacuoles of the mesophyll cells of leaves. Selenate in the vacuoles is not easily reduced or remobilized, as happens with sulfate, and thus, it accumulates unchanged. This selenate stored in the vacuoles is innocuous to the plant, and so, it does not promote an oxidizing environment, which results in methylated selenoamino acids in the grain [14], [21], [28].

Significantly, the different amino acid synthesis serves as a plant defense mechanism. It is known that SeMet, which is the major species produced in cereal seeds, can be non-specifically incorporated into proteins, leading to protein misfolding and thus, altered protein structure and function [29], [30], [31]. Therefore, excess of selenium results in plant toxicity. Oppositely, other plant species store selenium in other forms as a mechanism of selenium tolerance. Hyperaccumulators produce MeSeCys, which cannot be incorporated into proteins and is found as a free amino acid, thus reducing the selenium induce toxicity and allowing the plant to tolerate much higher selenium concentrations.

A similar mechanism is encountered in this case. Plants counteract the toxicity induced by selenite enrichment by the formation of selenocystine, which, as MeSeCys, cannot be incorporated into proteins and is stored as free amino acids. Consequently, the toxicity of selenite, which is already affecting the overall biomass yield, is compensated in seed production by the formation of less harmful forms of selenium.

Furthermore, little or no inorganic selenium is translocated to the grain. Although all the conditions have an important amount of selenate and selenite accumulating in the shoots, very little of it reaches the reproductive organs, with only organic selenium being effectively translocated. This is highly beneficial in order to produce a food product with the selenium enriched grain, since it is known that inorganic selenium is more toxic than organic counterparts [32].

Validation of the selenium speciation in grain

In order to confirm the significance of the obtained results, it was necessary to determine the reproducibility of the speciation analysis through different plants and cultivations.

Grains from different plants, biofortified with the same Se treatments and conditions as the previously discussed samples, were studied in CLAESS beamline, at ALBA synchrotron.

In order to be able to compare with the results from BM25A at the ESRF, considering the different beamline configuration in each synchrotron, the Se references and one previously studied sample were analyzed again at CLAESS, showing a good agreement.

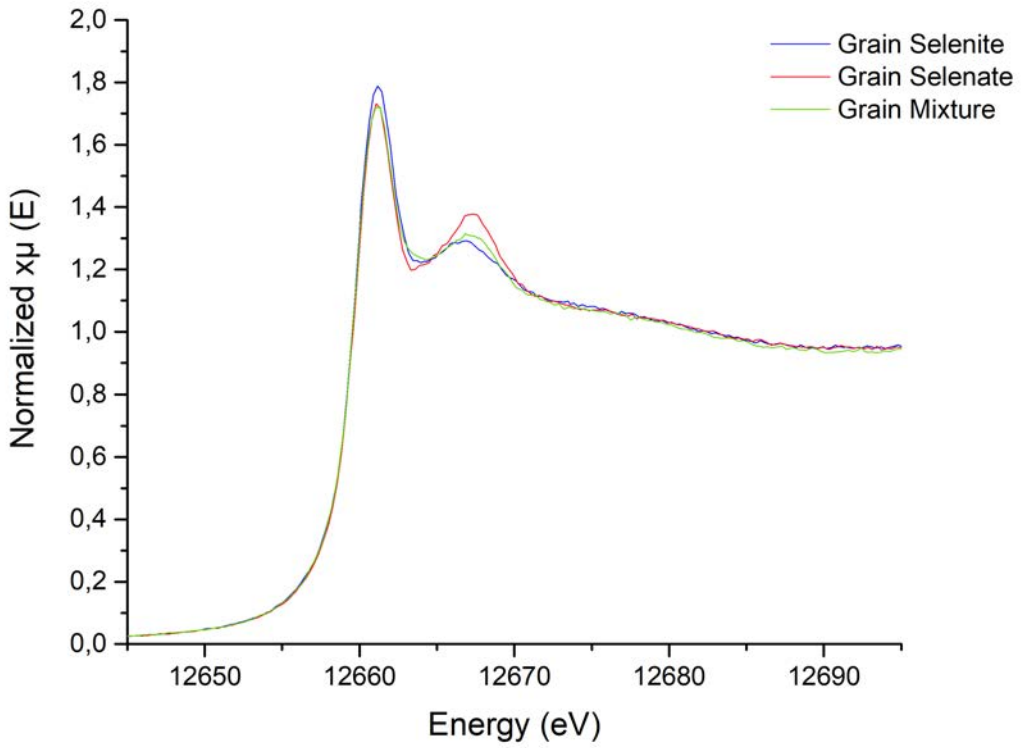

Figure 13: Normalized Se K-edge XANES spectra of grain from different plants, enriched in the same conditions*, enriched with selenite (blue), selenate (red) and mixture (green) treatments.*

In general, the same trends were observed in the samples (Figures 11 and 13): Selenite had a more intense white line than selenate and mixture enrichments. Also, selenite had a less intense second feature, whereas selenate presented the more intense one.

Finally, the biggest difference between the measurements in different plants are found in the edge position. In the results from CLAESS beamline, changes in edge position were less noticeable at the chosen scale, even if the trend of the position follows the same pattern of selenite < mixture < selenate.

Results were quite reproducible between plants. Obviously, there were small differences present, however, plants are different individuals with slightly different metabolic responses to selenium. Therefore, results will always have to consider relatively big variability. However, the changes noticed in the XANES spectra between selenium treatments always resulted in the same trends, irrespective of the plant individuum chosen. Selenite resulted in all cases in E_0 at smaller energies, more intense

white lines and less marked second features; selenate resulted in E_0 at higher energies and more marked second feature and mixed treatments had an intermediate behavior between those of selenite and selenate.

These differences between treatments are explained by the different selenium speciation depending on the Se specie used in the enrichment, as previously discussed.

Conclusions

XAS techniques allow the proper identification of selenocystine, C-Se-C aminoacids, selenite and selenate, although they fail to distinguish among C-Se-C amino acids due to the similarities of the XANES spectra of SeMet and MeSeCys.

Furthermore, XAS techniques provide speciation results by direct analysis of the sample, without the requirement of species extraction, and therefore ensuring that all species in the sample are considered, independently of their state. Besides, the stability of the species is ensured as XAS does not require a chemical pretreatment of the sample, and the use of fast measurements at liquid nitrogen temperatures prevents any possible radiation damage.

Thus, XAS confirmed the presence of elemental selenium in selenite-enriched roots, which is difficult to be determined by conventional methodologies due to its low solubility. The accumulation of elemental selenium in roots could have important implications in selenite metabolism, and proper quantification is required in order to assess the implications of selenite enrichment of wheat plants.

Furthermore, XAS results confirm and further extend the findings from chapter 3. It is seen how roots accumulate mainly organic selenium in the form of C-Se-C. Selenite enriched shoots show little inorganic selenium, due to this specie being quickly reduced, but selenate enriched shoots show an important accumulation of inorganic selenium, because a slower selenate reduction than selenate translocation.

This shows a very different behavior of the two inorganic selenium salts, which needs to be considered when considering the appropriate enrichment strategy. Selenium induced plant toxicity may be reduced by changing the enrichment conditions, so wheat cultures may be able to tolerate higher amounts of selenium. But other factors resulting from speciation information needs to be considered as well. Selenite results in higher toxicity to the plant and reduced yield at 10 µM, however, it leads to less accumulation of inorganic selenium in wheat shoots. These shoots could be used as straw to feed cattle. Then, the amount of selenium accumulation in shoots should be considered carefully, in order to determine the range in which selenium enrichment benefits the animals without reaching the limit when inorganic selenium accumulation leads to toxicity related symptoms in the cattle.

Moreover, this different metabolization of the two salts also results in different C-Se-C/C-Se-Se-C ratio in the grain, and accordingly different proportion of selenoamino acids. These different results depending on the enrichment condition also has relevant implications, since SeMet can be incorporated into proteins, and therefore lead to impair the plant reproductive success, but SeCyst and MeSeCys are accumulated as free amino acids and can be stored in higher amounts without causing toxicity.

Furthermore, the diverse amino acids also have different implication for human health, as they have different activity in the body. Therefore, a functional food preparation could benefit from a determined amino acid proportion depending on the application desired.

The use of the two salts, selenite and selenate, results in an intermediate ratio of the amino acids. Thus, the use of a mixture in a certain proportion would allow the tuneability of the resulting proportion of the amino acids as desired.

Moreover, in real field conditions the selenite/selenate ratio would depend on the pH and pE conditions of the specific soil. The use of mixtures of the two salts would permit to adjust the initial proportion in order to maintain the amino acid production and ensure the reproducibility of this proportion in different culture conditions.

PART II. Grain study by XRF and µXAS

Results and Discussion

The application of X-ray fluorescence mapping and X-ray absorption spectroscopy using a microfocused beam allows the obtaining of more detailed speciation information. It permits to differentiate not only between tissues (like roots, shoots and grain) as with bulk XAS, but also to distinguish within tissue parts, differentiating certain regions where an element or a chemical species accumulates preferentially.

It is relevant to identify the specific areas of the grain where the selenium species accumulate since most flour preparations only contain the endosperm of the wheat grains, i.e. the bran and the germ are discarded in the refining process [33], [34]. The amount and speciation of selenium in these regions may be different, and thus, the possible selenium enriched food prepared would be significantly different depending if a conventional preparation or a whole-grain flour preparation was selected.

Hence, a further detailed study of grain was required in order to complement the information obtained from the XAS experiments on ground grains. The main objective was to fully understand the metabolization and accumulation pathways of the different selenium enrichment conditions (selenite, selenate and mixtures) that lead to the accumulation of different selenium species in wheat grain, such as a different

selenoamino acid proportion. Moreover, it was investigated whether this proportion is maintained through the whole grain, or if a specific region of accumulation is responsible of the observed values.

Distribution of selenium and other nutrients in wheat ears

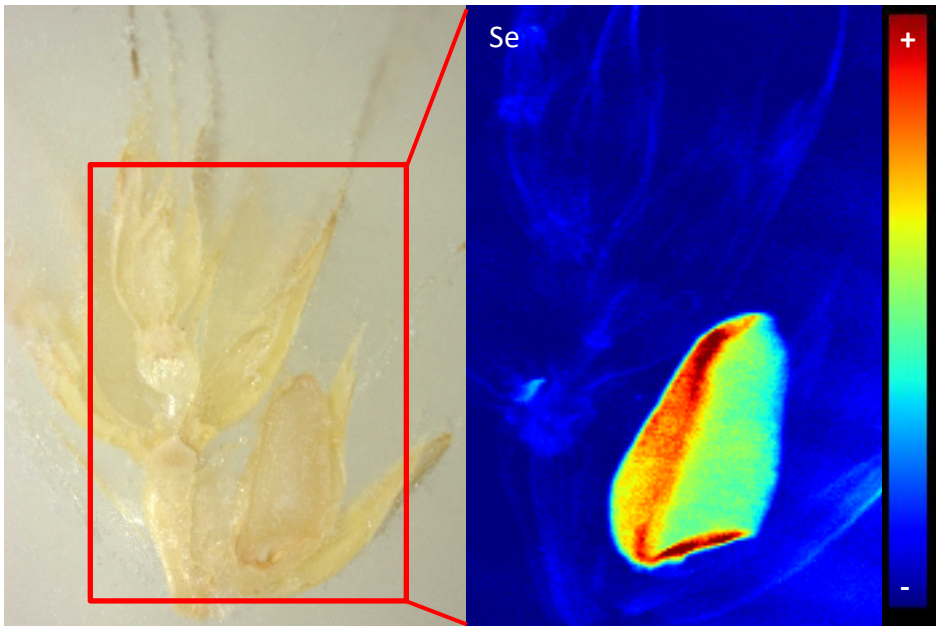

Figure 14: Study of the selenium distribution in a section of the ear of a senescent plant, containing one grain. Visible image obtained by a microscope, with the area studied by XRF marked by a red square. Selenium fluorescence map, where higher concentrations are shown in warmer colors and lower concentrated regions appear in blue, as indicated in the legend.

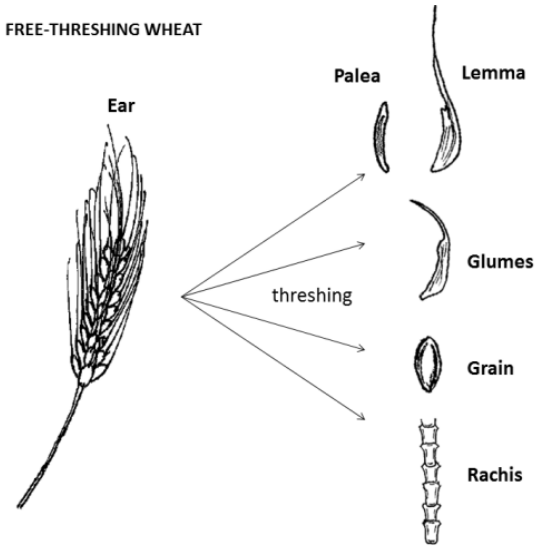

Figure 15: Parts on a wheat ear. Reproduced from [35].

A section of a wheat ear containing a grain was mapped in order to identify the distribution of selenium through the reproductive organ when the ear has reached maturity and ripe grains are present inside spikelets. In order to perform the study, the sample was embedded in paraffin and cut with a microtome, resulting in a flat surface with the interior of the ear and the grain visible. The prepared sample and the acquired XRF map are shown in Figure 14, and the corresponding parts in a wheat ear are detailed in Figure 15.

The results showed that there was very little remaining selenium in the ear rachis and the spikelet husk. On the contrary, selenium accumulated preferentially in the grain. Therefore, translocation of selenium was efficient, and all selenium reaching the ear was successfully stored in the grain. There it could serve as a nutrient for the development of the embryo in a future plant seedling or as a nutrient in flour preparations.

Besides, it can be noted that the distribution of selenium in the grain was not homogeneous. There was an uneven concentration of selenium through the grain endosperm, and there was as well an important accumulation in the grain germ. For this reason, it is important to perform a detailed μXRF experiment to determine the distribution of elements in the grain.

In addition, the different distribution of selenium respect to other nutrients was also investigated. Thus, K, Ca, P, S, Mn, Fe, Cu and Zn were also monitored during the XRF mapping (see figure 16).

From the images it could be inferred a different accumulation strategy on ears for selenium and for different nutrients:

K and Ca presented similar intensity in the ear rachis, the spikelet husk and the grain. Therefore, these two macronutrients were not predominantly stored in the seed but were distributed through the entire ear. This could mean that they have a role as a structural constituent of the ear and not only as a nutrient for the future seedling. Regarding the grain, they were mostly visible in the grain bran and germ, with practically no presence in the grain endosperm. This is due to endosperm being mainly composed of starch and non-starch polysaccharides, but very few nutrients [36].

P and S, also wheat macronutrients, were similarly stored mainly in grain bran and germ, with scarce presence in the endosperm. In contrast to K and Ca they do not serve as an important constituent of the ear, as no presence was detected in the rachis and just a little was found in the tips of the spikelet lemmas.

The micronutrients Mn, Fe, Cu and Zn were also preferentially stored in the germ, as occurred with the macronutrients. But, in contrast, they were not significantly located in the bran, and tended to be more homogenously spread through the endosperm. Mn and Fe were also located in parts of the spikelet husk but Cu and Zn were not.

Otherwise, the intensity measured for all nutrients was relatively smaller than the measured for selenium. Although, the fluorescence is proportional to the amount of element present in the sample, comparing intensities of different elements to extract quantitative concentration information is not straightforward. This is because the fluorescence yield decreases with the atomic number [1]. Therefore, elements with lower atomic number, such as P and S, resulted in lower fluorescence signal than elements with higher atomic number such as Se, disregarding the concentration. For this reason, P and S also presented higher levels of background noise.

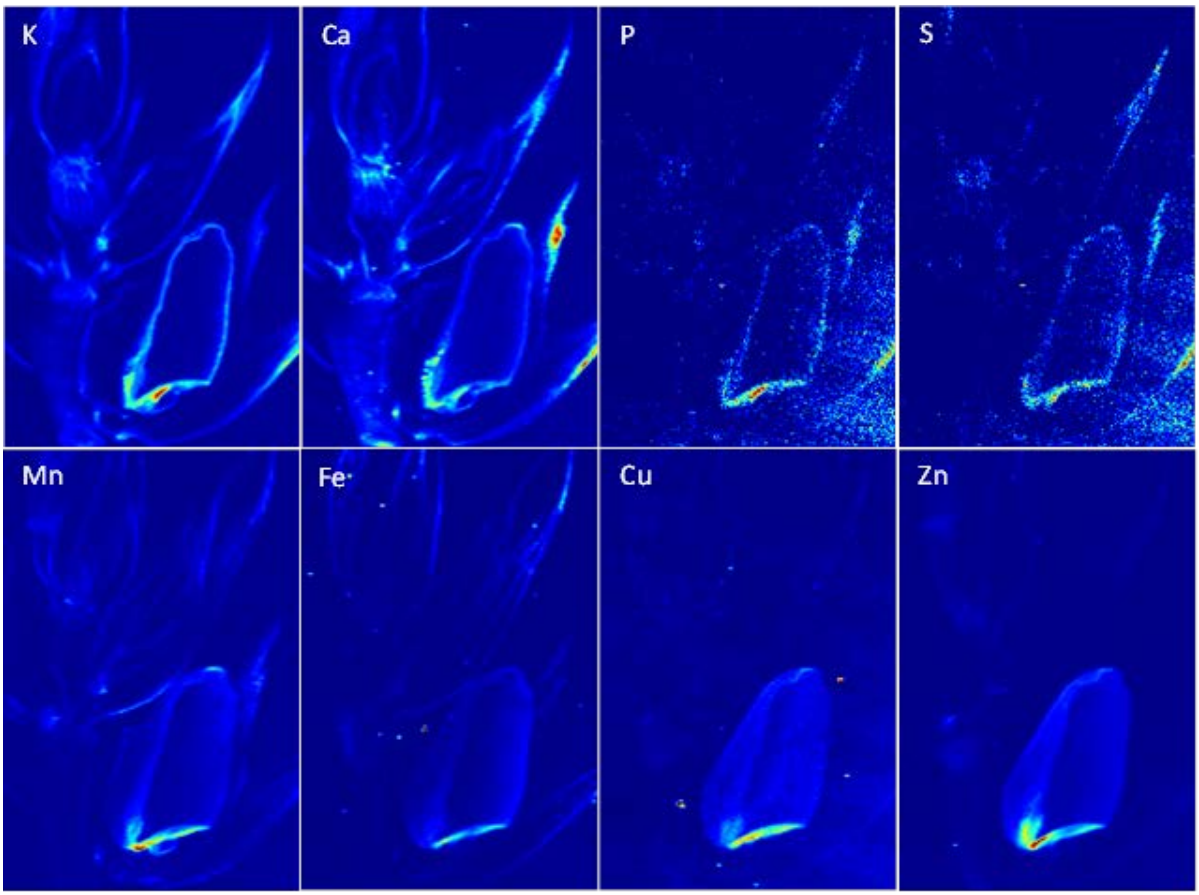

Figure 16: Elemental distribution in a section of the ear of a senescent plant, where one grain is visible, for K, Ca, P, S, Mn, Fe, Cu and Zn respectively. Warmer colors indicate higher concentration of the element.

Distribution of selenium and other nutrients in grain

From the XRF images of three grains enriched with the three different selenium conditions, shown in Figure 17, it was possible to distinguish similar accumulation patterns, through the grain regions detailed in Figure 18.

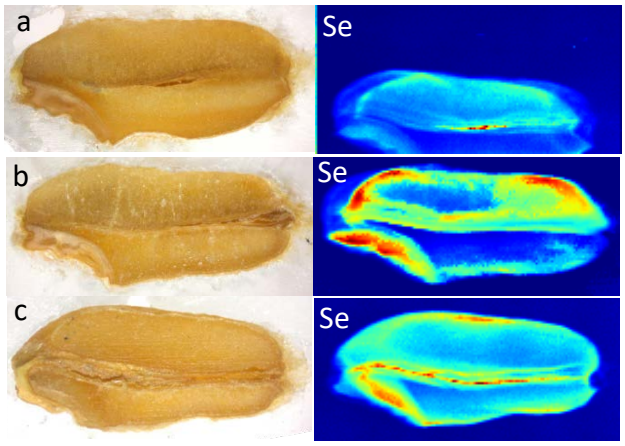

Figure 17: Selenium distribution in sections of wheat grains, enriched with a) selenite, b) selenate and c) mixture of selenite and selenate. Visible image obtained by a microscope, and selenium fluorescence map at 12677 eV, where warmer colors indicate higher concentration.

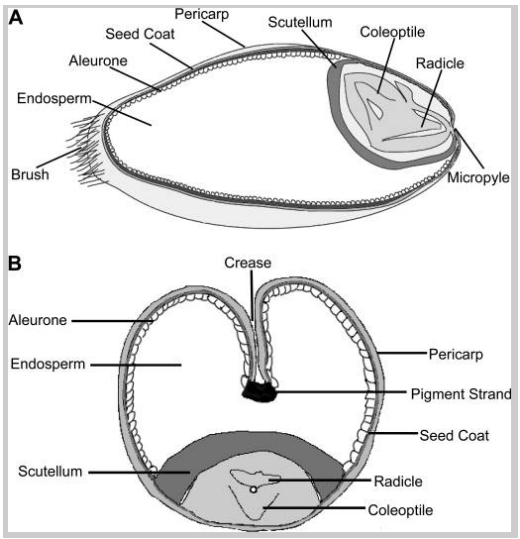

Figure 18: Parts on a wheat grain, from two different perspectives. Reproduced from [37].

The three grains showed higher accumulations in the germ, the bran and the pigment strand, and little selenium presence in the endosperm. However, there were also some detectable differences.

In the grain enriched with selenite, the majority of the selenium was very localized, with a significant amount present in a small area of the pigment strand. Despite the fact that the grain was not fully mapped due to issues during the measurement, part of the germ was visible, and it showed that the concentration of selenium there was lower in comparison to the two other enrichment conditions.

The grain biofortified with selenate presented a significant amount of selenium located in the germ, as well as in the bran and in the pigment strand. On the other hand some areas of the endosperm were practically empty of selenium, which was not as evident in the other treatments.

The distribution of selenium in the mixed enriched grain showed also a significant accumulation in the germ, the bran and the pigment strand. In the endosperm there was much less selenium amount, even if some radiation is still detected through all the endosperm, contrarily to what was observed with selenate treatment.

In conclusion, there was an uneven distribution of selenium through the grain in all the treatment conditions. The germ, the bran and the pigment strand were high accumulating parts of the grain. Oppositely, the grain endosperm tended to store lower selenium content.

The trend of selenium storage inside the grain was compared to that of other nutrients, in order to see if the distribution followed a common pathway for all nutrients or there were appreciable differences between them.

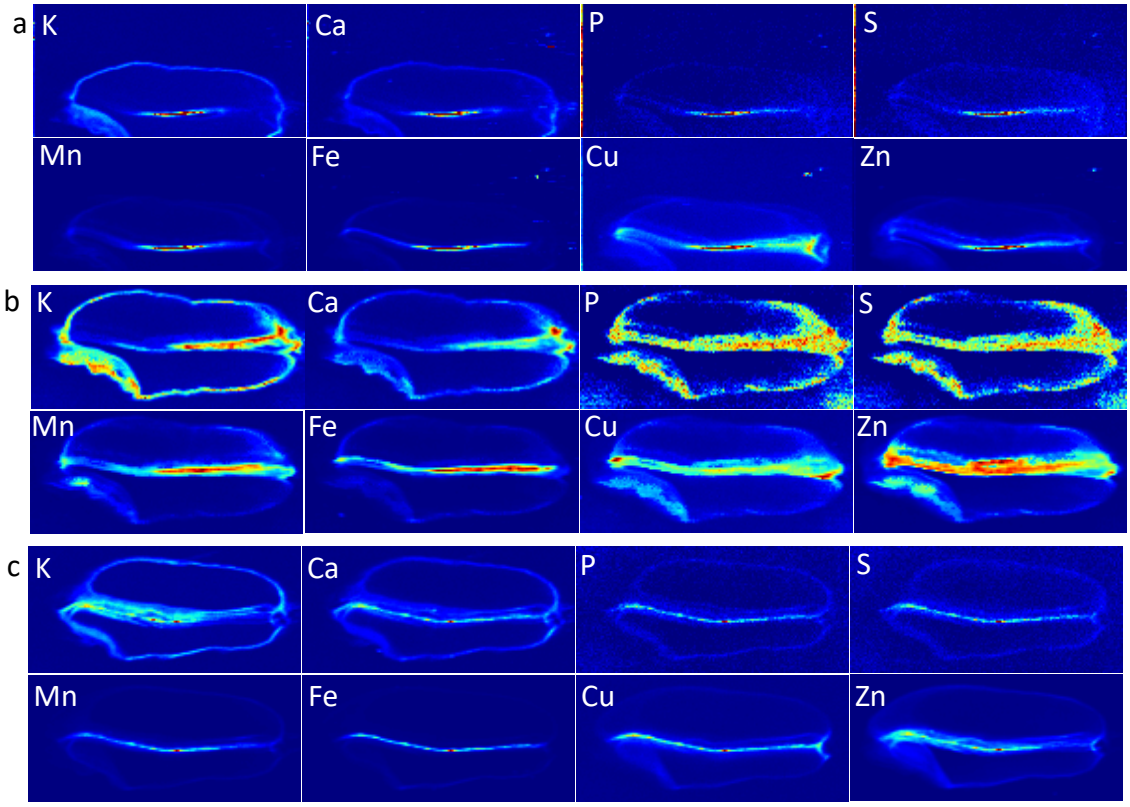

Figure 19: Elemental distribution in sections of wheat grains, enriched with a) selenite, b) selenate and c) mixture of selenite and selenate. Images of K, Ca, P, S, Mn, Fe, Cu and Zn fluorescence. Warmer colors indicate higher concentration of the element.

As shown in figure 19, nutrients accumulated in similar points as selenium, such as the germ, the bran and the pigment strand. However, none of the nutrients were found within the endosperm tissue.

K and Ca were the most abundant in the bran and homogenously distributed through it, and as well, were found in the center of the pigment strand and in the germ. P and S were located through the entire pigment strand and in the germ, and also in parts of the bran. Mn and Fe were present mostly in the pigment strand, and very little was found in the germ and the bran. Cu and Zn showed higher accumulation in the pigment strand and also in the germ.

Moreover, Se and S distributions presented similar distributions, because these elements have common metabolization pathways and selenium tends to accumulate substituting S positions in proteins [38].

Besides, selenate treatment seemed to enhance the accumulation of nutrients in the grain endosperm and germ, for all the studied nutrients.

The different distribution of selenium and other nutrients through the grain tissues is due to their different chemical composition. The endosperm accounts for an 80-85% of the dry mass of the grain and is known to be rich in polysaccharides, such as starch. The bran contains the pericarp, the seed coat and the aleurone layer and it represents the 13-17% of the grain dry mass. The bran contains a lot of fiber, but the aleurone layer is rich in proteins and enzymes. Finally the wheat germ (or embryo) contains the 2-3% of the grain dry mass and is richer in proteins and fats than the other parts [39], as shown in detail in table 3.

Table 3: Composition of wheat endosperm, bran and germ as found in the USDA database [40].

Grain Part	Protein (g/100g)	Lipid (g/100g)	Carbohydrate (g/100g)	Fiber (g/100g)
endosperm	10,33	0,98	76,31	2,7
bran	15,55	4,25	64,51	42,8
germ	23,15	9,72	51,8	13,2

Selenium is highly associated with protein fractions, as the selenoamino acids, the main selenium species in grain, can be incorporated in the protein structure or bonded to it [29]. Other nutrients are also part of proteins, such as P and S, or can form part of metallothioneins, such as Zn and Cu [41]. Since proteins are present in all the grain tissues, the elements associated to them will be more evenly distributed to the grain that nutrients that are not [42].

The germ is the part of the grain that will develop into a new plant and, for this reason, there is an important accumulation of nutrients in this tissue. Moreover, this part is richer in proteins than the rest of the grain, and thus, is able to store both selenium and nutrients.

The bran is composed of several layers. The aleurone layer is particularly rich in proteins and is known to accumulate Se, P, S, Fe and Zn among other mineral nutrients [38], [43], [44]. A preferential accumulation of K in bran was also previously reported in barley grains [45].

The pigment strand is part of the vascular bundle (which contains the xylem and phloem [46]) and is located in the crease area in the ventral part of the grain. It has also been reported in barley grains to accumulate high amounts of nutrients [45].

The wheat endosperm contains mainly polysaccharides and macromolecules. The sugars will serve as a supply of carbon in the development of the embryo into a new plant [36], [47], [48]. However, it contains much less nutrients (0,5-1,5%) than the germ (4,5%) [47].

This lack of nutrients in the endosperm result in poor flour preparations from cereals, due to the removal of the bran and the germ in the milling process. Consequently, in rice, a loss of 43% to 92% of the total nutrient content has been reported during milling [33], and in wheat, a 41–80% of loss of Zn, Fe, and K in front of an approximate 27% of loss of selenium during milling [34], [42].

As it is known, there is a protein region surrounding the starch granules in the starchy endosperm cell. Despite selenium not being detected inside the starch granules, it is present significantly in the surrounding protein [38]. This fact explains why some selenium fluorescence was also detected inside the endosperm, and this element was more evenly distributed throughout the whole grain than other nutrients [42].

Speciation of selenium in grain by µXAS measurements

Speciation was studied in the different areas inside the grain that had been identified as relevant in the selenium storage, i.e. germ, bran and the part of the endosperm near the pigment strand. The use of a microfocused beam allows the access to detailed speciation information in different parts of the grain, with a spatial resolution of 2x2 µm that is unmatched by other techniques that enable speciation [49].

µXAS spectra at Se K-edge were acquired in 6 points in each sample. Then the selenium speciation is studied by linear combination fit in each position.

Due to the limited beamtime available, it was only possible to investigate two grains, treated with two of the enrichment conditions, in the present experiment.

Selenite enrichment at 10µM cause bigger toxicity to wheat plants, smaller biomass production and a significantly smaller yield of grain. Thus, selenate and mixed enriched grains were considered more relevant of a possible realistic selenium fertilizer treatment.

Moreover, the selected grain sample representative of selenite enrichment showed, in the XRF studied of Figure 18, a smaller selenium content than the selenate and mixed enriched grains. Smaller concentrations result in spectra with bigger noise, and a much bigger measurement time is required to achieve acceptable data to obtain speciation information.

For these reasons, only selenate and mixed enriched grains were selected for this experiment, although selenite would also be included in a further more detailed study, that will be discussed in the following sections of the present chapter.

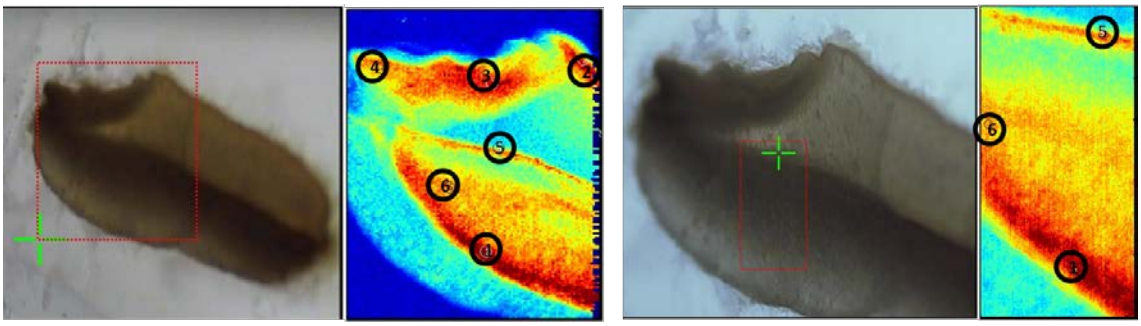

Figure 20: Selenium distribution in a selenate enriched wheat grain. The visible images present a red square in the areas that were mapped with a 12677 eV incident radiation, obtaining the corresponding X-ray fluorescence images, where warmer colors indicate higher selenium concentration. The black circles indicate the points where µXAS spectra were acquired, ordered by a number from 1 to 6. The second image shows a more detailed area inside the same region, and the same measurement points.

In the selenate enriched sample, shown in Figure 20, points 1, 5 and 6 are found in the endosperm, near or in the pigment strand. Points 3 and 4 are found in the germ of the grain and point 2 in the outer bran. The spectra obtained in the points are found in Figure 22. A linear combination fit is done with the spectra of the references and the results in % of Se species are found in Figure 24 and Table 4.

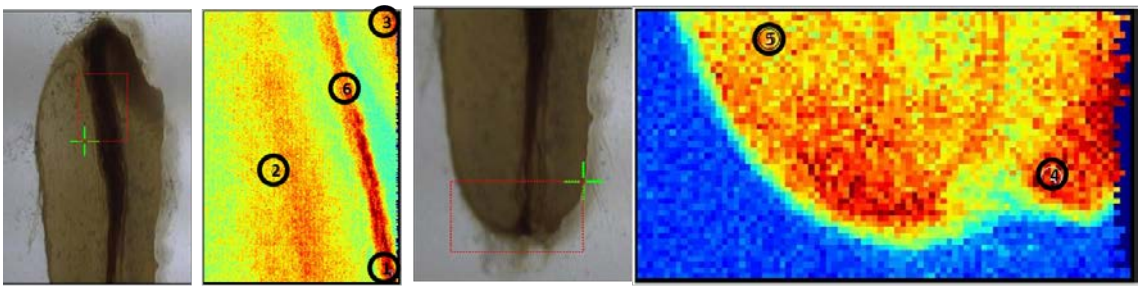

Figure 21: Selenium distribution in a wheat grain enriched with a mixture of selenite and selenate. The visible images present a red square in the areas that were mapped with a 12677 eV incident radiation, obtaining the corresponding X-ray fluorescence images, where warmer colors indicate higher selenium concentration. Two different regions inside the grain sample were analyzed. The black circles indicate the points where µXAS spectra were acquired, ordered by a number from 1 to 6.

In the mixed enriched sample, presented in Figure 21, two regions were mapped. In the first region 4 points were selected: points 1, 2 and 6 in the endosperm, near or in the pigment strand, and point 3 in the germ. In the second region 2 points were chosen: pionts 4 and 5, in areas of the bran. The spectra obtained in those points are found in Figure 23 and the linear combination fit results in Figure 25 and Table 4.

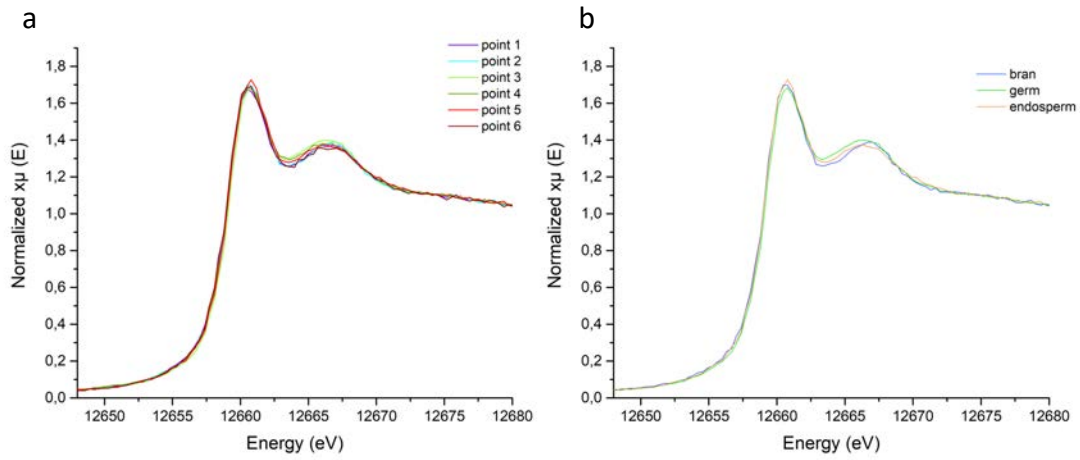

Figure 22: Normalized Se K-edge microXANES spectra of the points measured in a selenate enriched wheat grain: a) all points measured in the sample and b) one point selected in each grain region for easier comparison.

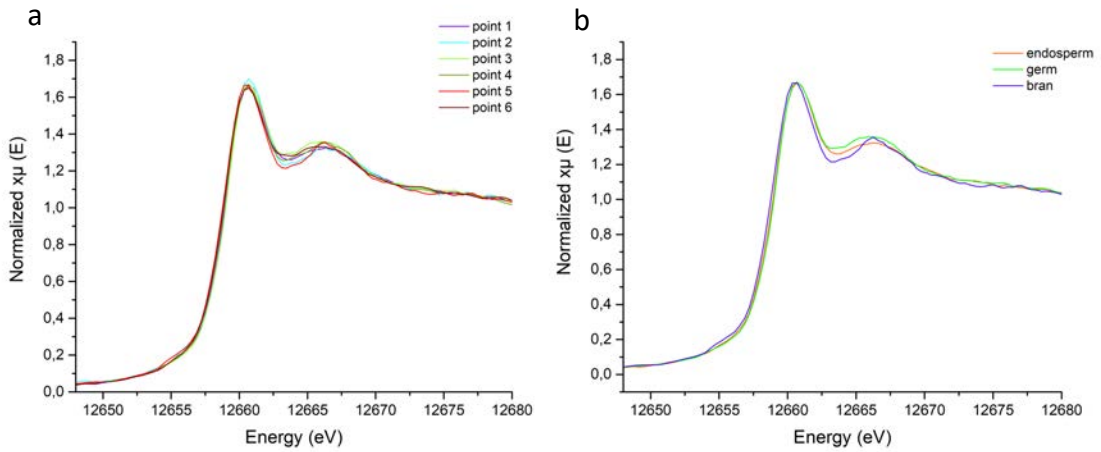

Figure 23: Normalized Se K-edge microXANES spectra of the points measured in a mixed enriched wheat grain: a) all points measured in the sample and b) one point selected in each grain region for easier comparison.

In the μXAS spectra obtained for each sample, it is possible to see that there are significant differences in the shape of the spectra acquired at the different points. Consequently, there are also significant differences between the chemical states of selenium in each of those points.

When comparing spectra collected in the same area of the grain between different treatments, it is found that, despite the possible differences due to the selenium enrichment, there are some common trends.

In both cases, the spectra acquired in the germ had the E_0 at higher energies than the endosperm and the bran. In addition, the decrease in intensity after the white line less marked, broader and slightly shifted towards lower energies. Besides, the germ also showed a higher intensity in the second feature.

On the other hand, the spectra measured in the bran had the more marked decrease in intensity after the white line, whereas the spectra in the endosperm had less intensity in the second feature.

In order to quantify these differences, the spectra from all the points were analyzed through a liner combination fit of the selected references: SeCyst for C-Se-Se-C amino acids, MeSeCys and SeMet for C-Se-C amino acids, Se (IV) and Se (VI). Results are found in Figures 24 and 25 and Table 4. The obtained linear combination fits descrived very well the sample spectra, with small R-factor and reduced χ2, and also a small residual.

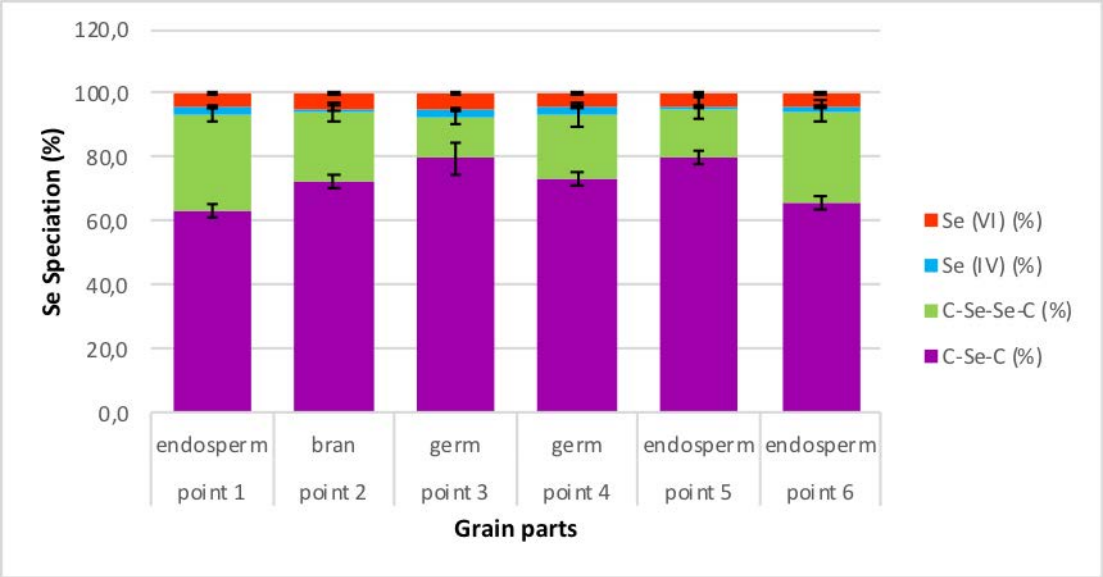

Figure 24: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas inside a selenate enriched grain.

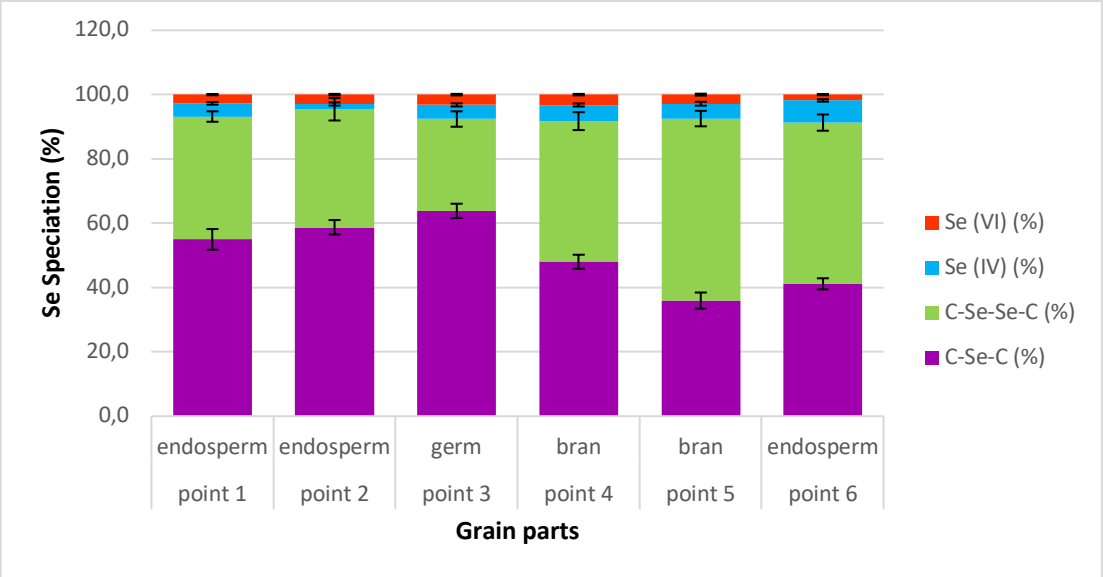

Figure 25: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas inside a grain enriched with a mixture of selenite and selenate.

Table 4: Results from least-square linear combination fits of samples with selenium standards. R factor and reduced chi square indicate the goodness of the fit.

Grain	Point	Part	R factor	reduced chi-square	Se (IV) (%)	Se (VI) (%)	C-Se-C (%)	C-Se-Se-C (%)
Grain Selenate	point 1	endosperm	0,00053	0,00016	2,6±0,4	4,3±0,2	63±2	30±2
	point 2	bran	0,00069	0,00021	0,9±0,5	4,6±0,2	73±2	22±3
	point 3	germ	0,00089	0,00027	2,8±0,5	5,1±0,3	80±5	12±2
	point 4	germ	0,00056	0,00017	2,9±0,4	3,8±0,2	73±2	20±3
	point 5	endosperm	0,00063	0,00019	0,8±0,4	3,9±0,2	80±2	15±3
	point 6	endosperm	0,00060	0,00018	1,8±0,4	3,8±0,2	66±2	29±4
Grain Mixture	point 1	endosperm	0,00055	0,00016	4,1±0,4	2,7±0,2	55±3	38±2
	point 2	endosperm	0,00079	0,00023	1,6±0,5	2,9±0,2	59±2	37±3
	point 3	germ	0,00075	0,00023	4,4±0,5	3,2±0,2	64±2	29±2
	point 4	bran	0,00074	0,00022	5,0±0,5	3,2±0,2	48±2	44±3
	point 5	bran	0,00123	0,00036	4,6±0,6	2,8±0,3	36±3	57±2
	point 6	endosperm	0,00046	0,00014	6,9±0,4	1,8±0,2	41±2	50±3

The differences in the features in the spectra detected between germ, bran and endosperm correlate with the linear combination fit result for each part. In all cases, the germ presents the biggest amount of C-Se-C amino acids within the sample, which results in the observed E_0 at higher energies and less marked features, as seen as well in the reference spectra of selenomethionine. Oppositely, bran and endosperm tend to have less amount of C-Se-C amino acids and a bigger proportion of C-Se-Se-C.

As mentioned before, this observation is due to the fact that C-Se-C, such as SeMet tend to be found incorporated into proteins [29]. Accordingly, a bigger proportion of this type of amino acid is located in the region that is richer in proteins. Oppositely, C-Se-Se-C amino acids are not incorporated in proteins, and although they can be found associated with protein rich areas, they can be found as free amino acids in areas containing less proportion of proteins, such as the bran and the endosperm.

Furthermore, the obtained selenoamino acid ratio depends on the type of enrichment, and follows the same trend observed for bulk ground grains in table 2. The selenate enriched grain contains a bigger C-Se-C/ C-Se-Se-C ratio than the mixed enriched grain. In all points in the selenate enriched grain C-Se-C amino acids were the predominant species. In contrast, mixed enriched grains had points with predominant C-Se-C and points in which C-Se-Se-C was the main species.

As discussed for the results found in table 2, the bigger toxic stress caused by selenite resulted in an enhanced ROS production in the plant and thus, a more oxidizing environment in the wheat tissues, that lead to the production of a bigger amount of the oxidized C-Se-Se-C. In contrast selenate is innocuously stored in leaf vacuoles, and

causes less stress, resulting in a less oxidizing environment and enhanced formation of C-Se-C amino acids.

Additionally, also in agreement with the results from bulk grains, it is observed that very little inorganic selenium is translocated to the grain, being the maximum total content found in the selenate sample 7,9% and 8,7% in the mixed enriched grain.

On the other hand, the values found in the different points (table 4) respect to the bulk grains (table 2) do not match exactly. The differences are mainly due to the fact that single locations of 2x2 μm inside an individual grain were measured in the latter experiment, instead of the analysis of a homogeneous mixture of different grounded grains, which will always include the variability between individual grains and ears.

Moreover, the fact that the measurements were carried out at different synchrotrons with different beamlines, also makes difficult to get a perfect agreement.

Study of the reproducibility of μXAS speciation in different grains

Due to the specific localization of the μXAS measurements in a single grain sample, it is necessary to study the variability between different grains enriched in the same conditions. It is a requirement in order to determine whether the results encountered in a single sample can be representative of the selenium speciation in all grains from a plant, and, as well, of grains from different plants enriched with the same treatment.

Three grains enriched with a mixture of selenite and selenate were measured at BM25A in order to the variability in selenium speciation. The same grain that was studied in the previous analysis at the SSRL synchrotron was analyzed again. It was compared with two other grains, one obtained from the same ear, and the obtained from a different plant.

Besides, the differences obtained in grains from the same treatment were compared to the diversity in speciation between grains from different treatment, in order to confirm that the difference in speciation depending on the selected enrichment is bigger than the variability between individual grains from one treatment.

Samples named A and B are grains from the same ear and sample C is a grain from another plant. In order to perform the experiment, 5 μXAS points were acquired in each of the three samples. Those points were evenly distributed including sections of the endosperm the germ and the bran. However, in sample C one of the scans did not have enough quality and was discarded in the present analysis.

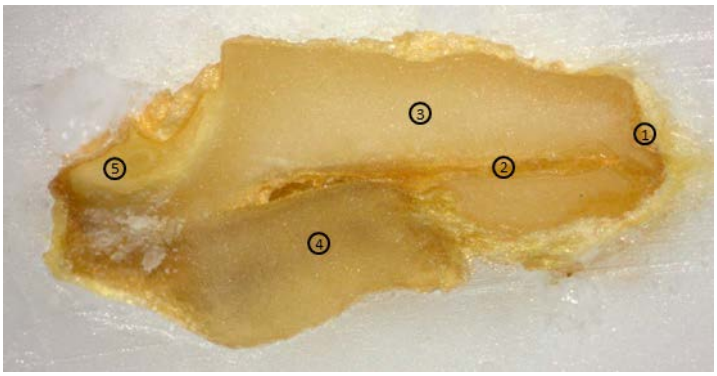

Figure 26: Example of the distribution of points within samples in the present experiment. Visible image, obtained by a microscope, of sample A, enriched with a mixture of selenite and selenate. The five points measured by µXAS are marked by black circles.

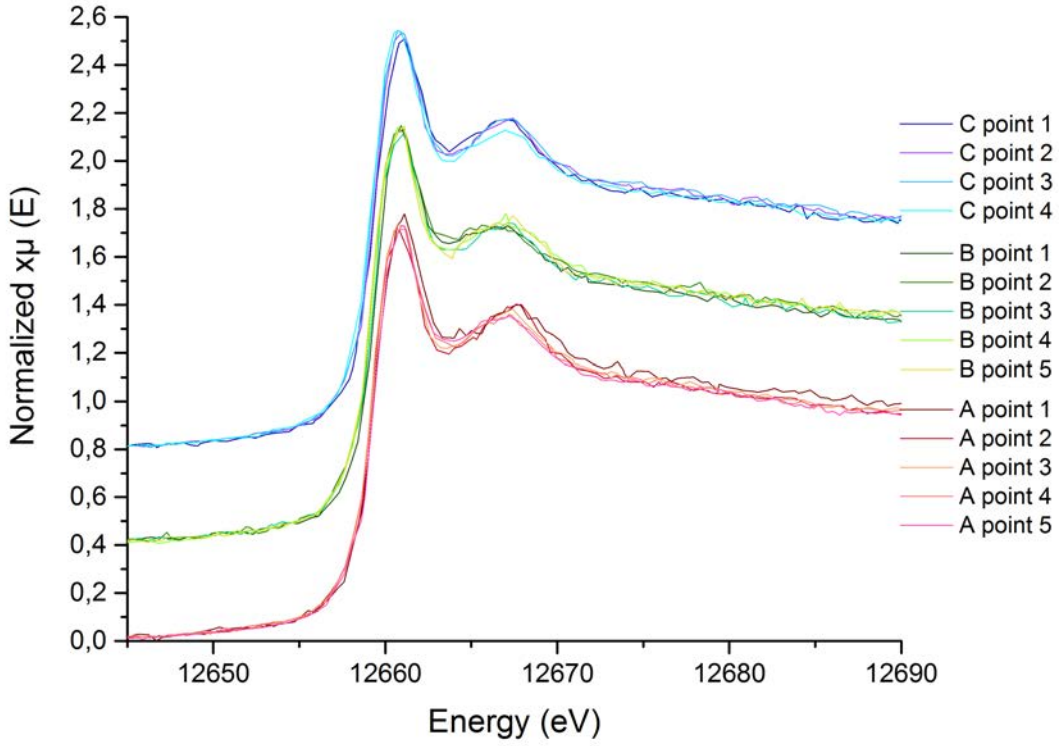

Figure 27: Normalized Se K-edge microXANES spectra of the points measured in the three wheat grains enriched with a mixture of selenite and selenate.

In the first place the variability of the obtained spectra within a sample was studied. In order to do so, the spectra of the 5 different points (4 in the case of sample C) were merged, obtaining an average spectrum. Then, the spectrum from each point was compared to the average for that sample in Figure 28.

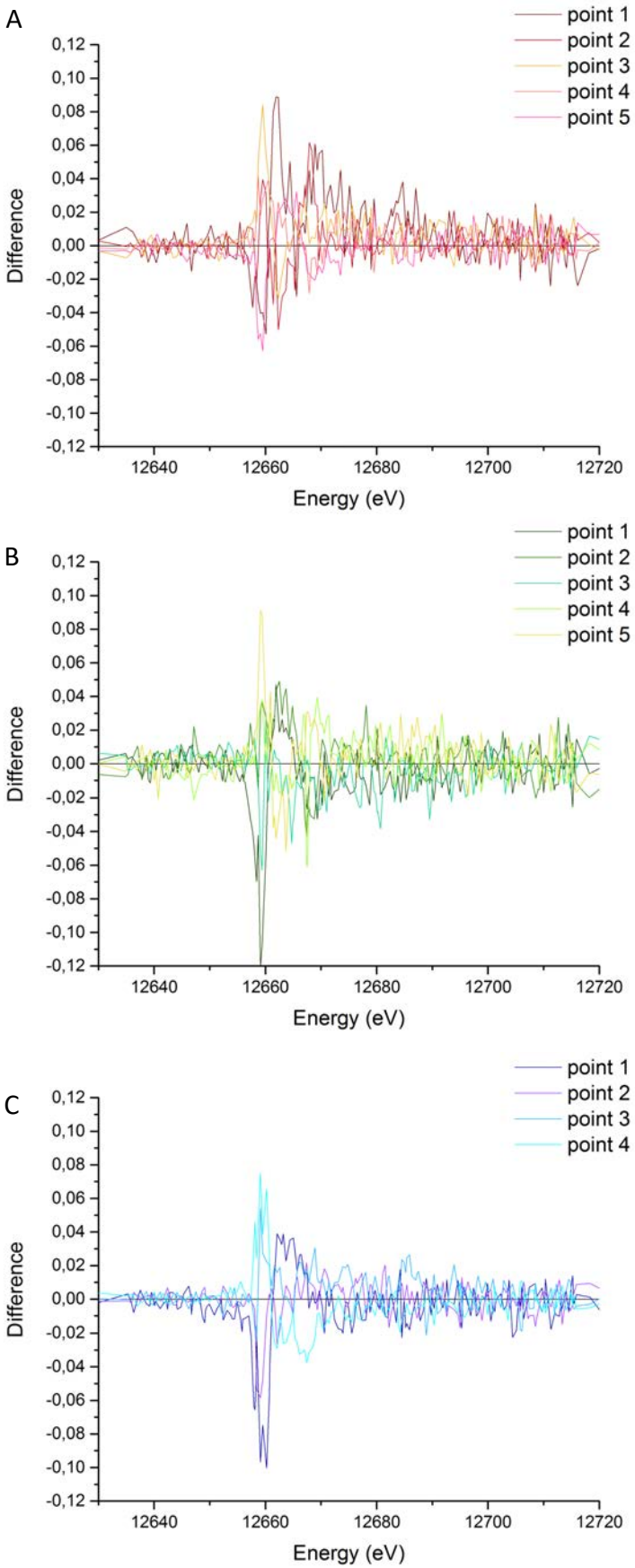

Figure 28: Difference spectra between the indicated point of a sample to the average spectra of that sample, for grains A, B, and C.

There is a significant variability within a sample, which is bigger than the levels of noise in the energy positions of the edge, the white line and the visible features in the spectra, from approximately 12655 eV to 12680 eV. The observed differences have magnitudes between +0,10 and -0,12.

The averaged spectra were used in order to compare the differences between samples. Sample A was used as a reference to do the differences, as shown in Figure 29.

The differences between sample A and sample B, both grains from the same ear, were seen to be very small, with differences lower than 0,04, and with a similar magnitude than the level of noise.

Moreover, observed differences between sample A and C, which were grains coming from different plants, but which have been exposed to the same conditions and selenium treatment, were also very small. In addition, differences in speciation between plants are analogous to those within the same ear.

Consequently, the differences between grains enriched with the same treatment, disregarding if they come from the same plant or from different plants, were smaller than the observed variability in the different parts inside a grain (endosperm, germ, bran).

Accordingly, for the μXAS experiments, in which the objective is to study the speciation localized in several positions inside a grain sample, it is possible to study a single grain as representative of all the grains from plants treated in a certain manner. Therefore, speciation results can be considered reproducible between grains.

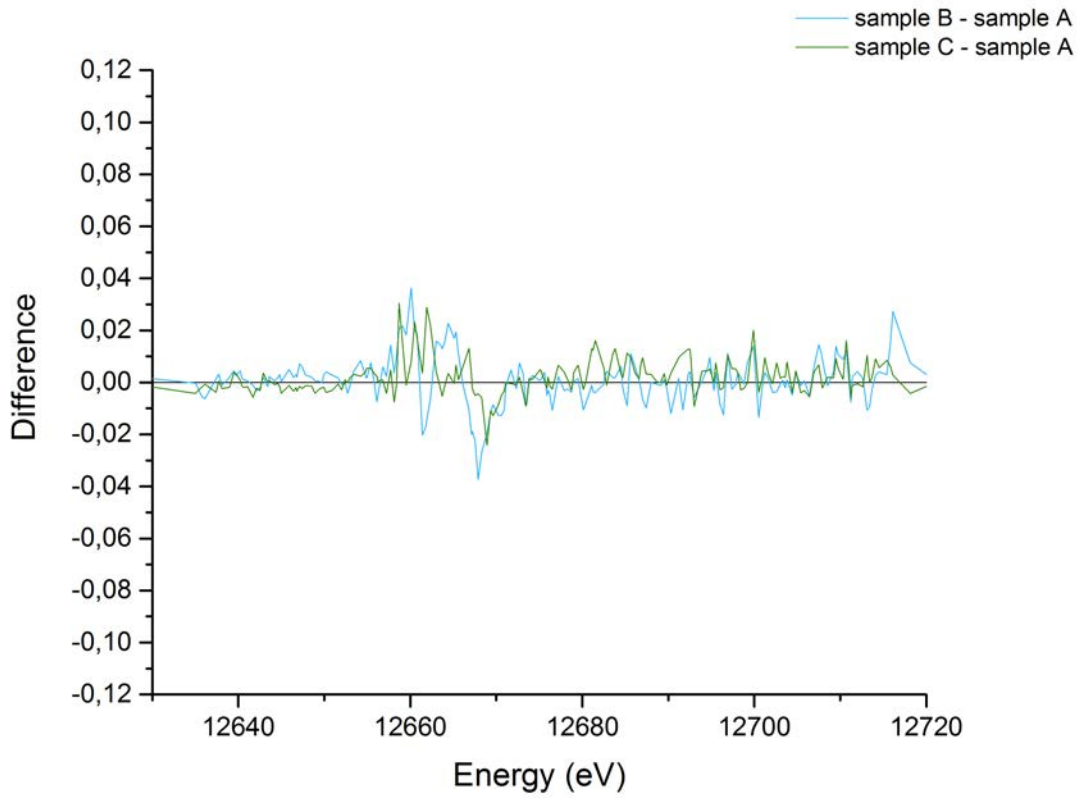

Figure 29: Difference spectra between the merge spectra of the points of sample B and C respect to the points in sample A, being A and B grains from the same ear and A and C grains from different plants, all enriched in the same conditions with 10µM of a mixture of selenite and selenate.

Finally, the differences within parts inside a grain (Figure 28) and between different grains (Figure 29), were also compared with the divergence between plants exposed to different selenium treatments.

Two more grains were chosen for this experiment, one enriched with selenite (sample D) and one enriched with selenate (sample E). They were analyzed in the same manner, with the acquisition of µXAS spectra in 5 different points within a sample, which were later merged for comparison between samples. The average spectra of sample D and sample E were compared to that of sample A.

It is shown in Figure 30 that grains treated with different selenium conditions have notorious differences. Those differences are much bigger that the ones coming from grains from the same treatment (Figure 29) and therefore, the modification of the selenium enrichment results in a significant modification of the speciation in the grain considered as a whole.

However, the differences between different enrichments are equal or smaller than those observed inside an individual grain (Figure 28). Consequently, the variability of

selenium speciation between endosperm, germ and bran is comparable to that resulting from an enrichment using different selenium species.

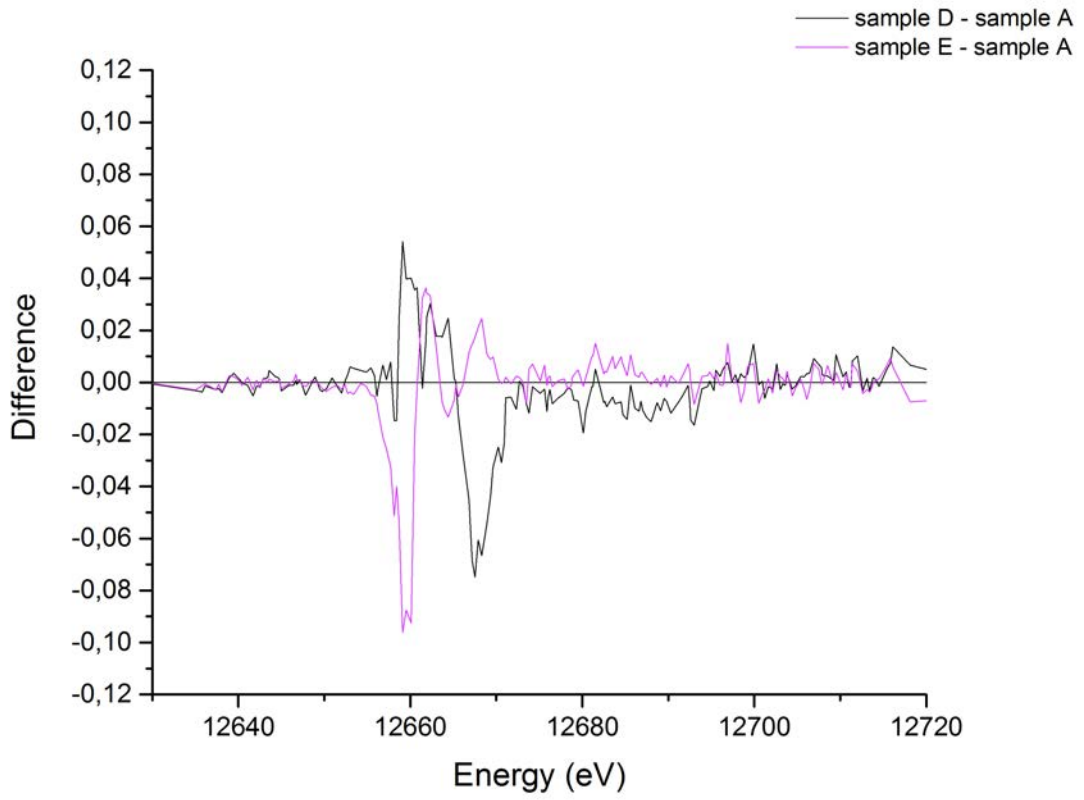

Figure 30: Difference spectra between the merge spectra of the points of sample D and E respect to the points in sample A, being A a grain enriched with a mixture of selenite and selenate, D a grain enriched with selenite and E a grain enriched with selenate.

Moreover, the differences were again observed in the energy range between approximately 12655 eV and 12680 eV. These energies correspond to the positions of the edge and the visible features in the spectra.

Figure 31 display the average of all the spectra within a gran. The comparison of the different treatments presents the same trends that were observed in the bulk experiments of selenium speciation in grain. The selenate treated grain have the edge position at higher energies, which is characteristic of higher content of C-Se-C amino acids. Besides, the selenite treated grain shows less intensity in the second feature, which is characteristic of a bigger content of C-Se-Se-C amino acids.

Consequently, µXAS experiments selecting of 5 points evenly distributed in a sample that acquire speciation information from all parts (endosperm, germ and bran), provides results that match those obtained from the bulk analysis of a ground grain. This observation serves to validate the adequacy of µXAS experiments to study selenium speciation in grains and to ensure the robustness of the obtained results.

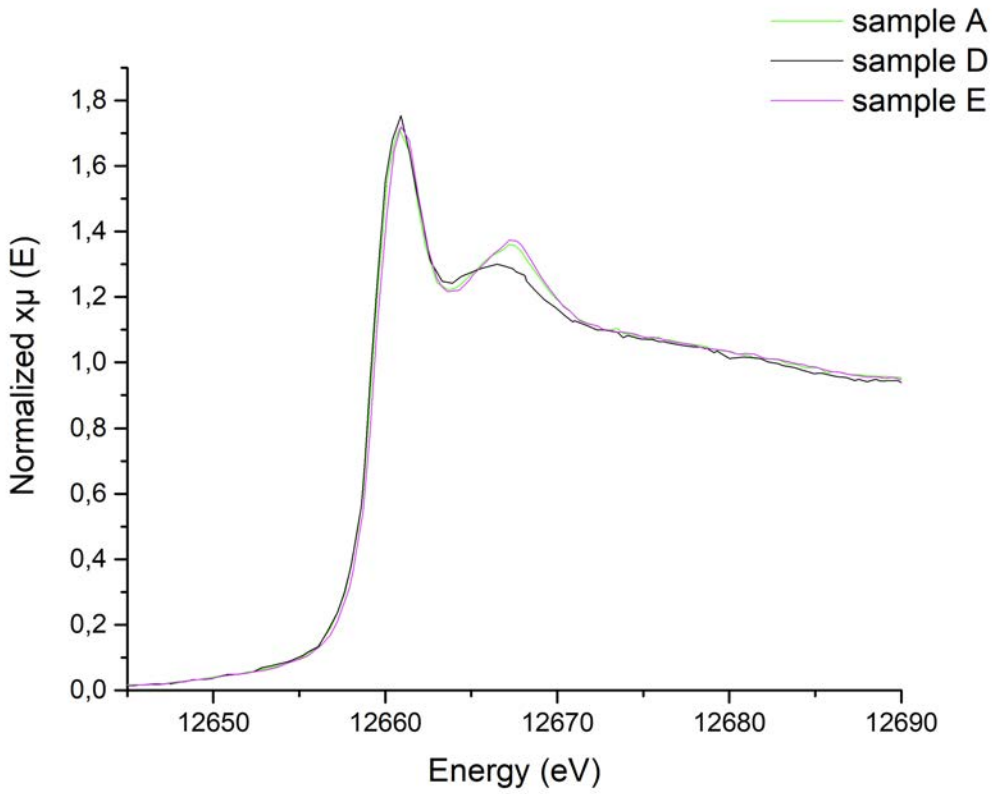

Figure 31: Normalized Se K-edge microXANES spectra of the merge spectra of the points of sample A, a grain enriched with a mixture of selenite and selenate, sample D, a grain enriched with selenite and sample E, a grain enriched with selenate.

Development of a detailed study of the selenium speciation in grain

As described above, a significant variability was detected between different points inside a grain, which was of the same magnitude that the differences between different treatments.

The previous μXAS experiments only considered 5 or 6 points in the whole grain. Therefore, only between 1 to 3 points were obtained for each specific tissue, such as germ, bran and endosperm. The speciation information obtained with this approach failed to provide complete information of the distribution of the species in the grain, with just few points analyzed, which could not be representative of the whole sample.

Besides, it also lacked to possibility to take into account the variability within a tissue. The tissues of the grain classified here as endosperm, germ and bran, also contain sub-regions within, such as the pericarp, seed coat and aleurone layer in bran, or the radicle, coleoptile and scutellum in the germ [37]. The acquisition of spectra in some of these sub-regions, which could not be easily distinguished by eye, could lead to added variability in the speciation results, as observed for example in Figures 24 and 25.

For this reason, to properly study the localization of selenium species in the different parts inside the grain, it was decided to analyze each sample by mapping the whole

inner surface by acquiring µXAS spectra in approximately 100, in order to cover the entire sample, in a experiment performed at CLAESS beamline, at ALBA synchrotron. The strategy used to acquire µXAS spectra is presented in Figure 32 in one grain as a representative example of all the samples studied.

In order to facilitate the analysis and to improve the spectra quality, adjacent points within the same grain tissue (i.e. germ, pigment strand, bran and endosperm) were merged if the spectra presented similar features. This procedure resulted in the division of the grain in several regions, as shown in Figure 33. Each sample was distributed in a slightly different number of characteristic areas in order to group a similar number of points to have comparable signal to noise ratio (between 7 and 11 areas were selected for each sample).

Then, each of these regions resulted in one average spectra. The normalized spectra from each area can be seen in Figures 34, 35 and 36.

Those spectra were analyzed by least squares linear combination fit of the selenium references. Results are encountered in Figures 37, 38 and 39 and Table 5

Finally, all the points were also merged in a single average spectrum, which was also analyzed by linear combination fit as an average of the sample speciation and is found in table 5 as well. In figure 40, the similarities between the data and the obtained fits are observed. Additionally, this average was used to compare in an easier way between the chosen mapping approach and the bulk results, as seen in Figure 41.

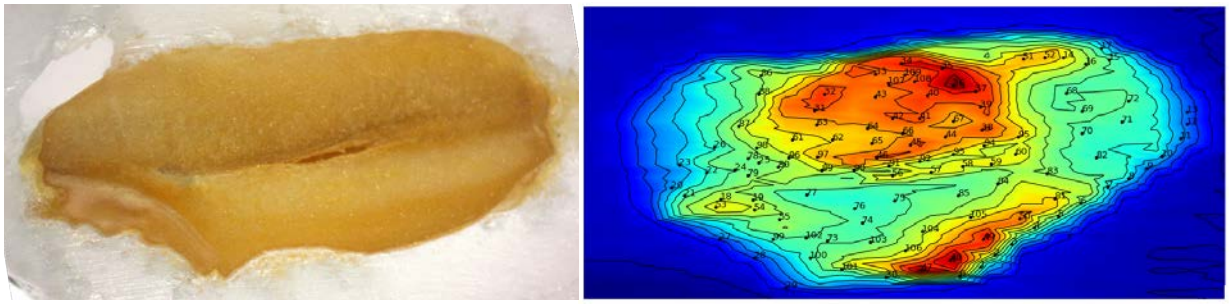

Figure 32: Example of the distribution of points within samples in the present experiment. Visible image obtained with a microscope and X-Ray fluorescence coarse image of a sample enriched with selenite, where warmer colors indicate higher selenium concentration. µXAS spectra were acquired successively on the numbered black points.

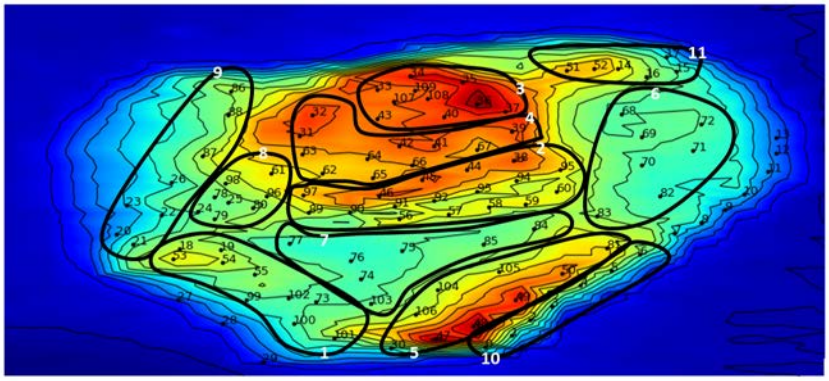

Figure 33: Example of the arrangement of adjacent points with the same features in diverse areas. Areas are marked by a black circle and numbered in white. In the present example, area 1 corresponds to the grain germ, 2 to the pigment strand, 3 to 8 to the endosperm and 9 to 11 to bran.

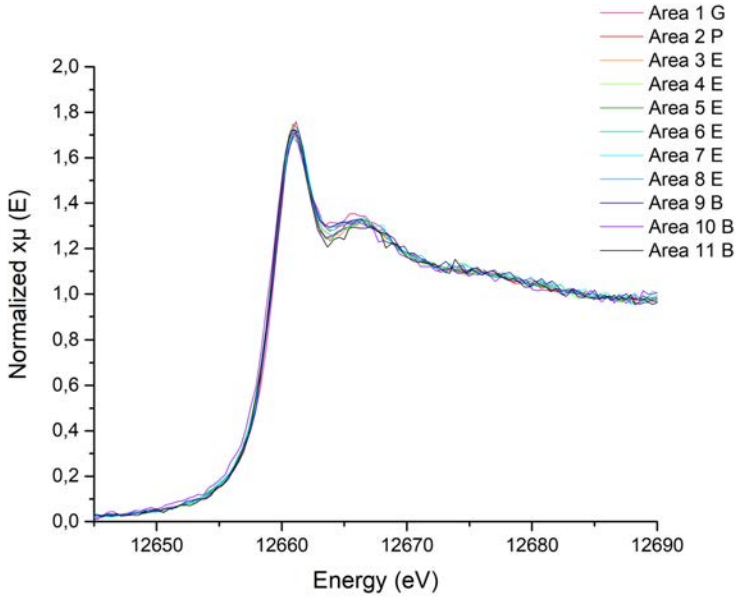

Figure 34: Normalized Se K-edge microXANES spectra of the areas of a grain enriched with selenite, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

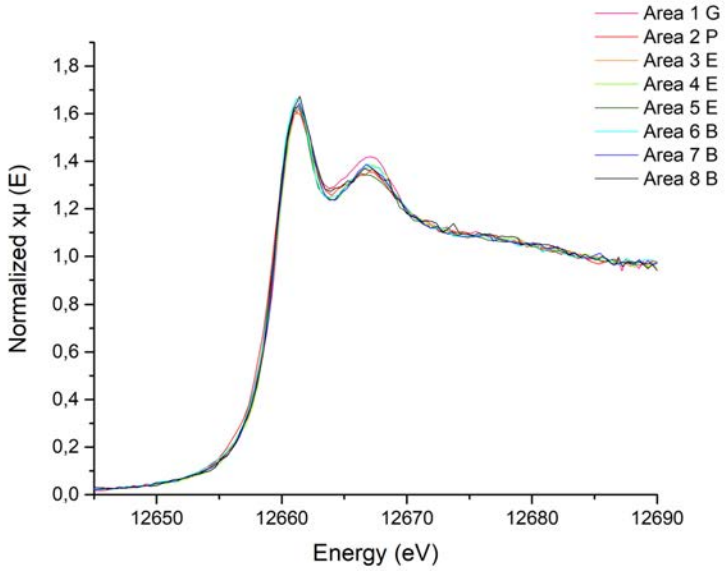

Figure 35: Normalized Se K-edge microXANES spectra of the areas of a grain enriched with selenate, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

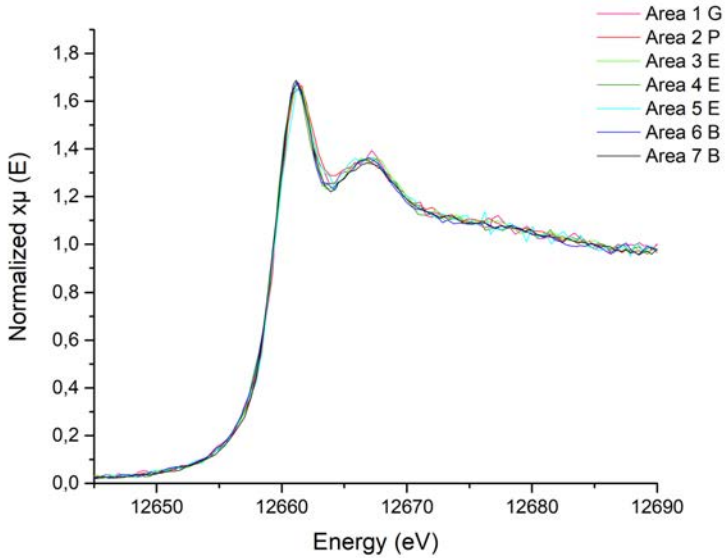

Figure 36: Normalized Se K-edge microXANES spectra of the areas of a grain enriched with a mixture of selenite and selenate, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

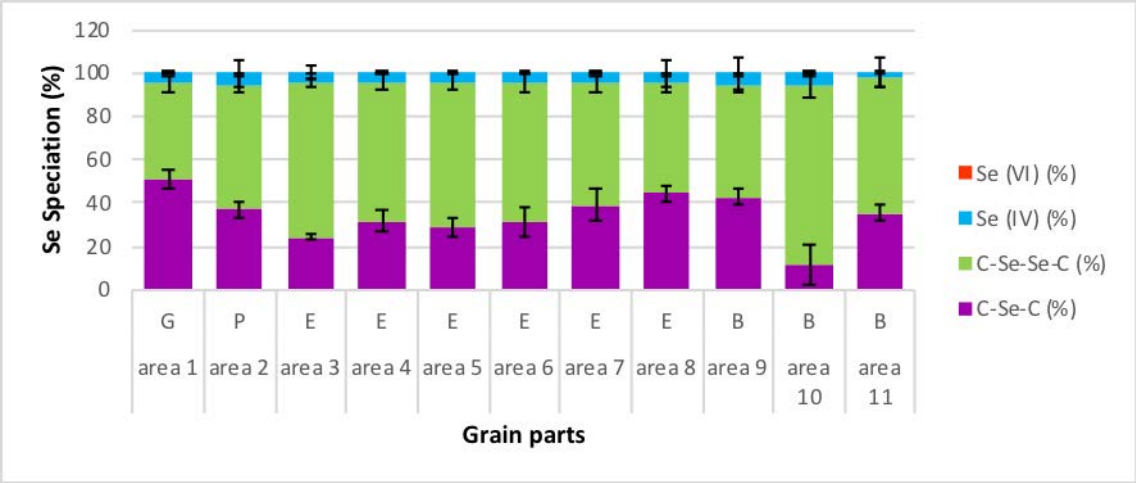

Figure 37: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas of a grain enriched with selenite, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

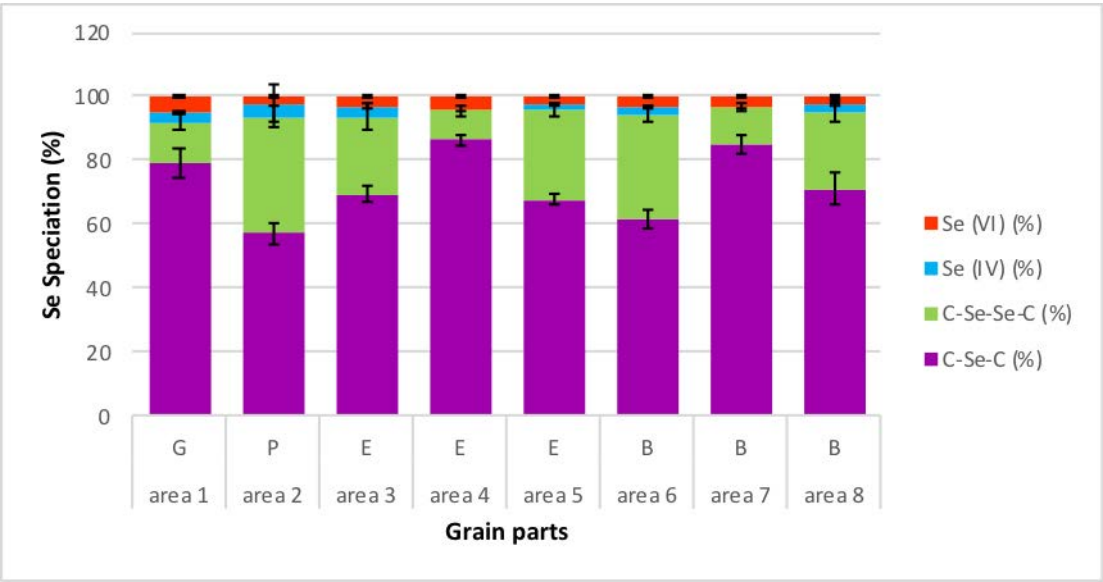

Figure 38: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas of a grain enriched with selenate, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

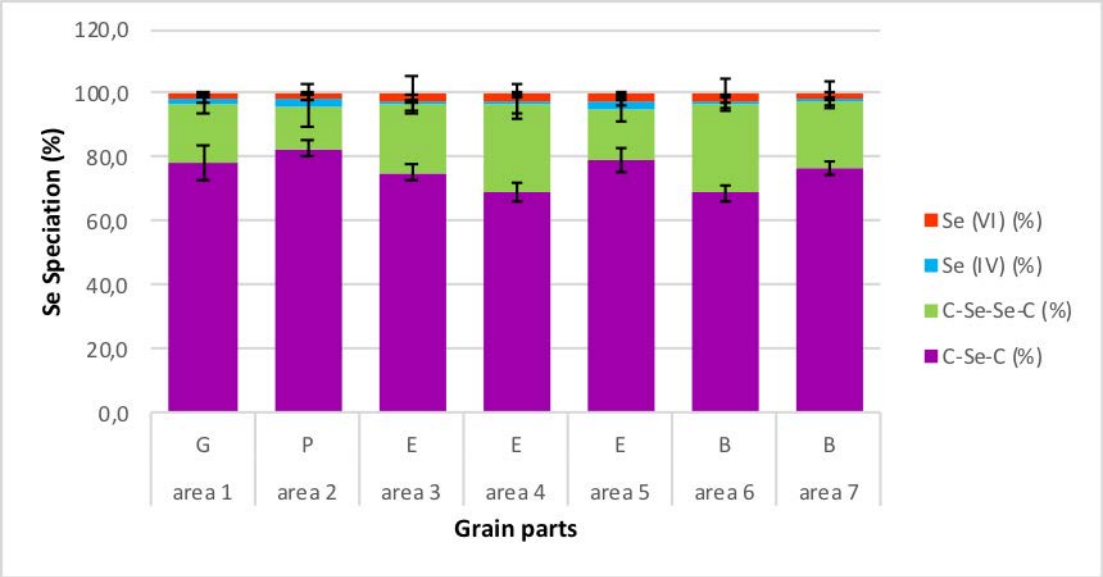

Figure 39: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas of a grain enriched a mixture of selenite and selenate, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

Table 5: Results from least-square linear combination fits of samples with selenium standards. R factor and reduced chi square indicate the goodness of the fit

Grain	Area	Part	R factor	reduced chi-square	Se (IV) (%)	Se (VI) (%)	C-Se-C (%)	C-Se-Se-C (%)
Grain Selenite	all points	-	0,00168	0,00041	4,9±0,7	n.d.	35±4	61±4
	area 1	G	0,00192	0,00047	5,0±0,8	n.d.	51±4	44±4
	area 2	P	0,00153	0,00037	5±6	n.d.	37±3	58±3
	area 3	E	0,00179	0,00043	5±3	n.d.	24±1	71±2
	area 4	E	0,00196	0,00048	4,6±0,8	n.d.	32±5	64±4
	area 5	E	0,00200	0,00049	4,4±0,8	n.d.	29±5	67±4
	area 6	E	0,00215	0,00052	4,9±0,8	n.d.	31±7	64±4
	area 7	E	0,00247	0,00060	5,3±0,9	n.d.	39±7	56±4
	area 8	E	0,00163	0,00040	5±6	n.d.	44±4	50±3
	area 9	B	0,00201	0,00049	5±7	n.d.	43±4	52±4
	area 10	B	0,00384	0,00090	6±1	n.d.	12±9	82±5
	area 11	B	0,00195	0,00048	3±7	n.d.	35±4	62±13
Grain Selenate	all points	-	0,00062	0,00015	2,1±0,4	3,2±0,2	69±4	26±2
	area 1	G	0,00109	0,00027	3,2±0,6	4,8±0,3	79±5	13±3
	area 2	P	0,00127	0,00031	4±6	2,3±0,3	57±3	36±3
	area 3	E	0,00088	0,00021	3,4±0,5	3,0±0,3	69±3	24±4
	area 4	E	0,00061	0,00015	n.d.	4,5±0,2	86±2	9±1
	area 5	E	0,00055	0,00013	1,2±0,4	2,7±0,2	68±2	28±2
	area 6	B	0,00096	0,00023	2,2±0,5	3,3±0,3	62±3	33±3
	area 7	B	0,00077	0,00019	n.d.	3,4±0,2	85±3	12±1
	area 8	B	0,00133	0,00032	3,0±0,6	2,2±0,3	71±5	24±3
Grain Mixture	all points	-	0,00052	0,00013	1,0±0,4	2±4	78±2	19±2
	area 1	G	0,00135	0,00033	1,4±0,6	2,0±0,3	78±5	18±3
	area 2	P	0,00101	0,00025	2,6±0,6	1,3±0,3	83±3	13±7
	area 3	E	0,00107	0,00026	0,7±0,6	3±5	75±3	21±3
	area 4	E	0,00112	0,00027	1±5	2,7±0,3	69±3	27±3
	area 5	E	0,00154	0,00037	2,2±0,7	2,8±0,3	79±4	16±3
	area 6	B	0,00092	0,00022	1,0±0,5	2±5	69±3	28±3
	area 7	B	0,00063	0,00015	0,1±0,4	2±4	77±2	21±2

Anova statistical analysis showed that the content of C-Se-Se-C was significantly higher and C-Se-C amino acids were significantly lower in the areas of selenite enriched grains, respect to areas in grains treated with other selenium species. These results, agree with that previously found in bulk samples (Table 2), due to a more oxidizing

environment in wheat tissues from the enhanced production of ROS species in plants exposed to selenite induced stress.

However, Anova analysis showed that levels of C-Se-Se-C and C-Se-C amino acids were not significantly different between the areas in grains treated with selenate or mixture of selenite and selenate. This outcome differs from the previous results emerging from both bulk experiments and preliminar μXAS studies at SSRL (Table 2 and Table 4). Yet, this later difference is due to the big variability in speciation observed within a sample.

Regarding accumulation of inorganic selenium in grain, grain enriched with selenite exhibited a significantly higher Se (IV) content in the different areas than the other two treatments, but showed no presence of Se (VI). This species was not detected in any tissue in the plant (Table 2), indicating that Se (IV) is not oxidized to Se (VI) in wheat, and accordingly is also not found in any part of the grain. Thus, it seems that, since there is no Se (VI) to be translocated into the grain, there is a slightly higher amount of Se (IV) translocated, which may indicate that despite Se (IV) and Se (VI) having different uptake pathways in roots, the pathways of translocation of these species from shoots to grain could have some interfering effects.

Moreover, content of Se (IV) was not significantly different between selenate and mixed treated grains, but the amount of Se (VI) was significantly higher in selenate treated grains in the diverse regions, due to the higher content of this species in the plant.

Regarding the speciation variation within a sample, it can be seen that the germ contained a higher proportion of C-Se-C amino acids, as shown by results collected in Table 4. As previously discussed the higher protein content in the grain germ is the reason for the higher content of C-Se-C amino acids, since, SeMet is generally encountered incorporated into proteins, in contrast to SeCyst, which is not incorporated in proteins and is found as a free amino acids. This effect is especially significant in the selenite treated grain, which had the less amount of C-Se-C through the sample, but an important percentage of these species was concentrated in the germ. However, the difference within the grain is less marked for selenate and mixed enriched grains, which had a high content of C-Se-C in many regions and then the preferential accumulation of C-Se-C amino acids in the germ was not significant.

Regarding the pigment strand, the bran and the endosperm areas, not a constant trend is detected in the grains, and the differences between them in the three treatments are not significant. This is due to the big variability within those tissues, which also contain diverse sub-regions, and thus, the acquisition of just single points within one tissue can lead to a misinterpretation of the results. However, it is observed that there is slightly less variability in those areas in the grains treated with a mixture of species: selenite treated grains presented C-Se-C contents between 12-44%, selenate treated grains 57-86% and mixture threated grains 69-83%.

On the other hand, the comparison of the average spectra from all points to the spectra obtained from the bulk analysis of the homogeneous powder of ground grains showed a good match between the spectra, as seen in Figure 40. This is a good indication of the adequacy of the selected approach of acquiring µXAS spectra in many points within a sample, in order to account for the speciation in the entire grain, over the common µXAS approach that tends to acquire only few points within a sample sunrface, which fails to resolve within sample variability. Furthermore, since variability within grains enriched with the same conditions is not too big, the acquisition of many points to map the whole sample, matched the results from the mixture of several grains in a powder.

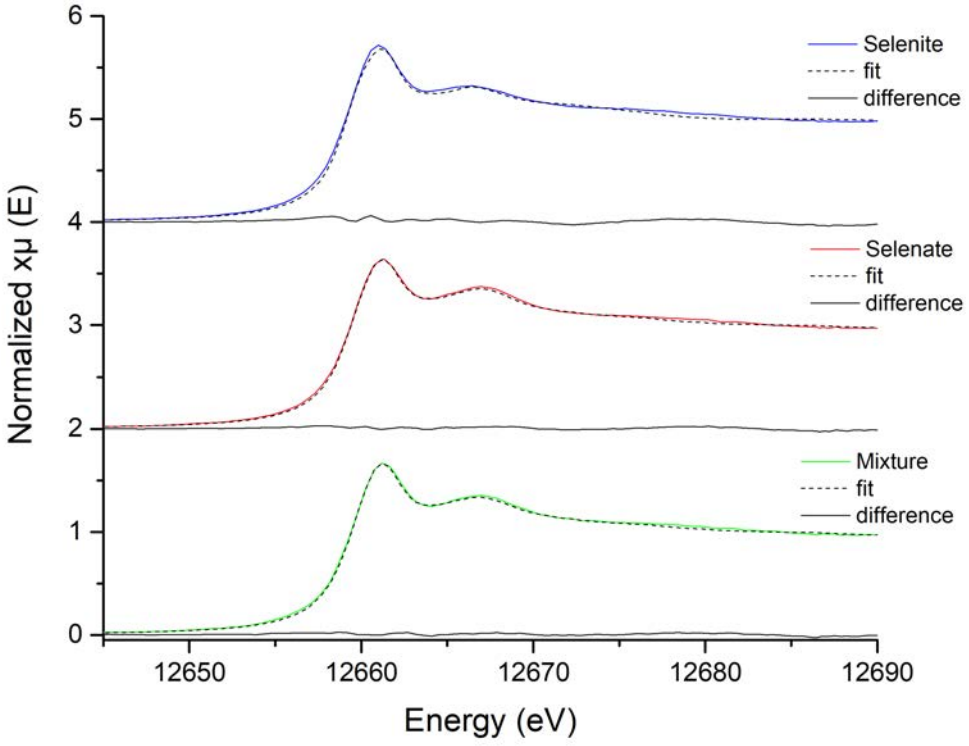

Figure 40: Normalized Se K-edge µXANES spectra of the average of all points acquired in each grain (solid line), result from the least-square linear combination fit of the spectra with pure selenium compounds (dash line), and difference between the real spectra and the fit.

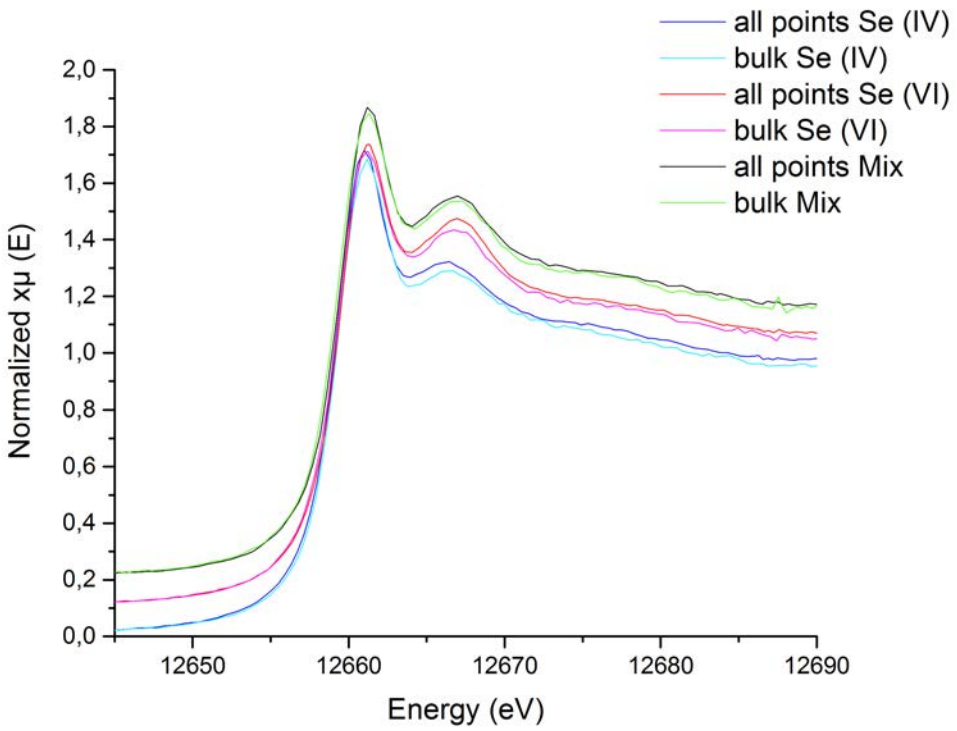

Figure 41: Normalized Se K-edge microXANES spectra showing the merge of the spectra of all points taken within a grain sample respect to the spectra obtained from a bulk powder of grains enriched in the same conditions, for selenite, selenate and mixture treated grains. Results show a good agreement between μXAS mapping and bulk analysis.

Conclusions

The information obtained by μXRF and μXAS has served to obtain novel information on the distribution of selenium species, as well as other nutrients, within wheat grain. This information complements the knowledge on the reproductive strategy of wheat, and above all, is essential in the design a selenium enriched functional food, in order to maximize the selenium and nutrient content in the flour preparation and ensure the adequacy and bioavailability of the selenium species.

It was clearly observed in the X-Ray fluorescence maps that the accumulation of selenium and nutrients through ears and grains is not homogenous. Grains fulfill the function of nutrients reservoirs for the development of a future seedling, and thus, they are the main accumulation pool of elements when the plants are mature, such as K, Ca, P, S, Mn, Fe, Cu, Zn and Se. Oppositely, the rest of the ear is depleted of nutrients and just serves as a husk for the grain, where only K and Ca and in smaller amounts Mn and Fe, are significantly present.

Within a grain, there are also several structures that accumulate selenium and nutrients in different amounts. Se, as well as the rest of the elements, is preferentially

located in the germ, the bran and the pigment strand, and very few nutrients are found in the endosperm. The different chemical composition of each tissue is responsible for this irregularity. Proteins are able to bond or incorporate several elements in their structure, including Selenium and other nutrients such as P, S, Zn and Cu. Germ contains the highest protein amount within the grain, bran contains protein-rich aleurone cells and the pigment strand the vascular bundle, and all these regions have been found to contain plenty of nutrients. Contrarily, the endosperm is rich in polysaccharides, and is formed by starch granules that do not accumulate selenium or the other nutrients.

Moreover, there are some detectable differences in the accumulation of selenium depending on the selenium specie used in the enrichment, however, since the observation was only performed on one grain for each condition, a further study increasing the number of samples is necessary in order to confirm the obtained findings.

The speciation within the grain is also not homogeneous. The variability of the resulting selenium speciation within grain tissues is seen to be as big as the variability between different selenium enrichment conditions. However, the variability between different grains from the same ear, or even from different grains from different plants treated with the same conditions, is much smaller. Therefore, the speciation obtained in grain is reproducible through all the grains of plants exposed to the same selenium treatment and one grain can be used as a representation of all the grains in the μXAS experiments.

However, since the variability within a sample has been seen to be very important, the proposed approach of mapping many points within a sample and then organizing these into regions with similar features is better than the common μXAS methodology of acquiring few points in each sample, which could fail to properly consider the changes in speciation within the sample.

Regarding the resulting speciation of selenium depending on the selenium species used in the enrichment, it has been seen that C-Se-C amino acids, followed by C-Se-Se-C amino acids, are the predominant compounds in wheat grain. There is a small inorganic content, but this is always between 2-8% of the total selenium, irrespective of the species used in the enrichment.

The results encountered in the present study match previous studies performed with XAS in wheat [20], [50]. However, in general, it is considered that wheat stores selenium mainly as SeMet, with several studies reporting values between 55-100% of the total selenium regardless of the selenium source (native or supplemented), the amount of selenium used for the biofortification, the chemical form (selenite or selenate), and the application method (foliar or soil application) [51]–[54].

In contrast, previously SeCyst was only detected in small amounts in wheat and wheat flour [53], [55], [56] as well as in other plants [56], [57]. However, in our work we have demonstrated that selenocystine can account for up to a 61±4% of the total selenium content in enriched grains. Moreover, the present study has shown an influence of the enrichment conditions over the selenomethionine/selenocystine ratio. To our knowledge, the present effect has not been previously reported.

Specifically, selenite treated plants form grains where the content of C-Se-Se-C amino acids, such as selenocystine, is equal or bigger than that of C-Se-C amino acids, such as selenomethionine, therefore, demonstrating the significance of selenocystine in enriched grains.

The reason for the underestimation of selenocystine content in other works is the difficulty in the quantification of this species by conventional techniques such as HPLC-ICP-MS, as discussed in chapter 3. However, XAS is able to quantify the species of selenium without the need of pretreatment or extraction, therefore ensuring the total quantification instead of a limited extraction, and the stability of the measured species.

The enhanced synthesis of C-Se-Se-C species in selenite treated grains is due to the bigger toxic stress that selenite cause to the plant compared to selenate, as seen by the decrease on biomass production. This results in the production of reactive oxygen species in the plant that create a more oxidizing environment within the cells. Accordingly, selenium groups tend to be oxidized, and selenocysteine residues form selenocystine species instead of being incorporated into proteins.

Regarding plants enriched with selenate or a mixture of selenite and selenate, it has been shown that they contain significantly higher amounts of C-Se-C compounds that selenite treated grains. However, the differences encountered between them depend on the methodology used in the analysis. If only 6 points are analyzed within each sample, grains treated with a mixture of selenite and selenate present a higher content of C-Se-Se-C amino acids, due to the influence of selenite in the enrichment. However, if the entire sample is analyzed covering it many points, the difference in content between mixture treated grains and selenate treated grains is not significant. Therefore, in this case, the variability within a sample is more relevant than the effect of choosing either selenate or a mixture of selenite and selenate 1:1, and only the enrichment with selenite has provided a significant and reproducible variability of the selenomethionine/selenocystine ratio.

Finally, this work has revealed the need of a special procedure to prepare functional foods based on Selenium enriched wheat: firstly, the necessity to use whole flour preparations in the elaboration of a selenium enriched functional food. Normal flour preparations remove the bran and the germ, leaving only the endosperm. However it has been demonstrated that the endosperm contains a small selenium amount, which

would not be adequate to increase selenium levels in deficient populations. Alternatively, the selection, exclusively, of a determined grain part (nowadays technically feasible) to prepare special enriched flours when the flour content is addressed to have a higher content of C-Se-Se-C against C-Se-C or vice versa.

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5. Interactions between mercury pollution and wheat selenium biofortification

Introduction

Heavy metal toxicity and phytoremediation

Heavy metals are mobilized due to anthropogenic activities, such as extraction from ores, leading to a release into the environment. They accumulate in soils and water bodies, posing a risk to living organisms through bioaccumulation and biomagnification [1].

In order to minimize the content of heavy metals and other pollutants several physical, chemical, and biological methods have been proposed. However, physical and chemical procedures are often complicated to implement and expensive [1]–[3]. On the other hand, plants have the capacity to uptake metals from contaminated media, and extract, stabilize, degradate or volatilize them [4]. The use of plants and the associated soil microbes to reduce the concentrations or toxic effects of contaminants in the environments is called phytoremediation [1], and is a simple, effective, cheap, not intrusive and environmentally friendly methodology [2], [5]. Specifically, the plant ability to uptake and translocate heavy metals to aboveground tissues allows the permanent removal of metals from the site at harvest and is called phytoextraction [6]. However, it is important to consider not only the bioavailability of the metal for plant uptake and translocation but also the plant tolerance to the pollutant concentration [2], [6].

The toxicity of trace metals for a great range of plant species follows the trend: Pb=Hg>Cu>Cd=As>Co=Ni=Zn>Mn, with the median toxic concentrations of 0,30 μM for Pb, 0,47 μM for Hg, 2,0 μM for Cu, 5,0 μM for Cd, 9,0 μM for As, 17 μM for Co, 19 μM for Ni, 25 μM for Zn and 46 μM for Mn [7].

Mercury

Mercury is a heavy metal and a highly toxic element. It is naturally present in the geosphere, but the industrial revolution has led to a large-scale mobilization and many anthropogenic activities have resulted in a global increase of the levels of mercury in the environment [8], [9].

Significant amounts of Hg have been found in soils several kilometres away from Hg-mines, where agriculture is extensively exploited [10]–[14]. Furthermore, cases of poisoning of the population have occurred in several occasions [15], [16]. Therefore, concern about its presence in crops has been raised [17]. Furthermore, mercury

occurrence in the environment, both in water bodies and in agricultural lands, may result in its mobilization and bioaccumulation through the trophic chain, and consequently, lead to a significant presence in certain food sources for humans [13], [18].

In the environment, mercury is found with an oxidation state of Hg (I) or Hg (II). It is present, in general, in the form of inorganic salts and oxides, such as HgS, $HgCl_2$, HgO and $Hg(OH)_2$ [19]. Besides, in living organisms, organic forms of mercury are also common, such as methylmercury.

Mercury exhibits its toxicity through different mechanisms: it blocks essential functional groups in biomolecules or replaces metal ions in them and it enhances the production of reactive oxygen species (ROS) [20]. Among the different Hg compounds, methylmercury is the most neurotoxic form that can be found, as it is able to cross the placental barrier in animals [21].

Both inorganic and organic forms of Hg are toxic to plants but at different levels due to distinct patterns of uptake and organ distribution [21].

Furthermore, plants have been shown to be able to uptake and translocate mercury, but this capacity depends on the form of mercury. Hg (0) in the soil seems to be not taken up by plant roots [14], but organic mercury, such as MeHg, and inorganic mercury, such as Hg^{2+} compounds are effectively absorbed [13], [14], [22], [23].

The amount of selenium to which the plant is exposed is equally important. It has been reported that a concentration of 2µM mercury inhibit by 50% the root elongation growth of wheat seedlings [19].

Selenium and mercury interactions

Selenium has been shown to interact with other elements, especially with heavy metals, when they occur simultaneously in a living organism. Antagonistic effects have been shown with S, P, F, As, Tl, Hg, Fe, Mn, Zn, Cu, Pb, Sn, Cd, Mg, W and other xenobiotics. Selenium counteracts the toxic effects caused by excessive accumulation of these elements. Specifically for heavy metals, three different mechanisms have been proposed to explain the detoxifying role of selenium: the formation of insoluble metal selenides, the alteration of the metabolism of the heavy metals and the minimization of the secondary effects of heavy metal poisoning [24].

Accordingly, selenium has a high potential in reducing the toxicity of heavy metals in plants [23], [25], [26] and animals [27]–[29], including humans [30].

Regarding mercury, the occurrence of both elements is effectively antagonistic, as the protective effect of one element against the other has been observed in many different organisms [31]–[33].

Therefore, an important interaction between selenium and mercury exists [34], and Se has been shown to protect from both inorganic and organic forms of Hg [35].

Mercury toxicity is based on its high affinity for sulphur compounds as, for instance, the thiol groups of the cysteine residues of many essential proteins and enzymes, affecting their functions [29].

Due to the similarities between selenium and sulphur chemistry, selenium can also create complexes with Hg, forming mixed compounds. In this form, Hg is less toxic. This detoxification process has been shown by the high correlation in tissues of the two elements: the contaminant (Hg among others) and selenium [21].

However, the exact mechanism for such detoxification is, up to date, still unknown. The metabolization of Hg and Se when simultaneous exposure takes place, the biomolecules involved, and intermediate and final compounds formed need to be deciphered.

Several compounds have been suggested to explain the detoxification mechanism, such as a mercury-selenide species [22], [23]. The formation of a Se–Hg–protein complex, biologically inert and with a 1:1 molar ratio of Se:Hg, has been widely proposed in literature [32]. More in depth studies have suggested several candidates as potential Se-Hg compounds: i.e., bis[methylmercuric]selenide, methylmercury selenocysteinate, selenoprotein P-bound HgSe clusters, and biominerals $HgSe_xS_{1-x}$ [21]. However, more direct analytical evidences are needed to confirm their presence in biological systems [21], [32].

Selenium and mercury coexistence in crops

Soil sites are often contaminated simultaneously with several pollutants [36], [37]. Coexistence of mercury pollution from mining in a naturally seleniferous area has been found, for example, in some regions of China, where rice crops have been affected [10].

However, mercury soil pollution is a worldwide problem. It has been shown that the uptake of Se and Hg by plants is modified when both are present in the medium [10], being their speciation also affected [19].

Therefore, the increasing use of selenium rich fertilizers to biofortify crops in Se-deficient areas can enhance mercury mobilization. Furthermore, selenium biofortification of a crop can be hampered by the presence of a heavy metal such as mercury, whose presence may also hinder the health benefits of Se-enriched functional foods and raise concern regarding food safety.

For these reasons, it is important to optimize the strategy of Se biofortification to obtain a desirable accumulation of selenium in edible tissues, while restricting mercury accumulation [36]. In the opposite case, if selenium addition led to a high

concentration of the heavy metal in the plant tissue, the strategy could be used for phytoextraction purposes, but then the crop would not be admissible for animal consume.

Objectives

The main goal of the present chapter is to describe the plant response when mercury and selenium are present simultaneously in the substrate and thus, available to the plants, identifying their interactions in the plant metabolism, in order to understand the possible mechanism of detoxification in wheat. In order to complete this main objective, the following tasks will be accomplished:

- ✓ To determine the effect of mercury exposure on plant development and to identify a possible protective effect of selenium simultaneous exposure.
- ✓ To measure the absorption and translocation of mercury in wheat, and to determine the effect of selenium in them.
- ✓ To measure the effect of the exposure to mercury on selenium uptake, distribution and speciation.
- ✓ To localize common accumulation areas and to identify interactions between the two elements
- ✓ To obtain further knowledge on the formation and the structure of a possible Se-Hg complex

Materials and Methods

Wheat culture and treatment:

Plants of common wheat (*Triticum aestivum* cv. Pinzón purchased from Semillas Fitó S.A.) were grown in hydroponic culture, in the conditions detailed in chapter 2. The nutrient solution (without or with the addition of the selected treatments) was renewed weekly in order to maintain stable the levels of water, nutrients, Se and Hg until the harvest.

At harvest, roots where washed with ice-cold $CaCl_2$ solution to remove the elements in the root apoplast, and then rinsed with deionized water. Then, plant material was dried in an oven at 45^{o}C during four days until stable weight, with air drying preferred over lyophilization in order to maintain the chemical structure for X-ray absorption spectroscopy (XAS) investigations [38].

Plants were exposed to selenium (as Na_2SeO_3, Na_2SeO_4 or a 1:1 mixture of the two salts) and/or mercury (as $HgCl_2$). The mercury–induced toxicity was studied first alone, without selenium application, and then in combination with the different selenium enrichment conditions i.e. selenite, selenate and mixture. Since the different behavior

of the selenium species without mercury exposure was already studied in previous chapters, only one selenium condition was used as a comparison for the individual Se treatment. The mixture condition was selected as the more relevant, which included the diverse metabolic processes of both species.

Two different cultures were performed, which are detailed in table 1:

In the first experiment the treatments were carried out with 25 µM of Selenium and 2 µM of Hg. After germination and two weeks of preculture time, at approximately in the vegetative stage (veg) num. 13, with 3 leaves unfolded (see chaper 2, table 1), Se and/or Hg were added to the nutrient solution. After 18 days of treatment, wheat plants were harvested, so the uptake of the elements was studied during the seedling stage of the plant. The exposure time was a little longer than the study of Se absorption in time (see Chapter 2) to ensure that the effects of the interaction of the two elements on the wheat plants could be seen.

In the second experiment, the final effect of Se and Hg over senescent plants and grain production was studied: the two elements were applied at the plant florescence stage (flor), num. 53, when approximately 1/4 of the inflorescence had emerged. Then the treatment was kept during the rest of the plant life cycle until senescence, and thus the treatment lasted approximately 2 months. Selenium and mercury were applied at 25 µM and 0,5 µM respectively. Lower Hg concentrations were chosen due to the excessive toxicity symptoms observed in seedlings with 2 µM, which could really hamper wheat growth and totally impede grain formation when applied for a longer period of time.

Table 1: Selenium and mercury treatments used in the present work and concentration and species used of each element.

Treatment	Seedlings 18 days, veg stage	Senescent plants 2 months, flor stage
Control	-	-
Hg	Not included	0,5 µM Hg as $HgCl_2$
Selenite + Hg	25 µM Se as Na_2SeO_3 + 2 µM Hg as $HgCl_2$	25 µM Se as Na_2SeO_3 + 0,5 µM Hg as $HgCl_2$
Selenate + Hg	25 µM Se as Na_2SeO_4 + 2 µM Hg as $HgCl_2$	25 µM Se as Na_2SeO_4 + 0,5 µM Hg as $HgCl_2$
Mixture +Hg	25 µM Se as Na_2SeO_3 + 25 µM Se as Na_2SeO_4 + 2 µM Hg as $HgCl_2$	25 µM Se as Na_2SeO_3 + 25 µM Se as Na_2SeO_4 + 0,5 µM Hg as $HgCl_2$
Mixture	25µM Se as Na_2SeO_3 +25µM Se as Na_2SeO_4	25µM Se as Na_2SeO_3 +25µM Se as Na_2SeO_4

Total elemental analysis:

As in previous experiments, an acid digestion was performed in closed HP500 PFA vessels in a microwave digestion system (Mars 5, CEM, USA) under EPA 3052. Plant material (50 mg) was placed in the digestor tube with 4ml of HNO3:H2O2 (3:1), and a gradient of temperature and pressure was applied up to 180 ºC and 1.9 atm for 45 minutes. Samples were stored at 4 ºC until analysis (see chapter 2).

The plant digestates were adequately diluted and the contents of ^{23}Na, ^{24}Mg, ^{31}P, ^{55}Mn, ^{56}Fe, ^{64}Zn, ^{65}Cu, ^{78}Se, ^{98}Mo and ^{202}Hg were measured by Induced Coupled Plasma Mass Spectrometry, ICP-MS, (X Series 2, Thermo Elemental, UK). Commercial standard solutions of 1000 mg L^{-1} of the elements were used for the calibration procedure. ^{45}Sc, ^{69}Ga, ^{89}Y and ^{115}In were monitored as internal standards.

The macronutrients S, Ca and K were measured by Induced Coupled Plasma atomic emission spectroscopy (ICP-AES). These analyses were performed by the external analytical service at “Scientific and Technological Center of the University of Barcelona”.

CVAFS:

Dried plant material (100mg) was placed in glass vessels with 5ml of concentrated nitric acid. A pre-digestion at room temperature was performed overnight, and later on, the samples were digested in a hot plate at 65^{o}C for 24h.

Mercury was analyzed by Cold Vapor Atomic Fluorescent Spectrometry, CVAFS, (2600 series CVAFS, Tekran, Canada) following the EPA method 1631.

The sample digestate was diluted with 0.5BrCl, a strong oxidizer that preserves the Hg in solution as Hg^{2+}. Before analysis, the excess of BrCl was neutralized with hydroxylamine hydrochloride 0.2%. During the analysis, the mercury is reduced with $SnCl_2$ to produce Hg (0), which is carried as gas phase using an Argon flux. This mercury was concentrated with two Au coated quartz sand trap, and detected using a UV detector.

XAS, µXAS and XRF:

- **SSRL:**

Elemental distribution was analyzed in wheat by X-Ray Fluorescence (XRF) with synchrotron radiation at Stanford Synchrotron Radiation Lightsource (SSRL), at beamline 10-2.

Energy calibration was performed on metallic selenium, at Se K-edge energy of 12658 eV. Beamline 10-2 was equipped with a 30 pole, 1.45-Tesla Wiggler ID end station and a double crystal Si (1 1 1) monochromator. A beam size of 50x50µm was used, with a dwell time of 50ms/point.

The distribution of elements was obtained selecting regions of interest at the Kα emission energies of Se, P, S, K, Ca, Mn, Fe, Cu, Zn and Lα of Hg, with an excitation energy of 12677.0 eV, over the Se K-edge energy.

The data was extracted and analyzed using the software SMAK [39], rendering images where higher elemental concentrations are shown in warmer colours and lower concentrated regions appear in blue, such as the one that can be seen in figure 2.

Two full seedlings, exposed to mixture of Se species at 25 µM and Hg at 2 µM, were mapped by XRF: one was fresh, measured immediately after harvest, and the other had been dried in the oven at 45^{o}C. They were directly placed in the sample holder with double-sided scotch tape for the measurements.

Two grains were also mapped by XRF, one treated with the mixture of species at 25 µM Se and the other with the mixture at 25 µM and Hg at 0,5 µM. Grains were embedded in paraffin and cut using a microtome. The precise microtome cuts were done vertically in order to expose selected regions of interest of the interior of the

grain, where the main features of grain physiology were visible, mainly the germ, the bran and the endosperm, and subfeatures inside them, as the pigment strand.

Spatially resolved µXAS (micro X-ray Absorption Spectroscopy) measurements were performed on a root sample, treated with a mixture of selenite and selenate at a concentration of 25 µM and mercury at 0,5 µM. Measurements were carried out at beamline 2-3 of SSRL synchrotron, equipped with a 1.3 Tesla Bend Magnet and a double crystal Si (1 1 1) monochromator, with a beam size of 2x2 µm. Fluorescence data was acquired with a vortex detector. Three points of interest were selected and three repetitions were acquired per point, for Se K-edge and for Hg LIII-edge. The three measurements were normalized and averaged using the Athena software [40], including energy calibration and background subtraction. The sample data was compared with spectra acquired in the same manner on pure references.

- **ESRF:**

Bulk XAS analyses were performed on the European Synchrotron Radiation Facility (ESRF) at beamline BM25A, the Spline.

The beamline is situated in a bending magnet source and is equipped with a double crystal Si (1 1 1) monochromator. Measurements were performed at Se K-edge and Hg L3-edge, after energy calibration with metallic selenium.

References were prepared with pure commercial standards in the form of pellets, diluted to the adequate concentration with cellulose. Their spectra were analyzed in transmission mode with gas ionization chambers.

The roots and shoots of wheat seedlings and senescent plants treated with the mixture+Hg conditions were selected for the analysis. Plant material was ground in a automatic mortar and pressurized in the form of a pellet, without dilution. Samples were acquired by fluorescence mode with a 13-element Si detector.

All measurements were carried out in liquid nitrogen cryostat at approximately 77K in order to avoid radiation damage. In each sample, 5 scans were acquired. The resulting spectra were normalized and merged with the software Athena, and the species content was analyzed by a least-square linear combination fit with the reference spectra.

- **ALBA:**

Selenium speciation was studied in grains by µXAS at CLAESS beamline, at ALBA synchrotron.

CLAESS beamline is equipped with a multipole wiggler source and measurements were performed with a double crystal Si (1 1 1) monochromator. Energy calibration was

performed on metallic selenium, at Se K-edge energy of 12658 eV. Selenium references were analyzed in transmission mode, with gas ionization chambers. Grains were analyzed by fluorescence mode with a silicon drift detector, with a beam size of 100x200 µm. All measurements were performed in a liquid nitrogen cryostat at 77K to avoid radiation damage.

Four grains were analyzed, which were representative of 4 treatments: Mixture of selenium species at 25 µM, Mixture of selenium species at 25 µM and mercury at 0,5 µM, selenite at 25 µM and mercury at 0,5 µM and selenate at 25 µM and mercury at 0,5 µM. These grains were also prepared by paraffin embedding and cut with a microtome in order to expose the regions of interest selected.

For each sample, a coarse XRF maps was acquired. Then, µXAS spectra were measured in approximately 60 points of interest covering the main grain features. Those points were grouped in areas, merging points in neighboring regions within the same grain tissue (i.e. germ, pigment strand, bran and endosperm). A slightly different number of areas resulted from each sample, in order to group a similar number of points to have comparable signal to noise ratio. The selection and grouping of points was performed with the software PyMCA [41]. The spectra normalization and least-squares linear combination fit analysis was performed with the software Athena [40].

IR:

A selection of control and treated roots were sectioned with a scalpel under a microscope, in order to separate the root epidermis (external surface) and endodermis (between the cortex and the inner vascular system).

The samples, mounted on a CaF_2 window, were analyzed by Fourier Transform Infrared (FTIR) spectroscopy, at MIRAS beamline, at ALBA synchrotron, in transmission mode with a microscope equipped with a 64x64 Focal Plane Array detector.

Different areas on the sample surface were mapped, with the acquisition of approximately 100 spectra per sample. Spectra were collected in the 4000-800 cm^{-1} mid-infrared range. Data acquisition was performed with the OPUS software (Bruker), and data treatment with Unscramble X (Camo Analytics).

A region of interest (ROI) was selected where characteristic absorption bands of the selected functional groups were more intense, specifically in the range of 1778-1536 cm^{-1} for proteins [42]–[45]. The spectra were treated with the Savintzky-Golay 2nd derivative and normalized. Principal component analysis (PCA) was used to reduce the number of variables into fewer dimensions, finding similar patterns within the data [46]. Furthermore, the spectra were also average in order to facilitate visual comparison.

Results and Discussion

PART I. Plant response to the presence of selenium and mercury

Wheat seedlings: affectation of plant growth

The effect of selenium and mercury application on the growth of wheat seedlings was studied through the comparison of the plant dry weight and the root elongation between treated plants and control plants, as seen in figure 1.

The addition of selenium and mercury to seedlings for 18 days did not result in a significant alteration of overall plant weight, but it did affect root elongation.

Selenium-induced root growth inhibition was not statistically significant, however, the addition of mercury resulted in a significant hampering of root elongation.

The remarkable effect of mercury on root elongation but the lower impact on plant weight has also been previously observed in wheat seedlings [19]. This is due to the fact that the presence of mercury is causing an increase in the production of ROS, known as oxidative burst, and a depletion of antioxidant compounds, e.g. ascorbate and GSH [20], [47]. This situation induces the plant to initiate secondary defense reactions, that include the strengthening of the cell wall through lignification, which results in the reduction or inhibition of root elongation [20], and it can even lead to damage to the cell membranes and/or cell death [19], [47].

Selenium is expected to decrease the damage caused by mercury to the plant; however, the study of plants exposed to only mercury should be included in order to estimate this effect. Regarding selenium speciation, it can be seen that there were no significant differences in root elongation, although the combination of selenite with mercury caused the highest inhibition.

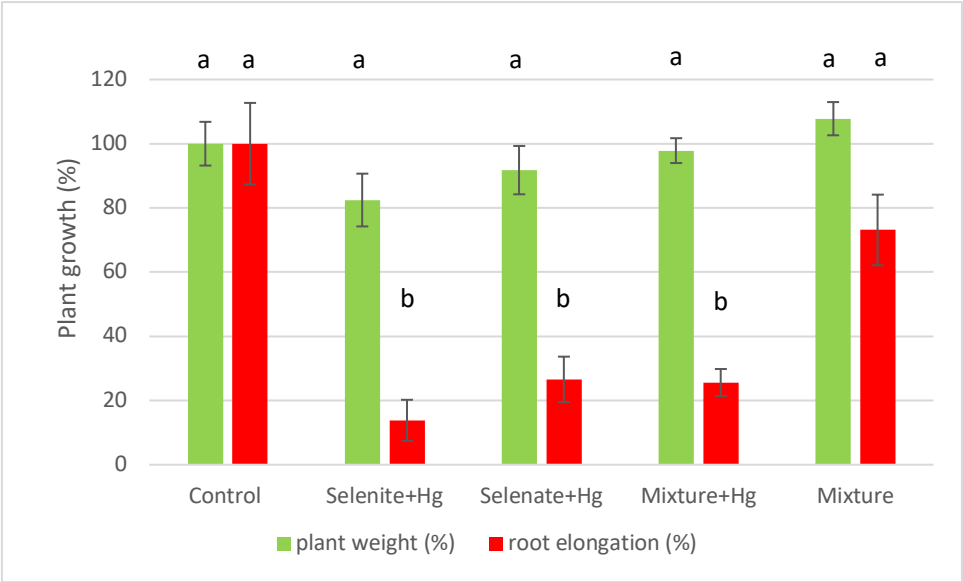

Figure 1: Overall plant weight and root elongation (%) of wheat seedlings after 18 days of selenium and/or mercury exposure. Given results are mean±SE (n=8). Letters indicates significance ($p<0.05$) between different treatments.

Wheat seedlings: elemental distribution

In order to begin understanding the metabolic uptake by wheat seedlings of selenium and mercury, and their effects on other nutrients, a full mapping of the elemental distribution through a plant was acquired. A seedling treated with a mixture of the two inorganic selenium species and mercury was selected.

In figure 2, a picture the full dried plant and a map of the corresponding selenium fluorescence signal are shown. In the picture roots (right side) and shoot, i.e. stem and leaves (left side) can be seen, divided in the middle by the presence of the old seed from where the plant germinated. In the fluorescence map, higher selenium concentrations are shown in warmer colors than low selenium concentration regions.

Selenium is mostly accumulated in roots and at the base of the stem, but there is also low concentration present in leaves. Thus, selenium was distributed through all the plant in 18 days.

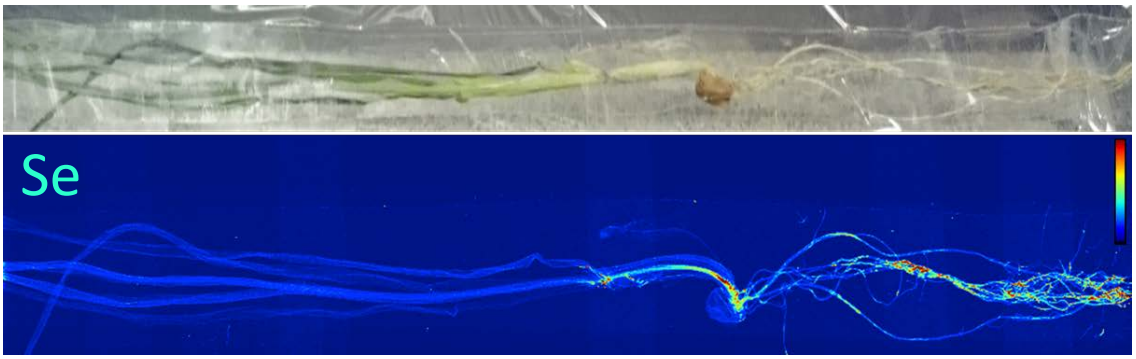

Figure 2: photography and Se XRF map of a full dry seedling acquired at 12700 eV.

The distribution of Se was compared to the distribution of Hg in figure 3. Selenium is shown in blue, and mercury in red, although the color becomes pink when they are overlapped.

It can be seen that mercury is accumulated in roots, and that the translocation to shoots is either not occurring or lower than the limit of detection of XRF.

Therefore, wheat root plants can absorb free mercury in the form of Hg^{2+} if they are exposed to a certain concentration of it in the substrate. However, it can be seen that most of the mercury is present in the lower part of the roots, which were submerged in the hydroponic solution, and almost none has reached the upper part of the roots, which were not submerged.

Accordingly, mercury is not mobilized through the plant xylem, neither within roots nor to shoots. These results are of special interest for both crop safety and environmental purposes, as the low plant mobilization may ensure the safety of consuming crops grown in soils with some mercury contamination.

The differences between the distribution of selenium and mercury were also compared to those of some relevant nutrients (S, P, K, Ca, Mn, Fe, Cu and Zn) in figure 4, where the fluorescence signal of the nutrient is presented in red, overlapped with selenium signal in blue.

Some important trends that can be extracted from the images: all elements analyzed are accumulated in roots, but the distributions are not identical.

S and P are more equally distributed through the roots, stem and leaves. These two elements are macronutrients and essential constituents of life, as they are present, among other molecules, in DNA, ATP and phospholipids in the case of P, and amino acids in the case of S, which will be found within all the cells through the plant [48].

Both K and Ca are more present in upper parts of the roots, and K also in stem and leaves. The top roots are known as crown roots and are thicker and shorter and have a structural purpose, used by the plant in order to strongly attach to soil. Calcium is a constituent of cell walls and is involved in plant growth and stem and root elongation

of plant [49], [50]. Potassium is generally concentrated in growing tissues and reproductive organs, due to its enzymatic, osmotic activity and its function in carbohydrate metabolism [49], [51].

Oppositely, Mn, Fe, Cu and Zn are found in the seminal roots, in the lower part which was immersed in water. They are micronutrients, and although they are essential, because they have a wide range of diverse metabolic functions in plants, only small amounts of them are required [52], [53].

The fact that such small concentration is needed to the plant can explain why their translocation to aboveground tissues is smaller than the quantity available for uptake in the hydroponic solution, and they accumulate in the lower part of the roots. However, a significant concentration of them is always translocated, as some fluorescence signal is observed in the stem in all the cases.

Oppositely, mercury fluorescence is not observed in the stem. Accordingly, there is no correlation, which means that the metabolic uptake of mercury does not follow the same mechanism of uptake and transportation through xylem that those essential metals.

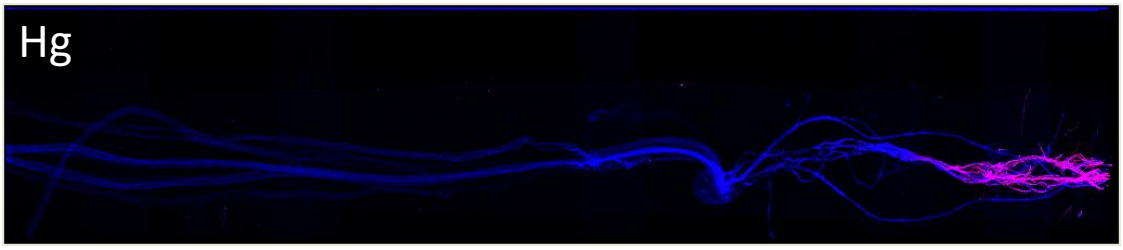

Figure 3: Se (blue) and Hg (red) overlapped XRF maps of a full dry seedling acquired at 12700 eV.

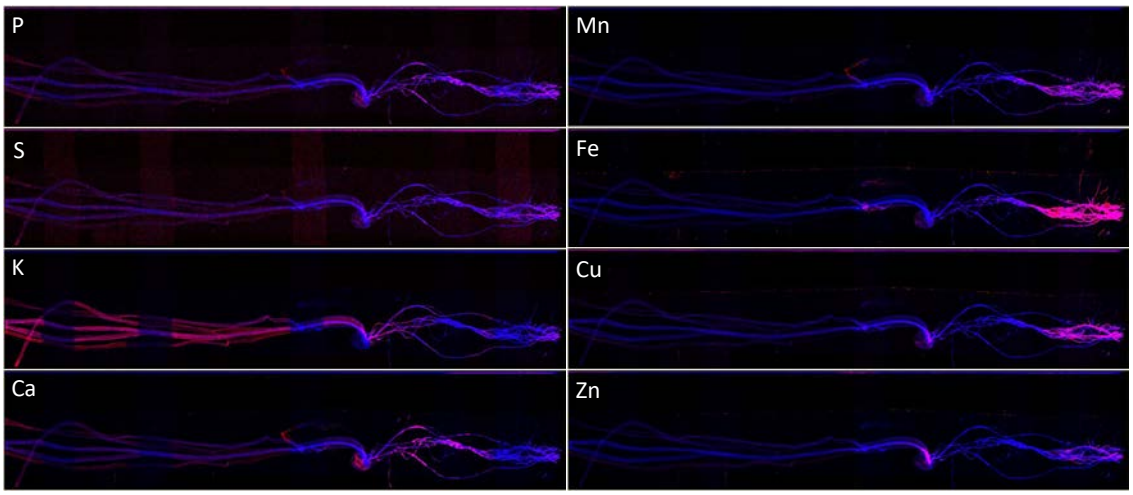

Figure 4: Se (blue) and indicated nutrient (red) overlapped XRF maps of a full dry seedling acquired at 12700 eV. The vertical stripes visible in some of the maps (P, S and K) correspond to the tape used to hold the samples.

Furthermore, the effect of the sample treatment in the observed distribution was studied, in order to determine that the washing and drying processes during harvest produced no alterations. A second entire plant was mapped, in this case a fresh seedling, which was placed directly in the beam immediately after harvest and root rinsed with distilled water. There were no significant differences in the distribution results between fresh and dried material, allowing the conclusion that the drying at 45ºC does not affect the results.

However, detailed images of this fresh plant in the intersection between roots and shoots, figure 5 a), and between stem and a leave, figure 5 b), confirmed that selenium is preferentially located and translocated through the vascular system of the plant [54], in comparison with other elements, such as Ca, that are seen in the exterior of the stem and the leaves due to their role as constituents of the cell walls.

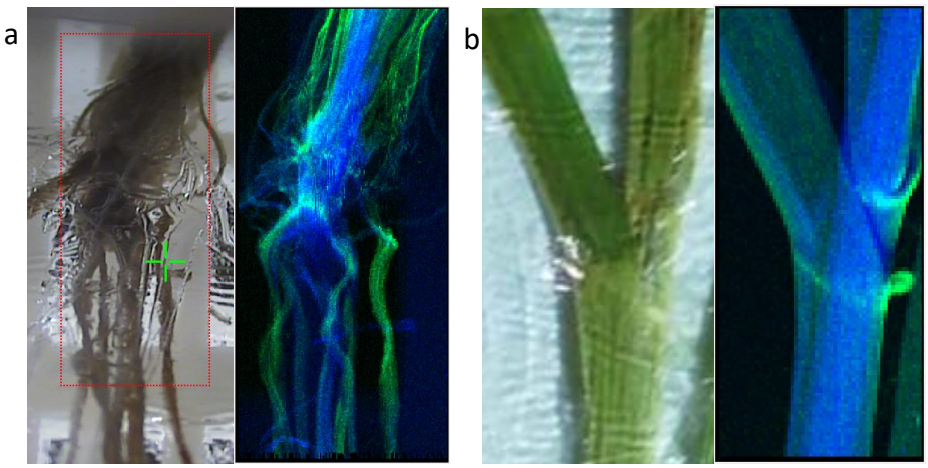

Figure 5: Se (blue) and Ca (green) overlapped XRF maps in a fresh wheat seedling acquired at 12700 eV in a) section between the root and shoot and b) between the stem and a leave.

Wheat plants: affectation of plant growth

The changes in growth of senescent plants by the exposure to selenium and mercury were also studied, as seen in figure 6.

As it was also the case with the seedlings, overall plant weight was not significantly affected with any treatment, and neither was the individual root or shoot weight. The exposure had a visible effect on root elongation, however, the measurement of this parameter is not straightforward as plants produce many entangled roots, difficulting an accurate quantification.

However, the grain weight was significantly affected by the mercury treatment, due to the toxicity of this element and its potential to produce elevated levels of ROS that will hamper plant development.

On the other hand, individual selenium exposure did not affect significantly the grain yield.

Furthermore, the protective effect of selenium against mercury was observed. In the conditions in which wheat was exposed to both elements, the grain weight did not decrease significantly, and the final yield was similar to that of selenium alone. Besides, there were no differences depending on the selenium species.

Therefore, it was confirm that there was a selenium mercury interaction that counteracted, at least partially, the mercury induced phytotoxicity, and this interaction took place with both selenite and selenate.

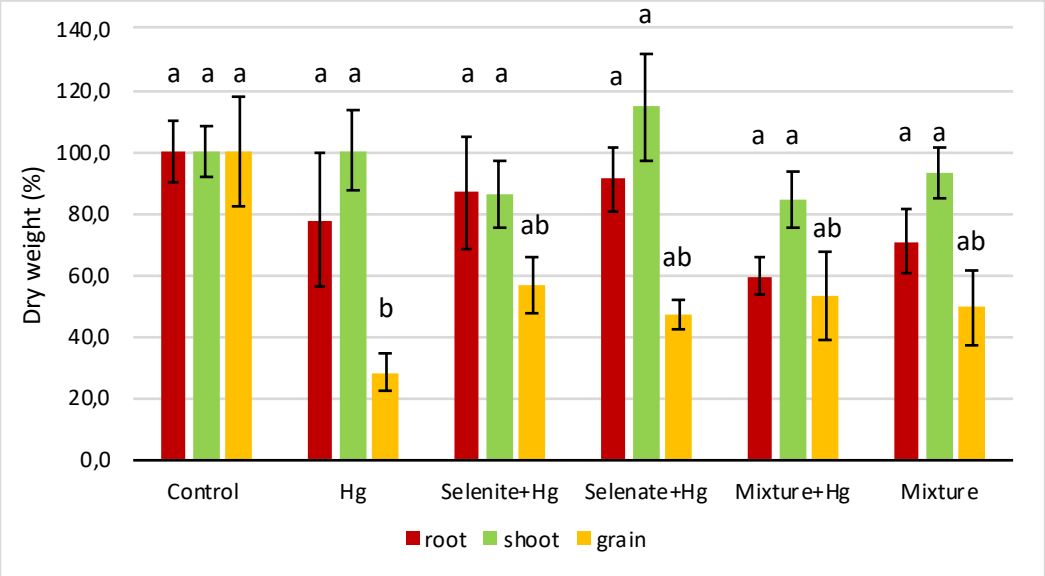

Figure 6: Root, shoot and grain weight (%) of wheat plants after selenium and/or mercury exposure. Rresults are mean±SE (n=6). Letters indicates significance (p<0.05) between different treatments.

Wheat plants: changes in selenium concentration by mercury

The changes of selenium concentration and distribution in the presence of mercury were studied:

Figure 7 shows that the simultaneous presence of selenium and mercury increases significantly the uptake of selenium through roots, which then results as well in an enhance translocation to the aerial tissues, and thus the selenium content increases in all tissues i.e. roots, shoots and grain. Therefore, the total selenium content in the plant is much higher when both elements are combined, and accordingly, the interaction of selenium and mercury is enhancing the selenium uptake by wheat. This effect is in agreement with what was observed in other plants for simultaneous selenium and mercury exposure [55].

Regarding the effects of the specific Se species, it can be seen in figure 8 how selenite interaction with mercury results in a biggest enhancement of root and shoot Se accumulation than seleante, but, however, significantly less accumulation in grain.

In comparison, the mixture of the two species leads to an even higher root selenium accumulation. Since the resulting concentration is not the average of the individual species concentrations, the effects of them are not simply additive. This can indicate that selenite and selenate have separate processes of interaction with mercury.

Selenite already results in higher Se accumulation in roots, as seen in Chapter 2, but in the case of a combined application with mercury, both root and shoot levels increase, rising the translocation factor to 0,81, which is much higher than the 0,09 found for selenite individually (at 25 µM florescence stage, see chapter 2). Therefore, the interaction between selenite and mercury is an additional process, which enhances the overall Se uptake and speeds up the translocation of selenite to shoots. The reason for that can be the formation of a Se-Hg complex, which is much more mobile than selenite itself, and can slow down the reduction to also less mobile organic selenoamino acid species.

The same effect is not seen in selenate treatment, whose individual translocation to shoots is already favored. Its translocation factor when both selenate and mercury are combined is 1,22, which is much lower than the individual selenate value of 3,32 (see chapter 2). Therefore, the Se-Hg complex formed with selenate may have lower mobility than selenate itself.

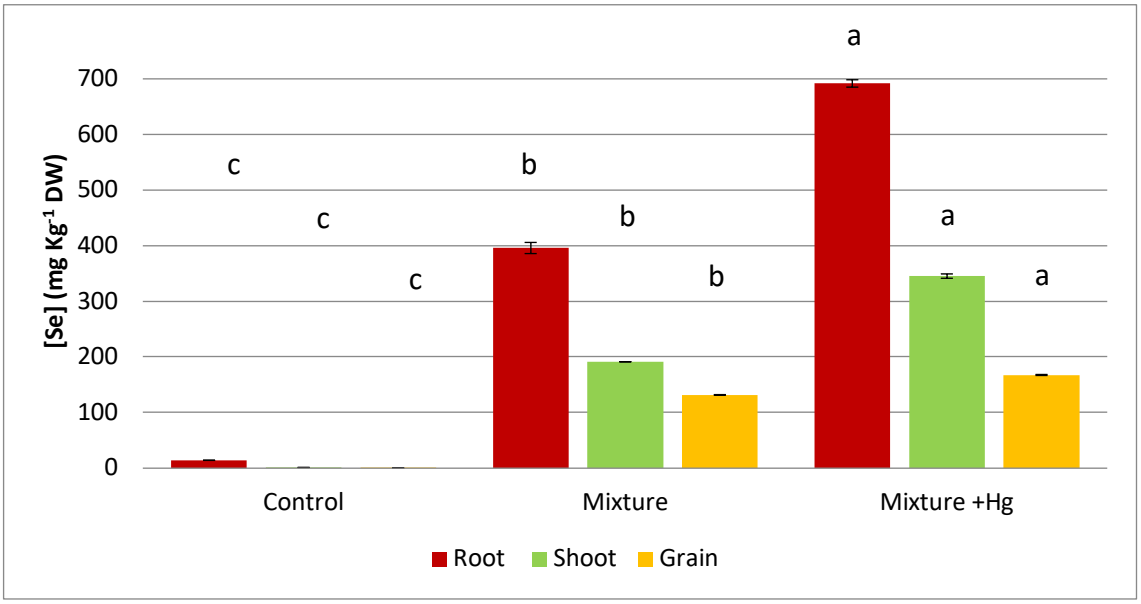

Figure 7: Selenium concentration in root, shoot and grain (mg Se/Kg DW), represented as mean±SD (n=6). Letters indicates significance (p<0.05) between treatments.

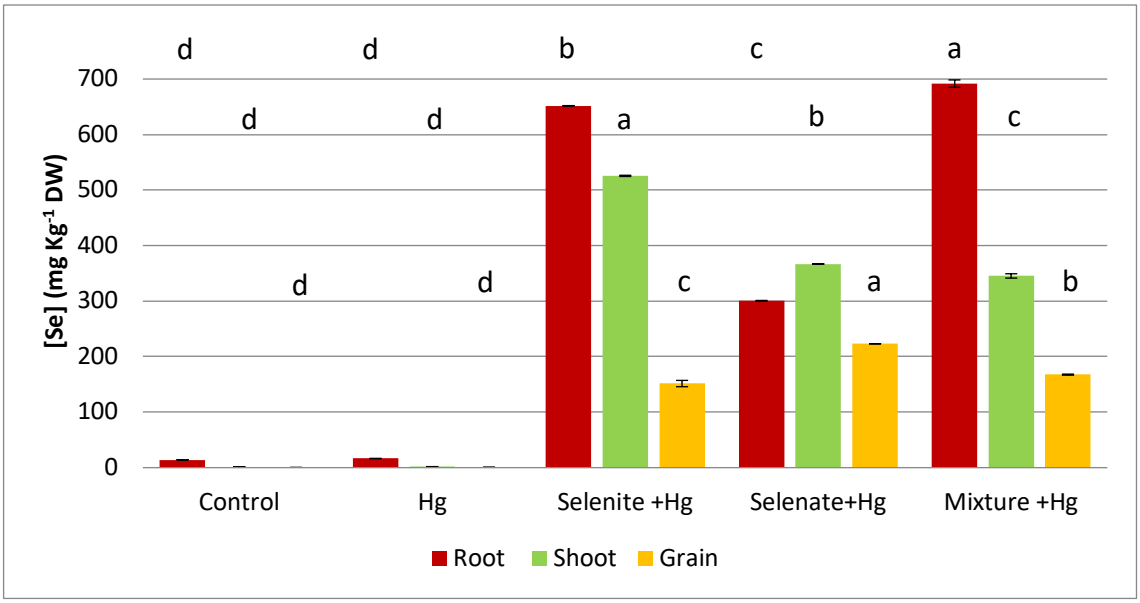

Figure 8: Selenium concentration in root, shoot and grain (mg Se/Kg DW), represented as mean±SD (n=6). Letters indicates significance (p<0.05) between treatments.

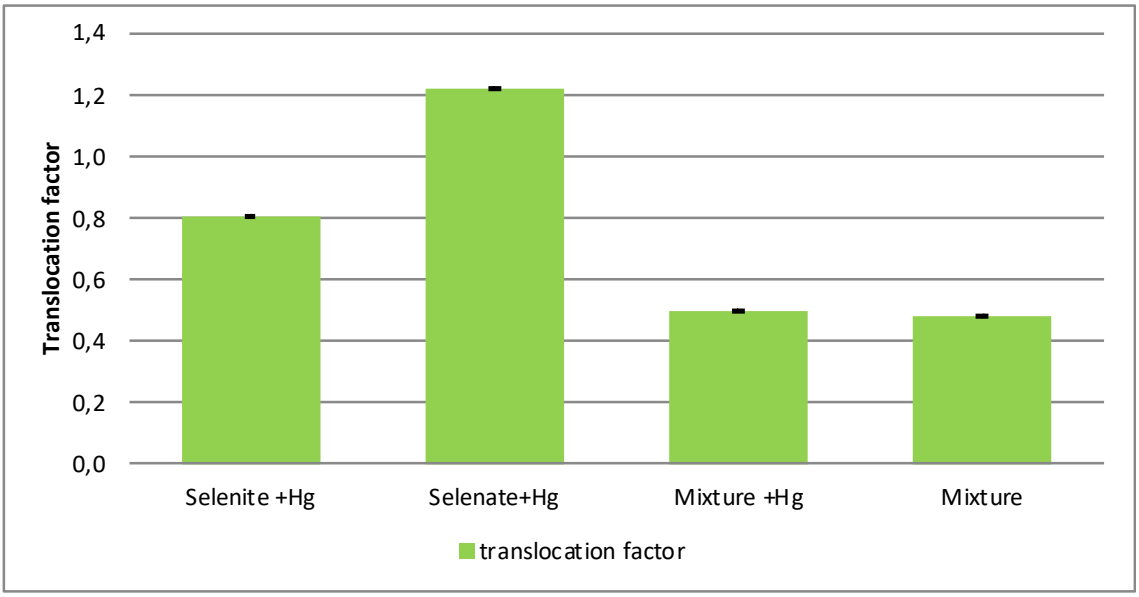

Figure 9: Selenium translocation factor (shoot Se concentration/root Se concentration) for each treatment, as mean±SD.

Wheat plants: changes in mercury concentration by selenium

The results of figure 10 show that mercury is highly accumulated in wheat roots, and very little of it is translocated to wheat shoots and grain, which confirms the previously observed Hg distribution in wheat seedlings. In fact, the translocation factor of Hg in these mature plants is smaller than 0,01 in all cases. For this reason, the Hg content in shoots and grain is plotted separately in Figure 11.

The simultaneous occurrence of mercury and selenium enhances mercury uptake. Since also the uptake of selenium was enhanced, probably part of the uptake is taking place involving the formation of a Se-Hg complex.

Regarding the specific selenium species, selenate enhances mercury accumulation in roots more than selenite, despite decreasing the content in shoots. As previously discussed, probably the Se-Hg complex has an intermediate mobility, and thus its formation is enhancing the mobility of selenite, while decreasing that of selenate.

However, the mixture is the condition that mostly raises Hg accumulation, both in roots and in shoots. Therefore, this is in agreement with the idea selenite and selenate interactions with mercury are not simply additive, but they are separate processes that are accumulative and can enhance Hg uptake as well as Se.

In contrast, grain Hg concentration is the lowest in the case of the mixture treatment. It is significantly lower than the exposure to mercury alone as well as to mercury and selenite and mercury and selenate.

Consequently, the application of a mixture of the two selenium salts is significantly enlarging Hg accumulation in roots and shoots. However, since the translocation to grain is effectively decreased, this treatment will result in the lowest mercury accumulation in grain and thus the least harmful for animal or human consume.

It is possible that the interaction with the mixture of the two species, which both promote root Hg uptake, result in enhanced transport through root cell transporters. Besides, with the complex of Se-Hg formed having an intermediate mobility through xylem, the accumulation in shoots is coherently higher. However, the Se-Hg complex may further react in shoots, being metabolized to a different species with a lower mobility, which cannot be transported through the phloem into the reproductive organ and the grain.

Accordingly, the application of a mixture treatment can be advisable for the growth of wheat crops in agricultural fields with risk of mercury pollution.

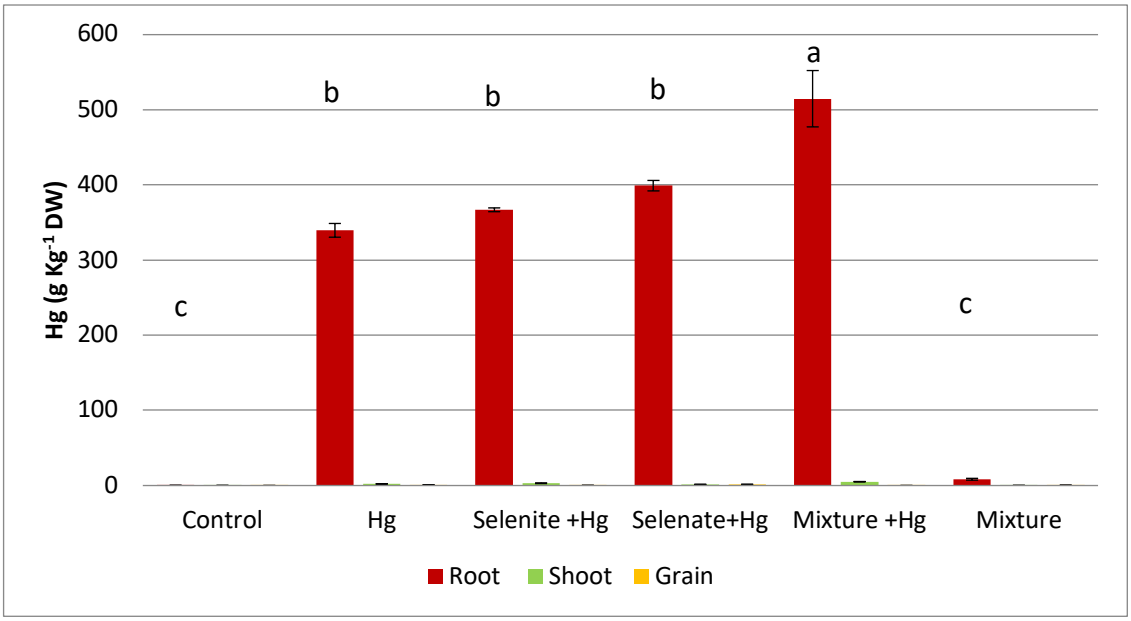

Figure 10: *Mercury concentration in root, shoot and grain (g Hg/Kg DW), determined by ICP-MS, and represented as mean±SD (n=6). Letters indicates significance (p<0.05) between treatments in roots.*

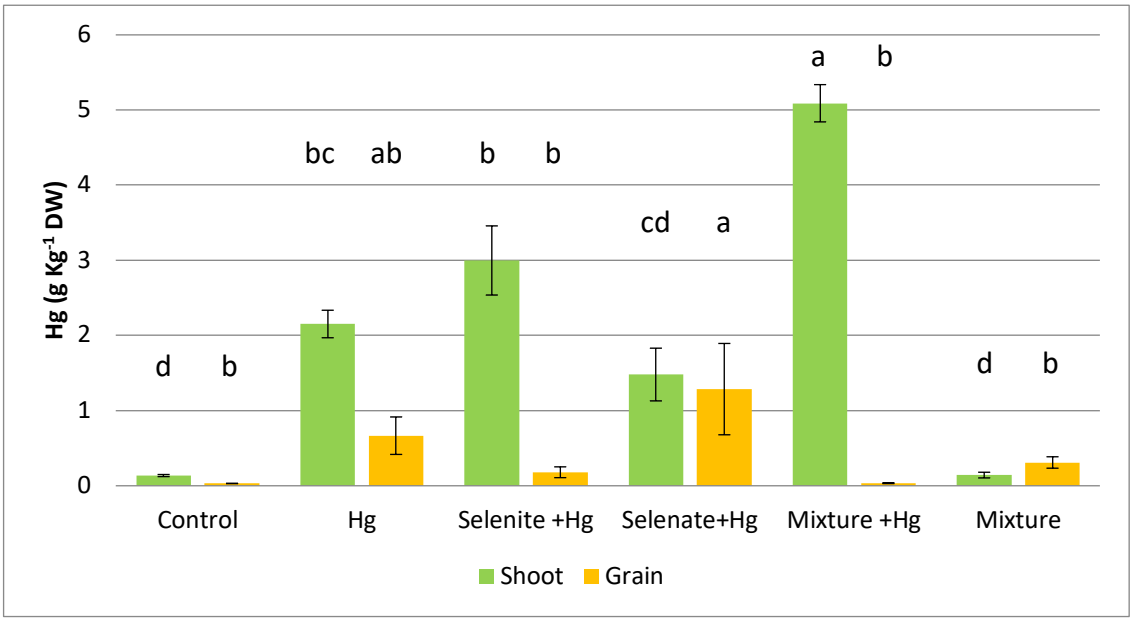

Figure 11: *Mercury concentration in shoot and grain (g Hg/Kg DW), determined by ICP-MS, and represented as mean±SD (n=6). Letters indicates significance (p<0.05) between treatments, in shoots and in grain.*

Wheat plants: mercury analysis by CVAFS

ICP-MS is not the most suitable technique for the analysis of the Hg concentration, as part of the mercury content can be lost during sample pretreatment and analysis, due to the absorption of a part of the mercury content in several materials.

Thus, mercury was analyzed by CVAFS, which is especially sensitive to mercury due to the concentration of the mercury by amalgamation in Au before detection.

Mercury levels determined in wheat by CVAFS were higher than the analyzed by ICP-MS.

Roots showed a higher amount, around 1330-1401 mg/g of DW, while shoots and grain level did not change significantly, with values between 1-10 mg/g and 0,02-0,3 mg/g respectively.

However, the results from CVAFS, even partial, confirm the trend previously observed: the interaction between selenium and mercury caused an increase of the levels of mercury uptake and accumulation in roots respect to those of individual mercury treatment, however, it decreased the translocation of mercury to the shoots and the grain, thus reducing the damage to the crop production and the safety concern.

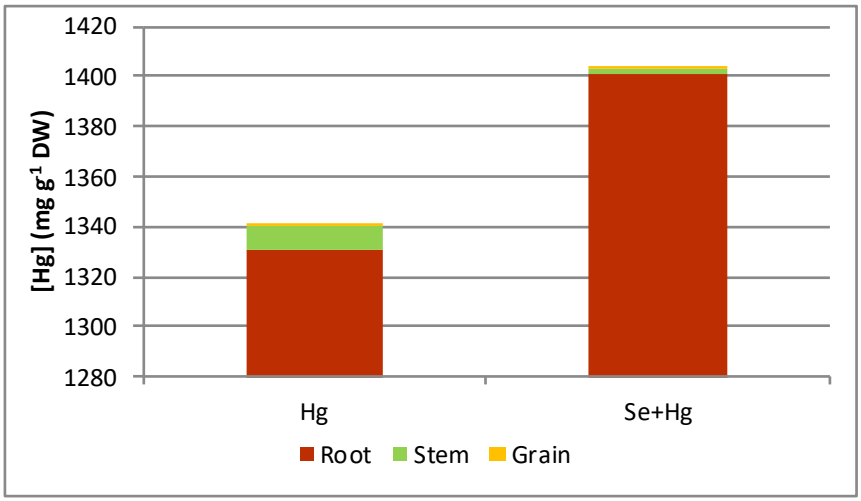

Figure 12: *Mercury concentration in root, shoot and grain (mg Hg/g DW), determined by CVAFS, and represented as mean±SD (n=6).*

Wheat plants: nutrient concentration

The affectation of selenium and mercury treatments over the overall plant health status was investigated through the analysis of changes in nutrient homeostasis. The alteration of macronutrients (S, P, K, Ca and Mg) is represented in figure 13 and that of micronutrients (Fe, Mn, Cu, Mo and Zn) in figure 14:

Sulfur concentration increases with mercury treatment in roots, shoots and grain. Selenium treatment decreases the concentration in roots but does not have a

significant effect in shoots and grain. The combination of selenium and mercury also tends to decrease S in roots and increase it in shoots, but no effect is seen in grain.

Mercury is known to have a big affinity for sulfur. When mercury is applied to the plant, there is a significant enhancement of the levels of accumulated in wheat. Therefore, most probably there is a formation of a S-Hg complex, and thus the plant S uptake increases to counteract the levels of Hg. However, when selenium is added together with mercury, the increment of the S concentration is not observed anymore. Therefore, Se is replacing sulfur in reacting with mercury to form a complex, and S metabolism is no longer severely affected, which will serve to decrease the induced toxicity.

Phosphorus concentration decreased in roots with all treatments. In shoots it also decreased with mercury and selenium and selenate+Hg and mixture+Hg, but increased with selenite+Hg. Oppositely in grains, it increased with selenium and with all selenium+Hg treatments, but there was no change with mercury treatment respect to the control.

Potassium concentration in plants treated with mercury individually increases in roots and decreases in shoots, contrary to the results with the presence of selenium individually. The treatment with a combination of both elements decreases K amount in both roots and shoots. Besides, potassium in grain decreases with mercury alone, as well as, with selenate+Hg.

Calcium in roots significantly decreases with all conditions assayed. Oppositely, Ca in shoots decreases with mercury and with selenium and mercury combined. However, no significant changes were observed in Ca levels in grains.

Magnesium levels in plants with mercury exposure increases in roots and in grains, with no significant changes in shoots. With selenium, Mg decreases in roots, but there are no changes in shoots and grain. With both elements, Mg decreases in roots only in the case of applied as mixture+Hg, increases in shoots in all cases and decreases in grain with selenate+Hg.

Regarding micronutrients, mercury raises the iron accumulation in roots, shoots and grain. Selenium increases the Fe levels in roots, but not in shoots or grain. The combination of the two elements increases the Fe in roots (except for selenite+Hg) and shoots, with selenate+Hg increasing also Fe grain concentration.

Manganese concentration significantly decreases in roots and shoots with mercury and selenium either individually or in combination. However, no significant canges are observed in grain Mn concentration except for the selenate+Hg treatment.

Mercury individually causes a significant increase in the copper levels of roots, shoots and grain. Selenium on the other hand, decreases levels in roots, while it has no effect on shoots and grain. The combined exposure to the two, decreases Cu levels in roots

and increases it on shoots. Moreover, selenate+Hg also cause a significant higher Cu amount in grain.

Molybdenum increased in roots with all conditions (except for selenite+Hg) and decreased in all treated shoots. However, no significant changes were observed in grain Mo concentration.

Finally, zinc accumulation increases in roots and grain with mercury treatment and decreases in shoots. With selenium treatment it decreases in roots, but there is no alterations of shoots and grain Zn. Finally, the combination of mercury and selenium also decreases root Zn level, while producing no change in shots and grain as in the case of selenium by itself.

Consequently, the modifications that occur to the nutrient uptake and accumulation processes after selenium and mercury treatments do not follow a common path, in agreement with what was seen for the selenium treatments in chapter 2. In general, mercury produces some changes in the plant nutrient concentration that are very different to that of selenium alone. Thus, a different mechanism of metabolization, interference with nutrient uptake pathways and plant-induced toxicity is expected from mercury respect to selenium.

Furthermore, the changes on the level of nutrients when both elements are combined does not result in the same plant response than any of two applied individually, therefore, the mechanism of plant metabolization of the complex Se-Hg should be different to the individual elements. However, the responses are generally more similar to those of selenium. Therefore, this complex metabolization is expected to follow more closely the mechanism of the different selenium species, and this will result in the prevention of some of the toxicity caused by mercury.

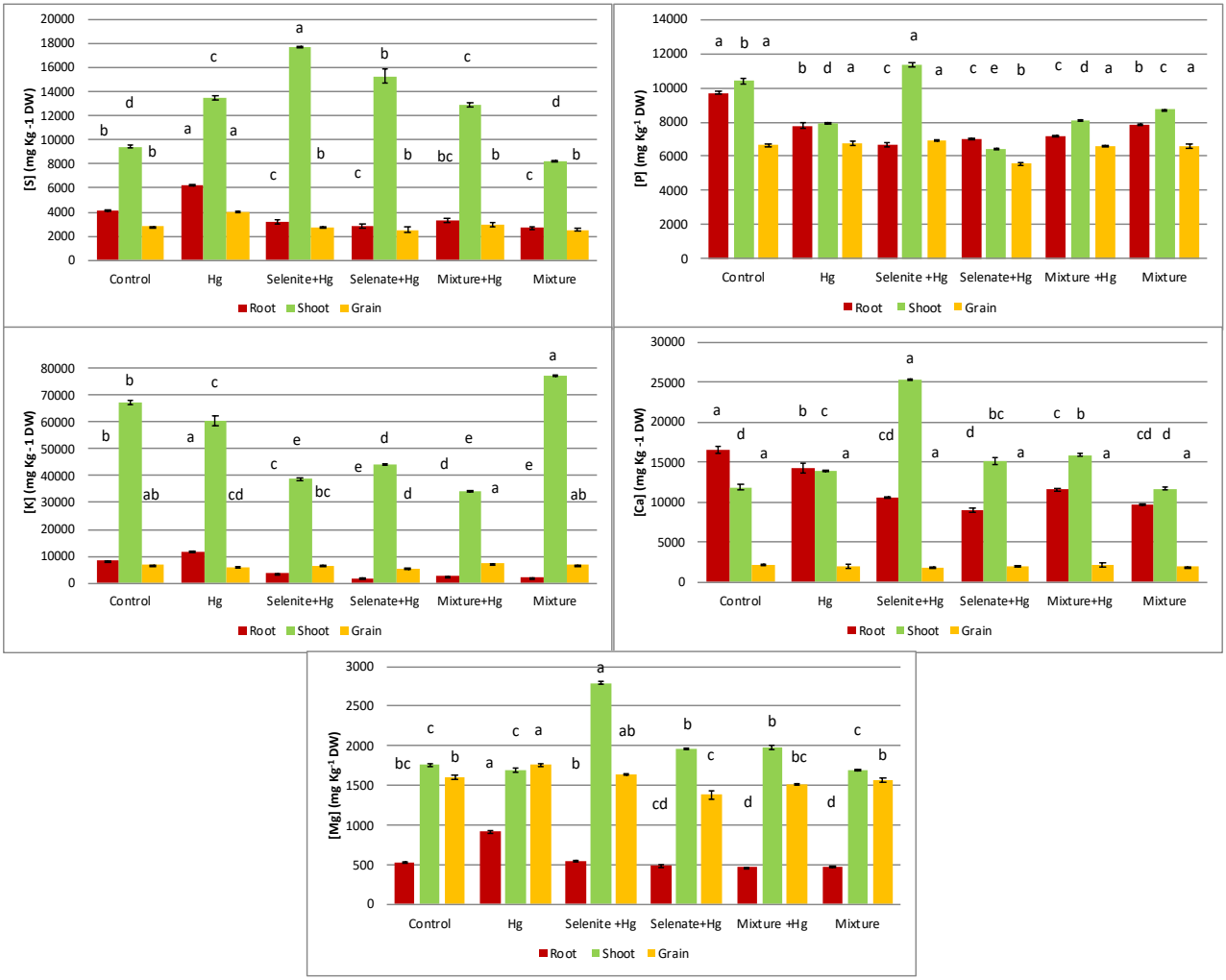

Figure 13: Macronutrient (S, P, K, Ca and Mg) concentration in the root, shoot and grain (mg/Kg DW), represented as mean±SD (n=6). Letters indicates significance ($p<0.05$) between treatments, in the cases where significant differences have been found.

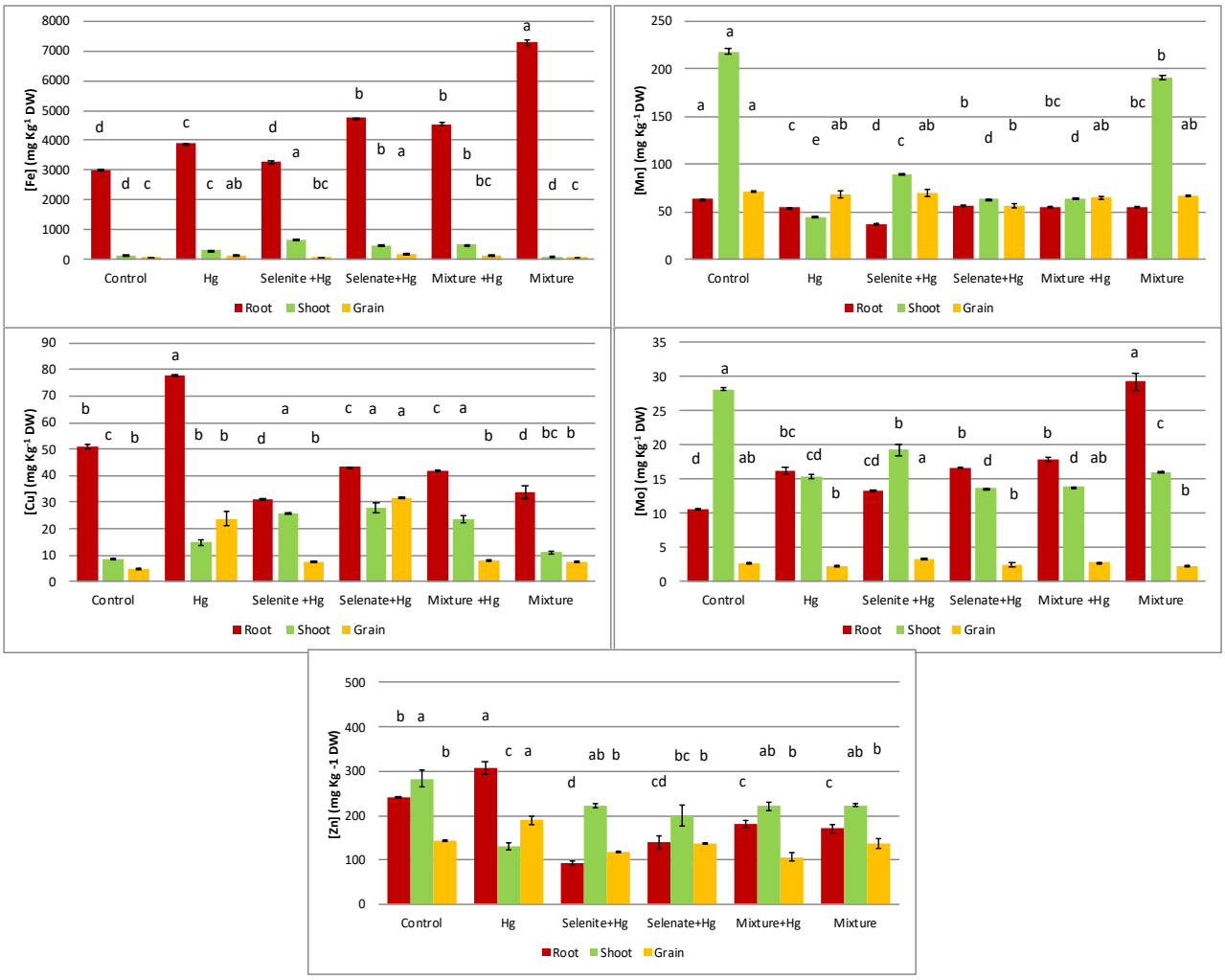

Figure 14: Micronutrient (Fe, Mn, Cu, Mo and Zn) concentration in the root, shoot and grain (mg/Kg DW), represented as mean±SD (n=6). Letters indicates significance ($p<0.05$) between treatments, in the cases where significant differences have been found.

Wheat seedlings and wheat plants: effect of mercury on selenium speciation

The speciation was studied by means of bulk analysis on the roots and shoots of the wheat seedlings and the wheat plants treated with a mixture of selenite and selenate at 25 µM and Mercury at 2 µM for seedlings and 0,5 µM for senescent plants. Speciation in grain will be discussed in depth in a further section, and accordingly, is not included here.

To do so, the spectra obtained for the samples was compared to that of pure references, which can be seen in figure 15.

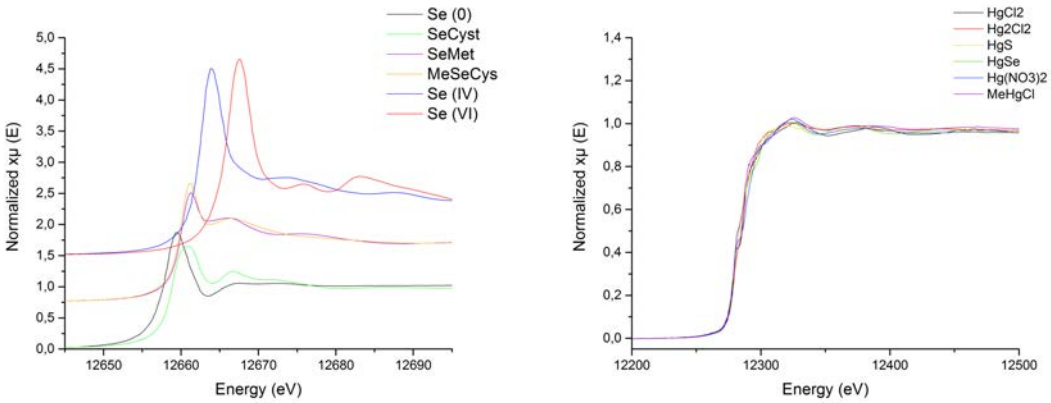

Figure 15: a) Normalized Se K-edge XANES spectra of the reference compounds: elemental selenium, selenocystine, methylselenocysteine, selenomethionine, sodium selenite and sodium selenate. b) Normalized Hg LIII-edge of the reference compounds: mercury (II) chloride, mercury (I) chloride, mercury sulphide, mercury selenide, mercury (II) nitrate and methylmercuric chloride.

Selenium compounds produced distint features, which can be easily identify in a sample.On the other hand, all mercury references produce very similar XANES spectra. Therefore, the liner combination fit of the XANES region of the spectra may not be the most adequate aproach in order to obtain information about the chemicalstructure of Hg in the plants.The analysis of the EXAFS region, may provide more valuable information, but it requires the availability of high quality spectra in the samples, which was not feasible to be obtained due to the low concentrations of Hg present.

However, the analysis of the Se XANES spectra provides relevant information.

In figure 16 and table 2, it can be seen how there is a significant fraction of selenium in the form of mercury selenide in roots. For senescent plants, this form comprises the 52±1 % of the selenium present in the sample. This value was obtained through least-squares linear combination fit with pure references. The obtained fit descrived very well the oscillations observed in the spectra (figure 17). However, the similarities of the spectra acquired in the sample to the pure HgSe standard are easily observed in figure 17. Therefore, it was confirmed that the two atoms interacted by the formation of a Se-Hg bond.

Besides, the behaviour of selenite and selenate metabolization has changed with mercury addition, respect to what was measured in chapter 4.

Selenate does not accumulate in roots, as was the case without mercury exposure, however, the detected levels of selenite accumulation in roots are higher. Therefore, the reduction of selenite into organic selenium occurs at a slower speed due to the presence of mercury, which is also confirmed by a lower amount of C-Se-C compounds formed in roots.

This hampered synthesis of C-Se-C compounds is balanced by the significant formation of HgSe in roots. This effect is already occurring in seedlings exposed during 18 days to Se and Hg, but it is much more evident in mature plants that were exposed to the elements for a longer time, and thus slower processes were also observed: less amount of selenite was accumulated, which was substituted mainly be HgSe and some Se (0).

Regarding shoots, no HgSe is seen to be translocated in any case. Thus this enables slightly higher C-Se-C concentrations. However, the predominant compounds in shoots are the inorganic selenium anions, in the same manner than in lack of mercury presence.

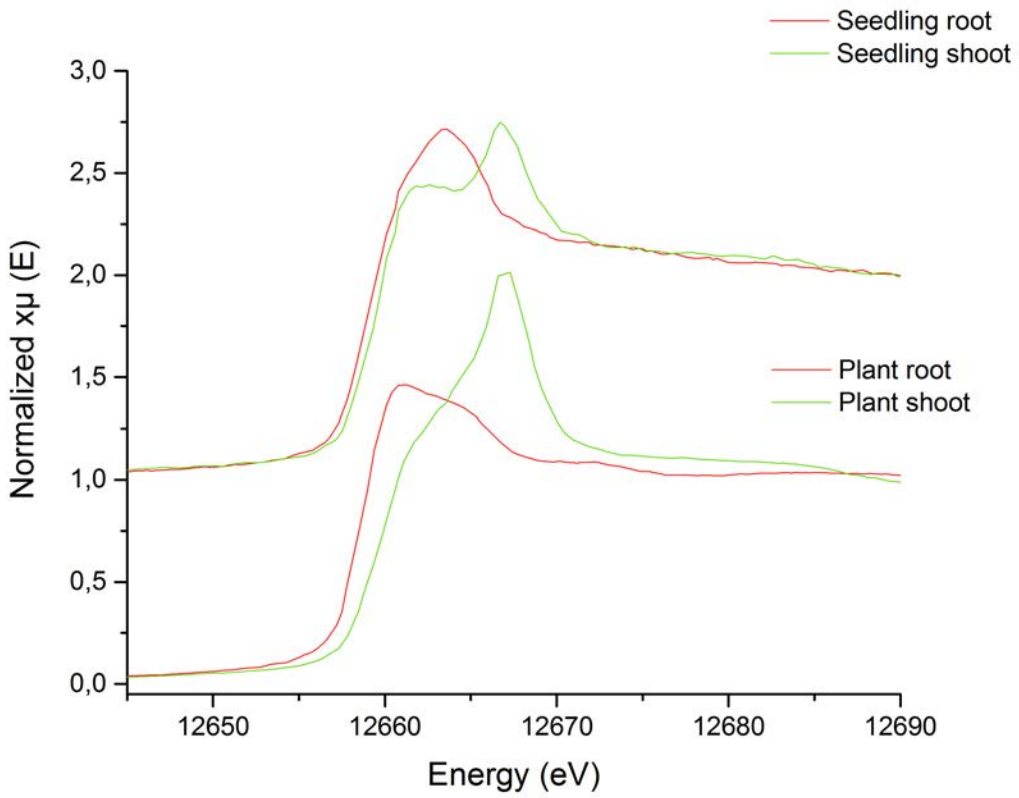

Figure 16: Normalized Se K-edge XANES spectra of roots and shoots from seedlings (top) and senescent plants (bottom) treated with a mixture of selenite and selenate and mercury.

Table 2: Results from least-square linear combination fits of samples with selenium standards. R factor and reduced chi square indicate the goodness of the fit.

	R factor	reduced chi-square	**Se (IV) (%)**	**Se (VI) (%)**	**C-Se-C (%)**	**C-Se-Se-C (%)**	**Se (0) (%)**	**HgSe (%)**
Root seedling Mixture+Hg	*0,003504*	*0,000974*	*18±1*	*n.d.*	*60±8*	*n.d.*	*n.d.*	*21±2*
Shoot seedling Mixture+Hg	0,002387	0,000666	*8±1*	*16,2±0,5*	*76±8*	*n.d.*	*n.d.*	*n.d.*
Root plant Mixture+Hg	0,000385	0,000095	*3,0±0,4*	*n.d.*	*36±3*	*n.d.*	*9,1±0,9*	*52±1*
Shoot plant Mixture+Hg	0,003470	0,001038	*20±1*	*30,6±0,7*	*40±9*	*n.d.*	*n.d.*	*n.d.*

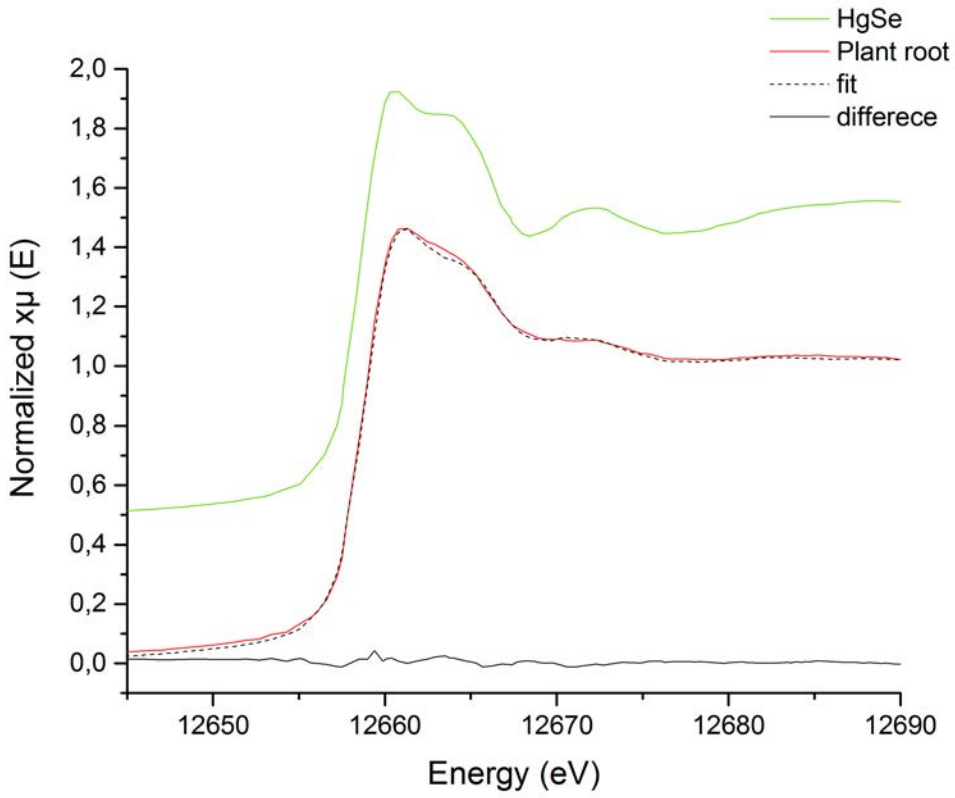

Figure 17: Normalized Se K-edge XANES spectra of a mercury selenide reference (top) and a sample of roots from a senescent plant treated with a mixture of selenite, selenate and mercury (bottom). Results include the least-square linear combination fit of the spectra with pure selenium compounds (dash line), and difference between the real spectra and the fit.

PART II. Detailed study of the effects on roots

Elemental distribution and speciation in a Se biofortified root with mercury

A detailed XRF study was performed in the crown roots of a senescent plant. The study of the top roots of a seedling had not shown mercury presence, with this element only being found in the part of the roots that were immersed in the solution. Accordingly, the translocation of Hg could be either a too slow process to be observed in 18 days, or inexistent, with Hg forming insoluble HgSe.

The results of the ICP-MS analysis in figure x and CVAFS in figure x, suggest that, even if small, there is some concentration of mercury present in shoots, and therefore, translocation through xylem is expected to some extent. The XRF of the crown roots of the senescent plant confirmed these results.

As seen in figure 18, selenium is present through the entire root. A relevant amount of it is seen in the interior of the root, where the transport to shots through the xylem is taking place. Furthermore, some selenium is found as hotspots in the root surface.

When compared to mercury distribution in figure 19, it can be observed that Hg is accumulated in the same hotspots in which selenium was found, but not in the root interior.

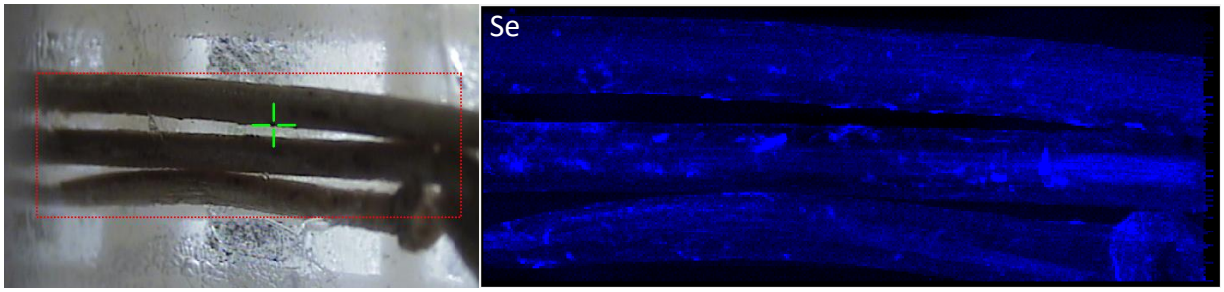

Figure 18: Selenium distribution in wheat roots enriched with a mixture of selenite and selenate and mercury, with [Se]=25µM and [Hg]=0,5 µM. Visible image obtained shows the sample placed in the beamline holder, and the XRF map shows selenium distribution in blue.

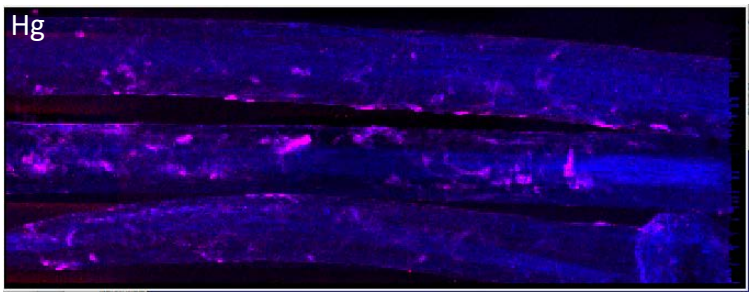

Figure 19: Selenium (blue) and mercury (red, in pink when overlapped) distribution in wheat roots enriched with a mixture of selenite and selenate and mercury, with [Se]=25µM and [Hg]=0,5 µM.

When this distribution was compared to that of other nutrients, different results where encountered (figure 20).

In this case, in a senescent plant, all nutrients were found to be present in these upper roots, even the micronutrients lacking in wheat seedlings, and therefore, transfer to above-ground tissues is occurring.

P, S, K and Cu are shown to be more homogeneously distributed through the root. Fe and Zn seem to follow a distribution pattern in the form of hotspots, as with Se and Hg. It is especially relevant in the case of Fe, where, despite the spots not matching perfectly, as was the case for Se and Hg, they are closely associated, as seen in figure 21.

The formation of the hotspots containing Se and Hg can be explained by the release of root exudates, which are normally composed of organic acids, in the case of root injury. Thus, these exudates lower the pH surrounding the roots, which could promote reduction of both selenite and selenate to mercury selenide [33], [56]. This mercury selenide will precipitate in the root surface forming the observed hotspots, with the perfect Se and Hg overlapping of the fluorescence signal.

On the other hand, Iron is also known to form iron plaques in roots surfaces, for example in rice grown in flooded areas, precipitating iron oxides and hydroxides [57]. These iron plaques can interact with metals and metalloids, thus affecting their uptake and translocation. It is known that they can enhance selenite uptake, although reduce that of selenate [57].

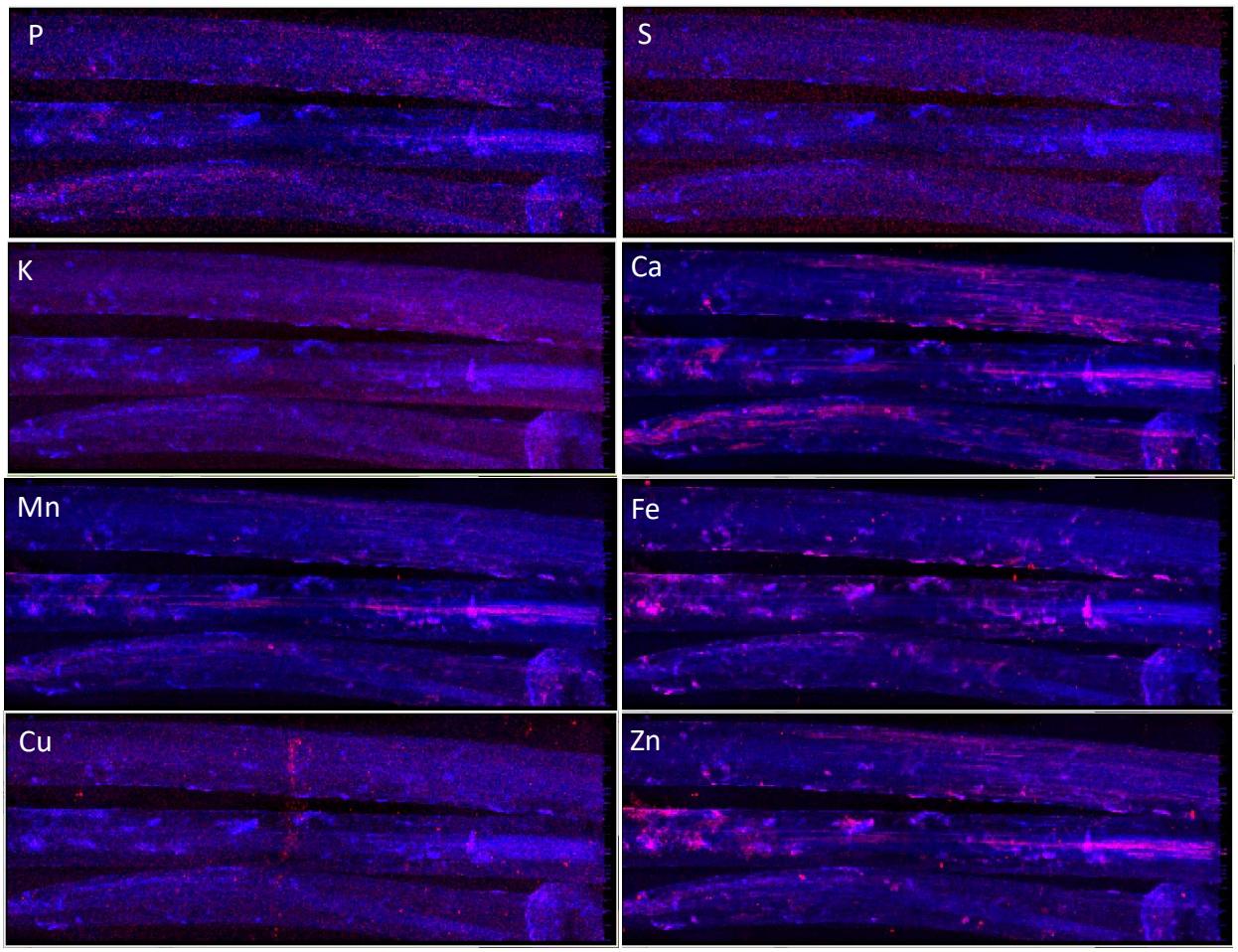

Figure 20: Se (blue) and indicated nutrient (red) overlapped XRF maps of enriched top roots of wheat acquired at 12700 eV.

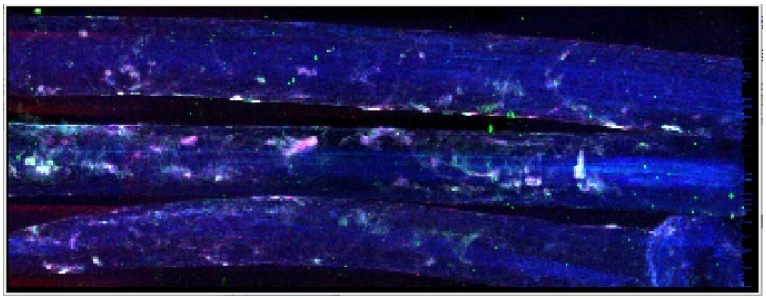

Figure 21: Selenium (blue), mercury (red) and iron (green, in white when the three are overlapped) distribution in enriched top roots of wheat.

The speciation in these Hg-Se hotspots was tested with µXAS measurements.

However, the concentration of mercury and selenium in those spots was found to be detectable, but too low to obtain good spectral quality in order to obtain structural information of Se and Hg, by comparison of the XANES region of the sample to that of

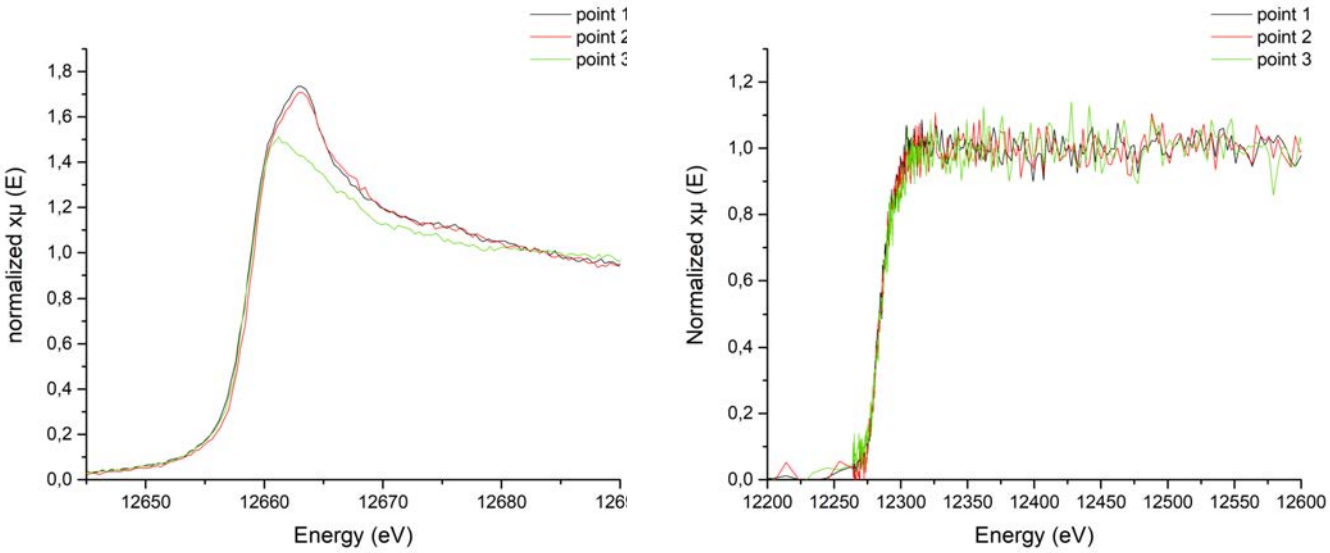

Figure 22: Normalized spectra of selenium (left) and mercury (right) µXAS measurements of 3 points in roots (with three repetitions per spectra).

Mercury and selenium interaction with root proteins

The study of the effect of selenium, mercury and simultaneous application of both elements on protein structure in wheat roots was performed by comparison to control roots. Besides, the root exterior epidermis and the root central endodermis were analyzed separately, in order to investigate the differences in protein structure during the uptake process, to discover changes that could enlighten the metabolic process of Se and Hg uptake and transport to root xylem. An example of the spectra acquisition procedure in root samples can be seen in figure 23, and of the spectra obtained in figure 24.

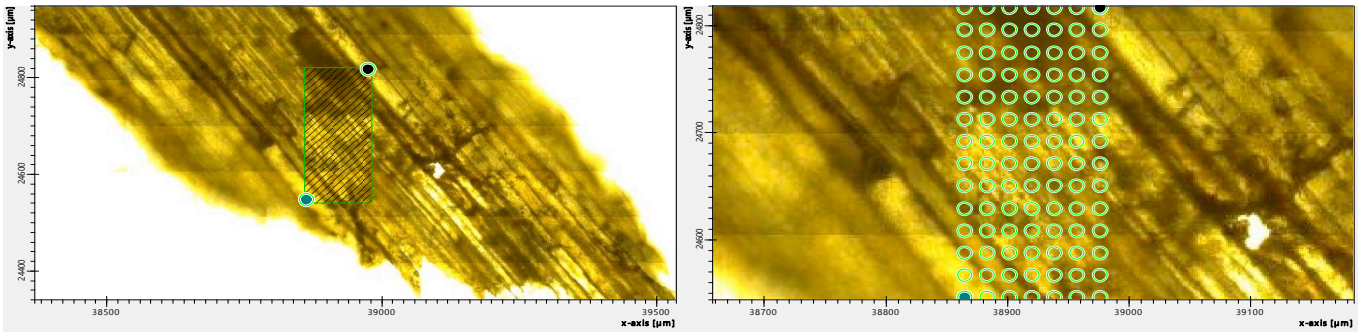

Figure 23: Example of spatially resolved IR spectra acquisition in root sample sections, where spectra were acquired at each green circle, resulting in approximately 100 spectra per sample.

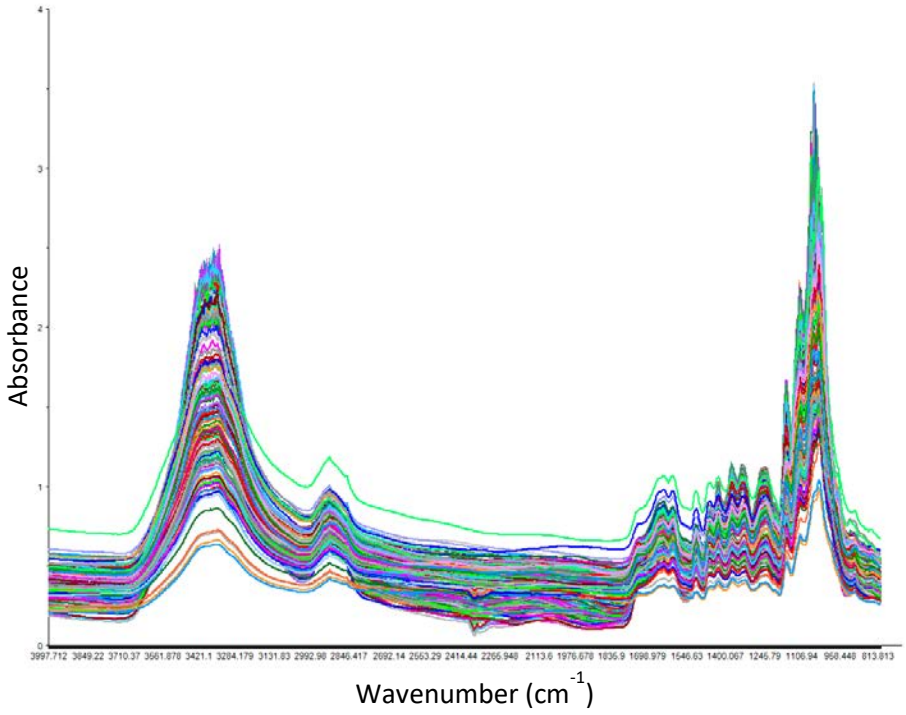

Figure 24: Example of spatially resolved IR spectra acquired in root sample sections.

All treatments caused a significant effect on root proteins, both in root epidermis and endodermis: there were significant differences in the positions and intensities of the absorption bands, as can be seen in the average spectra. Moreover, the PCA analysis also resulted in clusters separated from the control one, and thus, different relevance of each principal component for the different treatments, as seen in figure 25.

Selenium is known to be able to interact with proteins mainly by two mechanisms: by creating bonds with the sulfur contained in proteins or by substituting the sulfur atoms in the amino acids.

Oppositely, only one mechanism is possible for mercury: this element is known to form bonds with sulfur, however, it cannot be incorporated into proteins by replacement.

On the other hand, mercury interaction with selenium is favored over the interaction with sulfur, due to the higher polarizability of selenium that makes this element more reactive [58]. Thus, when both S and S are available, Hg is expected to bond preferentially to selenium.

In the root epidermis, it can be observed the similarities in the average spectra of the individual mercury and selenium conditions, as well as their cluster overlapping in the PCA analysis, which indicate similar behaviors in the PCA-1 and PCA-2. Accordingly, in the proteins in roots epidermis, selenium and mercury have a similar effect to the vibrations of their functional groups. For this reason, a similar interaction mechanism is foreseen, which indicates that both Se and Hg form bonds to the S atoms of the proteins in the root surface.

However, in the root endodermis, the same similarity between individual application of Se and Hg is not observed. Therefore, a different mechanism of interaction with proteins is expected for the two elements, such as the Se replacement of a S atom in proteins.

This is confirmed with the comparison of figure 26. The protein absorption bands in root epidermis (blue) are relatively similar between control and selenium treatments. However, in root endodermis, the changes observed are remarkably distinct. Therefore, even if there are interactions occurring in both cases, those in root endodermis are severely affecting proteins, such as their functional groups and/or the protein tertiary structure composed of disulfide bonds.

It is known from the speciation information previously discussed in this work, that the selenite uptaken by wheat roots is rapidly reduced to selenoamino acids, and selenate is easily translocated into wheat shoots. Therefore, it is expected that the selenium that has been uptaken in the mixture treatment, has been reduced to a big extent to organic selenium, which has been incorporated into the amino acids replacing sulfur, thus producing the changes in proteins observed.

Oppositely, the selenium in the root surface is in the process of being uptaken by the transporters in the cells walls, and thus it has not been metabolized yet and it is still found in the inorganic forms of selenite and selenate. Therefore, this forms will not be incorporated into proteins in the place of sulfur but just interact with the protein functional groups with relatively weaker bonds.

Regarding the simultaneous application of selenium and mercury, there is a big variability in protein vibrations in the roots epidermis, which do not match any of the process of the individual treatments. Therefore, it can be expected several complex interactions in these initial stages in which they will be found as Hg^{2+} and inorganic Se.

However, in root endodermis the selenium mixture and the selenium mixture with mercury have the similar effect on the absorption bands and an overlapping clustering in PCA-1 and PCA-2. Thus, a similar interaction with proteins is expected.

We have concluded that selenium applied individually will be found reduced to organic forms and incorporated into protein amino acids replacing sulfur. Thus the same effect on proteins could only be found if the same process is true for the simultaneous application. Moreover, in this case, mercury will not be directly reacting with the protein, as the vibrations of the protein groups are not further impaired. However, since the interaction of selenium and mercury is known from the previous results, and due to the high affinity of mercury for selenium, the interaction of the mercury atom to the incorporated selenium can be expected in a protein-Se-Hg complex.

Therefore, this protein-Se-Hg seems to be the most feasible candidate for the selenium mercury interactions, which confirms the hypothesis of preliminary works in plants [55] and animals [21].

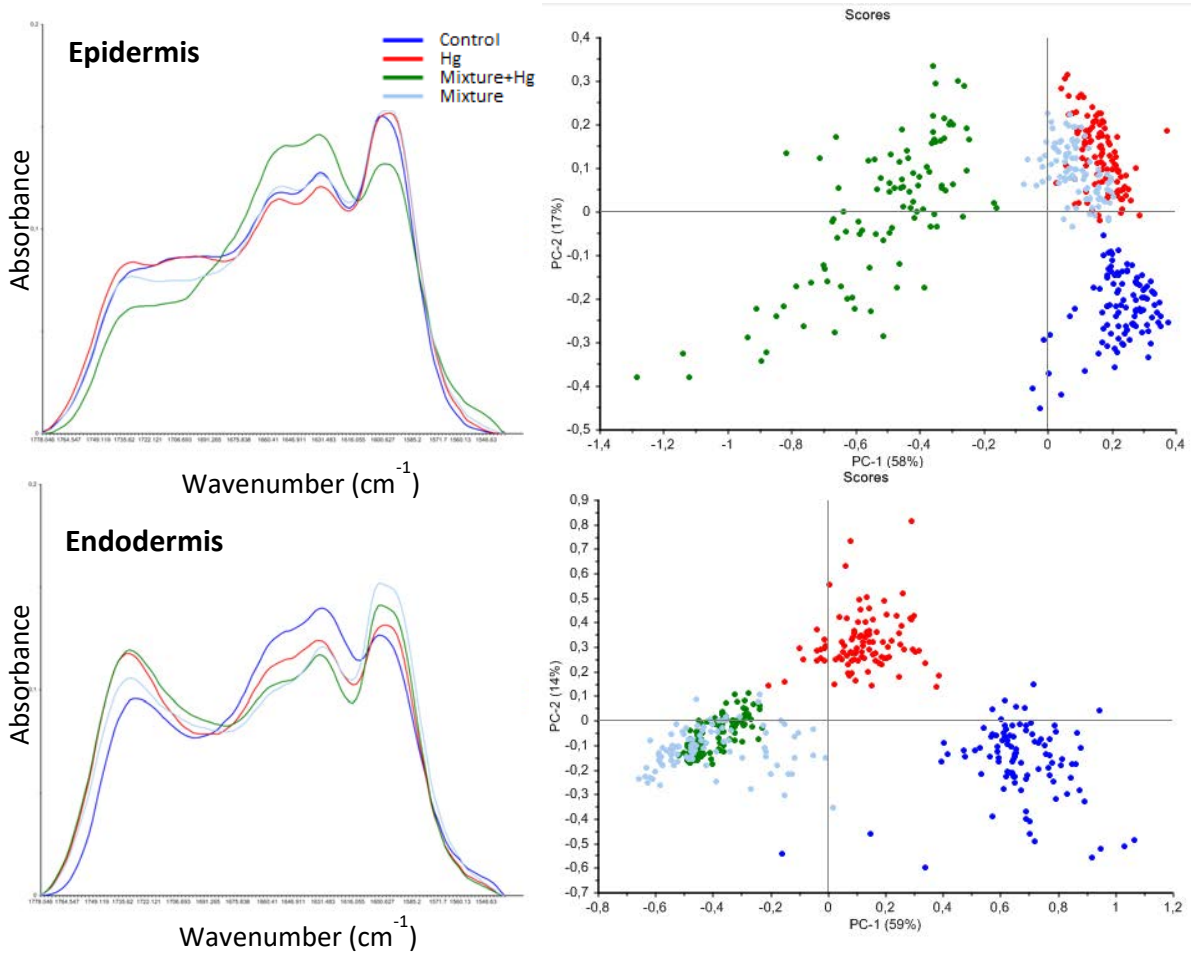

Figure 25: Protein ROI (1778-1536 cm^{-1}) averaged IR spectra, and PCA of the region, of root epidermis (top) and root endodermis (bottom), with the following treatments: control (blue), mercury (red), mixture of selenite and selenate and mercury (green), and mixture of selenite and selenate (light blue).

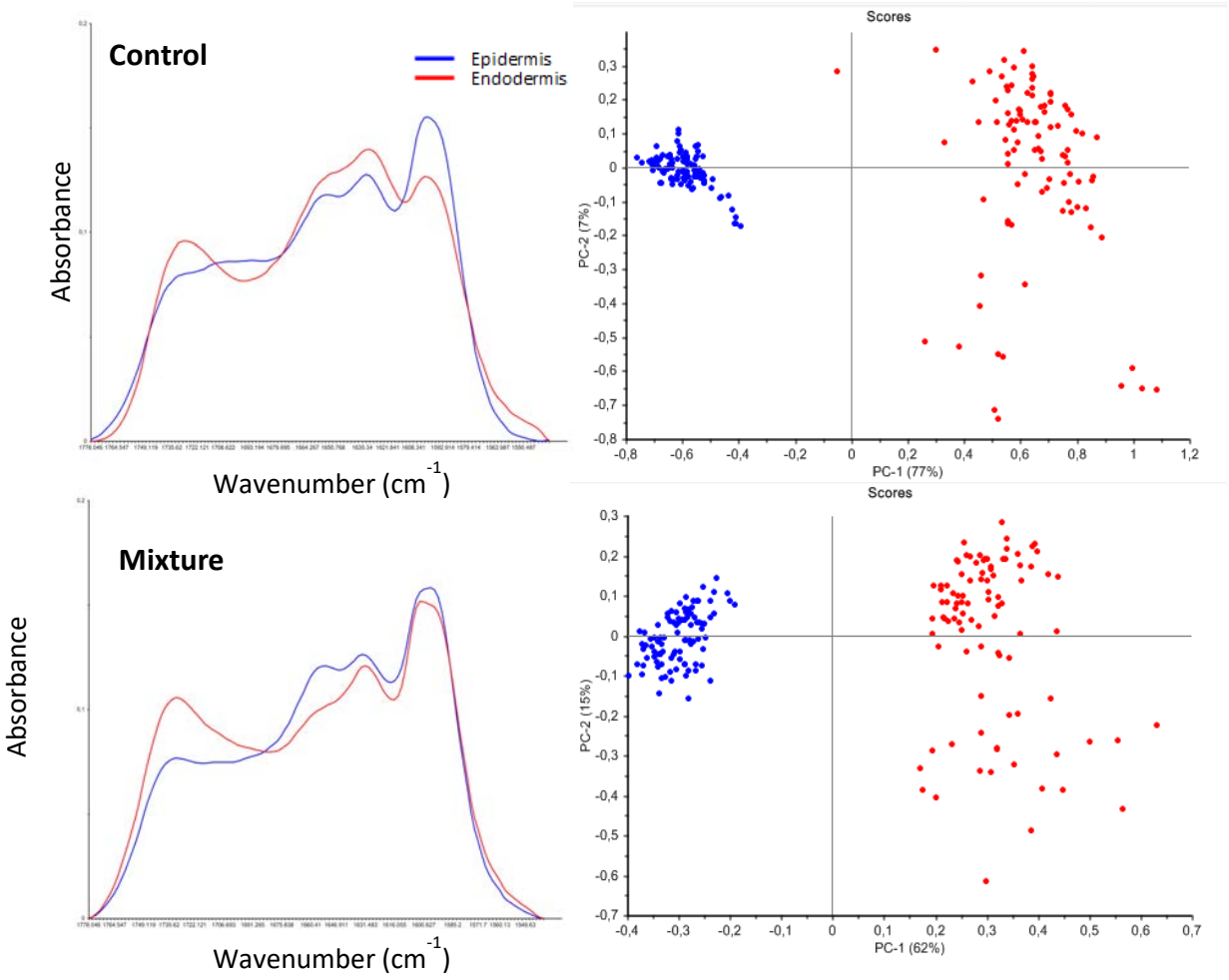

Figure 26: Protein ROI (1778-1536 cm^{-1}) averaged IR spectra, and PCA of the region, in a control root (top) and in a root treated with a mixture of selenite and selenate (bottom), from the root epidermis (blue) and endodermis (red).

PART III. Detailed study of the effects on grain

Elemental distribution in a Se biofortified grain with mercury

The elemental distribution within a wheat grain was studied in order to determine whether the exposure to mercury, which has been shown to affect the uptake and accumulation of selenium and nutrients in different tissues, affects as well the patterns of distribution inside the wheat grain.

Two grains were mapped, one with a mixture of selenite and selenate, figure 27 a), and figure 28 a), and one with the simultaneous exposure to the mixture plus mercury figure 27 b) and figure 28 b).

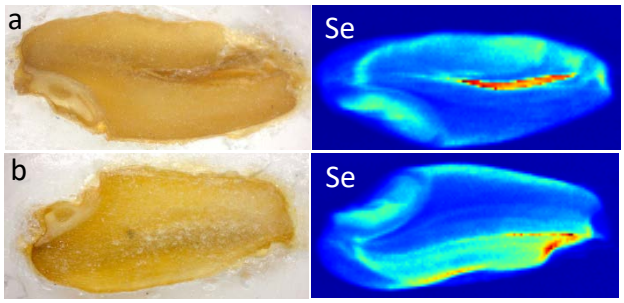

Figure 27: Selenium distribution in sections of wheat grains enriched with a) mixture of selenite and selenate with [Se]=25µM, and b) mixture of selenite and selenate plus mercury, with [Se]=25µM and [Hg]=0,5 µM. Visible image obtained with a microscope and selenium fluorescence map at 12677 eV, where warmer colors indicate higher Se concentration.

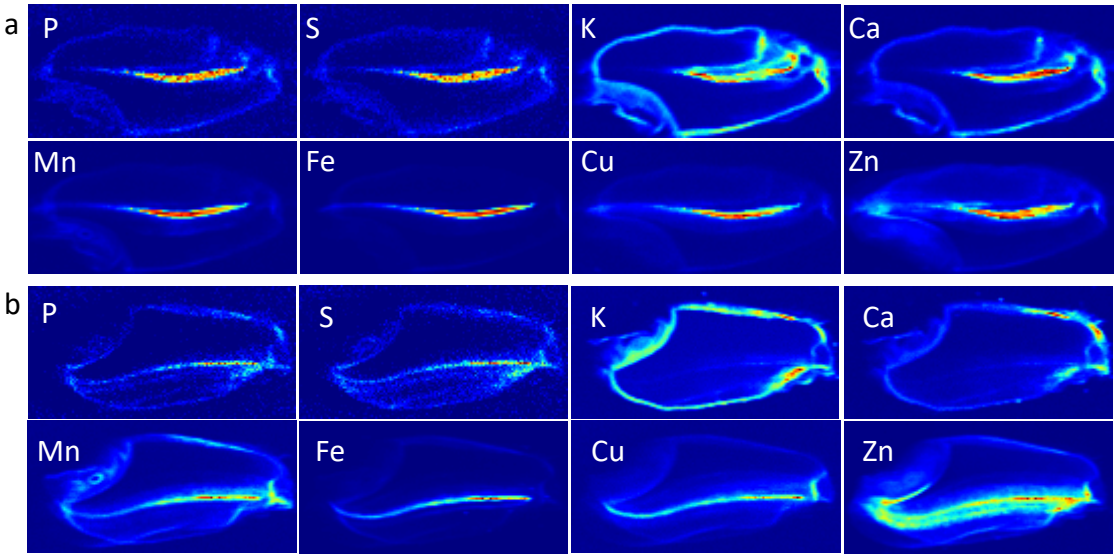

Figure 28: Elemental distribution in sections of wheat grains, enriched with a) mixture of selenite and selenate and b) mixture of selenite and selenate plus mercury. XRF maps of K, Ca, P, S, Mn, Fe, Cu and Zn fluorescence at 12677 eV, where warmer colors indicate higher concentration.

The XRF maps showed that mercury application did not modify selenium distribution within the grain. Following the same trend of the results reported in chapter 4 with a [Se]=10µM, selenium is mainly accumulated in the germ, the bran and the pigment strand. In contrast, a lower selenium presence is found in wheat endosperm.

As previously discussed this is due to the higher association of selenium in protein rich regions, which are located in the germ and the bran, but not in the endosperm, with is principally composed of carbohydrates [59].

Oppositely, mercury seemed to result in the modification of K and Ca distribution. After the mercury treatment, they are mainly concentrated in the grain bran, with

some presence in the grain germ, but they are not present in the pigment strand. However, in the treatment with selenium individually, their biggest concentration was in the pigment strand.

Moreover, the other nutrients such as Mn, Fe, Cu and Zn continued to be preferentially accumulated in the pigment strand and therefore mercury did not change their distributions.

However, these results, being obtained only from a single grain for each condition, cannot be considered conclusive. Thus, the modification of K and Ca grain distribution with mercury exposure should be considered an indication for further studies.

Effect of enrichment conditions on selenium speciation in XAS and μXAS analysis

The effect of mercury exposure on selenium speciation within a grain was studied. Selenite+Hg, selenate+Hg and mixture+Hg treated grains were analyzed, in addition to a grain with mixture of selenium species but without mercury for comparison. The present data was also compared to some of the spectra obtained from chapter 4, in this case, the grain enriched with mixture and the SeMet CRM.

Both bulk and μXAS analyses were performed, showing the same trend in the results, as can be seen in figure 29 (bulk XAS spectra), figures 30 to 35 and table 3 (μXAS spectra)

Regarding the treatment with the mixture of selenium species without mercury, in comparison from the previous results discussed in chapter 4, bigger selenium concentration, in this case, 25 μM at florescence time, resulted in the displacement of the edge position and E_0 to higher energies and thus, an enhanced C-Se-C accumulation than at 10 μM at vegetative time. SELM-1 a selenium enriched yeast CRM containing exclusively SeMet (with a C-Se-C structure) was added to the figures to facilitate the comparison.

The increment of C-Se-C amino acids in the plants treated with the mixture at 25 μM is due to the higher amount of selenium accumulated in plant tissues (25 μM: 396±10 mg/kg in roots, 191,4±0,3 mg/kg in shoots and 131,8±0,5 mg/kg in grain vs. 10μM: 252±23 mg/kg in roots, 64±8 mg/kg in shoots and 95±11 mg/kg in grain as mean±SD) which then results in a bigger number of unspecific substitutions of sulfur atoms in proteins, and thus a more elevated amount of protein-bound selenoamino acids, such as SeMet and MeSeCys. Oppositely, despite the higher selenium concentration, the shorter application time meant that the plant phytotoxicity did not increase proportionally, and thus the stress-induced ROS production was less significant, and for a shorter period, resulting in a less oxidizing environment that did not promote the formation of SeCyst amino acids.

Furthermore, in general, the addition of mercury also increased the amount of C-Se-C amino acids, respect to the individual selenium treatment. As previously discussed, the formation of a Hg-Se-protein complex is enhanced in the present of both elements as a mechanism of mercury detoxification.

Therefore, the once the reduction of the inorganic selenium has taken place, the mercury present, which has a high affinity for selenium, will bind to it, blocking positions for further oxidation and formation of Se-Se containing organic compounds. However, the unspecific incorporation of this selenium into proteins is still feasible and will result in the Hg-Se-protein that has been observed in roots.

On the other hand, the translocation of mercury to shoots and grain is limited, while selenium is effectively transported. Therefore, the remaining selenium not bounded to mercury, will be translocated selenium and further metabolized, and the higher Se concentration during a shorter time will also facilitate the C-Se-C forms in grains as previously discussed.

Finally, it must be considered that of the three combinations of selenium species with mercury, the mixture treatment provides a smaller amount of C-Se-C than selenite or selenate. Therefore, with the use of the mixture, the presence of selenium has been divided into two species that have some differences in their metabolism, with faster reduction rate for selenite and faster translocation rate for selenate. Accordingly, the mercury reactivity with each selenium species will not be the same, thus resulting in separated pathways of selenium and mercury interactions that result in a less acute enhancement of the formation of C-Se-C compounds, than with a single selenium species.

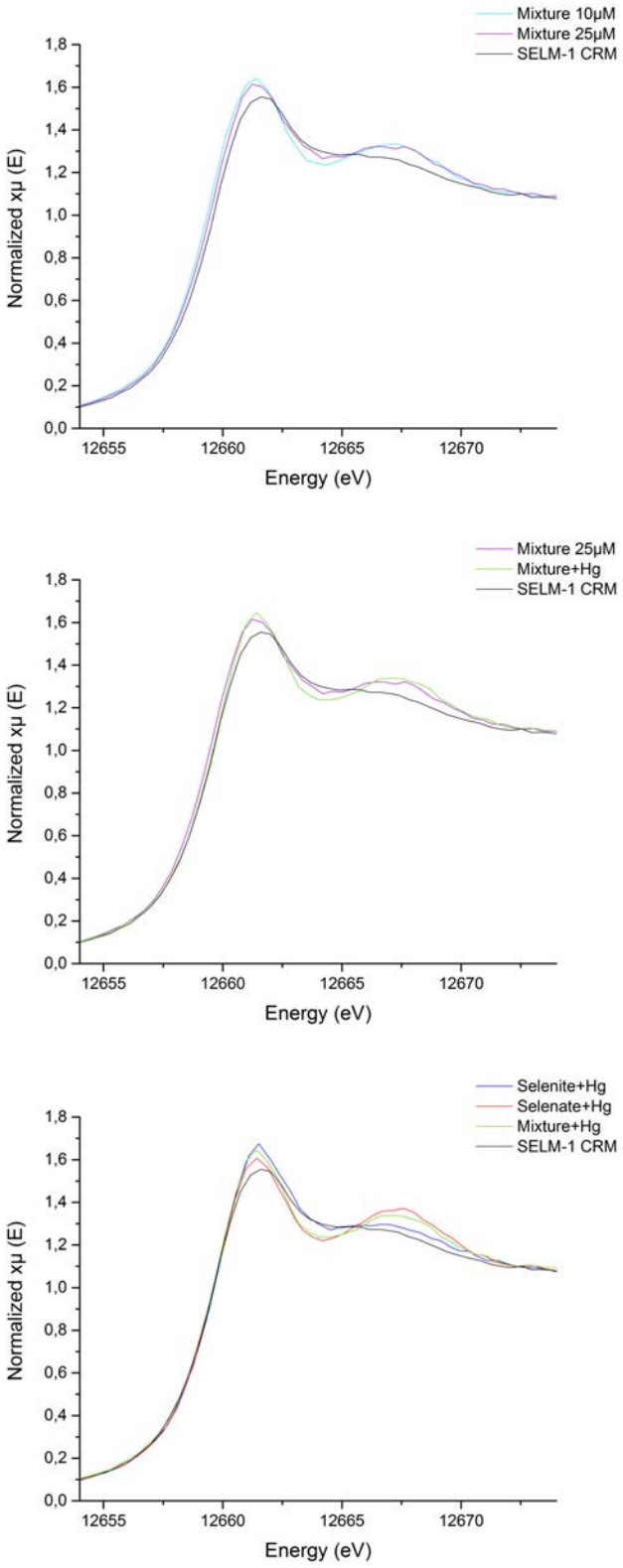

Figure 29: Normalized Se K-edge XANES spectra of bulk grains enriched with mixture 10 µM (light blue), mixture 25 µM (purple), mixture 25 µM and mercury (green), selenite 25 µM and mercury (blue), selenate 25 µM and mercury (red) and SELM-1 enriched yeast CRM containing selenium in the form of selenomenthionine (black).

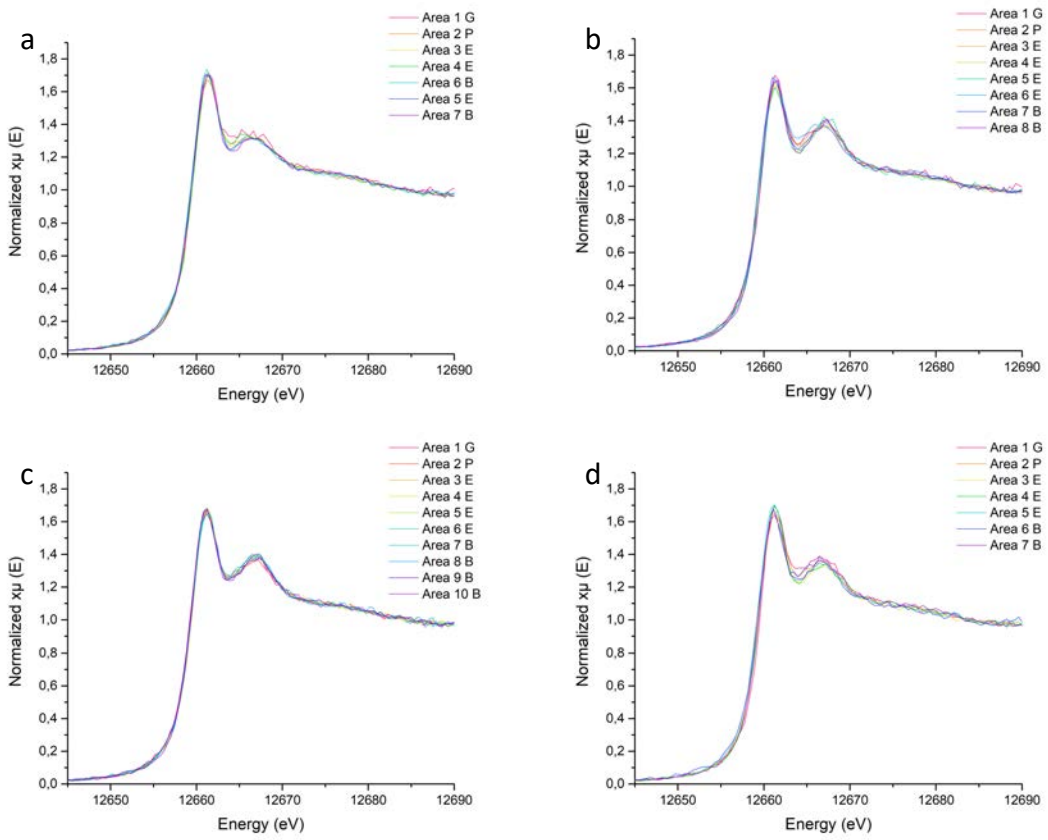

Figure 30: Normalized Se K-edge μXANES spectra of the areas of a grain enriched with a) selenite+Hg, b) selenate+Hg, c) mixture+Hg, d) mixture. Each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicates the germ, P the pigment strand, E the endosperm and B the bran of the grain.

Table 3: Results from least-square linear combination fits of samples with selenium standards. R factor and reduced chi square indicate the goodness of the fit.

Grain	Area	Part	R factor	reduced chi-square	Se (IV) (%)	Se (VI) (%)	C-Se-C (%)	C-Se-Se-C (%)
Grain Selenite+Hg	all points	-	0,00049	0,00012	1±1	n.d.	84±2	16±2
	area 1	G	0,00139	0,00034	3,3±0,6	n.d.	83±3	14±3
	area 2	P	0,00064	0,00015	1,8±0,4	n.d.	83±4	16±2
	area 3	E	0,00056	0,00014	1±1	n.d.	86±2	13±2
	area 4	E	0,00060	0,00015	1,4±0,4	n.d.	95±2	4±2
	area 5	E	0,00087	0,00021	0,6±0,5	n.d.	76±3	24±2
	area 6	B	0,00132	0,00031	1±1	n.d.	73±3	27±3
	area 7	B	0,00073	0,00017	n.d.	n.d.	87±3	13±1
Grain Selenate+Hg	all	-	0,00063	0,00015	1±1	4,3±0,2	80±2	15±2
	area 1	G	0,00193	0,00047	2,4±0,8	3,2±0,4	75±6	19±4
	area 2	P	0,00047	0,00011	1,1±0,4	4,7±0,2	87±2	8±5
	area 3	E	0,00052	0,00013	0,6±0,4	4±3	84±2	11±2
	area 4	E	0,00052	0,00012	n.d.	5±3	82±2	14±1
	area 5	E	0,00106	0,00025	n.d.	6,2±0,3	75±2	19±2
	area 6	E	0,00061	0,00015	2,1±0,3	3,8±0,2	94±2	n.d.
	area 7	B	0,00093	0,00023	0,6±0,5	2,6±0,3	74±4	23±3
	area 8	B	0,00083	0,00020	n.d.	4,9±0,3	84±3	11±2
Grain Mixture+Hg	all points	-	0,00102	0,00024	3,6±0,5	2±3	56±3	38±3
	area 1	G	0,00160	0,00038	3,9±0,7	1,8±0,3	55±4	39±7
	area 2	P	0,00093	0,00022	3,4±0,5	2,1±0,3	54±4	40±3
	area 3	E	0,00102	0,00025	2,4±0,6	3,0±0,3	70±5	25±3
	area 4	E	0,00137	0,00033	3,2±0,6	3±3	57±3	37±3
	area 5	E	0,00125	0,00030	4±4	2,1±0,3	55±33	39±3
	area 6	E	0,00110	0,00027	2,6±0,6	3,4±0,3	63±5	31±3
	area 7	B	0,00141	0,00034	4,4±0,6	3±4	56±3	37±3
	area 8	B	0,00182	0,00044	3,4±0,7	3±5	58±4	36±4
	area 9	B	0,00107	0,00026	2,6±0,6	3±4	57±3	38±3
	area 10	B	0,00178	0,00042	3,7±0,7	3±5	46±4	48±4
Grain Mixture	all points	-	0,00061	0,00015	2±3	1,4±0,2	67±2	30±2
	area 1	G	0,00076	0,00019	3,5±0,5	1,8±0,2	82±4	12±2
	area 2	P	0,00094	0,00023	3,3±0,5	1,6±0,3	66±4	29±3
	area 3	E	0,00049	0,00011	n.d.	1,7±0,2	71±2	27±1
	area 4	E	0,00068	0,00017	0,3±0,3	1,2±0,2	77±2	22±4
	area 5	E	0,00131	0,00032	2,7±0,6	0,7±0,3	50±4	47±3
	area 6	B	0,00206	0,00048	5,4±0,8	1,5±0,4	42±7	50±4
	area 7	B	0,00115	0,00028	1,4±0,6	2,6±0,3	70±3	26±5

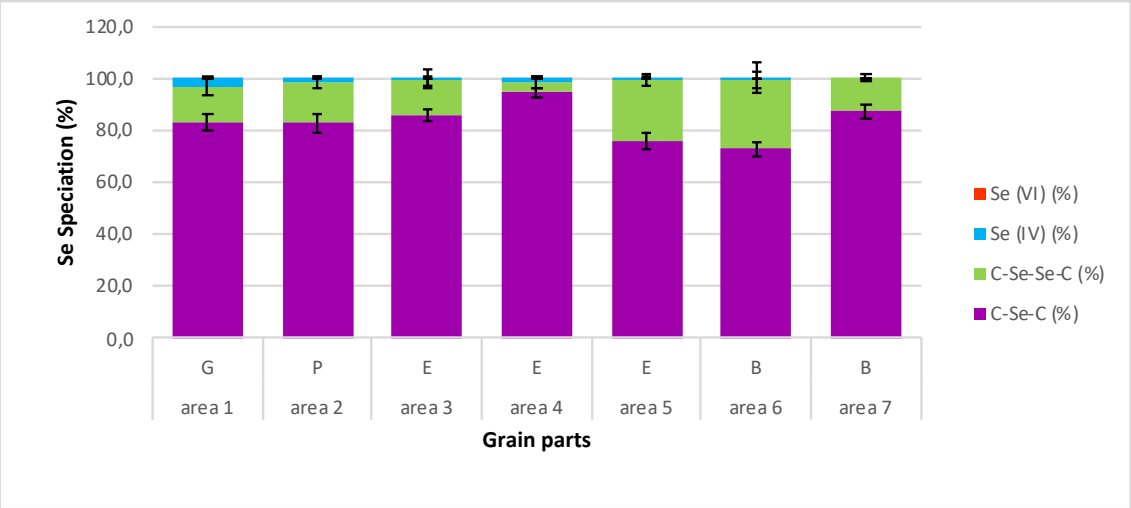

Figure 31: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas of a grain enriched with selenite+Hg, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

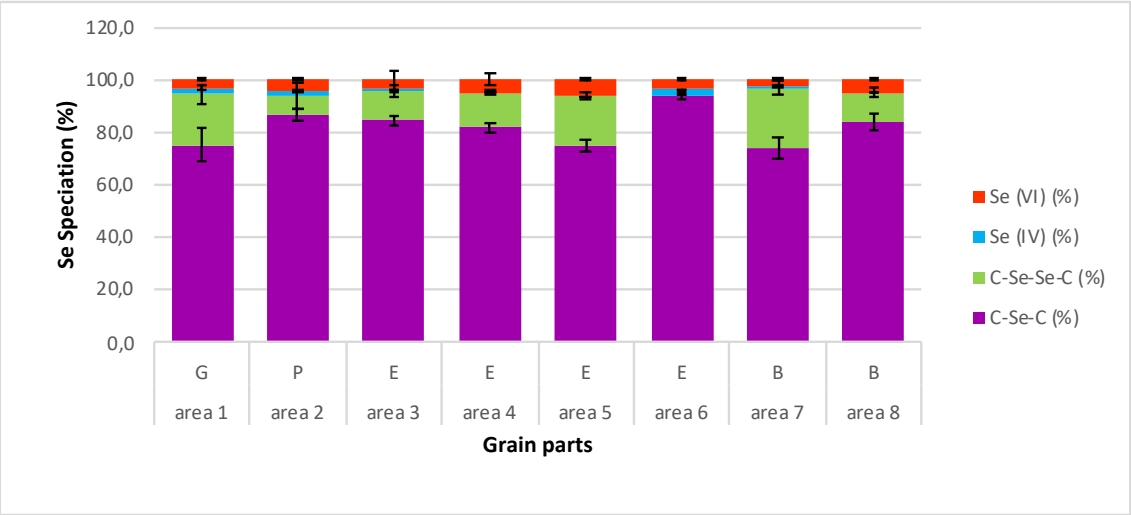

Figure 32: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas of a grain enriched with selenate+Hg, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

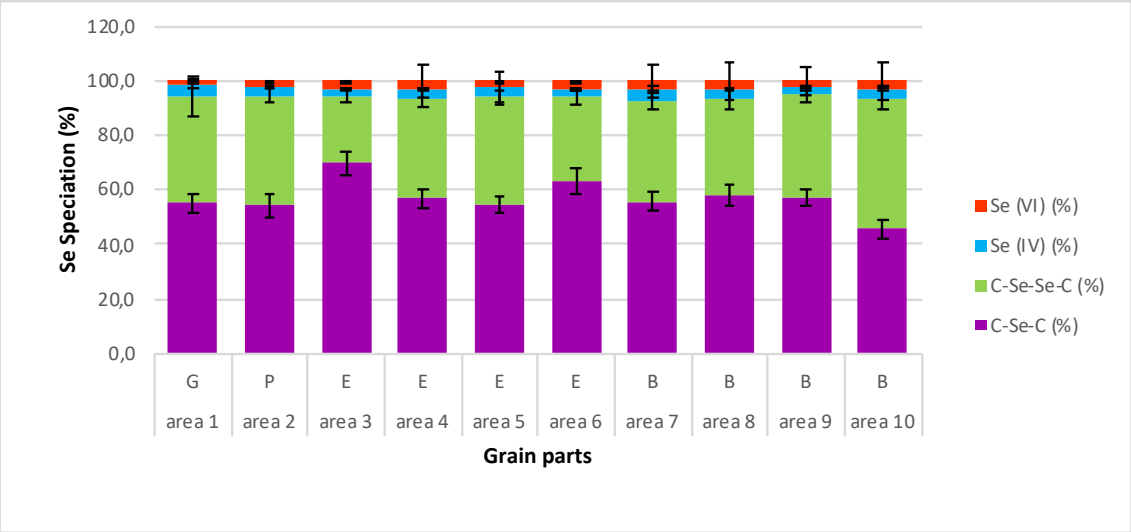

Figure 33: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas of a grain enriched with mixture+Hg, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

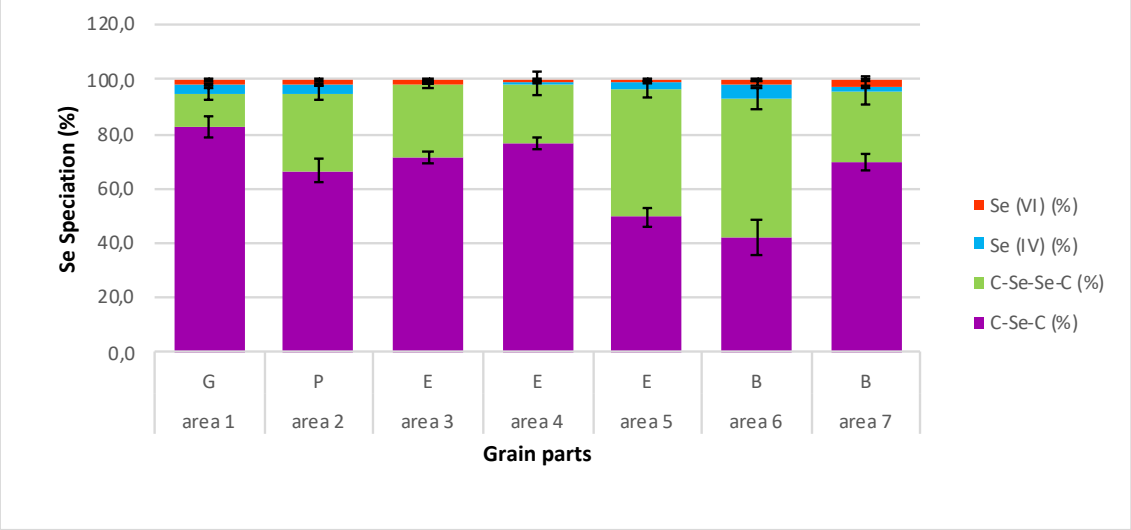

Figure 34: Linear combination fit results in % of Se specie contributing to the spectra measured in different areas of a grain enriched with mixture, where each spectrum is obtained from merging the points acquired within that area. Letters for each area correspond to the tissue: G indicate the germ, P the pigment strand, E the endosperm and B the bran of the grain.

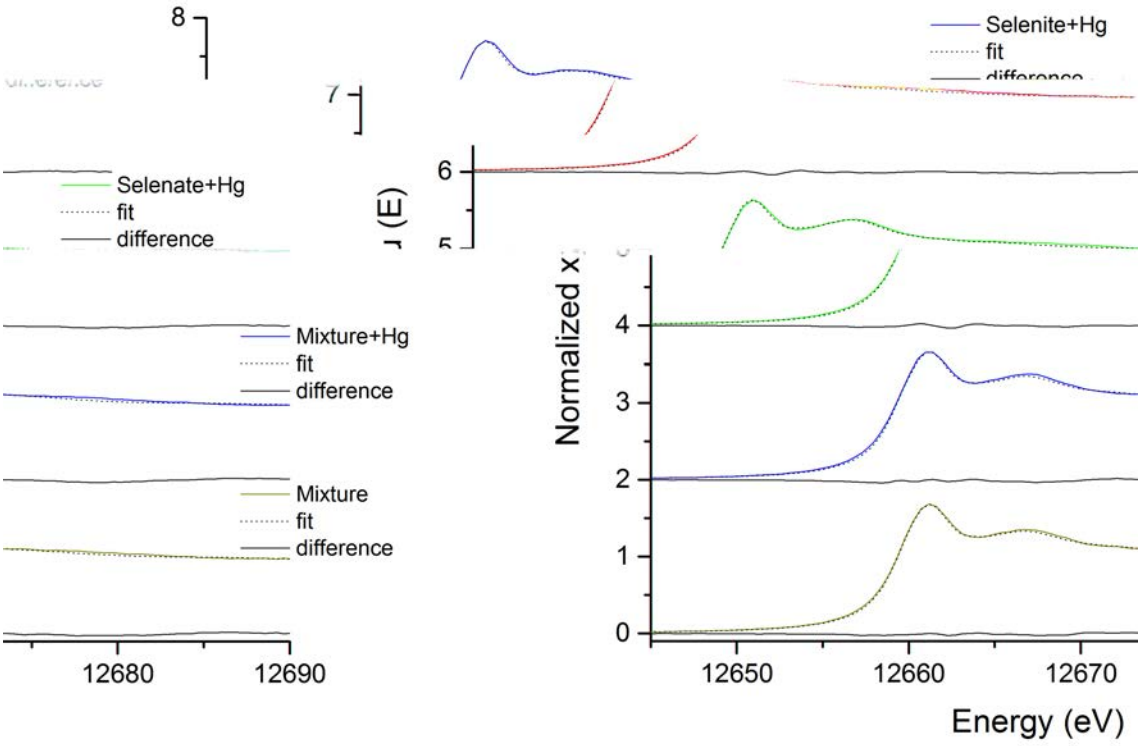

Figure 35: Normalized Se K-edge µXANES spectra of the average of all points acquired in each grain (solid line), result from the least-square linear combination fit of the spectra with pure selenium compounds (dash line), and difference between the real spectra and the fit.

Conclusions

Mercury exposure at the levels employed in this chapter did not affect plant overall weight but it hampered root elongation as well as grain production due an enhanced ROS production and a reduction of the antioxidant system in the wheat plants. However, selenium treatment counteracted mercury toxicity and resulted in higher grain yield.

Furthermore, it was observed that selenium and mercury were effectively taken up by wheat roots. However, mercury translocation was very limited, and thus this element was accumulated in under-ground tissues. Specifically, mercury was observed to be present in the roots in the form of hotspots, which also contained selenium, and thus, reduction of the two elements to form deposits of HgSe was hypothesized, as it is known to happen with iron. Afterwards, the measurement of selenium speciation in roots, confirmed the presence of HgSe, although this specie was not detected in shoots.

However, selenium translocation was still significant, although the behavior is modified from that of the individual Se exposure: selenate translocation is lowered, due to a fraction of it being retained in roots in the form of HgSe. Oppositely, selenite translocation is enhanced by mercury presence. The HgSe compound formed prevents its reduction to organic selenium, as seen by the lower C-Se-C content, thus favoring translocation.

In grain, the high selenium content, for a small application time (florescence stage) and in the presence of mercury, resulted in enhanced number of selenium substitutions of sulfur atoms, and a lower oxidizing environment, which increased the proportion of C-Se-C amino acids over C-Se-Se-C ones.

Finally, the evaluation of the effect of the exposure to mercury and selenium on the root protein content shows that, in root epidermis, both elements have a similar effect, due to both interacting with proteins through bonds with the S atoms in the amino acids. However, in root endodermis, the behavior of the elements differs, and thus, selenium is bonding to proteins by a different mechanism, by the substitution of a S atoms. However, the exposure of mercury in addition to selenium is not further modifying this structure and therefore, a complex with the form of Hg-Se-protein is expected.

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6. Conclusions

From results previously reported in Chapters 2, 3, 4 and 5, the following conclusions can be summarized as follows:

i. Selenium biofortification of wheat plants effectively results in a selenium accumulation in plant tissues, i.e. roots, shoots and grain, and thus the applied methodology is suitable for the production of a possible functional food from wheat flour.
ii. Both selenite and selenate anions can be used in wheat biofortification, although they have been shown to have a different behavior in plant metabolism: Selenite is readily reduced to Se (0) and to organic selenium in wheat roots and thus it significant accumulates in under-ground tissues. Selenate reduction is slower, with selenate reduction to selenite being the limiting step in selenium metabolism. However, selenate is easily transported from root epidermal cells into plant xylem, where it is rapidly translocated to above-ground tissues, causing a high accumulation of this species in shoots and grain.
iii. Excessive selenium accumulation in one of the tissues results in phytotoxicity. In general, non-accumulator plants are able to tolerate 100 mg Se/kg dry weight in their organs, and, although wheat plants have been shown to be able to store selenium well above that threshold, the selenium build-up induces stress to produce both a decrease of plant growth and biomass production and to hinder grain formation.
iv. 10 µM selenite resulted in excessive root Se accumulation, which caused a stress response in the plant, with an enhanced ROS production and a more oxidizing environment in wheat tissues. Therefore, selenite treatment also resulted in a 43% and 55% decrease in shoot and grain dry weight, respectively. Oppositely, plants treated with 10 µM selenate had lower Se accumulation in roots; the Se translocation to shoots was enhanced, where a certain amount of Se was innocuously stored in leaf vauoles, and thus selenate did not cause significant reductions in plant development.
v. In addition, the use of higher selenium concentrations in the biofortification resulted in higher toxicities. In this case, 25 µM of selenite caused a 86% decrease of grain weight. The same concentration of selenate resulted in an excessive selenium concentration in shoots that caused severe toxicity and thus plants were unable to produce grain.
vi. On the other hand, the use of a mixture of selenite and selenate, at both 10 µM and 25 µM Se, resulted in a more equilibrated selenium distribution through the plant, with intermediate concentrations in roots and shoots, which reduced plant toxicity for the same total selenium concentration.
vii. Furthermore, selenium uptake and translocation were observed to be a fast process with the enrichment being already significant 12 hours after the exposure. For this reason, the application of selenium at the plant florescence stage, instead of at a vegetative stage, and thus for a smaller period of time during the time of grain

formation and ripening, decreased the Se toxicity while maintaining, in general, a stable selenium concentration in grain.

viii. Selenium biofortification also affected the uptake and metabolization of sulfur, with which selenium share common pathways, and other nutrients, such as the macronutrients P, K, Ca, Na and Mg and the micronutrients Fe, Mn, Cu, Mo and Zn. Although their concentrations in roots and shoots were significantly affected after selenium exposure, their content in grain was less altered. Consequently, the nutritional value of wheat grain was not changed by selenium enrichment.

ix. Furthermore, all Se enriching conditions results in the metabolization of the inorganic selenium species into selenoamino acids. Selenite enrichment results in an almost quantitative conversion of this species into organic selenium, which is then found through the plant. Oppositely, due to the slower metabolization of selenate, this species is found to accumulate in shoots. However, very little of such inorganic selenium reaches the grain, with the inorganic fraction being always less than 8% of the total selenium.

x. Regarding the content of selenoamino acids in grains, previous studies have just reported selenomethionine to be the major component. Although this has been found to be true with the application of selenate, the selenite induced oxidizing environment resulted in an enhanced formation of selenocystine, in even higher proportions than selenomethionine.

xi. This mechanism can further serve to reduce the phytotoxicity experienced in the selenite treatments. Selenomethionine can be incorporated into proteins, producing alterations in the protein structure, folding and functionality, and therefore impairing the plant reproductive success. Selenocystine, in contrast, cannot be unspecifically incorporated into proteins, thus it is accumulated as free amino acids, which can be stored in higher amounts with lower toxicity.

xii. Furthermore, an approach with the mixture of the two species can enable the use of different ratios in order to tune the proportion of selenium species produced in grain to the desired application of the enriched flour. Besides, in real field conditions the selenite/selenate ratio would depend on the pH and pE conditions of the specific soil. The ratio could also be adjusted depending on the particular environmental parameters, in order to maintain constant the produced selenoamino acid ratio irrespective of seasonal variability.

xiii. On the other hand, the spatial distribution of the selenium species was not homogeneous within the grain. It was determined that most of the selenium species accumulated in the germ, bran and pigment strand of the grain, and a small concentration was localized in the grain endosperm. A similar behavior was found for other mineral nutrients, with the endosperm containing smaller quantity of those elements than other tissues due to its higher carbohydrate content. Oppositely, the higher protein content is localized in the germ, therefore, this tissue has been observed to accumulate preferentially selenomethione bonded to proteins.

xiv. Since common flour preparation techniques use only the grain endosperm, removing the bran and germ in the process, whole-flour preparations would be advised in order to produce an enriched functional food without significant selenium losses.

xv. Finally, selenium demonstrated a potential to counteract mercury toxicity. The phytotoxicity symptoms observed in mercury treated plants, such as impaired root elongation and grain formation, were lowered in presence of selenium. Furthermore, the root uptake of selenium and mercury was enhanced in the presence of both elements, and common accumulation areas were seen in the form of hotspots in roots surfaces, which indicates their interaction and the possible formation of a Se-Hg complex. On the other hand, the translocation of mercury to shoots and grain decreased in the presence of selenium, reducing the risk for the consumption of the crop in case the application of selenium biofortification is performed in soils contaminated with mercury. Besides, the formation of the Se-Hg complex reduced selenate mobility and translocation to the shoot, but in the same manner, it also hindered selenite conversion into selenoamino acids, resulting in more translocation in selenite cultures. Moreover, root protein content was also affected by the selenium and mercury exposure, and the obtained infrared spectra indicate the formation of a complex in the form of protein-Se-Hg.