



Influence of selenium speciation on its bioavailability from diet and food supplements

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Summary

Like the moon after which it has been named – *Selene* in Greek –, selenium is an element that has a bright and a dark side. This oligo-element is essential at a low to moderate level of intake, but becomes toxic at high doses. In Belgium, the recommended values for Se intake have been estimated to be 60 and 70 µg per day for adult women and men respectively. This level of intake is supposed to ensure the maximal activity of the antioxidant selenoenzymes glutathione peroxidases. The Se status of people differs considerably according to the regions of the world because of the heterogeneous repartition of Se in soils, resulting in variable Se contents in food. In Europe, Se content of soils is naturally low and Belgian people could therefore be at risk of Se deficiency. In addition to the dose, another important determinant of the Se impact on the organism is its speciation, *i.e.* the chemical form of Se. Different species have been shown to have distinct degrees of bioaccessibility, bioavailability, bioactivity and toxicity. This work aimed to improve the current knowledge about how the nature of Se species influences the degree of Se bioavailability, in the context of the Se status and food habits of the Belgian population. This research required the development of an analytical method of Se speciation analysis. This method consisted in an enzymatic ultrasonic-assisted extraction of Se, followed by a chromatographic separation of Se species by HPLC in an ion-pairing reversed-phase partitioning mode, and the final detection of ^{78}Se by ICP-MS in the presence of H_2 as a reaction gas. This work first permitted us to calculate that the average Se intake by the general adult Belgian population is 60 µg Se day⁻¹, which is at the lower end of the range of recommended intake. Secondly, we measured decreasing degrees of bioavailability for SeMet > MeSeCys > selenate > selenite, and we showed that these degrees of bioavailability were the consequence of distinct modes of intestinal transport among Se species. Finally, we observed that the bioavailability of Se from different Se-enriched food supplements was generally low, especially for selenite-based ones. This work allowed us to formulate an opinion about the suitability of distinct forms of Se in the context of a Se enrichment of the diet, and opened the door to further research in relation to the species-dependence of Se health effects.

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List of abbreviations

AP	apical
AE	anion exchange
BL	basolateral
CRM	certified reference material
DMEM	Dubbelco's modified Eagle medium
EGTA	ethylene glycol tetraacetic acid
FBS	foetal bovine serum
FS	food supplement
γ -glut-MeSeCys	gamma-glutamyl-methylselenocysteine
GPx	glutathione peroxidase
HBSS	Hank's buffered salt solution
HPLC	high performance liquid chromatography
ICP-MS	inductive coupled plasma – mass spectrometer
IP	ion pairing
LDH	lactate deshydrogenase
LY	lucifer yellow
MeCys	methylcysteine
MeOH	methylselenol
MeSeCys	methylselenocysteine
Met	methionine
P_{app}	apparent permeability (coefficient of -)
RBC	red blood cells
RDA	recommended daily allowance
ROS	reactive oxygen species
RP	reverse phase
RT	retention time
S	sulfur
Se	selenium
SeCys	selenocysteine
SeCys ₂	selenocystine
Se(IV)	selenite
SeMet	selenomethionine
SeP	selenoprotein P
Se(VI)	selenate

TEER	transepithelial electrical resistance
TMSe ⁺	trimethylselenonium ion
TRx	thioredoxin reductase
TUI	tolerable upper intake
V _{max}	maximal velocity of transport

Chapter 1

**Foreword,
context of the thesis**

Chapter 1 - Foreword, context of the thesis

Formerly known only by specialists, selenium (Se) has today become familiar to a great part of the average population. This metalloid element finds its main applications in the industry, in cosmetics and in food. Industrial applications make use of the semi-conductor properties of Se for electronics, and of its photoelectric abilities for photovoltaic cells. Selenium is also employed for photographic toning and coloration of glass. In cosmetics, Se is incorporated to anti-dandruff shampoos as Se disulfide, or in skin care creams for its anti-aging properties. The question of Se in food was the concern of this work.

Since its discovery in 1817 and until the mid fifties, Se was exclusively regarded as a poison. In 1957, Se was recognized as an essential oligo-element and from this moment, discoveries about healthful effects of Se came one after another. This made the public opinion radically change about the element. Positive effects of Se are for the most part related to antioxidant events. In the context of the modern life that is associated with stress, low quality of food, diminution of physical expenditure and increasing recourse to pollutants, the oxidative stress has turned into a common concept in people's mind, and antioxidant products (omega-3 fatty acids, phenolic compounds, vitamins,...) have gained in popularity. Nowadays in occidental countries, antioxidant compounds are incorporated to a variety of dietary supplements freely available for the population. But, in contrast with drugs and medicines, consumers are usually not aware of the risk of an overdose associated with food supplements, and those products might be taken in excess in order to guarantee or accelerate the expression of beneficial effects. In case of Se, this could be problematic because the particularity of this element is to have a narrow nutritional margin between deficiency and toxicity. Hence, Se could rapidly switch from the status of essential element to the one of toxic compound if it is taken in high quantities.

It must also be mentioned that people are not equal face to the need of a Se-enrichment of the diet. This need is mediated by diet habits and by the Se content of the ingested food. Selenium content of food reflects Se concentrations of local soils by a mechanism of Se transfer through the soil-plant system. The distribution of Se in soils is not uniform around the world. Two typical examples of extreme Se concentrations in soils are the Punjab area

in India, where drink and cooking water is continuously contaminated by excessive Se concentrations, and the Keshan region in China, where people suffer from endemic severe cardiomyopathy related to their dramatically low Se intake. To less extreme extents, Se content of US soils is globally high while soil Se concentrations are naturally low in Europe.

The dose of ingested Se is not the only factor determining the essential or the toxic side of the element. Over the last two decades, it has become clear that the chemical form of Se (*i.e.* Se speciation) is also of critical importance. Speciation influences Se fate in the organism from its liberation from the food matrix to its ability to be incorporated to active selenometabolites. In a general way, it is believed that organic species are more bioavailable and less toxic than inorganic ones. However, there is still a lack of information about the effects associated with individual species, and about the mechanisms of action mediating their impact on the organism.

This sets the scene where the present thesis takes place. Owing to the situation of low Se content of Belgian soils, people are expected to readily recourse to Se-enriched food supplements in order to benefit from Se essential functions. The question of the species-dependent health impact of Se in case of consumption of food supplements requires particular attention because they often contain a single or a few number of Se species at high concentrations. In this thesis, we wanted to know if there was an actual need for Se supplementation in Belgium and, in case of supplementation, establish the performances of distinct types of food supplements with regard to their speciation. For that purpose, we focused on the questions of bioaccessibility and bioavailability of distinct Se species in the context of a nutritional level of intake. The questions of bioactivity and toxicity were not experimentally addressed in this work, but were considered through a survey of the literature.

Chapter 2

General introduction on selenium

Chapter 2 - General introduction on selenium

Foreword

Before entering the core of the chapters, it seems useful to present the main characteristics of Se with a focus on its chemistry, biochemistry and metabolic reactivity. Selenium behavior is indeed particularly complex and not easily predictable because of its high reactivity and of the exogenous factors likely to influence it. This introduction aims at presenting some key information that will allow the good understanding of the present work as well as of the general problematic of Se.

2.1. Brief history of selenium

Selenium was discovered in 1817 by a Swedish chemist named J.J. Berzelius who named the element after *Selene*, the Greek moon goddess, to note its homology to tellurium (*tellus*, earth in Latin) (Trofast, 2011). At the beginning, Se was regarded exclusively as a toxic element because it had caused poisoning to livestock and people in several places around the world where Se content of soils and plants was high (Navarro-Alarcon & Lopez-Martinez, 2000). Opinion about Se radically changed 140 years later when it was discovered that, at appropriate doses, Se was actually an essential element able to prevent several kinds of necrosis in Vit E-deficient rodents (Schwarz & Foltz, 1957). In 1973, the beneficial effect of Se was related to the antioxidant enzyme glutathione peroxidase (GPx) in which Se occupies the active centers (Rotruck *et al.*, 1973; Gromer *et al.*, 2005). During the following years, at least 25 additional categories of proteins and enzymes were recognized as “selenoproteins” in the human body (Gromer *et al.*, 2005). Those include thioredoxin reductases (TRx), deiodinases, selenoprotein P and numerous minor proteins (for a list, see Behne & Kyriakopoulos, 2001). In the early seventies, the first epidemiological studies suggested a relation between Se deficiency and the risk of cancer (Shamberger & Willis, 1971; Shamberger & Frost, 1969). A decade later, the concept of elemental speciation was introduced and the existence of species-dependent health effects began to be considered (Thompson *et al.*, 1982). It was the start of Se species quantification analyses (Robberecht & Van Grieken, 1982). In the nineties, rapid advances were made in the field of elemental

speciation analysis with the introduction of hyphenated techniques coupling HPLC to ICP-MS (Thomas, 2008). In 1996, the fact that Se could be beneficial at supranutritional level of intake was pointed out by an interventional study revealing an anti-cancer effect associated with high doses of Se (Clark *et al.*, 1996). Today, the hot topics of Se research mostly refer to the identification of anti-cancer mechanisms (Algotar *et al.*, 2012; Yu *et al.*, 2012; Zeng, Cheng & Johnson, 2012), and to the determination of minor Se species with state-of-the-art techniques (Klein *et al.*, 2011; Preud'homme *et al.*, 2012).

2.2. Chemistry of selenium

2.2.1. Generalities

Figure 2-1: Periodic table of chemical elements (illustration from Google image)

In the periodic table (figure 2-1) Se has the atomic number 34 and it is classified as a non-metal while usually referred to as a metalloid. The standard atomic mass of Se is 78.93 u but Se is declined under six naturally occurring isotopes with proper relative abundance: ^{80}Se (49.8%), ^{78}Se (23.5%), ^{82}Se (9.2%), ^{77}Se (7.6%), ^{76}Se (9.02) and ^{74}Se (0.87). Selenium lies in the box crossing Period 4 and Group 16/VIIa. By belonging to Group 16 (chalcogens), Se shows an external electronic configuration (*i.e.* $4s^23d^{10}4p^4$) that lacks two electrons compared to the stable configuration of rare gases. Thereby Se tends to react in a way favoring the gain of these electrons in order to reach the selenide (Se^{2-}) state, *e.g.* Na_2Se or FeSe (Reilly, 2006). In addition, Se has a close atomic radius, high electronegativity and elevated ionization energy (Atkins & Jones, 1998). In Group 16, Se lays just under sulfur (S), which means that the above-mentioned properties of Se are very similar for S. The majority of Se-

containing molecules are analogues of S compounds: they have exactly the same molecular structure except for an atom of Se replacing S, *e.g.* methionine vs selenomethionine or sulfite vs selenite (Birringer, Pilawa & Flohé, 2002). The metabolic outcome of Se also follows for a great part the sulfur pathways, often using the same enzymes that do not discriminate between S and Se. Some slight differences nevertheless exist between S and Se compounds, including notably a lower pKa for Se species compared to their S counterparts. This greater acid strength makes that selenol groups (-SeH) of selenoenzymes are readily dissociated at physiologic pH, which makes them very reactive for their catalytic role in antioxidant reactions (*cf.* section 2.3. "Selenium and human health") (Arteel & Sies, 2001; Johansson, Gafvelin & Arner, 2005).

2.2.2. Selenium speciation

The concept of speciation of an element refers to its occurrence in or distribution among different species. A species is defined by a specific and unique molecular, electronic, or nuclear structure of an element (Lobinski & Szpunar, 1999). Different species can vary in terms of oxidation state, of stoichiometry or with the possible presence of ligands (Reeder, Schoonen & Lanzirotti, 2006). Over the past 30 years, evidence has been brought that different species of Se do not affect a living organism in the same manner. Differences have been reported in terms of efficiency of absorption (Rider *et al.*, 2012; Zeng *et al.*, 2001), of Se retention, of ability to induce selenoenzymatic activity (Wang & Lovell, 1997; Wang & Xu, 2008) and of toxicity (Tiwarly *et al.*, 2006). It has thus become clear that it is not the total element content but that of Se species that must be considered in order to obtain information on its nutritional essentiality or toxicity.

The common oxidation states of Se are -2 (selenide), 0 (elemental Se), +4 (selenite) and +6 (selenate). Most organic species of Se contain Se at the selenide state. The higher oxidation states are mostly encountered in inorganic species. Tables 2-1 and 2-2 present some relevant inorganic and organic species of Se, with their principal characteristics.

Chapter 2 - General introduction on selenium

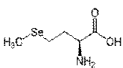
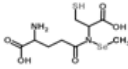
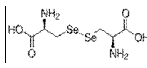
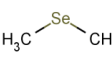
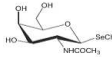
Table 2-1: Common inorganic Se species and their principal characteristics

INORGANIC					
Oxidation state	Name	Molecular formulae	Structure	Relevant characteristics	Sulfur analogue
0	Elemental Se	Se		Solid, red or gray, insoluble in water, non toxic.	Elemental S
	Selenide ion	Se ²⁻		Gas, highly toxic but rapidly oxidized to elemental Se in presence of air.	Sulfide ion
-2	Hydrogen selenide ion	HSe ⁻		Important product of Se metabolism, occurring in physiologic pH range.	
	Hydrogen selenide	H ₂ Se		Volatile, toxic, odiferous. Occurring in acidic conditions.	
	Metal-selenide	<i>e.g.</i> HgSe, ZnSe, PbSe			
	Se dioxide	SeO ₂			S dioxide
+4	Selenite ion	SeO ₃ ²⁻			Sulfite ion
	Selenite salts	<i>e.g.</i> Na ₂ SeO ₃ , Ag ₂ SeO ₃		Solid, soluble in water	
	Selenous acid	H ₂ SeO ₃			
	Selenate ion	SeO ₄ ²⁻			Sulfate ion
+6	Selenate salts	<i>e.g.</i> Na ₂ SeO ₃		Solid, soluble in water	
	Selenic acid	H ₂ SeO ₄			

Sources: Atkins & Jones, 1998; INRS, 2002; Whanger, 2002. Illustrations: www.wikipedia.org, www.chemspider.com

Chapter 2 - General introduction on selenium

Table 2-2: Common organic Se species and their principal characteristics

ORGANIC				
Name	Molecular formulae	Structure	Relevant characteristics	Sulfur analogue
Selenomethionine	C ₅ H ₁₁ NO ₂ Se		Essential selenoamino acid. Main Se-compound in food, particularly abundant in cereals. Not distinguishable from Met regarding incorporation in body proteins.	Methionine
Methyl-selenocysteine	C ₄ H ₉ NO ₂ Se		Non-proteogenic selenoamino acid derivatives. Enzymatic substrates for methylselenol synthesis. Readily excreted due to their methylated group. Present in <i>Allium</i> and <i>Brassica</i> plants.	
γ-glutamyl-methyl-selenocysteine	C ₉ H ₁₆ N ₂ O ₅ SSe			
Selenocystine			Diselenide: two molecules of Se-cysteine mutually oxidized. Very stable. Low solubility in water.	Cystine
Selenocysteine	C ₃ H ₇ NO ₂ Se		Selenoamino acid, form under which Se is present in active centers of selenoenzymes. Considered as the 21st amino acid as it is genetically coded by UGA codon. Unstable if isolated because its selenol group (-SeH) is readily oxidized in Se-cystine or other compounds.	Cysteine
Methylselenol	CH ₃ SeH		Intermediate of Se metabolism. Probable specific anti-cancer agent. Unstable, readily oxidized in dimethylselenide and excreted by methylated excretion pathway.	
Dimethylselenide	(CH ₃) ₂ Se		Gas, minor excretion product of Se metabolism (in breath). Typical odour of garlic.	
Trimethylselenonium ion	(CH ₃) ₃ Se ⁺		Excretion product of Se metabolism.	
Selenosugars			Main excretion products of Se metabolism in urine.	

Sources: Stadtman, 1990; Atkins & Jones, 1998; INRS, 2002; Whanger, 2002. Illustrations: www.wikipedia.org, www.chemspider.com

2.3. Selenium from the environment to the food

Selenium is present in the natural environment essentially under inorganic form. Water contains Se in the form of selenite and selenate, elemental Se^0 can be additionally found in soils, and selenium dioxide and some volatile organoselenium compounds produced by industries (through coal and oil combustion) can be present in the air. Selenium concentration is approximately $0.45 \mu\text{g kg}^{-1}$ in sea water and $0.2 \mu\text{g kg}^{-1}$ in stream water (Rezanka & Sigler, 2008). In soils, average Se concentrations approximate 1 mg kg^{-1} but, as previously pointed out, the Se soil content is highly variable from a region of the world to another, ranging from values inferior to 0.1 mg kg^{-1} in Se-deficient areas to more than 100 mg kg^{-1} in seleniferous regions. Figure 2-2 provides a global view of the areas where Se has been reported to be in excess or in too low amounts in soils (Fishbein, 1991; Oldfield, 1999; Navarro-Alarcon & Cabrera-Vique, 2008).



Figure 2-2: Worldwide cartography of Se in soils (illustration adapted from Oldfield, 1999). Areas in red: Se toxicity reported. Areas in blue: Se deficiency reported.

Selenium enters the food chain through the plants that take up inorganic Se from the soil. Selenate is very soluble and mobile, and is thus highly available for plants. Selenate occurs mostly in aerobic and alkaline soils (Rezanka & Sigler, 2008). The first cases of Se intoxication to cattle were reported on these types of soils, and for this reason, this affliction was primarily referred to as

“alkali disease” before knowing that Se was the cause (Navarro-Alarcon & Cabrera-Vique, 2008). Selenite dominates in most types of soils and is less available than selenate due to its lower solubility and its propensity to form (oxy)hydroxides with Fe, Mn or Al, and to adsorb on humic substances present in soils (Seby *et al.*, 1997). Elementary Se is insoluble and inert (Sager, 2006). In plants, selenite is rapidly metabolized mostly into SeMet or SeCys, and stored under these forms directly in the roots. In contrast, selenate is highly mobile in the xylem. It is previously translocated in above-ground plant parts (Sager, 2006) and less readily transformed into organic species than selenite (Terry *et al.*, 2000). Selenium concentrations in most plants remain usually low ($\sim 10 \mu\text{g kg}^{-1}$) except in “Se-accumulator” plants that can tolerate Se concentrations up to 10^3 times higher than usual plants (Dumont, Vanhaecke, *et al.*, 2006). This particularity comes from the fact that these plants can convert mineral Se into non-proteinogenic selenoamino acids derivatives, *i.e.* MeSeCys and gamma-glutamyl-MeSeCys (γ -glut-MeSeCys), instead of the usual SeMet and SeCys. MeSeCys and γ -glut-MeSeCys have low reactivity, do not follow the metabolic pathway of selenoprotein synthesis like usual selenoamino acids, and thereby do not generate selenotoxicity normally induced by these species (Ogra *et al.*, 2005). Se-accumulator plants are typically those that belong to *Brassica* and *Allium* families.

As a result of the mineral-to-organic conversion of Se in plants, most food and feed consumed by humans and animals contain Se under organic form. The principal way of metabolization of Se-compounds in living organisms is through the synthesis of several selenoenzymes and selenoproteins whose main functions will be detailed in the next section.

2.4. Selenium intake and health functions

2.4.1. Guideline values for selenium intake

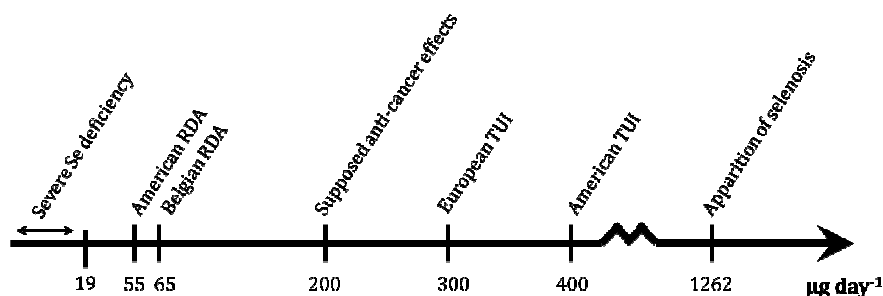


Figure 2-3: Principal reference values for Se daily intake of adult individuals.

Figure 2-3 presents the principal reference values for daily Se intake of normal adult individuals. As reported on this figure, the US Food and Nutrition Board has set the recommended daily allowance (RDA) for Se at 55 µg for both sexes. This level of intake is supposed to guarantee the maximal activity of GPx enzymes (Monsen, 2000; Thompson, 2004). In Belgium, higher values of recommended intake have been fixed in order to guarantee the absence of Se deficiency, *i.e.* 60 µg day⁻¹ for women and 70 µg day⁻¹ for men (SHC, 2009). At levels of intake below the recommended values, the risk of Se deficiency is likely to appear. The minimum Se requirement to prevent severe deficiency (*i.e.* Keshan disease) has been set at 19 µg Se day⁻¹ (WHO/FAO/IAEA, 1996). On the other hand, the tolerable upper intake (TUI), *i.e.* the highest daily level of Se intake that is supposed to cause no adverse effect to the majority of individuals, has been advised at 400 µg Se day⁻¹ in the USA (Goldhaber, 2003; Pedrero, & Madrid, 2009) and 300 µg Se day⁻¹ in Europe (SCF, 2000). The Environmental Protection Agency of the USA defined 1262 µg Se day⁻¹ as the level of intake at which clinical Se intoxication appears (Navarro-Alarcon & Cabrera-Vique, 2008), but adverse effects due to Se excess have been reported for lower values of intake (~800-900 µg day⁻¹) (Reid *et al.*, 2004).

2.4.2. Functions of selenium

Selenium's functions are mediated by the action of several selenoproteins and selenoenzymes.

2.4.2.1. Antioxidant function

About half of the characterized selenoproteins have been reported to have antioxidant function (Tapiero, Townsend & Tew, 2003), thereby general oxidative stress responds directly to dietary Se (Rayman, 2000). From Se antioxidant action will derive many of the Se-associated health effects described below. Antioxidant selenoproteins mostly involve the GPx and TRx selenoenzymatic families. In particular, GPx selenoenzymes represent about 30% of the body's Se content (Mezes & Balog, 2009). The GPx family includes at least four isoforms that have their proper function and/or tissue distribution. GPx1, or cellular-GPx, is the most abundant selenoenzyme in the organism. It is expressed in every cellular type, but predominates in the liver and in erythrocytes (Behne & Kyriakopoulos, 2001). GPx2, or gastrointestinal-GPx, is found predominantly in cells of the gastrointestinal epithelium. GPx3, or extracellular/plasma-GPx, is the second most abundant selenoprotein in the plasma after selenoprotein P. GPx4, or phospholipid hydroperoxide-GPx, specifically reduces the fatty acid hydroperoxides that are esterified to phospholipids. GPx4 has been identified in both cytosol and mitochondria (Tapiero, Townsend & Tew, 2003). Unlike the three other GPx isoforms that are constituted of four SeCys-containing subunits, GPx 4 is a monomer containing a single molecule of SeCys (Behne & Kyriakopoulos, 2001). All GPx isoenzymes act for the prevention of oxidative damages by catalyzing the reduction of peroxides and hydroperoxides in a GSH-dependent process. The TRx family also includes several isoforms: TRx1 (cytosolic and the most abundant TRx), TRx-2 (specific to testes), and TRx-3 (mitochondrial) (Gromer *et al.*, 2005). Additionally to GPx and TRx, selenoprotein P (SeP), although its main function is to be a plasmatic Se transporter, has also been reported to express at least two types of antioxidant enzymatic-like activities, *i.e.* phospholipid hydroperoxide glutathione peroxidase and peroxynitrite reductase activities (Arteel & Sies, 2001; Steinbrenner & Sies, 2009). Besides, SeP has heparin-binding sites that enable the coating of the membranes of endothelial cells which can serve as a protective barrier against oxidants (Arteel & Sies, 2001).

2.4.2.2. Role in the immune system and inflammation

Selenium deficiency has been linked to a loss of immunocompetence through impairment of both cell mediated immunity and B-cell function (Rayman, 2000). Selenium shortage decreased the proliferation of lymphocytes

T and B in response to antigens, the activity of natural killers and the production of IgG and IgM (Kiremidijan-Schumacher *et al.*, 1994). In contrast, supranutritional intake of Se (200 $\mu\text{g d}^{-1}$ as sodium selenite) in Se-replete populations has marked immunostimulant effects including increased ability to develop into T lymphocytes, to proliferate and to destroy tumor cells, and increased activity of natural killers. These effects appeared to be related to the ability of Se to upregulate the expression of receptors of the growth-regulatory interleukin-2 on the surface of T lymphocytes and natural killers, therefore facilitating their interaction with the immunostimulant cytokine (Kiremidijan-Schumacher *et al.*, 1994). Besides, a high rate of selenoenzymatic synthesis has been observed in the cells of the immune system (Rayman, 2000). Selenium has also been shown to be beneficial in the treatment of several inflammatory diseases. It reduced the pain and the gravity of symptoms caused by rheumatoid arthritis (Rayman, 2000) and chronic pancreatitis (McCloy, 1998), reduced the mortality from acute pancreatitis (Kuklinski *et al.*, 1991), and decreased the occurrence of asthma (Shaheen *et al.*, 2001).

2.4.2.3. Prevention of viral infection

Selenium deficiency has been reported to promote HIV infection and its progression to AIDS (Look *et al.*, 1997). This effect has been attributed to the increased oxidative stress, the decreased immunity and the induction of inflammatory responses occurring in condition of low Se status for HIV patients (Tapiero, Townsend & Tew, 2003). Besides, in Se-deficient mice, it was observed that harmless coxsackie virus could mutate into a pathogenic form inducing myocarditis. This effect was associated to a defect in GPx1 and could derive from an increased oxidative stress (Beck *et al.*, 1998). Whether other RNA viruses are able to achieve similar mutations has not been highlighted (Rayman, 2000). On the other hand, supranutritional intake of Se has been shown to prevent the infection from hepatitis B virus and its progression into liver cancer (Yu, Zhu & Li, 1997).

2.4.2.4. Role in cardiovascular disease

An inverse correlation has been observed between serum Se concentrations and death from coronary heart disease in humans (Salonen *et al.*, 1982). Selenium may be protective against cardiovascular diseases owing to GPx4 ability to reduce phospholipid and cholesteryl ester hydroperoxides associated

with lipoproteins, preventing the accumulation of oxidized low-density lipoproteins in the artery walls (Sattler, Maiorino & Stocker, 1994). Selenium might also contribute to reduce the inflammatory state induced by atherosclerosis. However, most epidemiological studies failed to demonstrate a clear link between the level of Se intake and the cardiovascular risk (Rayman, 2000).

2.4.2.5. Thyroid function

The role of Se in thyroid function is mediated by the selenoenzymes iodothyronine deiodinases that activate by deiodination the thyroidal precursor thyroxine (T₄) into the active circulating hormone triiodothyronine (T₃) (Tapiero, Townsend & Tew, 2003). Three types of deiodinases have been identified (Behne & Kyriakopoulos, 2001).

2.4.2.6. Keratinocyte function

Selenium deficiency has been linked to dry skin, erythema and brittle hair, and these symptoms were shown to disappear upon dietary supplementation with Se (Sengupta *et al.*, 2010). In experimental conditions, mice with suppressed ability to express selenoproteins in epidermal cells (*via* targeted Sec tRNA knock out) showed several abnormalities such as hyperplastic epidermis, aberrant hair follicle morphogenesis and alopecia (Sengupta *et al.*, 2010). In humans, supplementation conducted with Se in combination with other antioxidants led to increased skin density, and improved roughness and scaling (Heinrich *et al.*, 2006). In addition, Se disulfide is commonly used in anti-dandruff shampoos (The American Society of Health-System Pharmacists, 2012). By being positioned at the interface between the body and the external environment, skin and hair are exposed to high level of oxidative environmental stress from UV rays and chemical pollutants. It is therefore probable that the beneficial action of Se on these body areas are related to an improved antioxidant status.

2.4.2.7. Cerebral function

The fact that during Se depletion, the brain receives a priority supply seems to indicate that Se should be crucial for this organ (Rayman, 2000). Low Se intake has been shown to disturb the turnover rate of several

neurotransmitters (Castaño *et al.*, 1997), and brain Se concentrations of Alzheimer's patients were reported to be 60% that of controls (Hawkes & Hornbostel, 1996). Selenium has also been reported to impact several parameters related to the mood. A US study observed on healthy men a diminution of hostility, confuseness, anxiety, unsureness, depression and tiredness associated with high level of Se intake ($226.5 \mu\text{g d}^{-1}$) compared to low level ($32.6 \mu\text{g d}^{-1}$) (Finley & Penland, 1997).

2.4.2.8. Role in reproduction

Selenium plays a role in male reproductive functions by being required for normal morphological development of testicles, for testosterone biosynthesis and for the maturation of spermatozoa (Behne, Weiler & Kyriakopoulos, 1996). The role of Se in sperm maturation has been associated to the presence of GPx4 that evolves from its selenoenzymatic form in early spermatids to a structural protein of considerable importance in mature spermatozoa (Ursiny *et al.*, 1997).

2.4.2.9. Role in cancer

Numerous epidemiological and prospective studies have provided signs of an inverse relation between Se intake and cancer incidence and mortality. The question of the role of Se in cancer prevention is complex and will be addressed in detail in section 2.4.6. of this chapter.

2.4.3. Selenium deficiency

Since the main functions of Se are mediated by antioxidant selenoenzymatic activities, most of the consequences of Se deficiency can be associated with increased oxidative stress (Rayman, 2000; Tapiero, Townsend & Tew, 2003; Thompson, 2004). The most serious health troubles related to Se deficiency are Keshan disease, a severe cardiomyopathy, and Kashin Beck disease (KBD), a distortive osteoarthritis, both endemically occurring in Chinese regions where Se content in soils and in food is extremely low. For example, in Keshan area, the daily Se intake can be inferior to $10 \mu\text{g}$ (Navarro-Alarcon & Cabrera-Vique, 2008). Regarding both pathologies, additional etiologic factors to Se deficiency are believed to be involved. As for Keshan disease, Se deficiency is suggested to lead to an enhanced sensitivity to the virulence of coxsackie virus causing

myocarditis (Beck *et al.*, 1997). Concerning KBD, the combination of Se deficiency with iodine deficiency, and the possible presence of fulvic acid and mycotoxins in food would be amongst the factors determining the appearance of the disease (Tapiero, Townsend & Tew, 2003). Both Keshan and KBD were shown to disappear with appropriate level of Se intake (Fairweather-Tait, 1997). Less overt Se deficiency has been associated with a diminution of immunocompetence, a promotion of some viral infections such as HIV, an accelerated degeneration of cognitive function, a greater incidence of depression, and hypothyroidism in case of concomitant iodine deficiency.

2.4.4. Selenium excess

Overt Se toxicity, *i.e.* selenosis, is less widespread than Se deficiency. Chronic selenosis has nevertheless been detected in some populations of seleniferous areas such as the Northern great plains of the USA or parts of Venezuela and Columbia (Rayman, 2008). Chronic Se intoxication is associated with anemia and disruption of immunologic functions, and is typically characterized by hair loss and nails brittleness. Acute intoxications have been reported to cause severe gastrointestinal and neurologic disturbances. Both types of intoxications share typical garlic odor in breath due to dimethyl selenide (Goldhaber, 2003; Tinggi, 2003).

2.5. Interaction of Se with the organism

Section 2.5. corresponds to a paragraph of a review article published in 2012 in *Food Chemistry* entitled “Current knowledge in species-related bioavailability of selenium in food” (Céline Thiry^{1,2}, Ann Ruttens¹, Luc Pussemier¹ and Yves-Jacques Schneider², 2012).

2.5.1. Concepts of bioaccessibility, bioavailability and bioactivity

The general journey of a nutrient from food begins with its release from the food matrix. The degradation of macromolecules into material that can be

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assimilated by the body occurs in the mouth, in the stomach and the small intestine upon the action of digestive enzymes under specific physico-chemical conditions. Once released, free nutrients can be absorbed from the lumen of the intestinal tract towards the interior of the body. Absorption occurs mostly in the small intestine whose epithelium displays villi and microvilli that extend its surface and maximize the absorption efficiency. At the center of each intestinal microvillus lay blood capillaries that encircle a lymphatic vessel (figure 2-4).

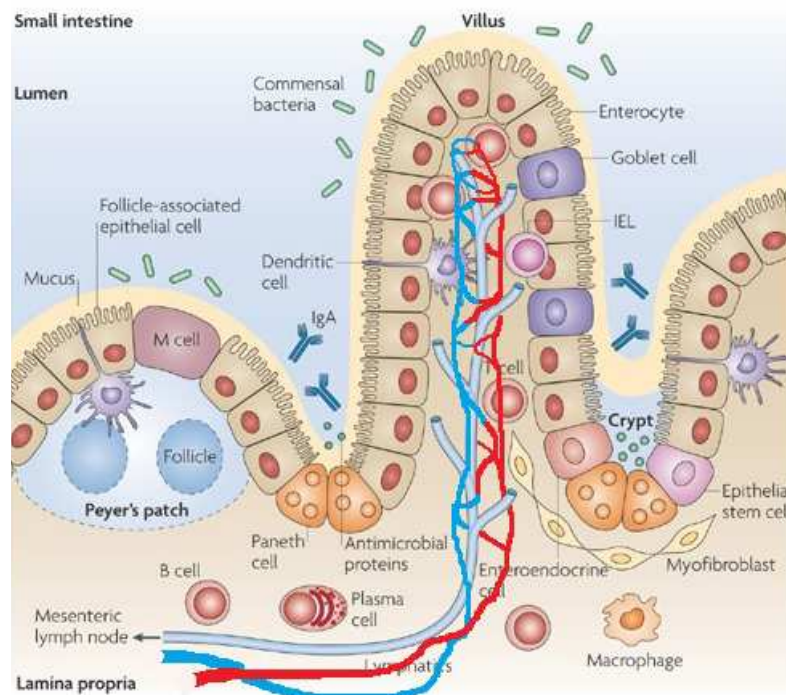


Figure 2-4: Anatomy of the small intestine epithelium (illustration adapted from Abreu, 2010).

Nutrients can be transported through the intestinal epithelium in a way depending on the nutrient's nature (*cf.* chapter 7), and then cross the unicellular wall of the blood capillaries (amino acids, hydrophilic vitamins, monosaccharides), or of the lymphatic vessel (hydrophobic vitamins and lipid compounds). Blood capillaries converge to the hepatic portal vein that goes directly to the liver. In the liver, a fraction of the nutrients can be metabolized. Non-metabolized nutrients and part of the newly-synthesized metabolites are then released into the systemic bloodstream that circulates from the heart to

the various areas of the body. There, nutrients can be taken out of the blood and finally be metabolized in order to exert a biological action (Campbell, 1995).

Bioaccessibility, bioavailability and bioactivity each relate to a particular stage in a nutrient's journey, and are determinants of the extent to which this nutrient will finally impact the organism. According to figure 2-5 that refers to Se, the bioaccessible fraction is the one that is released from the food matrix into the intestinal lumen, and that can thereby possibly be absorbed through the intestinal mucosa (Shen *et al.*, 1997; Ruby *et al.*, 1999; Stahl *et al.*, 2002). By opposition, the non-accessible fraction remains entrapped in the food matrix and ends up in the faeces. Bioavailability is a widely used concept that basically refers to the fraction of an ingested substance that is available for use by the organism. In practice, however, the concept of bioavailability remains rather vague because several definitions have been proposed according to authors and areas of research (Fernandez-Garcia, Carvajal-Lerida & Perez-Galvez, 2009). In this work, we refer to two definitions that are frequently encountered. The first one considers that the bioavailable fraction of a substance is the one that is absorbed through the intestinal barrier (Heaney, 2001). The other one considers the bioavailable fraction as the one that reaches the systemic bloodstream (*i.e.* that has passed through the liver) (Schümann *et al.*, 1997; Brandon *et al.*, 2005). The choice of one or the other definition depends largely on two criteria: the nature of substance that is considered, and the model of study that is used. Regarding the nature of the substance, when considering a drug, it is essential to refer to the second definition of bioavailability because the drug concentration can be critically affected by the first-pass metabolism of the liver. In case of nutrients, for which there is no such active hepatic metabolism, bioavailability is commonly associated with the absorbable fraction of a substance (Heaney, 2001). Regarding the model of study, for practical reasons, in *in vivo* models bioavailability is estimated by measuring a substance concentration in the systemic bloodstream (*cf.* chapter 6), while in *in vitro* models, which are more restricted, bioavailability is considered to be the percentage of absorption through a simulated intestinal barrier (*cf.* chapter 7). Finally, the bioactive fraction of a substance is the one that is converted into biologically active metabolites. Most of the time bioactivity is estimated by measuring the activity of metabolized enzymes in blood and tissues, such as glutathione peroxidase in case of Se.

One must be aware that, here, an effort was made to relate precise terminology to the different levels of interaction that a substance can have with the organism. However, in practice, a considerable number of studies use 'bioavailability' as a general term to refer to bioaccessibility, to actual bioavailability, or to bioactivity, and this reinforces the confusion regarding the concept of bioavailability.

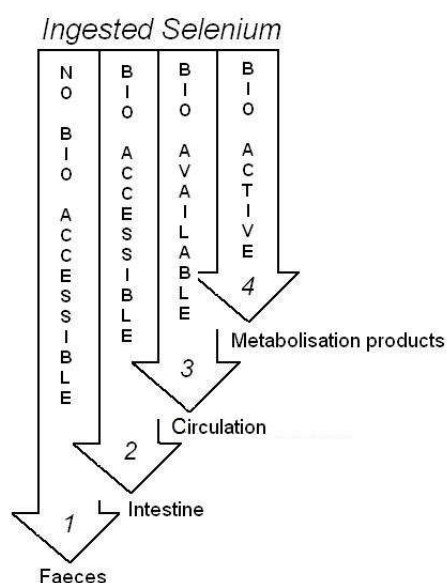


Figure 2-5: Ingested Se can be separated into different fractions:

1. *Non-bioaccessible fraction: Se remaining in the food matrix during its transit through the organism and ending up in the faeces.*
2. *Bioaccessible fraction: Se released from the food matrix in the intestinal lumen.*
3. *Bioavailable fraction: Se absorbed through the intestinal barrier and reaching the hepatic portal vein / the systemic circulation (function of the definition).*
4. *Bioactive fraction: Se transformed into active selenometabolites.*

Among the factors determining the degrees of Se bioaccessibility, -availability and -activity, the speciation plays a crucial role but additional factors can also be of importance. One of these factors is the nature of the other components of the food. For example, the presence of vitamin E acts synergistically with Se for the induction of GPx activity (Thompson, 2004). On the other hand, heavy metals and fibers tend to reduce Se intestinal absorption (Fairweather-Tait, 1997; Reeves *et al.*, 2007; Ralston *et al.*, 2008). The nature of the lipids present in food can also affect Se bioavailability (Mutanen & Mykkänen, 1984), as well as the presence of sulfur compounds (especially methionine) that can compete with Se for absorption and utilization (Waschulewski & Sunde, 1988). Besides, some parameters related to the person can influence Se bioavailability such as the initial Se status, the age, the sex, the health state or the lifestyle (Thompson, 2004). It is also relevant to examine if any processing was applied to the food. For example, cooking, boiling or freezing might cause the loss of certain selenocompounds, influence

their bond to the matrix, or induce changes in Se speciation that could impact on the degree of Se bioavailability (Navarro-Alarcon & Lopez-Martinez, 2000; Pedrero, Madrid & Camara, 2006; Pedrero & Madrid, 2009).

2.5.2. Species-dependent metabolism of Se

Selenium has a species-dependent fate in the organism which is related to distinct metabolic pathways followed by Se species in the body. Figure 2-6 gives a global view of the current knowledge about the metabolism of Se species in the human body.

2.5.2.1. Absorption, transport and distribution

The absorption of Se through the intestinal barrier has been reported to be species-dependent. This aspect of Se metabolism will be thoroughly addressed in chapter 7. Following its absorption, Se joins the bloodstream in order to be distributed to different body locations. Selenium transport is mediated by two major plasmatic transporters *i.e.* albumin and SeP. The transport has been suggested to occur upon two successive pathways (Suzuki *et al.*, 2009). During the first hour following its intestinal absorption, Se binds to albumin and is transported towards the liver where it serves for the synthesis of selenoproteins, notably SeP. Once formed, SeP integrates the bloodstream and becomes itself a Se transporter on the long term between the liver and other organs and tissues. After the liver, the kidneys are the second most important organs where selenoprotein synthesis takes place. While the liver mostly deals with the syntheses of SeP and cellular GPx, the kidneys synthesize extracellular GPx (Suzuki *et al.*, 2009). Liver and kidneys respectively contain 30% and 15% of the total Se content of the body (FAO/WHO, 2002). Other Se-containing organs and tissues are muscles (30% of total Se body content), plasma (10%), erythrocytes, spleen, pancreas and skeleton (Stahl *et al.*, 2002).

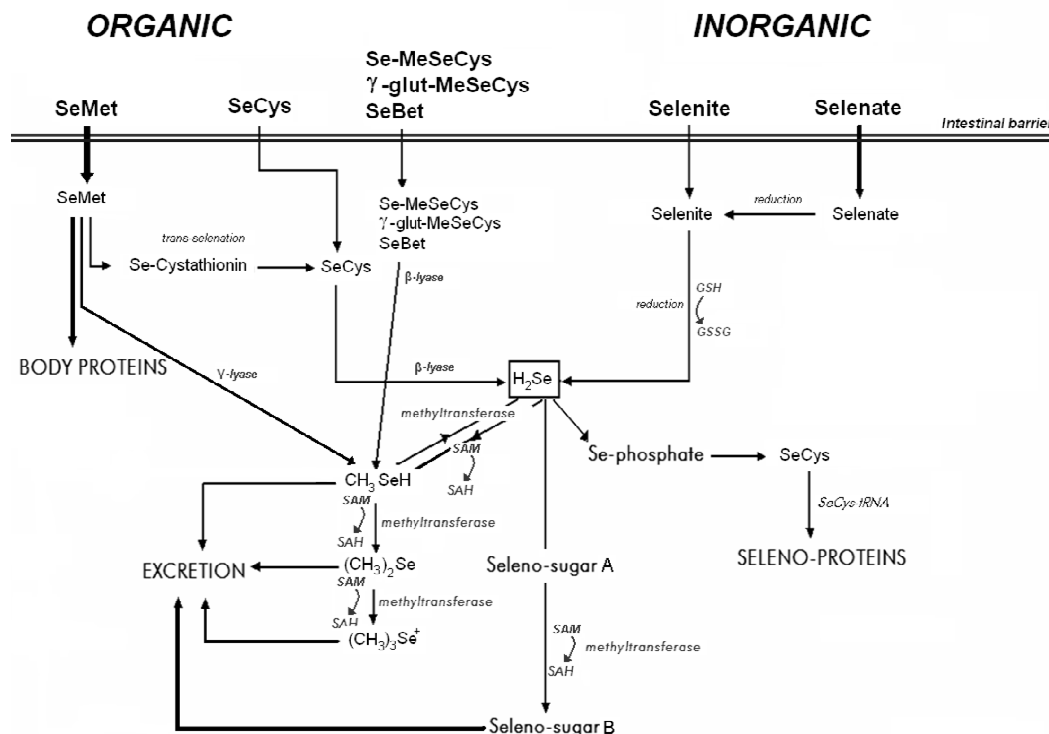


Figure 2-6: Proposed schematic view of normal metabolism of Se in humans (adapted from Suzuki, Doi & Suzuki, 2006; Suzuki, Kurasaki et al., 2006; Suzuki et al., 2008).

SeMet can follow three pathways: (by decreasing order of importance) unspecific incorporation to Se-containing proteins, trans-selenation into SeCys, and directly joining the methylated excretion pathway. SeCys is enzymatically converted into the common intermediate for utilization and excretion of Se: H₂Se. Selenite and selenate are transformed into H₂Se through successive reduction steps consuming GSH. Once formed, H₂Se can be used either for selenoprotein synthesis in a UGA-coding process, or be methylated and join the excretion pathway.

CH₃SeH: methylselenol ; (CH₃)₂Se: dimethylselenide ; (CH₃)₃Se⁺: trimethylselenonium ; γ-glut-methylselenocysteine: gamma glutamine methylselenocysteine ; GSH: glutathione ; H₂Se: hydrogen selenide ; MeSeCys: methylselenocysteine ; SAH: S-adenosylhomocysteine ; SAM: S-adenosylmethionine ; SeBet: selenobetaine ; SeCys: selenocysteine ; SeMet: selenomethionine.

2.5.2.2. Utilization

The kind of utilization made from Se by the body is once again dependent on its chemical form. All Se species that are presented on the top of figure 2-6 are able to enter the pathway of selenoprotein synthesis, but with different propensities. Selenate and selenite serve exclusively this function, as well as most SeCys. SeMet can also be used for selenoprotein synthesis but the majority of this species is unspecifically incorporated into body proteins that become in this way 'Se-containing proteins' (Navarro-Alarcon & Cabrera-Vique, 2008; Ducros & Favier, 2004). In contrast with selenoproteins, Se-containing proteins do not have proper functional properties related to the presence of Se (Stadtman, 1996). The integration of SeMet into Se-containing proteins occurs by the replacement of methionine in the amino acid chain due to the fact that methionyl-tRNA is not able to discriminate between the two analogues (Schrauzer, 2000). Tissues with high protein content such as the skeletal muscles can thereby be regarded as a kind of storage place for Se under the form of SeMet. Se-containing proteins with unspecific incorporation of SeCys also exist but exclusively in case of huge intracellular levels of free SeCys. The replacement of Cys by SeCys is not desirable because this can alter the functional properties of some sulfur-proteins such as *e.g.* iron-sulfur protein (Gromer *et al.*, 2005).

The synthesis of selenoproteins requires the formation of a precursor that is common for all nutritional source of Se: hydrogen selenide (H_2Se). Hydrogen selenide is also the intermediate between the pathways of utilization and of excretion of the element (Suzuki, Doi & Suzuki, 2006; Suzuki, Kurasaki *et al.*, 2006). The formation of selenide from inorganic forms of Se occurs by successive reduction steps involving cellular glutathione (*cf.* section 2.6.2. "The role of Se in promotion of oxidative stress"). The reduction of selenate into selenide generally occurs in the organ of selenoprotein synthesis, usually the liver, but selenite can be reduced directly in red blood cells (Suzuki *et al.*, 2009). Among organic Se species, SeCys and the fraction of SeMet that was not incorporated to Se-containing proteins, both reach the liver under their original form (Beilstein & Whanger, 1988; Suzuki, Doi & Suzuki, 2006; Suzuki *et al.*, 2008). There, SeMet follows the *trans*-selenation pathway (comparable to the *trans*-sulfuration pathway) to be transformed into SeCys. The formation of H_2Se from SeCys is then mediated by the β -lyase that cleaves the carbon-Se bond of SeCys.

Once formed, the common intermediate selenide can join the selenoprotein synthesis pathway. For that purpose, it is transformed into Se-phosphate, an activated form of Se that is a donor of SeCys (Stadtman, 1996; Suzuki, 2005). Selenocysteyl-tRNA inserts one or several (in case of SeP) SeCys residue(s) into the aminoacid sequence of the active center(s) of selenoproteins. The sequence of nucleotides coding for SeCys is the codon UGA. This is a usual termination codon, however, the presence into the mRNA, just after UGA, of the particular stem-loop structure SECIS (selenocystein insertion sequence) permits the recognizance of UGA as the SeCys-coding sequence rather than as a stop codon (Stadtman, 1996). SeCys is in that sense sometimes considered as the 21st amino acid. Selenoproteins contain Se exclusively under the form of SeCys (Suzuki, 2005) but it is interesting to mention that intact SeCys from food cannot be immediately used for selenoprotein synthesis. It must necessarily be transformed into H₂Se in order to synthesize *de novo* molecules of SeCys that will be incorporated into selenoproteins (Suzuki, Doi & Suzuki, 2006; Suzuki, Kurasaki *et al.*, 2006).

The pathway followed by methylated Se species such as MeSeCys, γ -glut-MeSeCys and selenobetaine, differs from those of the pre-cited Se species. After their absorption, methylated species enter directly the methylated pool of Se by being transformed into methylselenol (CH₃SeH) by the β -lyase. In case of very excessive intake, SeMet can also be directly transformed into methylselenol (Suzuki, Kurasaki *et al.*, 2006; Zeng, Botnen & Jonhson, 2008). The methylated pathway is above all intended for the excretion of Se. Only a fraction of methylselenol can be demethylated into H₂Se in order to join the selenoprotein synthesis pathway (Suzuki, Doi & Suzuki, 2006; Pedrero & Madrid, 2009). Thereby, methylated Se species usually produce very low levels of H₂Se. Since a large part of Se-induced toxicity derives from the pro-oxidative metabolism of H₂Se (*cf.* section 2.6.2. "The role of Se in promotion of oxidative stress"), this explains that MeSeCys and γ -glut-MeSeCys are less toxic than other Se species.

2.5.2.3. Excretion

The excretion of Se occurs either through successive methylation steps producing several intermediates, either through the formation of selenosugars (Se-methyl-N-acetylgalactosamide or selenosugar B). Urine is the principal mode of Se excretion in humans and contains the element as selenosugar or as trimethylselenonium ion. Under conditions of high Se intake, dimethylselenide

can also be excreted by breathing, leading to a characteristic odor of garlic in the breath (Suzuki, Doi & Suzuki, 2006; Suzuki, Kurasaki *et al.*, 2006). The excretion of Se is regulated by its intake, and methylation and demethylation reactions between selenide and methylselenol seem to be a critical step in this process (Suzuki *et al.*, 2008). When Se intake is poor, its excretion is reduced and Se supply is maintained exclusively to priority organs (brain, reproductive organs, endocrine glands). In parallel, the levels of GPx1 and, to a lower extent, GPx3 quickly decrease in the rest of the body (Ducros & Favier, 2004; Steinbrenner & Sies, 2009). On the contrary, when Se intake is high, its excretion increases. An enhancement of Se excretion is however possible only up to a certain limit over which Se begins to accumulate and to potentially generate toxicity (van der Torre *et al.*, 1991).

2.5.3. Models for the study of selenium bioavailability

2.5.3.1. *In vivo* models

Animal models were the firsts to be used, and are still nowadays the most often used models in order to investigate Se bioavailability directly on animals or to approximate what should occur in humans. Typically, animals are firstly made Se-deficient with a low-Se diet, to be fed afterwards with the selenized food of interest. During the period of repletion, the extent and the rapidity of the Se status recovery are measured. As a comparison, a parallel group of animals is often fed with a reference selenocompound such as selenite. In particular, the “slope-ratio assay” is a method that was reported by Suttle in 1974 and that generates a value of bioavailability by dividing the slope of the regression line generated by a dose-response relationship of a tested food item by the slope of the regression line generated by the reference selenocompound. The value of the resulting ratio can then be used to quantitatively compare the degrees of bioavailability of Se from different food matrices (Alexander, Whanger & Miller, 1983). This method has been largely applied to various food matrices (Shi & Spallholz, 1994; Alexander, Whanger & Miller, 1983; Ornsrud & Lorentzen, 2002; Reeves *et al.*, 2007).

When the purpose is to investigate Se bioavailability in the human body, working directly with human models permits to obtain more accurate information than by making approximations through animal models. However, working with persons involves extra-difficulties. Indeed, humans cannot

release as much information as animals that are usually sacrificed at the end of the experiment, allowing the analysis of biopsied tissues and organs. Besides, persons are not as standardizable as animals regarding their initial Se status and the composition of their diet. As for humans, it is useful to label the tested food with stable Se isotopes in order to differentiate the Se contained in the tested food items from the Se coming from other constituents of the diet or from endogenous secretions. It is also recommended to administer a reference dose with different isotopic label so that differences in individual absorption capacities can be eliminated (Fairweather-Tait, 1997).

As well in humans than in animals, two types of parameters are commonly measured in order to estimate the nutritional impact of Se on the organism after a period of Se enrichment. The first type of these parameters is the Se content of products of the metabolism and of various body locations. For example, the Se content of faeces and urine reflects the short-term Se status, while the Se content of blood and hair reflects Se status on a longer term. The second type of measured parameters is the extent of selenoenzymatic activity. Usually, the selenoenzymes being monitored are GPx because of their abundance in the body and their easiness to be quantified. Those two types of measurements relate therefore to both bioavailability and bioactivity but, as was mentioned earlier, in practice, authors rarely make the distinction between the different notions, and associate all these parameters with Se bioavailability.

2.5.3.2. *In vitro* models

In comparison to *in vivo* models, *in vitro* experiments have the advantages of being faster, cheaper and simpler. In addition, they are a manner to spare laboratory animals, and to carry out experiments that might be not feasible or practical on living beings.

In vitro models are extensively used to estimate bioaccessibility from a food matrix. Experiments typically consist in an enzymatic digestion of a food product under conditions of temperature and pH mirroring the *in vivo* situation (Fernandez-Garcia, Carvajal-Lerida & Perez-Galvez, 2009). Basically, this consists in a salivary digestion with amylase, followed by a gastric digestion at acidic pH with pepsin, and finally by an intestinal digestion with pancreatic enzymes and bile salts at neutral pH. The details of the method (*e.g.* duration, pH, enzymatic concentration, shaking method) can be subject to large

variations according to the authors. Although an optimized protocol has recently been proposed (Hollebeeck *et al.*, 2012), no real consensus exists to date about a standardized method of *in vitro* digestion. For this reason, one must be aware that the bioaccessible fraction can be dependent on the protocol used to perform the *in vitro* digestion (Oomen *et al.*, 2002). Also, the method used to separate the bioaccessible fraction from the remaining food matrix (sedimentation, centrifugation, microfiltration, ultrafiltration) may be of importance. For example, centrifugation has been reported to lead to a 10-times more important bioaccessible fraction of lead from soil than ultracentrifugation (Van de Wiele *et al.*, 2007). It is not clear what method is the best to isolate bioaccessible fraction, but ultrafiltration has been reported to be particularly appropriate because it represents the size fraction that is believed to cross the intestinal lumen (Van de Wiele *et al.*, 2007 ; Laird, 2010).

Regarding the *in vitro* study of bioavailability, cellular models making use of Caco-2 cells are among the most common and have been used in the context of the present thesis (*cf.* chapters 7 & 8). Caco-2 cells derive from a human colon carcinoma cell line and undergo spontaneous differentiation into enterocytes-like cells. Enterocytes are the most abundant cells of the small intestine epithelium (80% of total intestinal cells) (figure 2-4). They are responsible for the absorption of the nutrients present in the intestinal lumen. Enterocyte-like Caco-2 cells express several structural and functional characteristics of enterocytes including a 'apical-vs-basolateral' polarity, the presence of multiple microvillousities, and the induction of several enzymes specific to the brush border membrane. In culture, Caco-2 proliferate into a monolayer of cells that are inter-connected by tight junctions. Hence, this monolayer is very comparable to the *in vivo* intestinal epithelium (Simon-Assmann *et al.*, 2007). The Caco-2 model of the intestinal barrier is appropriate to study various processes occurring in the small intestine such as cellular uptake, retention, transport, metabolism and selenoprotein synthesis (Zeng, Botnen & Johnson, 2008).

This model suffers nevertheless from some weaknesses. Firstly, it does not consider cellular types other than enterocytes. The additional cells of the intestinal epithelium are goblet and Paneth cells, enteroendocrine cells and M cells. The role of goblet cells is to secrete the mucus that normally forms a coating at the apical side of enterocytes and that serves as a physical barrier to antigens. Paneth cells secrete antimicrobial agents such as lysozymes and

phospholipase A2. Enteroendocrin cells secrete several hormones stimulating the absorption processes: cholecystokinin (stimulating pancreatic enzymatic secretions), secretin (stimulating bicarbonate secretion), and the gastric inhibitory peptide (inhibiting acid secretion). M cells endocytate pathogens and deliver them into underlying lymphoid cells of Peyer's patches where immune responses can be initiated (Henderson *et al.*, 2010). All those cells have thus mainly a role of protection against pathogens and, in that sense, their presence is not crucial for the study of the mechanisms of nutrients absorption. A second deficiency of the described *in vitro* model is the absence of commensal microbiota. It was recently shown that colonic bacteria were able to induce speciation changes to arsenic species from rice (Alava *et al.*, 2012). It is reasonable to think that a similar species interconversion could occur to Se. Normally, the bulk of Se absorption occurs in the small intestine (Brandon *et al.*, 2006) where the microflora is poor (Ceuppens *et al.*, 2012), and Se should not reach the colon. In this case, the absence of microbiota in the cellular model is not a problem. But in particular cases where Se would be entrapped in a food matrix that cannot be degraded nor absorbed in the upper parts of the intestine, like fiber-rich products such as rice, it should be worth considering the microbial aspect of the digestion. A third weakness attributable to most *in vitro* models is the fact that cellular systems are usually static compared to *in vivo* conditions where there is a continuous flow of digested food arriving from the stomach on the apical pole of the cells and a stream of blood on the basolateral side. In *in vitro* conditions, the concentration gradient established between the culture medium and the inside of the cells is stable. This could tend to slow down Se transport and reduce the degree of Se absorption compared to a situation where nutritive medium would be moving and continuously renewed. Finally, the Caco-2 model of the intestinal barrier mimics exclusively the process of absorption and does not take into account the previous steps of the digestion that could impact on the liberation of Se from the food matrix (*i.e.* Se bioaccessibility). When considering the absorption efficiency of Se from food, it is thus necessary to carry out a prior *in vitro* salivary-gastro-intestinal digestion on the food product.

2.6. The ambivalent behavior of selenium

Almost all important health effects ascribed to Se derive from redox reactions. Whereas Se is appreciated for its antioxidant properties through

selenoenzymatic activity, the element can actually act as well as an antioxidant than as an oxidative agent. This double personality is at the origin of the narrow margin existing between Se essentiality and toxicity for a living organism. Basically, Se essentiality is related to its antioxidant action while Se toxicity derives from its oxidative properties. The dose of Se and its speciation are two critical factors determining the redox behavior of the element. To a lesser extent, additional factors can also come into the picture such as the other components of the diet (*e.g.* presence of Hg) or the health state of the person.

2.6.1. Role of Se in prevention of oxidative stress

In aerobic organisms, the cellular energy is produced through the mitochondrial respiratory chain. This process consists in a transfer of electrons originating from successive steps of glycolysis and Krebs cycle, along to successive complexes of the mitochondria inner membrane. The electron transfer is coupled to the pumping out of protons, which produces an electrochemical gradient on both sides of the membranes. By following their electrochemical gradient, protons get back into the mitochondrial cytosol, allowing the production of ATP by the ATP-synthetases. The final electron acceptor of the chain is O₂. By accepting four electrons, it causes the formation of H₂O and CO₂ that are easily handled by the body. In about 5% of the cases, however, O₂ accepts less than four electrons, which leads to the formation of partially reduced oxygen species that are called reactive oxygen species (ROS) (Lushchak & Semchyshyn, 2012). With reference to their name, these compounds are highly reactive and tend to rip off an electron from another liaison as quickly as possible. Most ROS include hydrogen peroxide (H₂O₂), (phospho-)lipid hydroperoxides, superoxide radical (O₂^{•-}), hydroxyl radical ([•]OH), nitric derivatives ([•]NO, ONOO⁻) and various organic radicals ([•]RO) (Lushchak & Semchyshyn, 2012). When present at physiological concentrations, ROS play a role as secondary messengers able to regulate apoptosis, to activate transcriptional factors and to modulate the expression of genes (Pincemail & Defraigne, 2004). But when present at high concentration (stress, smoking, pollution, radiation, etc.), these compounds can readily oxidize the cellular material within the first line the membranous lipids (especially polyunsaturated fatty acids), thiol groups of cell proteins and enzymes, and DNA bases. Consequences of these oxidations include the diminution of cellular exchanges, loss of some transcription signals,

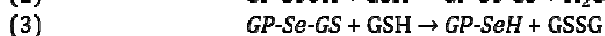
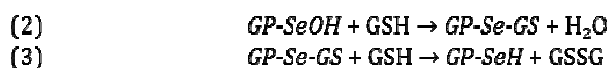
misfunction of certain proteins, loss of enzymatic activity, inflammation, DNA breaks, and occurrence of genetic mutations, possibly leading to cell death (aging) by apoptosis and necrosis, or to cancers (Kohen & Niska, 2002). Some ROS are more damaging than others. Hydroxyl radical is particularly dangerous because it is extremely reactive, not easily destroyed, and it is directly involved in DNA damage (Letavayova, Vlckova & Brozmanova, 2006). To a certain extent, oxidative damages from ROS can be prevented by the presence in the body of two major types of antioxidant compounds. The first class of antioxidants are low molecular weight compounds including glutathione, carotenoids, Vit E, and Vit C, that serve as substrates in the reactions of oxidation by ROS, thereby leading to less reactive reduced compounds. The second class of antioxidants contains enzymes that catalyze reactions of reduction of ROS (superoxide dismutases, glutathione peroxidases, catalases, peroxiredoxins). Among these enzymatic antioxidant systems, some are Se-dependent. This is the case of the two major categories of selenoenzymes: glutathione peroxidases and thioredoxin reductases. Both of them act by intercepting a pro-oxidative agent and reacting with it in order to reduce its reactivity.

- Glutathione peroxidases (Stadtman, 1996):

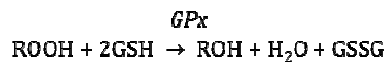
Glutathione peroxidases (GPx) selenoenzymes act in collaboration with the glutathione system in a 3-phases mechanism of action. At physiological pH, GPx are initially present under reduced form and express an ionized selenol group (-Se⁻). In the presence of peroxides or hydroperoxides (*e.g.* H₂O₂), the selenol group is oxidized into enzyme-selenenic acid (-SeOH), with formation of the corresponding alcohol or water:



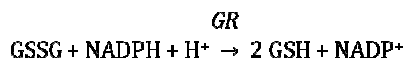
The reduced form of GPx is regenerated by a two-step process involving reduced glutathione (GSH) as a reductant:



The overall reaction is:

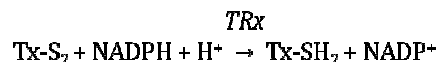


Reduced GSH can be regenerated by glutathione reductase (GR) in a NADP⁺/NADPH-dependent process:



- Thioredoxin reductases (Mustacich & Powis, 2000):

Thioredoxin reductases (TRx) selenoenzymes act in collaboration with the thioredoxin system. Thioredoxins (Tx) are dithiol proteins that, similarly to GSH, are naturally present in cells under reduced form and can react with oxidizing agents. In the same manner as glutathione reductases regenerate GSH from GSSG, the reduced form of Tx is regenerated by TRx enzymes in a NADP⁺/NADPH-dependent process:



TRx might also be involved in the recycling of ascorbate, another important actor in the protection of cells against oxidative stress.

In addition to its action through selenoproteins, Se acts against oxidative stress by binding metals and preventing them from generating ROS (Battin & Brumaghim, 2009). The existence of complexes using Se as a coordination center has been reported for Zn, Cu, Fe, As and Hg (Prince *et al.*, 2007; Ramoutar & Brumaghim, 2007; Battin & Brumaghim, 2009). Selenium protection against Hg (methyl-Hg) toxicity has been the most documented and was associated with a mercury-selenide complex containing approximately 100 atoms of each element (Prince *et al.*, 2007).

2.6.2. Role of Se in promotion of oxidative stress

Although the above-cited selenoenzymes have an undisputable antioxidant role, free Se is able to cause oxidative damages. Depending on their oxidation state and the environmental redox conditions, free Se compounds can be either reduced or oxidized by other cellular components. Selenocompounds at high

oxidation state (+4 and +6) usually act as oxidants and are easily reduced into the selenide state (-2). Reduction of Se occurs mostly upon oxidation of thiol groups of several molecules of reduced glutathione (GSH) (figure 2-7). Selenite has also been reported to be reduced into elemental Se in a similar way by ascorbic acid (Gosetti *et al.*, 2007). This depletion of the pool of cellular antioxidants can lead to an increased vulnerability to oxidative stress (Gad & Abd El-Twab, 2009). This constitutes a first source of Se-induced toxicity. Another one is the direct oxidation by Se of thiol groups of proteins or enzymes, leading to their inactivation (Letavayova, Vlckova & Brozmanova, 2006). But the most important mechanism of Se-induced toxicity occurs through the oxidation of hydrogen selenide (H_2Se) by O_2 , leading to formation of superoxide radical ($\text{O}_2^{\bullet-}$) (figure 2-7) (Gad & Abd El-Twab, 2009; Brozmanova *et al.*, 2010). The dismutation of $\text{O}_2^{\bullet-}$ can further generate H_2O_2 , which can in turn react with partially reduced metal ions such as Fe^{2+} or Cu^{2+} to form the highly damageable hydroxyl radical [$\bullet\text{OH}$] through Fenton reaction ($\text{Fe}^{2+}_{(\text{aq})} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+}_{(\text{aq})} + \text{OH}^{\bullet}_{(\text{aq})} + \bullet\text{OH}$) (Letavayova, Vlckova & Brozmanova, 2006).

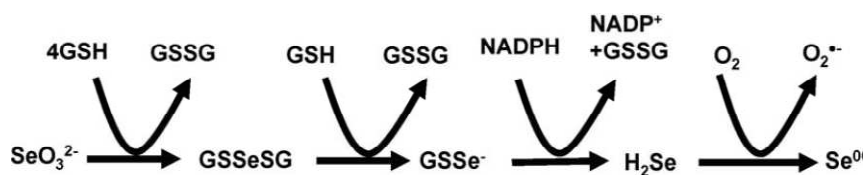


Figure 2-7: Selenium redox reactions from selenite (+4) to elemental Se (0), leading to formation of superoxide (illustration from Letavayova, Vlckova & Brozmanova, 2006).

2.6.3. Determinants of the redox behavior of Se

The major factors determining the pro- or antioxidant behavior of Se are the dose and the chemical form (Letavayova, Vlckova & Brozmanova, 2006).

Observations realised about the dose reveal that at recommended level of intake or under this level, Se acts in favor of antioxidant activity. In this situation, it is probable that the majority of Se is immediately and in totality integrated to selenoproteins and Se-containing proteins. At moderate supranutritional doses, Se compounds begin to exert pro-oxidant activity and some inhibition of cell growth can be observed (Letavayova, Vlckova & Brozmanova, 2006). This can be explained by the fact that when Se intake increases, the selenoenzyme synthesis pathway becomes saturated. Hence,

there is an important fraction of free Se intermediates in the cell, notably H_2Se that catalyses formation of superoxide $[\text{O}_2^{\bullet-}]$. At still higher level of intake, acute toxicity can occur due to ROS-mediated DNA strand breaks (Combs & Gray, 1998; Letavayova, Vlckova & Brozmanova, 2006).

Data concerning Se speciation reveal that SeMet is less toxic than selenite (Tiwary *et al.*, 2006). On rats, the LD_{50} value for intravenous administration of Se as SeMet has been reported to be four times that of selenite (Ammar & Couri, 1981). In contrast with inorganic species, organic compounds like SeMet and methylated species can follow several metabolic pathways that do not systematically end with the formation of H_2Se . As a result, lower quantities of H_2Se and of subsequent superoxide radical are generated (Brozmanova *et al.*, 2010). In addition, the toxicity of organic Se species is limited by the fact that their transformation into selenide is enzymatic, and does not impoverish the cellular stocks of GSH. On the other hand, it has also been suggested that organic Se species could specifically exert some toxicity by inactivating thiol enzymes and cofactors through the replacement of S in sulfhydryl functioning groups (SCF, 2000; INRS, 2005). This has however not been experimentally confirmed. Regarding inorganic species, selenate has been reported to produce less amounts of superoxide than selenite *in vitro* (Mezes & Balogh, 2009)

2.7. Selenium and cancer

2.7.1. Evidence of Se effect against cancer

The protective effect of Se against cancer has been highlighted in two scenarios: for Se intakes that correct the nutritional status of Se-deficient persons, and for Se intake above the recommended values to Se-replete persons.

The first scenario suggests that a low Se status may be among the determinants of cancer risk, at least for certain types of cancer. The epidemiological studies that firstly pointed out this relation reported the mortality due to lymphomas and cancers of gastrointestinal tract, peritoneum, lungs and breast, to be lower for people residing in areas of the US with moderate or high Se concentrations in crops than for those residing in areas with low-Se forage (Shamberger & Frost, 1969; Shamberger & Willis, 1971). During the following years, a number of additional epidemiological studies

confirmed the association between low nutritional Se status and the occurrence and severity of cancers (for a review of these studies, see Combs & Gray, 1998 and Rayman, 2000). The link between Se deficiency and the occurrence of cancer should result, at least partially, from a lack of antioxidant selenoenzymes, which can promote oxidative damage to DNA and cause mutations possibly inducing carcinogenesis (Ramoutar & Brumaghim, 2007).

The second scenario of Se anti-cancer action is associated with supranutritional Se intake. This phenomenon was brought up by several experimental studies on animals, and interventional trials on humans, that were initially inspired by the encouraging conclusions from epidemiological studies. Numerous studies conducted on animals with chemically or virally induced tumors concluded to an antitumorigenic effect of high-level Se exposure (Combs & Gray, 1998). Among these studies, the group of Ip reported SeMet (Ip & White, 1987) and SeCys (Ip *et al.*, 1991) to be less effective than selenite in preventing dimethylbenz(a)anthracene(DMBA)-induced mammary tumors in rats. In humans, at least three interventional trials are worth mentioning. The first of them is the vast Nutritional Prevention of Cancer (NPC) trial that was conducted in the nineties in the USA (Clark *et al.*, 1996). The authors reported a reduction by nearly 50% of the incidence of, and mortality from, carcinomas on several body sites due to a supplementation of 200 $\mu\text{g d}^{-1}$ of Se yeast in a Se-replete population. A second study conducted in the Qidong county of China supported these results by reporting a diminution of the incidence of liver cancer after a daily supplementation of 200 μg of Se yeast or of inorganic Se. It should be mentioned that in this study, people were initially deficient in Se (Yu, Zhu & Li, 1997). By way of contrast with these two studies, SELECT is a vast 12-years intervention trial started in 2001 that, to date, failed at demonstrating any effect of the element administered as SeMet (200 $\mu\text{g d}^{-1}$) (Lippman *et al.*, 2009).

2.7.2. Mechanisms of Se action against cancer

As for the mechanisms proposed to explain Se action against cancer, it appears that both antioxidant and pro-oxidant properties of Se play a role. Selenium acts as an antioxidant in the context of cancer prevention for people having low Se status. In this case, overcoming Se deficiency by elevation of the Se intake up to nutritional values ($\sim 60 \mu\text{g d}^{-1}$) permits to optimize the activity of selenoenzymes and to limit oxidative damages that could lead to cancer

(Combs & Gray, 1998). In contrast, it seems that the anti-cancer effects reported for supranutritional Se intake ($\sim 200 \mu\text{g d}^{-1}$) of Se-replete individuals would derive from pro-oxidant events (figure 2-8). Indeed, at this level of intake, selenoenzymes have reached their maximal level of antioxidant activity. Selenium is thus mostly present as a free compound, notably under the form of H_2Se , and this favors the formation of superoxide anion and other ROS. It was then suggested that the Se-induced oxidative damages that are normally deleterious for healthy people, would actually be beneficial for cancerous patients and could lead to a significant slowdown in cancer development by attacking tumoral cells and inhibiting their growth. This phenomenon is thought to occur through a Se-induced targeted apoptosis of cancerous cells (Letavayova, Vlckova & Brozmanova, 2006; Ramoutar & Brumaghim, 2007; Brozmanova *et al.*, 2010). Two processes were proposed to explain the induction of apoptosis. The first is the oxidation by Se of the sulfhydryl groups of cysteines present in the catalytic area of enzymes, and the subsequent inactivation of those enzymes, *e.g.* protein kinase C (Drakke, 2006). The second is a Se-induced break of DNA strands. In this scheme, highly oxidative Se species would be more efficient for cancer prevention than species with low reactivity such as SeMet. Among these oxidative species, the intermediate metabolite selenodiglutathione GS-Se-SG, deriving directly from selenite (figure 2-7), is thought to be particularly efficient against cancer. It was reported to block cell proliferation through the inhibition of protein synthesis, and to enhance apoptosis. These effects have been attributed to the pro-oxidative metabolism of H_2Se that is produced from the breakdown of the instable selenodiglutathione into the more stable glutathione selenol (GS-SeH) with the release of H_2Se (Lanfear *et al.*, 1994; Tapiero, Townsend & Tew, 2003).

In addition, there has been evidence since about twenty years that a particular organometabolite of Se, *i.e.* methylselenol, and indirectly its precursors (MeSeCys, γ -glutamyl-SeCys, Se-betaine) are more efficient against carcinogenesis than other Se species. This was firstly illustrated in a rat DMBA-induced mammary tumor model in which MeSeCys-based diet induced fewer tumors than selenite, SeMet and SeCys (Ip *et al.*, 1991; Ip *et al.*, 2000). Similarly, high-Se garlic and high-Se broccoli, two plants particularly rich in methylated species, were more efficient than selenite in reducing, respectively, the occurrence of chemically-induced mammary tumors and the formation of aberrant colonic crypts in rats (Finley *et al.*, 2001). The mechanism of action of

methyalted species is not utterly explained but, once again, it would be associated with a blocking of the clonal expansion of pre-tumoral cells and to an enhancement of their targeted apoptosis (Ip, Thompson & Ganther, 2000; Ip, Dong & Ganther, 2002). Additional mechanisms have also been proposed: Se-rich garlic has been observed to reduce carcinogens activation and metabolization in rats by stimulating the phase II detoxication enzymes (Ip & Lisk, 1997), and MeSeCys was reported to decrease angiogenesis in tumoral tissue (Jiang *et al.*, 1999).

Alternatively, the synthetic Se compound 1,4 phenylenebis(methylene) selenocyanate (p-XSC) has been shown to achieve great chemoprevention efficacy for minimal toxic side effects. The mechanism of action would not be associated with selenoprotein synthesis (El-Bayoumy *et al.*, 1992).

This pro-oxidative theory of Se against cancer has been suggested to explain the absence of Se effect in the SELECT interventional study, compared to the NPC trial and the Chinese study. In the case of SELECT, the supplementation has been conducted with SeMet while, in the other studies, Se-enriched yeast or selenite were used. The analysis of a sample of the yeast supplement used for the NPC trial revealed that the fraction of SeMet might have been as little as 27% of the total yeast Se content (Drake, 2006). This seems to indicate that SeMet was not the selenocompound at the origin of the anti-cancer effect, and that this effect would rather be due to the presence in yeast of other Se species, known or unknown, potentially more oxidative than SeMet (Yoshida *et al.*, 2002). Selenite is notably known to be present in yeast, although it is normally at low concentration (Rayman, 2004; Schrauzer, 2006).

Aside from the redox-related functions of Se, the fact that Se deficiency reduces immune responses and that supranutritional intakes increase the capacity to produce cytotoxic lymphocytes that can destroy tumor cells, could also play a role in the anti-cancer effect of Se (Kiremidijan-Schumacher *et al.*, 1994; Combs & Gray, 1998).

Figure 2-8 summarizes the potential mechanisms of action suspected to be involved in Se-induced protection against cancer.

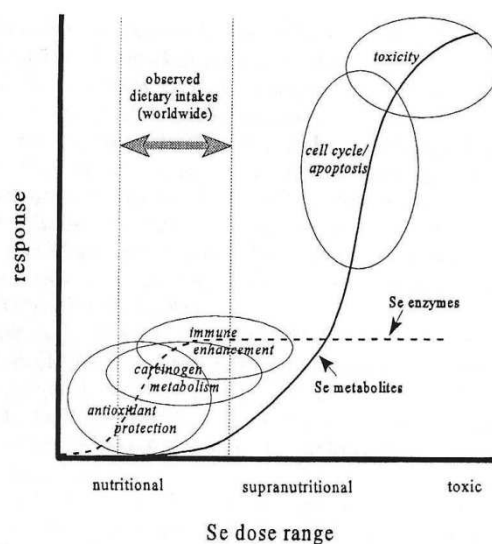


Figure 2-8: Two-stage model for cancer-related effects of Se in function of the dose (illustration from Combs & Gray, 1998).

2.8. Conclusion

In view of its reported either beneficial or harmful effects, Se is sometimes referred to as an 'essential poison'. This appellation derives from the dual redox behavior of Se, which conditions the actual effect of the element on the organism. It seems clear that any Se deficiency should be avoided since, below recommended Se intake, Se acts exclusively as an antioxidant and fulfils various functions that are essential for the organism. The consequence of supranutritional Se intake is less clear because, although it has been associated with some beneficial effects, high Se intake can cause oxidative damages. The result of such a Se intake will largely depend on the dose and the form of Se, as well as on the health status of a person.

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Chapter 3

Thesis aims

Chapter 3 - Thesis aims

Selenium is essential for the good function of the organism since it notably intervenes importantly in the process of defense against oxidative damages. The impact of Se on the organism has been shown to be dose-dependent: beneficial at low to moderate level but becoming toxic at higher level. In addition to the dose, the speciation of Se is the second crucial factor influencing the manner how Se affects the organism. Distinct forms of Se present differences in terms of absorption, of metabolization, and of toxicity. In contrast with the abundant data existing about the dose-dependence of Se health impact, there is a need of more in-depth investigation on the influence of Se speciation. It is still more important in Belgium where people are expected to face slight Se deficiency due to the low level of Se in local soils and food, and where people are thus expected to readily recourse to Se-enriched food supplements (FS). Indeed, in case of FS, the question of the species-dependence is particularly relevant because those products usually contain a single or a few number of Se species at high concentration.

Thereby, the general aim of this work is to improve the current knowledge about the influence of the nature of Se species on the degree of Se bioavailability, in the context of the Se status and food habits of the Belgian population.

In order to achieve this general aim, the work is divided into different chapters with proper specific objectives:

- **Development of an analytical method of selenium speciation in cellular material and in food products**

This first objective is the prerequisite to all realizations of this work. It consists in the development of a precise and reproducible method of quantification of total Se and of Se species by (HPCL-)ICP-MS. This requires the development of sample preparation processes (acid mineralization and enzymatic extraction of Se), of Se species separation by chromatography, and of optimization of Se detection by ICP-MS. All these points will be addressed in **chapter 4**.

- **Estimation of the current selenium status of Belgian people**

The second objective of this work is to set the scene concerning the Se status of the Belgian population. This will be realized in **chapter 5** through the calculation of the average intake of Se by the general adult population in Belgium. The calculated level of Se intake, when compared to guideline values for optimal Se intake, will highlight the eventual need of increase (or decrease) of the Se intake by the general population.

- **Identification of the major food sources of selenium**

In the context of the determination of the Se intake of Belgian people, an additional objective of **chapter 5** is to identify the food products that contribute the most to the Se intake. By knowing the major food sources of Se, it will be possible to identify certain category of persons with particular diet habits who could be at risk of deficiency or excessive intake of Se. Moreover, the speciation analysis of these products will reveal if some Se species are particularly involved in the Se intake.

- **Establishment of the selenium species profile of food**

The identity and the quantity of Se species present in food products are of importance in order to estimate the impact that the Se content of these products will exert on health. One of the objectives of **chapter 6** is thus to establish, through a large review of the recent literature, the Se species profiles of various types of food commonly consumed in Belgium.

- **Evaluation of the influence of the chemical form on selenium status**

Another objective of **chapter 6** consists in the study of the extent to which different Se species can affect the organism. It is intended to be realized through a survey of the relevant literature that will assess the impact of distinct Se species and Se-containing food on several biomarkers of health, *e.g.* Se blood and tissue concentrations, or induction of selenoenzymes. In addition, the metabolic pathways used

by different Se species and their specific outcome on the health will be reviewed.

- **Determination of the precise degree of bioavailability of several selenium species commonly present in food**

The information about Se bioavailability that can be obtained from the literature is associated with many different models and experimental conditions. This causes considerable variability among the results. For this reason, the objective of **chapter 7** is to determine precise values of bioavailability for distinct Se species through *in vitro* experiments.

- **Investigation on the mechanisms mediating the species-dependent bioavailability of selenium**

The other objective of **chapter 7** is to associate the degrees of bioavailability of different Se species with species-dependent mechanisms of transport through the intestinal mucosa. The occurrence of active or passive transport and the possible competition with sulfur compounds will be investigated for that purpose.

- **Determination of the degrees of bioaccessibility and bioavailability of selenium from Se-enriched food supplements**

Se-enriched food supplements are readily consumed in order to increase one's Se status. Those products exist under different types, with distinct Se species profiles. According to the form of Se that they contain, FS are expected to affect the health differently. The purpose of **chapter 8** is to clarify the differences existing among different types of Se-enriched FS by measuring the bioaccessibility and bioavailability of their Se content with *in vitro* methods.

The global methodology of this work is summarized in figure 3-1.

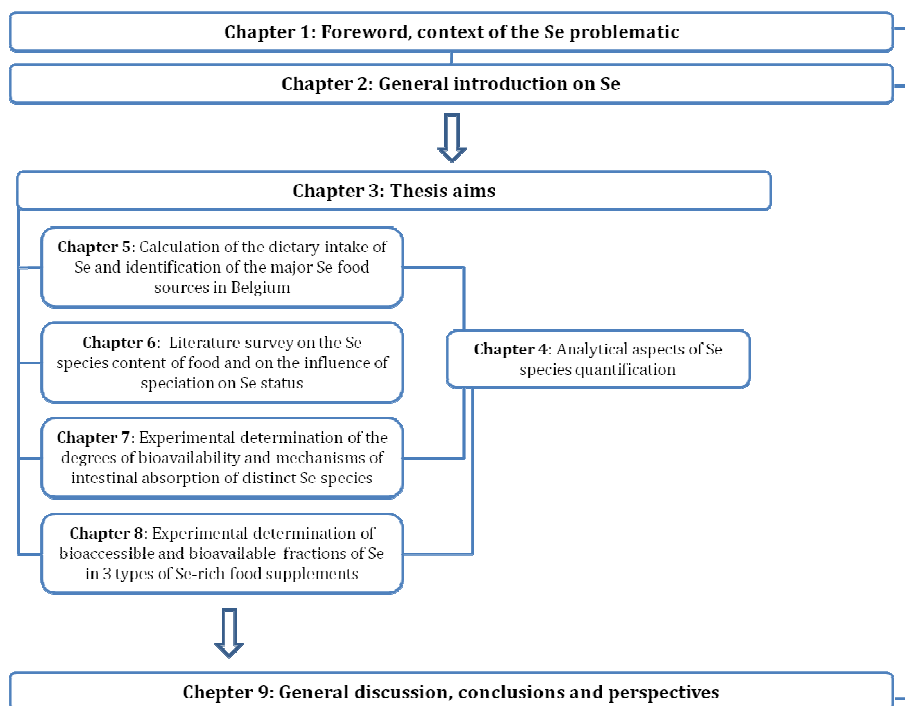


Figure 3-1: Schematic structure of the thesis.

Chapter 4

Selenium speciation analysis by HPLC-ICP-MS

Chapter 4 – Selenium speciation analysis by HPLC-ICP-MS

Foreword

Since all selenium analyses reported in this work were performed by ICP-MS (coupled to HPLC in case of speciation), and because the development of the analytical methods represented a large fraction of the workload of this thesis, it seemed relevant to address in depth the question of Se analysis. Hyphenated techniques like HPLC-ICP-MS consist in the combination of the high selectivity of chromatographic separation with the sensitivity of mass spectrometry (Lobinski & Szpunar, 1999; B'Hymer & Caruso, 2006). Nowadays hyphenated techniques have almost become the rule to analyze Se species because these are the only techniques sensitive enough to allow detection of the low concentrations of Se in food. In this chapter, the analytical devices and associated methods used in this thesis are presented, and their application and relevancy for Se quantification are discussed.

4.1. Global view on the analytical process

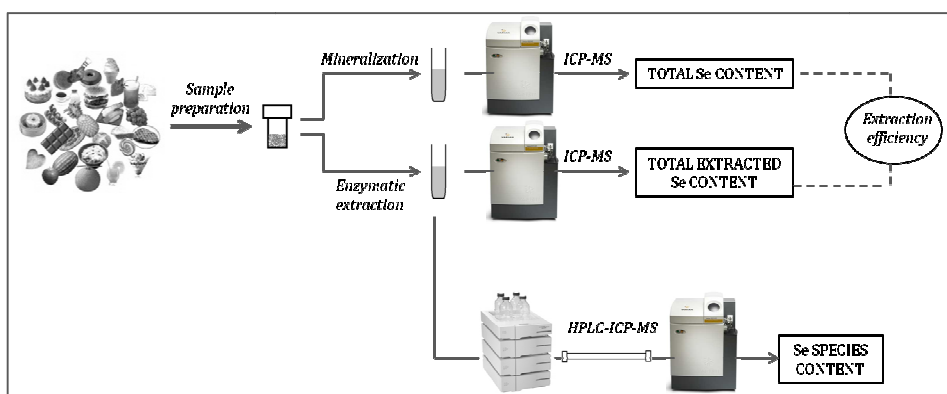


Figure 4-1: Global analytical scheme for total Se and Se species analysis in food samples.

Original samples were fresh food (fish, meat, vegetables, cereals, drinks,...), food supplements and certified reference materials (CRM). Samples were analyzed freshly or dried and were in all cases homogenized by chapping or grinding. For dried samples, the moisture content was determined and samples

were stored at room T°. Fresh samples were kept at -20°C. So-prepared samples could undergo two kinds of processes (figure 4-1). When the purpose was to measure their total Se content, without regard to the speciation profile, an acid mineralization of the sample was performed in a microwave oven and the mineralized solution was further analyzed by ICP-MS. When the purpose was to identify and quantify Se species, an enzymatic extraction softer than acid mineralization was realized. The enzymatic extract was analyzed firstly exclusively by ICP-MS in order to determine the extraction efficiency (total extracted Se / total mineralized Se content), and secondly by HPLC-ICP-MS, in order to separate Se species and then quantify them.

4.2. Analytical devices

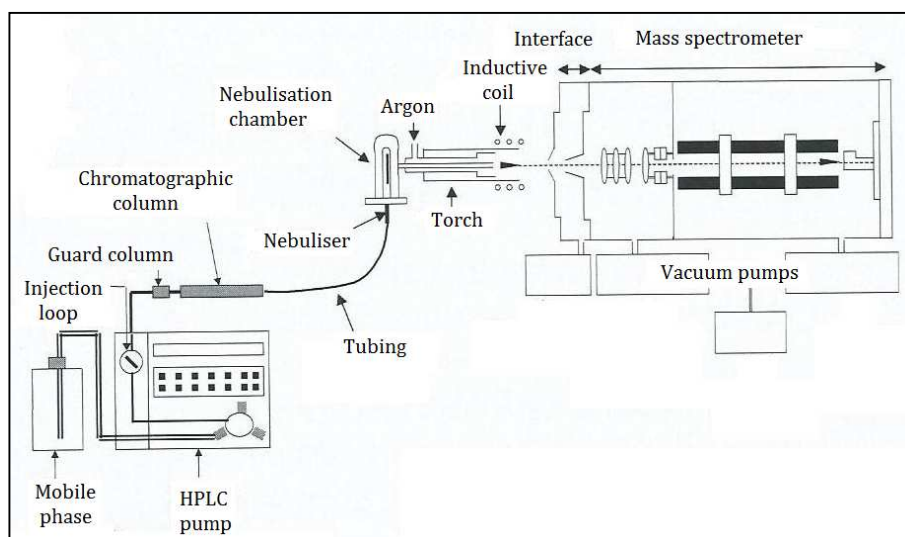


Figure 4-2: Global open view of typical HPLC-ICP-MS device (illustration from UT2A, university of Pau, France).

4.2.1. ICP-MS¹

4.2.1.1. Global view

Introduced in the early 1980', inductively coupled plasma - mass spectrometer (ICP-MS) is a device performing spectroscopic determination of atomic species. The precise entities that are detected and quantified are "m/z (mass to charge) ratios". These entities are formed in a plasma of argon situated at the entry of the ICP-MS device. Once in the plasma, the sample is completely destroyed and volatilized, resulting in a gas containing individual atoms. Simultaneously, atoms are ionized, most often with a single charge. Thereby the m/z ratio of an element is usually equal to its atomic mass. Once formed, monoatomic ions leave the plasma and are guided through a system of cones and lenses in order to form an ionic beam that is introduced into a mass analyzer where ions are separated according to their m/z ratio. Each of these entities can finally be detected and counted in the detector generating a mass spectrum. The strength of the ICP-MS in the context of this work is its ability to carry out **rapid** and **multi-mass** determinations at **trace** level.

4.2.1.2. Step by step

i. Admission, atomization and ionization

Most of the time, the sample to be analyzed is presented in aqueous solution. An important degree of dilution of the element of interest is desirable in order to limit matrix effects that could reduce the ionization efficiency of the analyte (Ito & Tsukada, 2002; Taylor, 2005). In addition, if sample has undergone acid mineralization prior to analysis, it must be further diluted in order to reach acid concentrations that are tolerated by ICP-MS (max 8% v/v). The liquid sample is introduced into the ICP device by a nebulizer which produces a continuous aerosol in a nebulization chamber (figure 4-2). In this chamber, droplets are sorted by size and the largest ones are stopped before reaching the plasma. Plasma refers to an ionized gas which, in case of ICP-MS, is constituted of ions of argon (Ar⁺) and their associated electrons. Plasma occurs

¹ Most of this paragraph can be referred to "Chimie analytique" (Skoog *et al.*, 2012). Additional references are included within the text.

at the end of a plasma torch consisting in three concentric quartz tubes through which different streams of Ar are circulating. The central tube conducts the gas transporting the nebulized sample, the most external tube hosts plasma in its end and furnishes argon for its alimentation at a flow rate of about 20 liters min^{-1} , and the intermediate tube conducts a tangential Ar flow whose role is to cool the external tube and to keep constant the plasma axis (figure 4-3). Argon ionization is initiated by a spark. This generates electrons ($\text{Ar}^0 \rightarrow \text{Ar}^+ + \text{e}^-$) that are accelerated by a fluctuating magnetic field produced by an alternating (radio frequency) current passing through an external induction coil. The magnetic field amplifies the phenomenon till total ionization of the Ar gas. By Joule effect, the expulsion of electrons generates liberation of thermal energy producing high temperature ($\sim 6000^\circ\text{C}$) that enables the successive steps of desolvation, vaporization, atomization and excitation necessary to obtain ionized elemental particles. Efficiencies of atomization and ionization occurring in plasma are critical to guarantee the reproducibility and efficiency of the ICP-MS measurements. These are dependent on the constancy of the nebulizer flow and the stability of the plasma, but also on the element to be analyzed. In case of Se, ionization is limited by its relatively high energy of ionization², i.e. 941 kJ mol^{-1} compared to other elements usually analyzed by ICP-MS, e.g. Pb, 716 kJ mol^{-1} ; Ni, 737 kJ mol^{-1} ; Cu, 746 kJ mol^{-1} or Fe, 763 kJ mol^{-1} .

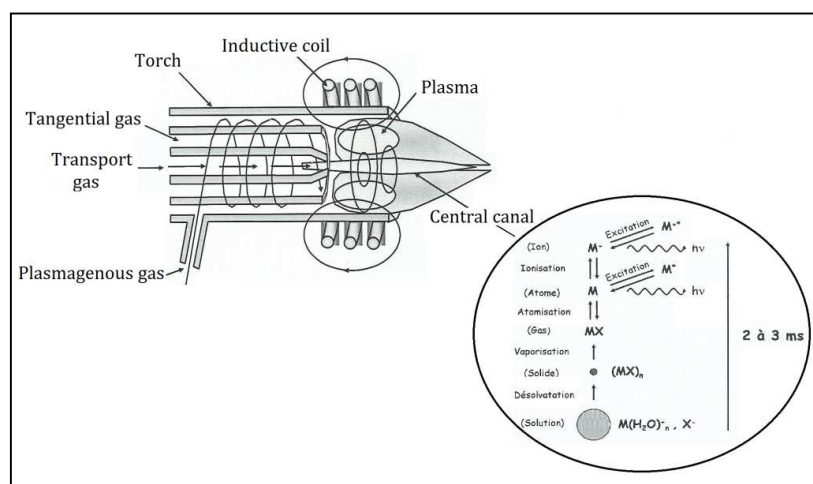


Figure 4-3: Plasma torch and process of production of monoatomic ions (illustration from UT2A, university of Pau, France).

² First energy of ionization = minimal energy required for the expulsion of one electron from the external electronic couch of the element (Atkins & Jones, 1998).

ii. MS interface and removal of spectroscopic interferences

While atomization and ionization occur upon atmospheric pressure, the mass spectrometer operates under vacuum, which creates a depression allowing the extraction of ions from the ICP area. The passage from ICP to MS occurs through an interface consisting in two successive metal cones, called sampler and skimmer, pierced on their top by a small orifice (~1 mm) allowing the passage of ions towards a succession of optical lenses that guide them towards the mass analyzer.

The beam formed in the plasma contains the analyte ions but also a variety of uninteresting compounds that need to be rejected before reaching the MS area. Among these 'parasite' compounds are particles such as neutrals and photons that, if they reached the detector, would cause an enormous background noise. Neutrals and photons are rejected from the ionic beam by the electrostatic lenses situated prior to the MS. In addition, some ionic species can be problematic when, upon post-plasma decrease of T° , they combine to form polyatomic compounds having similar m/z ratio than the analyte. Ionic species that generate these polyatomic combinations include various ions formed from the sample matrix and ions originating from the plasma such as Ar^+ , ArO^+ , ArH^+ , H_2O^+ , O^+ , O_2^+ or Ar_2^+ . Polyatomic combinations originating exclusively from the plasma, like $^{40}\text{Ar}^{38}\text{Ar}^+$ should in theory not affect the results as Se present in calibration standards must be impacted in the same manner as Se present in the sample. However, such interferences hugely increase the signal background noise and thus reduce the sensitivity of the analyte's response. The major problem is when an interfering compound is formed from a component of the sample because, in this way, only the response of Se contained in the sample is impacted, resulting in an overestimation of its Se content. For example, the atomization-ionization of a sample containing chlorine will result in the formation in the plasma of the polyatomic combination $^{40}\text{Ar}^{37}\text{Cl}^+$ that interferes with the analysis of ^{77}Se , which results in overestimated quantification of Se.

Interferences can be handled by a specific device named collision or reaction interface (CRI). The CRI consists in a post-plasma interface delivering a gas able to either break the interfering compounds (collision gas) or to react with them (reaction gas), aiming at changing the mass of the interfering ion into

a mass that is different from the analyte's one. More rarely, usually in case of multiple interferences, the analyte itself can have its own mass modified by the gas in order to reach a non-interfered mass. The CRI device is located prior to the mass analyzer, usually among the optics (Paucot, 2006) but in the Varian ICP-MS used for this work, the CRI was integrated to the skimmer cone that was pierced by a channel conveying the C/R gas. A large flow of C/R gas allows the destruction of a maximum of interfering molecules, however increasing C/R flow also goes with a decrease in the signal sensitivity. This is because C/R gas can react, to variable extent, with the analyte as well as with interferents. By consequence, the limitation of action of the CRI comes from the fact that there is a maximal gas flow that can be reached which still allows sufficient sensitivity on the mass of interest. Typically, CRI gas flows are close to 100 ml min⁻¹.

Choice of the less interfered isotope for Se analysis

Selenium naturally exists under six isotopes and each of them can serve for Se quantification. As an ICP-MS allows simultaneous multi-mass analysis, measurements of total Se can be realized on several isotopes (usually ⁷⁶Se, ⁷⁷Se and ⁷⁸Se). Getting similar results on distinct Se masses indicates that signals were not interfered by polyatomic species. By contrast with ICP-MS, HPLC does not allow multi-mass analysis and a single mass of Se had to be chosen for speciation measurements. Table 4-1 gives isotopes of Se with their respective abundance and the usual interferences susceptible to occur on these masses. Among Se isotopes, the choice of mass 74 for Se analysis was automatically rejected because of its too low occurrence (0.87%) making ⁷⁴Se hardly detectable. The choice was made between the most abundant isotopes according to the capacity of the CRI of our ICP-MS to remove interferences on these masses with hydrogen (H₂) as a reaction gas. Most of polyatomic compounds interfering with Se masses originate from the plasma and include Ar⁺ ion. Isotope 40 represents 99.6% of total occurrence of Ar, meaning that polyatomic interfering molecules containing ⁴⁰Ar are very abundant in the post-plasma ionic beam. For this reason, although ⁸⁰Se is the most abundant of Se isotopes, this mass could not be used for Se quantification because it was not possible to remove in totality the huge interference caused by ⁴⁰Ar⁴⁰Ar⁺ without using huge flow of H₂ that killed the signal of Se. By contrast, interferences on

mass 78 could be efficiently eliminated with a flow of 90 ml H₂ min⁻¹ and ⁷⁸Se was thus chosen for speciation analyses.

Table 4-1: Isotopes of Se with corresponding abundance and most important interfering ionic compounds (May & Wiedmeyer, 1998).

Isotope of Se	Natural abundance (%)	Interfering ionic compounds
⁷⁴ Se	0.87	³⁶ Ar ³⁸ Ar ⁺ , ³⁷ Cl ³⁷ Cl ⁺ , ⁴⁰ Ar ³⁴ S ⁺
⁷⁶ Se	9.02	³⁶ Ar ⁴⁰ Ar ⁺ , ³⁸ Ar ³⁸ Ar ⁺
⁷⁷ Se	7.66	⁴⁰ Ar ³⁷ Cl ⁺ , ³⁶ Ar ⁴⁰ Ar ¹ H ⁺ , ³⁸ Ar ² ¹ H ⁺
⁷⁸ Se	23.52	³⁸ Ar ⁴⁰ Ar ⁺ , ³⁸ Ar ⁴⁰ Ca ⁺
⁸⁰ Se	49.82	⁴⁰ Ar ₂ ⁺
⁸² Se	9.19	⁴⁰ Ar ₂ ¹ H ₂ ⁺

Figure 4-4 presents our methodology of optimization of H₂ flow for the detection of ⁷⁸Se. In this test, the two major types of polyatomic interferences occurring on mass 78 were considered: ³⁸Ar⁴⁰Ar⁺, formed in the plasma, and ³⁸Ar⁴⁰Ca⁺, formed from calcium potentially present in a sample. The removal of the interference caused by ³⁸Ar⁴⁰Ar⁺ was assessed by the maximization of the ratio “signal Se / background noise (the background noise corresponds to the signal measured on mass 78 without presence of Se)”, which guaranteed the minimum interfering signal for a maximal Se signal on mass 78. The removal of the interference caused by ³⁸Ar⁴⁰Ca⁺ was assessed by the equalization of the signals obtained for 10 ppb of Se and for a solution containing 10 ppb of Se and 1 ppm of Ca. This would correspond to a ratio “signal Se+Ca / signal Se” of 1. Among the values of H₂ flows matching both these criteria, the lowest flow was chosen in order to keep the highest sensitivity possible on mass 78.

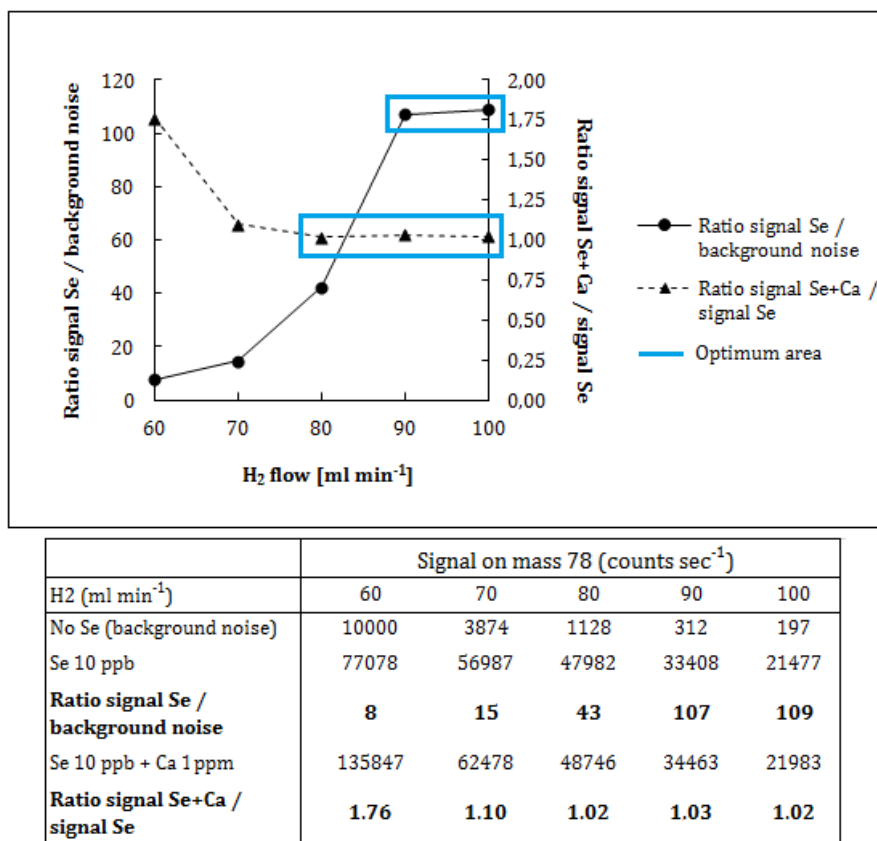


Figure 4-4: Optimization of H₂ flow for ⁷⁸Se detection by ICP-MS. The strategy consists in finding the minimal H₂ flow guaranteeing simultaneously the maximization of the ratio signal Se / background, and the similarity for signals of Se and Se+Ca (i.e. ratio signal Se+Ca / signal Se = 1). The areas corresponding to H₂ flows matching these criteria are indicated in blue on the chart. As shown on the table, the signal of Se decreases with increasing H₂ flow.

iii. Mass analyzer

Most mass analyzers of ICP-MS are quadrupoles, and this was the case of our Varian ICP-MS. Quadrupoles consist of four cylindrical rods, usually disposed as a circle, that are connected to a combination of a direct and a radio frequency alternating currents. According to the voltage applied to the rods, only ions with a determined m/z ratio are able to create their way through the quadrupole. An electron multiplier detector detects each ion that exits the quadrupole and amplifies its signal through a succession of dynodes producing

increasing amounts of electrons. For each ion, a pulse is registered in the detector and associated to a m/z ratio, generating a mass spectrum.

4.2.2. ESI-MS

Electrospray ionization-MS (ESI-MS) was not utilized in this thesis but it is worth mentioning because it is an interesting complement to ICP-MS analysis. In ESI device, there is no plasma and the sample is dispersed by electrospray producing a fine aerosol of ionized macromolecules. In contrast with ICP-MS, devices without plasma present the advantage to be more tolerant to high concentration of organic solvents that could be required in HPLC mobile phases. Indeed, the presence of organic solvents in an ICP-MS can destabilize or even extinguish the plasma (Kolbl *et al.*, 1995), and causes carbon deposits on the plasma torch which can lead to cross-contamination and clogging of cones (Bird *et al.*, 1997). But the major advantage of ESI-MS compared to ICP-MS is that the molecules are not destroyed like they are in a plasma, which allows the identification of compounds for which a standard does not necessarily exist (Casiot *et al.*, 1999; Goenaga-Infante *et al.*, 2004). In addition, with ESI tandem MS mode, fragmentation patterns can be obtained to get information about the structure of the molecule, and the isotopic composition can be determined (Casiot *et al.*, 1999). This makes of ESI-MS a method of choice for the identification and characterization of minor or unknown Se species. The major limitation of ESI-MS is its low sensitivity compared to ICP-MS (about 100 times lower). This is mostly due to the presence of the matrix load of the samples that has not been destroyed during ionization. This technique can thus not be used for determination of very low concentrations or analysis of complex matrices (Kotrebai *et al.*, 2000). Typically, ESI-MS devices have been used for the investigation of selenometabolites in Se-rich yeast since yeast has low matrix load for high Se concentrations (Casiot *et al.*, 1999; Kotrebai *et al.*, 2000; Preud'homme *et al.*, 2012).

4.2.3. HPLC

4.2.3.1. Global view on HPLC and its coupling to ICP-MS

High performance liquid chromatography (HPLC) was the device devoted to the separation of Se species prior to enter the ICP-MS. The end of the LC column was directly coupled to ICP nebulizer so that Se species enter one by one inside

the ICP chamber. In this coupling, ICP-MS replaces typical detectors of HPLC (UV, IR, electro, fluoro) which are not sensitive enough to allow determination of concentrations so low as those typically encountered for Se in food (ng g^{-1}). Basically, HPLC consists in one or several pump(s) working at high pressure (~ 100 bars in our case) to introduce a mobile phase containing the sample of interest through a narrow silica column covered by various binding sites (stationary phase) with more or less affinity for Se species. Se species interact with mobile and stationary phases and, according to their affinity with the phases, they leave the column at successive intervals and they can be detected and quantified. The efficiency of such a system relies on a game of associations of mobile and stationary phases in order to create an environment allowing the best separation possible of each of the Se species. Separation must also occur in an acceptable elution duration. More rarely, gas chromatography (GC) can also be used for separation of Se species, mostly for volatile compounds (dimethylselenide, dimethyldimethyldiselenide), or with a previous derivatisation step for non-volatile species (B'Hymer & Caruso, 2006).

4.2.3.2. Separation of Se species

Interactions of Se species with mobile and stationary phases can be of different nature, mostly electrical, hydrophobic or size-related. The kind of LC separation is dependent on the species of interest and the desired degree of selectivity.

Size exclusion chromatography (SEC) separates molecules according to their size and their shape. The resolution of SEC is generally too poor to allow discrimination of Se species with a molecular weight inferior to 10kDa which makes this technique more suitable as a purification step preceding a complementary chromatographic separation in a multidimensional scheme (Wrobel *et al.*, 2004; Bierla *et al.*, 2008).

Ion (anion or cation)-exchange chromatography (IEC) is based on the interactions of charged species with an oppositely charged stationary phase. In anion-exchange mode, the stationary phase is recovered by positively charged groups able to fix the sample anions, and the mobile phase is negatively charged. During the elution process, the mobile-phase anions (counteranions) compete with the sample anions for binding the positive sites of the stationary phase. Depending of the proper affinities of distinct species for mobile and

stationary phases, the sample anions are progressively replaced by the counteranions and eluted out of the column (figure 4-5). This technique is particularly efficient for separation of inorganic Se species that are often charged or easily ionisable (B'Hymer & Caruso, 2006).

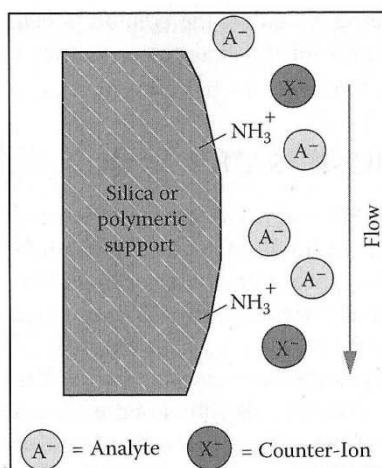


Figure 4-5: Principle of separation using anion-exchange chromatography (illustration from Thomas, 2008).

Partition chromatography, in normal or reversed phase, allows separation of compounds according to their polarity. In reversed-phase mode, the stationary phase is nonpolar and the mobile phase is more polar. Typically, stationary phases consist of carbon chains of 8 or 18 carbon atoms (C8, C18) and mobile phases consist of water mixed with organic solvent such as acetonitrile or methanol. This technique is particularly convenient to separate organic compounds but less appropriate for charged inorganic species that tend to rapidly elute with the hydrophilic mobile phase. The addition of an ion-pairing agent to the mobile phase permits to create a second equilibrium that allows a separation between charged compounds (B'Hymer & Caruso, 2006). Pairing ions have both an organic and an ionic end. Se species as negatively charged ions can form ion-pairs with positively charged pairing ions that are also able to link the stationary phase by hydrophobic interactions (figure 4-6). With this scheme, the elution of paired Se species functions like an anion exchange mode. This method seems to be the most appropriate for the separation of most Se compounds usually present in food (Bird *et al.*, 1997; Szpunar, 2000; B'Hymer & Caruso, 2006).

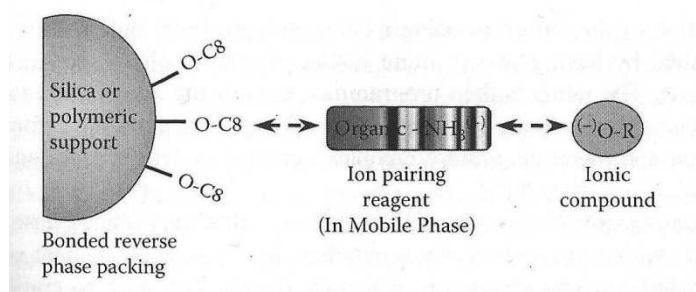


Figure 4-6: Principle of separation using ion pairing reversed-phase chromatography (illustration from Thomas, 2008).

Nevertheless, whatever the kind of separation, resolution of Se species in food samples remains quite low with a single column and it is often not possible to clearly distinguish more than three or four species within a run. To quantify more species, or to validate ambiguous results, it becomes necessary to combine successively two or more kinds of separation (Szpunar, 2000; Diaz Huerta, Fernandez Sanchez & Sanz-Medel, 2006). In the present work, speciation analysis concerned Se standards in cells and culture media, and Se in food supplements samples. None of these samples represented a large spectrum of different species, which allowed us to use a single mode of separation. We independently tested two modes of separation, *i.e.* anion exchange (IE) and ion pairing reversed-phase partitioning (IP-RP), to achieve the separation of four selenospecies: selenate (Se(VI)), selenite (Se(IV)), selenomethionine (SeMet) and methylselenocysteine (MeSeCys).

i. Anion exchange (AE) separation

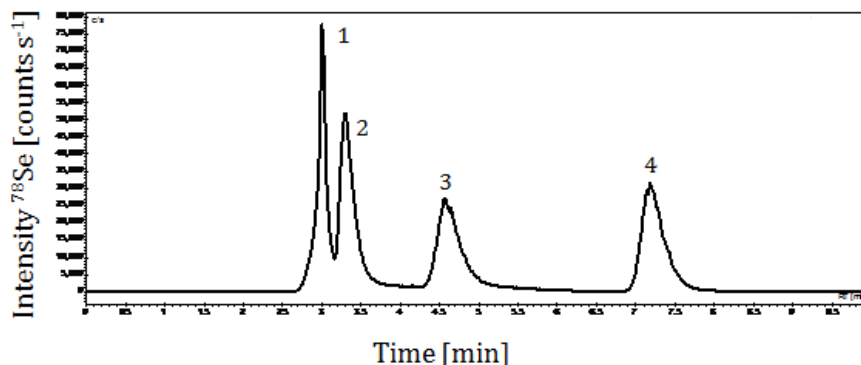


Figure 4-7: Chromatographic separation of Se species with anion-exchange column in a solution of standards (10 ppb). **1** MeSeCys, **2** Se(IV), **3** SeMet, **4** Se(VI).

The column used to achieve separation of Se species in the anion-exchange mode was a Hamilton PRP-X100 (Varian) in which the stationary phase was covered by positively charged quaternary ammonium groups. The mobile phase was made of citric acid 10 mM in milliQ water with 2% MeOH at pH 5.0 (Pedrero *et al.*, 2007). At this pH, citric acid is negatively charged and pushes progressively Se anions out of the column by replacing them on the ammonium groups (Stadlober, Sager & Irgolic, 2001). The presence of methanol served to amplify the signal of Se (Bird *et al.*, 1997; Stadlober, Sager & Irgolic, 2001). According to figure 4-7, total elution of Se standards could be achieved within 10 min in an isocratic mode at a flow rate of 1 ml min⁻¹, but peaks were globally broad and MeSeCys and Se(IV) were not well separated. This method was used for the determination of Se species in fish and meat but the results were not reported in this thesis due to the poor quality of the chromatogram obtained. Two examples of these chromatograms are presented in figures 4-8 and 4-9.

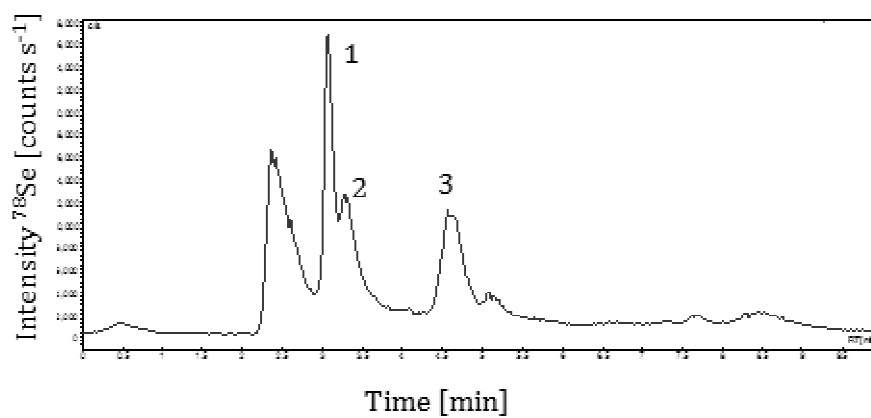


Figure 4-8: AE-HPLC-ICP-MS chromatogram of ^{78}Se in mussel. **1** MeSeCys, **2** Se(IV), **3** SeMet.

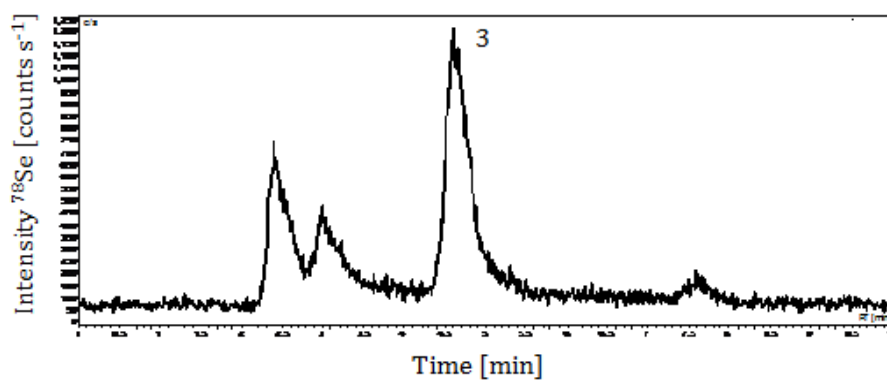


Figure 4-9: AE-HPLC-ICP-MS chromatogram of ^{78}Se in beef muscle. **3** SeMet.

ii. Ion pairing reversed-phase (IP-RP) separation

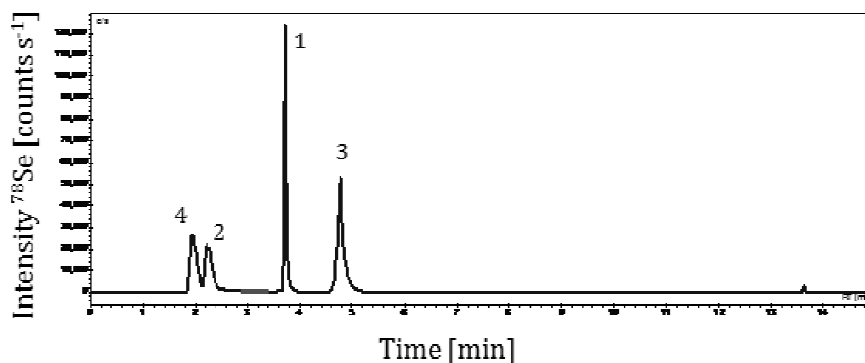


Figure 4-10: Chromatographic separation of Se species with ion pairing reversed-phase column in a solution of standards (10 ppb). **1** MeSeCys, **2** Se(IV), **3** SeMet, **4** Se(VI).

For the separation of Se species upon partitioning mode, the column was a C8 Alltima (Alltech). The mobile phase was made of milliQ water containing 0.2% (v/v) of heptafluorobutyric acid (HFBA) as pairing ion, at pH 6.0 (adapted from Montes-Bayon *et al.*, 2006). The elution of organic species occurred upon an increasing gradient of MeOH (50% v/v in H₂O) in 10 min (1 ml min⁻¹). By comparison with figure 4-7, peaks of Se standards presented on figure 4-10 were better shaped, especially the latest eluted. Here, the difficulty was to sufficiently retain and separate the two inorganic species. Indeed, in real samples, Se(VI) and Se(IV) often tended to overlap. However this was not problematic in the context of the present work as the samples analyzed (cells, culture media and food supplements) contained either selenite or selenate but never the two species simultaneously, which avoided overlaps. Examples of IP-RP chromatograms are provided in chapter 8, section 8.3.1. “Concentration of Se and its speciation in original FS”.

4.3. Selenium analysis

4.3.1. Total Se quantification: acid mineralization

The mineralization mediated by microwave oven is an aggressive process (high T°, acidic pH, oxidizing conditions) that completely destroys the matrix of the sample in order to release the entirety of its elemental content in solution. The detailed process of mineralization is described in the section 5.2.2.

“Selenium determination” of chapter 5. During mineralization, Se species are oxidized which modifies the original Se profile and makes speciation analysis no longer possible. In the present thesis, this process is used to determine the total Se content of various food products (*cf.* chapter 5) and to verify the Se content of food supplements investigated in chapter 8. The capacity of the mineralization process to release the totality of Se from samples was verified by concomitant analysis of certified reference materials (CRM) having authenticated Se content. Ideally a CRM of the same nature as samples should be systematically added to the sequence of analysis. For example, IAEA-407 (fish tissue) was added to fish samples and BCR-185R (bovine liver) to meat samples. A good recovery of Se content from CRM also permitted to verify that there was no interference on the measured mass. Examples of CRM recoveries are presented in section 5.3.1. “Quality control” of chapter 5.

4.3.2. Selenium species quantification: enzymatic extraction

As for Se speciation analysis, the first difficulty to overcome is to extract a maximum of Se from the solid matrix without affecting its original chemical structure. This requires methods of extraction that are softer than acid mineralization. Among existing solid-liquid methods, hot water extraction (85-90°C) was firstly considered because this method had been efficient in our lab for the extraction of As species from various complex matrices. This was however not the case for Se for which extraction efficiencies were systematically inferior to 20%. Enzymatic extraction was then considered owing to the fact that this process has been reported to allow the highest extraction efficiencies (Diaz Huerta, Fernandez Sanchez & Sanz-Medel, 2006; Montes-Bayon *et al.*, 2006). The choice of enzymes is dependent of the kind of matrix entrapping Se. As a large majority of Se is contained into proteins, proteolytic enzymes (usually non specific proteases) are systematically used, but lipase or driselase (mix of cellulases, hemicellulases and pectinases) can be added in case of fat or vegetal samples. Maximum potential of these enzymes is obtained at a temperature of about 37°C, at pH7.5, and with a device creating motion inside the sample in order to favour the action of enzymes. Such an environment can be set up by different means that have been tested in this work on different types of samples: shaking water bath, microwave (MW) with magnetic agitator, ultra sound (US) water bath or US probe. The characteristics and performances of these methods are synthesized in table 4-2.

Table 4-2: Advantages and disadvantages of Se enzymatic extraction methods.

Extraction methods	Advantages	Disadvantages
Shaking water bath	Good extraction efficiency. Good repeatability of replicates.	Time-consuming (3x16h) and risk of Se species degradation.
Microwave	Good extraction efficiency. Rapid (1 run of 24 samples = 30min).	Poor stability of MW low T° => high variability of replicates.
US water bath		Low extraction efficiency (10-20%).
US probe	Good extraction efficiency. Rapid (1 sample = 2min).	Risk of contamination with the probe.

According to table 4-2, shaking water bath, microwave and US probe-mediated methods all showed high extraction efficiencies, but the former two presented the disadvantages to be respectably time-consuming and of low repeatability. Thereby, the method that was retained was the combination of enzymes and US probe. This relatively recent and innovative method of extraction is nowadays one of the most often used to extract Se because it gives good extraction efficiencies in a very short time (Siwek *et al.*, 2006; Peachey, McCarthy & Goenaga-Infante, 2008; Vale *et al.*, 2008). It was showed that extraction of Se was three times more efficient with the conjunction of enzymes and US probe than with only enzymes or only the probe (Capelo *et al.*, 2004). No detailed mechanism has been proposed to explain the boosting effect on extraction generated by the combination of ultrasonication and enzymes, but it is likely that the cavitation produced by US enhances hydrolytic activity of enzymes. Our US extraction method is schematized in figure 4-11 in the context of a sample of Se-enriched food supplement. According to the type of sample, the nature of enzymes, the quantity of sample, the volume of Tris-enzyme solution and the duration of ultrasonication require adaptations.

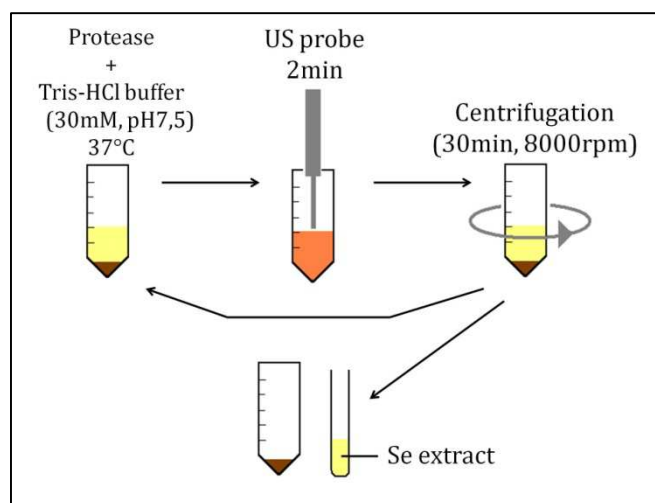


Figure 4-11: Methodological scheme of Se extraction by US probe in the context of a sample of FS. The sample is weighted in a Falcon tube and 10 ml of the buffered solution of Tris-enzymes is added. The sample is placed for 1h in a 37°C water bath. Ultrasonic probe (50 Hz) is applied for 2 min. The sample is centrifuged for 30 min at 8000 rpm (6650 x g). The supernatant is kept for analysis and a second extraction is applied to the pellet. Similarly, a third extraction will be further applied. The Se contents of the three supernatant are measured and summed up to obtain the value of extractable Se from FS.

To assess if there are differences in the efficiency of extraction among species, the existence of a CRM containing certified concentrations of Se species would have been ideal. Unfortunately, in case of Se, there is only one existing CRM of this type, *i.e.* a selenized yeast certified for its SeMet content (SELM-1, NRCC, Ottawa, CA). The analysis of this sample with our extraction method permitted to retrieve in average 92 ± 10 % of the certified SeMet content (N=6) (figure 4-12).

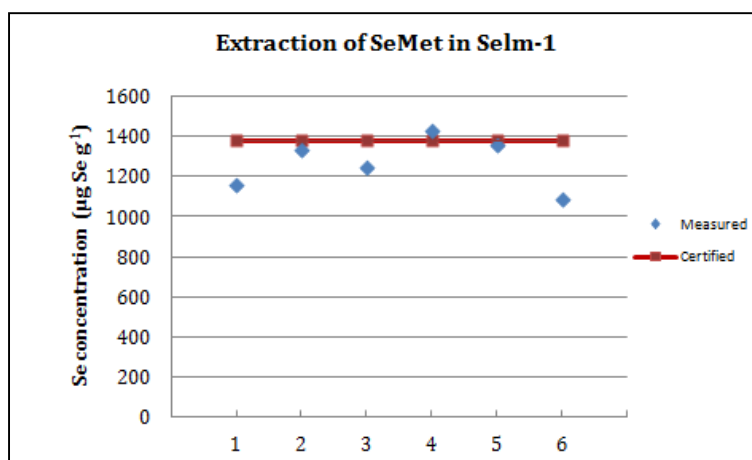


Figure 4-12: Recovery of SeMet in selenized yeast CRM Selm-1. Certified Se concentration as SeMet = 1379 $\mu\text{g Se g}^{-1}$.

For all Se species, the absence of speciation changes during the extraction process was assessed by the recovery of Se after spiking of Se species standards on SELM-1 (figure 4-13).

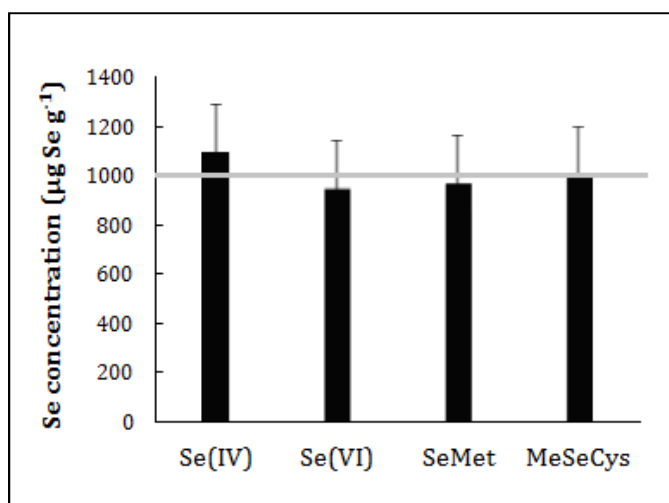


Figure 4-13: Post-extraction recovery of Se species standard spiked at 1000 ppm on Selm-1 ($n=3$, $N=3$). For each species, the entirety of the spike was retrieved after extraction, indicating that no change of speciation had occurred.

4.3.3. Calibration

An external calibration was realized in order to link the Se signal (counts per sec) to a concentration (ppb). An individual calibration must be achieved for each Se species since distinct species have different ionization efficiency resulting in different signal intensities. Stock solutions of standards (1000 ppm) were kept about 6 months at 4°C and appropriate dilutions were realized the day of analysis. We observed that ICP-MS responses for Se could vary with the nature of the solution containing the element. Therefore, standards were prepared in a liquid of composition as close as possible to the sample *i.e.* in HBSS for analysis of culture media and in enzyme solution for analysis of cells or FS extracts. Calibration curves systematically showed a coefficient of determination r^2 of at least 0.999 for each species.

4.3.4. Limit of detection-quantification

The limit values of detection (LOD) are 0.24 µg per liter of measured solution for total Se, and 1 µg l⁻¹ for Se species. These values were calculated respectively as 3 times the standard deviation of 20 blanco's, and as 3 times the baseline standard deviation. The limit values of quantification (LOQ) are equal to 2 times the LOD values. The LOD-LOQ values in original samples were obtained by multiplying the above values by the dilution factor of the sample.

4.4. Discussion and conclusion

Over the last twenty years, enormous progress has been made for the determination of Se species in various matrices. Yet, speciation of trace elements globally remains a delicate task, and it is still more the case for Se that presents certain characteristics making its determination more complex than simple routine analyses. Firstly, the fact that Se has a higher energy of ionization than most of other elements makes its ionization in ICP-MS plasma less efficient and less constant, leading to higher variability in measurements. Secondly, few standards of Se species are commercially available. This is also the case with CRM (especially for Se species) that could otherwise better validate analyses. Moreover, some Se species are highly reactive which makes them difficult to analyze. For example, SeCys, despite its biological importance is almost never quantified because its selenol group is so reactive that, on the one hand there is no commercial standard for SeCys, and on the other hand its

measurement necessitates a long and complex process to stabilize the molecule (Bierla *et al.*, 2008). Selenium is also known to be sensitive to oxidation, which prevents middle and long term storage of samples. Finally, Se measurement is highly dependent on the matrix it is entrapped in. For each kind of matrix, methods of extraction, of separation and of calibration, as well as the isotope to be measured must be thoroughly revised.

With the material and methods presented in this chapter, we could measure total Se in a wide range of food products (*cf.* chapter 5). One of the original aims at the beginning of this thesis was to also achieve Se speciation on these products in order to generate a large data table on Se species content of food. Attempts have been made to achieve Se speciation on fish, meat and vegetables. In better cases, SeMet could be identified (*cf.* figures 4-8 and 4-9) but, as just stated above, we realized that to obtain more complete information on Se species profiles in all those products, this would represent a colossal work of literature review, testing and adaptation of methods for each type of matrix. More realistically, this thesis therefore focused exclusively on Se speciation in cellular material (culture medium and cell pellet) and in three types of Se-enriched FS. The methods presented here permitted the identification of four Se species (selenate, selenite, SeMet, MeSeCys) in those matrices, and constitute a good basis for further development of speciation methods in more complex food matrices.

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Chapter 5

**Dietary intake of selenium by the
general adult population in
Belgium**

Chapter 5 - Dietary intake of selenium by the general adult population in Belgium

Foreword

Among the factors determining Se status (*i.e.* Se body content and activity of Se-dependent compounds), the most direct is the dose of Se intake. Selenium is almost exclusively taken through the food, and, as was previously mentioned, Se content of food products is influenced by their geographical location. We also previously pointed out that European soils are generally naturally poor in Se compared to other places in the world. On this basis, we expect the level of Se intake in Belgium to be rather low. On the other hand, a still increasing fraction of our food is nowadays imported and thus, Se level of local soils does not reflect Se food content with as much accuracy as in the past.

Thereby, it seems important to begin this thesis by clarifying the situation concerning the level of Se intake of Belgian people. This will enable us to consider the need of a Se enrichment of the diet. In addition, the identification of the major food sources of Se will permit us to further characterize the impact of these products on Se status with respect to their speciation profile (*cf.* chapter 6). In this study, we addressed exclusively the case of the adult population (>15 years) but a similar work could be done for children. In contrast with adults who are expected to suffer from Se deficiency, children (1-14 years) have recently been pointed out as particularly exposed to the risk of Se excess in line with an exposure to Se that decreases with the age (EFSA, 2010).

This chapter arises from an article that was accepted for publication in *Food Additives and Contaminants*, entitled “Dietary intake of selenium by the general adult population in Belgium”, and written by Nadia Waegeneers¹, Céline Thiry^{1,2}, Ludwig De Temmerman¹ and Ann Ruttens¹.

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Abstract

The total selenium content of about 800 food products purchased in Belgium was determined and combined with food records to determine the nutritional Se level of Belgian people. The mean dietary intake was calculated to be 60 $\mu\text{g Se day}^{-1}$, which is at the lower end of the range recommended by the Superior Health Council in Belgium (60-70 $\mu\text{g day}^{-1}$), but adequate according to the value of 55 $\mu\text{g day}^{-1}$ recommended by the Scientific Committee on Food of the European Commission. The largest Se concentrations ($> 1 \mu\text{g g}^{-1}$) were found in Brazil nuts and offal, of which the consumption is limited. The major sources of Se intake are meat and meat products (32%), fish and shellfish (20%), and potatoes and pasta (13%).

Keywords

Selenium, dietary intake, meat, fish, wheat, pasta

5.1. Introduction

Selenium was recognized as an essential oligo-element only four decades ago (Schwarz & Foltz, 1957; Rotruck *et al.*, 1973). The essentiality of Se is related to its presence in the active center of several selenoproteins that are involved for the most part in prevention of oxidative stress (Gromer *et al.*, 2005), in thyroidal function and in Se transport. There seems to be no real consensus on the recommendations for dietary intake of Se. In some regions, the recommended dietary allowance for Se has been determined on the basis of the maximization of selenoenzymes activity, and was fixed for both adult men and women at 55 $\mu\text{g d}^{-1}$ in the USA (Monsen, 2000) and by the European Commission (SCF, 2000), and within 60 and 75 $\mu\text{g d}^{-1}$ in the UK and in Belgium (Rayman, 2000; SHC, 2009). On the other hand, a WHO/FAO/IAEA expert group recommended an intake level of only 40 $\mu\text{g d}^{-1}$ (men) and 30 $\mu\text{g d}^{-1}$ (women) on the basis that only two-thirds of the full expression of selenoenzymes activity was required (Rayman, 2000). The actual Se intake of people varies importantly, notably depending on their geographical location. This phenomenon is explained on the one hand by the local food habits of the populations, and on the other hand by the fact that Se content of food is conditioned by Se content of local soils that is highly heterogeneous around the

world. Thereby, reflecting Se concentrations in soils, most people reach the recommended value for Se intake in the USA while, in Europe, people are generally considered to be slightly Se-deficient (Rayman, 2000). In this study, we considered the particular case of Se dietary intake in Belgium, taking into account the two above-mentioned parameters, *i.e.* food habits of Belgian people and Se content of their food. This study aims at establishing the general Se intake of Belgian adult people, as well as the degree of contribution of particular products to the Se intake. Such a study is of particular relevance in the case of Se since this element has a narrow margin between deficiency and toxicity.

5.2. Materials and methods

5.2.1. Samples and sample preparation

About 900 samples of food products (meat, fish, potatoes, vegetables, drinks, etc.) were bought in local stores, markets and supermarkets, or obtained from local sources. All samples were classified into nine food groups (table 5-1) corresponding approximately to the groups used in the Belgian food consumption survey (De Vriese *et al.*, 2005) that served for the estimation of food products intake. No strict relation was applied between the consumption rate of a product and the number of samples collected but, within a food group, products with higher consumption rate were generally collected in higher number. Samples were cleaned or treated according to normal kitchen practices whenever relevant, *e.g.* vegetables were washed, and potatoes, carrots and oranges were peeled. Bread, vegetable and fruit samples were dried in an oven at 70-90°C before homogenization. Homogenization was performed with an analytical grinder (IKA A11; for dry products such as pasta and rice), a kitchen mill (Fritel CN 9170; for fresh samples such as fish and meat), or a hammer mill (Culatti AG; for dried bread, vegetables and fruits). Dry products were stored in polyethylene pots at room temperature after homogenization while fresh samples were stored at -20°C.

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Table 5-1: Food groups, subgroups and products used to calculate Se intake by the general adult Belgian population.

Food group	Subgroup	Product
<i>Bread, toast (rusk), breakfast cereals (mean usual intake: 133 g d⁻¹)</i>		
	Bread (0.96)	
	Breakfast cereals (0.04)	
<i>Potatoes and pasta (mean usual intake: 146 g d⁻¹)</i>		
	Potatoes and potato products (0.67)	
	Pasta and rice (0.33)	Pasta (0.76), rice (0.24)
<i>Vegetables (excl. –soups & –juices) (mean usual intake: 138 g d⁻¹)</i>		
	Vegetables	Tomato (0.20), carrot (0.19), Belgian endive (0.13), onion (0.13), lettuce (0.08), leek (0.06), paprika (0.05), mushrooms (0.05), cucumber and courgette (0.04), cauliflower (0.04), broccoli (0.03)
<i>Fruit (excl. –juices & olives) (mean usual intake: 118 g d⁻¹)</i>		
	Fruit	Apple (0.30), orange (0.27), banana (0.23), mandarin (0.11), pear (0.09)
<i>Meat and meat products (mean usual intake: 61 g d⁻¹)</i>		
	Meat of pork, beef and lamb (0.54)	Meat of pork (0.55), beef (0.40), lamb (0.04), beef liver (0.01)
	Meat products (0.29)	
	Poultry meat (0.17)	
<i>Fish and shellfish (mean usual intake: 23.9 g d⁻¹)</i>		
	Fish (0.77)	Cod (0.25), salmon (0.22), tuna (0.21), herring (0.09), mackerel (0.08), plaice (0.08), trout (0.035), pollack (0.035)
	Shellfish (0.23)	Mussels (0.30), shrimp (0.28), scampi (0.20), langoustine (0.06), crayfish (0.06), crab (0.05), scallop (0.05)
<i>Eggs (mean usual intake: 10 g d⁻¹)</i>		
	Eggs	
<i>Milk products and Ca-enriched soy products</i>		
	Milk and dairy drinks (mean usual intake: 90.6 g d ⁻¹)	
	Yoghurt and pudding (mean usual intake: 63 g d ⁻¹)	
	Cheese (mean usual intake: 30 g d ⁻¹)	
<i>Drinks (mean usual intake: 1508 ml d⁻¹)</i>		
	Mineral water (0.44)	
	Coffee (0.24)	
	Soft drinks (0.14)	
	Beer (0.08)	
	Thee (0.05)	
	Wine (0.04)	

The mean usual intake is indicated per food group or subgroup. Weight factors used to calculate weighted means Se concentrations per food group or per subgroup are indicated within brackets.

5.2.2. Selenium determination

Selenium analyses were all performed in duplicate. Each batch of samples was accompanied by an appropriate certified reference material (CRM) in order to validate Se quantification. The reference samples used were NIST-1549 (milk powder) (NIST, Gaithersburg, MD, USA), NIST-1568a (rice flour), BCR-185R (bovine liver) (IRMM, Geel, Belgium), IAEA-407 (fish tissue) (IAEA, Vienna, Austria), and DORM-3 (fish proteins) (NRCC, Ottawa, Canada). Amounts of 250 mg of homogenized food samples and 150 mg of CRM were digested by microwave (MarsXpress, CEM, NC, USA) in PTFE tubes in the presence of 8 ml of a mixture of extra pure HNO_3 - bi-distilled H_2O (1:1, v/v). Digested samples were appropriately diluted in bi-distilled water before ICP-MS analysis (Varian 820-MS, Mulgrave, AU). Isotope 77 of Se was monitored, with 90 ml min⁻¹ of H_2 as reaction gas to remove interferences on this mass. The limit of quantification (LOQ) was calculated as six times the standard deviation of 20 procedure blanks and was equal to 0.12 $\mu\text{g Se}$ per liter of measured solution. Taking into account the respective dilution factors and moisture percentages of the samples, the LOQ value corresponded to 0.04 $\mu\text{g Se g}^{-1}$ in fish, meat and eggs, to 0.02 $\mu\text{g Se g}^{-1}$ in milk products, to 0.005 $\mu\text{g Se g}^{-1}$ for fruits, cereals and potatoes, and to 0.001 $\mu\text{g Se g}^{-1}$ for vegetables. All results were expressed in $\mu\text{g Se g}^{-1}$ on a whole weight basis.

5.2.3. Intake calculation

Food consumption data were collected from the Belgian national food consumption survey that was performed in 2004 (IPH, 2004). In this survey, information on food intake was collected from persons of 15 years and older by using two non-consecutive 24h recalls in combination with a self-administered food frequency questionnaire, in order to obtain “usual intake” values (De Vriese *et al.*, 2005). This survey is the first and unique of this type realized in Belgium, which justifies the use of relatively old consumption data (2004) in the present study. To be compatible with these food consumption data, all food products collected in the frame of this work were divided into nine food groups corresponding approximately to those of the food consumption survey (*e.g.* “fish and shellfish”; table 5-1). Several of these food groups were divided into subgroups (*e.g.* “fish”, “shellfish”) whenever consumption data were available

for these subgroups. Some subgroups were further divided into products (*e.g.* cod, salmon, etc.).

- The mean dietary intake (I ; $\mu\text{g d}^{-1}$) of Se by the general Belgian population (≥ 15 years) was calculated from the mean usual intake of the food groups ($\text{intake}_{\text{food group } i}$; g d^{-1}) and the average Se concentration per food group ($[\text{Se}]_{\text{food group } i}$; $\mu\text{g g}^{-1}$) as:

$$I = \sum_i \text{intake}_{\text{food group } i} \cdot [\text{Se}]_{\text{food group } i}$$

- In order to calculate the average Se concentration per food group, a weighted mean was calculated from the Se concentrations of the subgroups:

$$[\text{Se}]_{\text{food group}} = \sum_i [\text{Se}]_{\text{subgroup } i} \cdot F_w(\text{subgroup } i)$$

The weight factor (F_w) for a subgroup i was calculated based on the relative usual intake of the subgroup within its food group as:

$$F_w(\text{subgroup } i) = \frac{\text{usual intake } i}{\sum_i \text{usual intake within the subgroup}} \cdot 100\%$$

The usual intake of a food group does not necessarily equal the sum of the usual intakes of its subgroups.

- In order to calculate the average Se concentration of a subgroup consisting of different products, a weighted mean was calculated from the Se concentrations of the products:

$$[\text{Se}]_{\text{subgroup } i} = \sum_j [\text{Se}]_{\text{product } j} \cdot F_w(\text{product } j)$$

Since usual intake values were not available per product, the relative importance of the product within the subgroup was estimated from product sales data or other consumption data collected between years 2000 and 2010 (SEAFOODplus, 2010; VLAM, 2010). The weight factor (F_w) for a subgroup i was calculated as:

$$F_w(\text{product } j) = \frac{\text{sale or consumption (per capita) of product } j}{\sum_j \text{sale or consumption (per capita) of products within the subgroup}} \cdot 100\%$$

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Table 5-1 lists mean usual intake data and weight factors for food groups, subgroups and products.

For the food group “potatoes and pasta”, usual intake values for rice and pasta were converted to potato-equivalent values in the food consumption survey (20 g pasta was assumed equivalent to 70 g potatoes). This largely increased the usual intake value of the “potatoes and pasta” food group to 306 g d⁻¹ while the usual intake values for the “potato and potato products” subgroup was 98 g d⁻¹ and that for the “pasta and rice” subgroup was 48 g d⁻¹. In the present study, the Se usual intake from the food group “potatoes and pasta” was calculated as the sum of the Se intake of the subgroups “potato and potato products” and “pasta and rice”.

For the food group “milk products and Ca-enriched soy products”, the mean usual intake value was not provided for the entirety of the group, and the mean usual intake values for individual subgroups were therefore considered.

Selenium intake of high-level consumers of a certain subgroup was estimated by multiplying the 97.5th percentile intake for consumers of that subgroup with its respective Se concentration, and adding this value to the population mean Se intake from the rest of the diet (Tennant, Bequet & Jans, 2009).

5.2.4. Handling of data below the LOQ

Measurement results below the LOQ are associated with a large relative standard deviation between duplicate samples and with large measurement uncertainties. Nevertheless, even under the LOQ, the actual measured values of samples were used in the intake calculations as these values represent the best estimation of the true Se concentration in the samples. Using zero or the actual LOQ value for results below the LOQ would respectively lead to an under- or overestimation of the Se intake.

5.3. Results and discussion

5.3.1. Quality control

The mean of measured values of NIST-1549, NIST-1568a and BCR-185R did not differ significantly from their certified values (table 5-2). In contrast, Se

concentration measured in IAEA-407 (fish tissue) was significantly higher than the certified value. Similarly, Se concentration measured in DORM-3 (fish proteins) was elevated compared to its informative Se content, although this value is not certified. These slight overestimations of Se concentrations in fish suggest the presence of some interference that is not totally eliminated by the reaction gas. To prevent an overestimation of the dietary Se intake, the results of all fish measurements were corrected based on the mean recovery of Se in IAEA-407 (*i.e.* measurement result - 18%).

Table 5-2: Certified and measured ($n=2$) values, and their extended uncertainties, for Se in CRM's.

Reference material	Certified values	Measured values
NIST-1549 (milk powder)	0.11 ± 0.01	0.12 ± 0.03
NIST-1568a (rice flour)	0.38 ± 0.04	0.36 ± 0.01
BCR-185R (bovine liver)	1.68 ± 0.14	1.68 ± 0.06
IAEA-407 (fish tissue)	2.83 ± 0.13	3.28 ± 0.13
DORM-3 (fish proteins)	$(3.3)^{\$}$	4.00 ± 0.14

§ Informative value.

5.3.2. Selenium concentrations in different food products

The mean and range of Se concentrations measured in selected food items are shown in table 5-3. The largest Se concentrations ($> 1 \mu\text{g g}^{-1}$) were found in nuts, offal, mussel and crab. Selenium concentration in Brazil nuts ($2.5 \mu\text{g g}^{-1}$) was the highest measured. In the others nuts, Se concentrations accounted for $0.17\text{-}0.82 \mu\text{g g}^{-1}$ in peanuts and pistachios, and were inferior to $0.1 \mu\text{g g}^{-1}$ in cashew nuts, almonds and walnuts. In fish, mean Se concentrations varied between animal species from 0.16 to $0.82 \mu\text{g g}^{-1}$. The largest concentrations were found in tuna fish and mackerel, while the lowest concentrations were found in trout and salmon. Similar concentration ranges were found in crustaceans, with largest concentrations found in crab and langoustine, and lowest concentrations found in scallop and crayfish. One out of 15 mussel samples had a particularly high Se concentration of $2.3 \mu\text{g g}^{-1}$, which led to a mean concentration of $1.0 \mu\text{g g}^{-1}$ in mussels while the median was only $0.38 \mu\text{g g}^{-1}$. The Se concentrations found in fish and crustaceans correspond with those found in Denmark (Larsen *et al.*, 2002), the UK (Ysart *et al.*, 1999; Rose *et al.*, 2010), Ireland (Murphy & Cashman, 2001) and Slovenia (Smrkolj *et al.*, 2005).

However, in a French study (Leblanc *et al.*, 2005), Se contents found in fish ($0.17 \mu\text{g g}^{-1}$) and shellfish (below the detection limit of $0.022 \mu\text{g g}^{-1}$) were much lower than the values found in the present study.

According to table 5-3, several food products had Se concentrations around $0.20 \mu\text{g g}^{-1}$: eggs, poultry meat, cheese, mushrooms and pasta. In Denmark and the UK, similar concentrations were found in eggs (Ysart *et al.*, 1999; Larsen *et al.*, 2002; Rose *et al.*, 2010) but in Greece and France, lower concentrations were measured (0.04 - $0.17 \mu\text{g g}^{-1}$; Leblanc *et al.*, 2005; Pappa, Pappas & Surai, 2006). In several countries, the Se levels in poultry meat were lower than those measured in the present study, ranging from $0.07 \mu\text{g g}^{-1}$ in Spain to $0.184 \mu\text{g g}^{-1}$ in France (Díaz-Alarcón *et al.*, 1996; Murphy & Cashman, 2001; Larsen *et al.*, 2002; Leblanc *et al.*, 2005; Smrkolj *et al.*, 2005; Pappa, Pappas & Surai, 2006; Rose *et al.*, 2006). Also Se levels in mushrooms were higher than in Denmark and Ireland (0.058 and $0.033 \mu\text{g g}^{-1}$ respectively; Murphy & Cashman, 2001; Larsen *et al.*, 2002), but they were in accordance with the range of values reported in a review compiling results from several European studies (0.005 to $0.251 \mu\text{g g}^{-1}$, recalculation from results for dry weight by applying a dry matter content of 6.6%).

Except in offal, Se concentration in beef and pork meat was on average $0.15 \mu\text{g g}^{-1}$. Especially the levels found in beef were higher than in other countries such as the UK ($0.076 \mu\text{g g}^{-1}$; Ysart *et al.*, 1999), Ireland ($0.08 \mu\text{g g}^{-1}$; Murphy & Cashman, 2001) and Slovenia ($0.035 \mu\text{g g}^{-1}$; Smrkolj *et al.*, 2005). As all beef samples were from Belgian origin, the higher Se concentrations that were found might be due to Se supplementation of Belgian cattle (oral communication from Pr B. Goddeeris, faculty of bioscience engineering, KUL).

Finally, in other food products, Se concentrations were rather low: about $0.05 \mu\text{g g}^{-1}$ in bread, breakfast cereals and rice, less than $0.01 \mu\text{g g}^{-1}$ in most vegetables (with differences among species of vegetables) and less than $0.003 \mu\text{g g}^{-1}$ in beverages.

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Table 5-3 (to be continued): Se concentrations [$\mu\text{g g}^{-1}$ whole weight] in selected food products.

Food group	Subgroup	Products	n	Mean	Range
<i>Bread, toast (rusk), breakfast cereals</i>					
		Bread	22	0.05	0.01 - 0.12
		Breakfast cereals	5	0.06	0.03 - 0.09
<i>Potatoes and pasta</i>					
		Potatoes and potato products	120	0.006	<0.005 - 0.15
		Pasta and rice			
		Pasta	11	0.17	0.02 - 0.43
		Rice	28	0.06	0.02 - 0.23
<i>Vegetables (excl. -soups & -juices)</i>					
		Vegetables			
		Tomato	5	0.003	<0.001 - 0.004
		Carrot	152	0.003	<0.001 - 0.015
		Belgian endive	5	0.003	<0.001 - 0.005
		Onion	5	0.005	0.002 - 0.008
		Lettuce	4	0.006	0.004 - 0.012
		Leek	25	0.008	0.003 - 0.039
		Paprika	1	0.001	
		Mushrooms	3	0.18	0.11 - 0.33
		Cucumber and courgette	5	0.002	<0.001 - 0.002
		Cauliflower	3	0.008	0.003 - 0.019
		Broccoli	5	0.017	0.007 - 0.027
<i>Fruit (excl. -juices & olives)</i>					
		Fruit	6	<0.005	<0.005
		Nuts	9	0.50	<0.04 - 2.5
<i>Meat and meat products</i>					
		Meat of pork, beef and lamb			
		Meat of pork	10	0.15	0.12 - 0.20
		Meat of beef	21	0.14	0.04 - 0.39
		Meat of lamb	1	0.06	
		Beef and chicken liver	11	0.61	0.17 - 1.7
		Beef kidney	3	1.9	1.7 - 2.1
		Meat products	14	0.13	0.10 - 0.25
		Poultry meat	5	0.21	0.16 - 0.29

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Table 5-3:

Food group	Subgroup	Products	n	Mean	Range
<i>Fish and shellfish</i>					
	Fish				
		Cod	20	0.36	0.21 - 0.55
		Salmon	6	0.23	0.21 - 0.27
		Tuna	4	0.82	0.58 - 1.05
		Herring	3	0.27	0.19 - 0.32
		Mackerel	3	0.55	0.47 - 0.70
		Plaice	7	0.47	0.24 - 0.63
		Trout	3	0.16	0.13 - 0.20
		Pollack	15	0.38	0.28 - 0.46
	Shellfish				
		Mussels	15	1.0	0.32 - 2.33
		Shrimp	11	0.59	0.40 - 0.85
		Scampi	3	0.24	0.16 - 0.34
		Langoustine	3	0.80	0.62 - 0.94
		Crayfish	3	0.23	0.19 - 0.28
		Crab	3	0.90	0.31 - 1.30
		Scallop	3	0.17	0.09 - 0.25
<i>Eggs</i>					
	Eggs		15	0.23	0.21 - 0.27
<i>Milk products and Ca-enriched soy products</i>					
	Milk and dairy drinks		13	0.03	0.01 - 0.05
	Yoghurt and pudding		5	<0.02	<0.02-0.03
	Cheese		7	0.18	0.09 - 0.27
<i>Drinks</i>					
	Mineral water		7	0.001	0.0006 - 0.0016
	Coffee		6	0.002	0.0019 - 0.0026
	Soft drinks		3	0.003	0.0013 - 0.0032
	Beer		6	0.002	0.001 - 0.003
	Thee		3	0.002	0.0018 - 0.0021
	Wine		8	0.003	0.001 - 0.009

Sign < indicates values under LOQ.

5.3.3. Dietary intake of selenium

Based on the data in table 5-3 and on the weight factors in table 5-1, the mean dietary intake of Se by the general adult population in Belgium has been calculated to be 60 µg d⁻¹. This is within the range of 28 to 61 µg d⁻¹ that was previously measured in a duplicate meal study by Robberecht *et al.* (1994). This value is at the lower end of the range that is recommended by the Superior Health Council in Belgium (60-70 µg d⁻¹) (SHC, 2009), but adequate according to the value of 55 µg d⁻¹ that is recommended by the Scientific Committee on Food of the European Commission (SCF, 2000) and by the US Food and

Nutrition Board (in SFC, 2000). The mean dietary Se intake level in Belgium is slightly higher than that in the UK (48-58 $\mu\text{g d}^{-1}$, Rose *et al.*, 2010) and Denmark (48 $\mu\text{g d}^{-1}$, Larsen *et al.*, 2002), and but lower than the Se intake of 18-year-old Dutch males (72 $\mu\text{g d}^{-1}$, Van Dokkum *et al.*, 1989) as measured in the 1980's. The Belgian dietary Se intake corresponds to the intake measured in France in the duplicate meal study of Noël, Leblanc & Guerin (2003), *i.e.* 66 $\mu\text{g d}^{-1}$, and to the notice of the National Food Agency (Afssa, 2009), *i.e.* 53.7 $\mu\text{g d}^{-1}$, but is less comparable to the result of the total diet study of Leblanc *et al.* (2005), *i.e.* 42 $\mu\text{g d}^{-1}$.

According to our calculations, almost 32% of the daily Se intake in Belgium comes from the food group “meat and meat products” that are thereby the major sources of dietary Se. Meat (including meat products and offal) has been reported to be the primary source of Se in several other European countries including the UK (27%), Denmark (32%) and France (22%) (Rose *et al.*, 2010; Larsen *et al.*, 2002; Leblanc *et al.*, 2005). The second most important source of Se identified in the present study is the food group “fish and shellfish” that counts for almost 20% of the total Se intake. The third most important source is the food group “potatoes and pasta” (13%). The importance of this food group is not due to the high consumption of potatoes, like it is the case for some other trace elements such as Cd (Vromman *et al.*, 2010), but rather to the relatively high Se concentration in pasta (0.2 $\mu\text{g g}^{-1}$). Pasta contains much more Se than other wheat-based products, *i.e.* 0.05 $\mu\text{g g}^{-1}$ in bread and 0.06 g g^{-1} in breakfast cereals. A difference between all these products is that the wheat used in pasta is in general *durum* wheat (*Triticum turgidum* var. *durum*) while that used in bread is common wheat (*Triticum aestivum* var. *aestivum*). Therefore, one hypothesis to explain the difference in Se concentrations between bread and pasta might be a genetically determined difference in Se accumulation between both types of wheat. However, in a diversity screen trial where these types of wheat were grown at the same location, Zhao *et al.* (2009) did not find any significant difference in the Se concentrations between both types of wheat. *Durum* wheat is not commonly grown in Belgium, in contrast to common wheat. The difference in the origin of the wheat might thus be a second hypothesis to explain the difference in Se concentrations in the end product. Indeed, the Se concentrations in Belgian agricultural soils are low: 95% of the Se concentrations in wheat-grown soils are between 0.22 and 0.57 $\mu\text{g g}^{-1}$ ($n = 184$; unpublished data). This results in a mean Se concentration in common wheat of

0.048 $\mu\text{g g}^{-1}$ ($n = 184$; unpublished data). In Italy, an important producer of pasta, the import of wheat is significant (Spadoni *et al.*, 2007). Therefore, a widespread use of wheat imported from Se-rich areas in the industrial production of pasta could explain a high level of Se in commercial pasta (0.08-0.23 $\mu\text{g g}^{-1}$) (Cubadda *et al.*, 2009).

In France, drinks were reported to be an equally important source of Se as meat (daily Se intake of 9 $\mu\text{g d}^{-1}$ from drinking waters, alcoholic and non-alcoholic beverages, coffee and other hot beverages). However, in that study, Se concentrations in all beverages were below the detection limit of 0.022 $\mu\text{g g}^{-1}$ and were systematically set equal to a concentration of 0.011 $\mu\text{g g}^{-1}$ (50% of the detection limit). This may lead to a large overestimation of the actual contribution of drinks to the total Se intake as Se concentrations in drinks measured in the present study were below 0.003 $\mu\text{g g}^{-1}$.

Aside from the general population, people with particular diet habits might be at risk regarding their Se intake. On the one hand, high-level consumers of meat and/or fish and shellfish might have an excessive level of Se intake. This was however estimated at 75 $\mu\text{g d}^{-1}$, which is just above the recommended dietary allowance range according to the Superior Health Council in Belgium but within the recommended range in the UK. Similarly, high-level consumers of pasta have a mean dietary Se intake of 71 $\mu\text{g d}^{-1}$. Unfortunately, the food consumption survey did not consider the intake of offal and Brazil nuts which have been reported to be the food products with the highest concentrations in Se. High level consumers of those products could therefore be at considerable risk of excessive Se intake. It has notably been reported that the daily consumption of two Brazil nuts corresponds to an intake of 100 μg of Se (Thompson *et al.*, 2008). On the other hand, since meat and fish are the main sources of Se in Belgium, vegetarian people could be at risk of Se deficiency. Unfortunately it was not possible to make an estimation of the Se intake by vegetarians because typical vegetarian food has not been sampled and was not taken into account in the food consumption survey. However, in an anterior study (Roekens, Robberecht & Deelstra, 1986), the daily Se intake of Belgian vegetarians had been estimated to be as low as 13 $\mu\text{g d}^{-1}$, based on duplicate meals and food consumption records. But this study might be not relevant due to its oldness and the fact that nowadays vegetarian people are becoming more and more aware of the need of dietary supplementations for certain nutrients (Larsson & Johansson, 2005). Since the Se intake of Belgian people was

calculated to be at the lower limit of the recommended values, the risk of Se deficiency may also concern individuals with higher requirements in Se, such as pregnant and breast-feeding women (SHC, 2009), or people undergoing important level of oxidative stress (*e.g.* smokers, top-level athletes, people exposed to pollution or toxics, people that are deficient in antioxidant compounds) (Thompson, 2004; Rayman, 2008).

5.4. Conclusion

In this study, the Se intake of the general adult population in Belgium was calculated to be 60 $\mu\text{g d}^{-1}$. This value is at the lower end of the range that is recommended by the Superior Health Council in Belgium (60-70 $\mu\text{g d}^{-1}$), but adequate to the value of 55 $\mu\text{g d}^{-1}$ that is recommended by the Scientific Committee on Food of the European Commission and by the US Food and Nutrition Board. Based on these values, the majority of the Belgian population seems to be protected against acute Se deficiency or toxicity. In contrast, people with particular food habits or Se needs could be at higher risk.

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Chapter 6

Influence of selenium speciation on its bioavailability

Chapter 6 - Influence of selenium speciation on its bioavailability

Foreword

In chapter 5, we have stated the Se intake of the Belgian population at 60 μg day⁻¹. This information is very interesting since it indicates that the Se intake of Belgian people is rather low, but it is not sufficient to reflect people's status in Se. Indeed, only a fraction of the ingested Se will have an impact on the organism. This fraction is mediated by successive steps of bioaccessibility, bioavailability and bioactivity. A major determinant of the importance of Se released at each of these steps is the chemical form under which Se is present in food, and each type of food has its own profile in Se species. Thereby, if for a similar dose of Se we consider for example the consumption of a SeMet-rich food like wheat or the consumption of fish that has a varied Se profile, it is probable that the Se status of people having eaten wheat will be higher than that of people having eaten fish. The core of chapter 6 consists in the investigation of the species-dependence of Se bioavailability by means of a survey of the literature. This was realized through the construction of several tables containing published data about the degree of bioavailability of Se under different forms and from various types of food products. Note that, in this chapter, bioavailability could not be precisely defined due to the variety of definitions considered in the studies of interest. Therefore, depending on the study, Se bioavailability could be associated with various parameters like Se content in blood, Se concentration in tissues, Se capacity of GPx induction, Se absorption, Se retention,...

Chapter 6 is an update of an article that was published in *Food Chemistry* in 2012 and that was entitled "Current knowledge in species-related bioavailability of selenium in food" (Céline Thiry^{1,2}, Ann Ruttens¹, Luc Pussemier¹ and Yves-Jacques Schneider², 2012).

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Abstract

Selenium is an essential trace element that has raised interest because of its antioxidant and anticancer properties. Selenium becomes however toxic at high doses. The beneficial or toxic effect of Se is not only dose-dependent, but it also relates to the chemical form (*i.e.* speciation) of the element. In this review, we summarise a number of recently published data concerning both Se speciation and Se relative bioavailability in various foodstuffs. In parallel, the bioavailability of Se is put in relation with its species-dependent metabolism in humans. Thereby, we aim to better understand how distinct Se species present in commonly consumed food can impact on health. It is strongly suggested on the basis of higher retention and lower toxicity, that organic species of Se (especially SeMet, the major species in food) are more beneficial than inorganic species in the context of a balanced diet. Further research is however desirable concerning the characterization of still unidentified Se species in food, and the determination of their health effects.

Keywords

Selenium, food, speciation, bioavailability, metabolism

6.1. Introduction

Selenium is an essential oligo-element that exerts its action essentially through antioxidant properties of selenoenzymes such as glutathione peroxidases. On the basis of the maximum activity of these enzymes, the recommended value of Se intake for adult individuals has been fixed at 55 $\mu\text{g day}^{-1}$ by the US Institute of Medicine (Goldhaber, 2003). In parallel, several reference values for Se optimal intake and Se diet enrichment have been proposed (*cf.* chapter 2, section 2.4.1. "Guideline values for Se intake"). But none of these values takes into account the fact that Se is present in food under different chemical forms, organic or inorganic, and that these forms do not exert the same effect on the organism (Meltzer *et al.*, 1990; Amoako, Uden & Tyson, 2009). For example, different Se compounds will result in different degrees of Se retention in the body, of Se concentrations in blood and organs, of selenoenzymes activity, and of toxicity (Whanger & Butler, 1988; Wang & Xu, 2008; Rider *et al.*, 2010). From previous publications, it appears that organic

forms are more retained (Zeng, Botnen & Johnson, 2008; Rider, 2009) and less toxic than inorganic ones (Ammar & Couri, 1981; Tiwary, *et al.*, 2006). Among organic forms, some have been reported to have additional specific properties like the capacity of integration to proteins for SeMet (Schrauzer, 2000) or a probable anti-cancer action for methylselenol (Ip, Thompson *et al.*, 2000). These differences among species lead to an 'uncorrelation' between Se intake and Se health effects. This phenomenon can be explained, at least partially, by the fact that different species are not equally bioavailable for the organism (Meltzer *et al.*, 1990). The species-dependence of Se bioavailability is therefore a crucial determinant of the Se status of persons. In this context, the present paper provides a summary of existing information about Se speciation and Se bioavailability in food, and aims at investigating the relations that can exist between the two of them.

6.2. Selenium speciation

In general, speciation refers to the physical and chemical forms (*i.e.* species) under which an element can exist. Despite the analytical advances that have been made in this field during the last decades, there are still a relatively limited number of studies dealing with the quantification of Se species in food. One reason is because of the difficulty to measure the low concentrations of Se species in food that are often in the range of the ng g^{-1} . Moreover, most of existing studies focus exclusively on the few most abundant and easily measurable species, even though dozens of different selenocompounds can occur in biological tissues (for a list of selenocompounds, see, for example, Dumont, Vanhaecke & Cornelis, 2006). Most published data concern the inorganic forms selenite and selenate, and some organic species such as selenomethionine (SeMet), selenocysteine (SeCys) and methylated selenocysteine (MeSeCys).

A global view of the total Se content of various food products has been provided in chapter 5. This gives a primary idea about the global Se richness or poorness of commonly consumed food products. As for the content of Se species in food, an important compilation of published results has been realised in 2008 (Rayman, 2008) and is presented in the appendix of the present thesis. Table 6-1 is a complement and an update of this compilation with the most relevant data from recent literature. As far as possible, attention was paid to

select studies in which the percentage of Se extraction was mentioned or could be calculated, since it could influence the values reported for Se species quantification.

According to table 6-1, inorganic forms of Se were rarely present in food. In contrast, SeMet was identified in almost all types of samples, and usually as a considerable fraction of the total Se content. Concentrations of SeMet varied according to the category of food product, but also within a category in function of the animal or the plant species. Meat products from table 6-1 contained almost the entirety of their Se content under organic form, either as SeMet or as SeCys (Bierla *et al.*, 2008). The fractions of SeCys were the highest in the liver and in kidneys, probably because those are areas with high rate of SeCys-containing selenoenzymes synthesis. In fish, trimethylselenonium ion (TMSe⁺) - an intermediate in the Se excretion pathway - was detected in addition to SeMet (Quijano *et al.*, 2000), but a large fraction of Se remained unidentified. In a previous study unmentioned here, Cappon and Smith (1981) reported the presence of selenate in various species of fish at concentrations ranging from 14 to 36 % of the total Se content. In cereals, almost the totality of the Se content was under SeMet form, as well in cereals cultivated naturally as in those that received a Se enrichment. In other types of plants, most of the data on Se speciation arose from enrichment experiences that were conducted on Se-accumulator varieties. It must therefore be kept in mind that the obtained Se species profiles did not necessarily reflect the natural Se status of those plants. In table 6-1, *Allium* plants revealed important concentrations of MeSeCys and γ -glutamyl-MeSeCys. This illustrates the particular ability of these plants, when grown on seleniferous soils, to accumulate huge quantities of Se under forms that do not generate Se toxicity (Wrobel *et al.*, 2004; Ogra *et al.*, 2005; Diaz Huerta, Fernandez Sanchez & Sanz-Medel, 2006). Inorganic Se was also detected in these plants but in small quantities. This should be some rest of inorganic Se from fertilizers that has not been fully metabolized by the plant organism. Among the rare plant products that are naturally rich in Se, Brazil nuts have been reported to have a majority of their Se content under the form of SeMet (Thompson, *et al.*, 2008), and, in sesame seeds, SeMet accounted for 80% of total Se (Kapolna *et al.*, 2007). No data were found for Se species in fruit and vegetables from non-accumulator plants. This is probably because the very low Se concentrations in those products make speciation analyses almost

impossible. Finally, Se-enriched yeast, that is widely consumed as a food supplement, also contained high amounts of SeMet.

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Table 6-1 (to be continued): Recent published data about concentrations of Se species in food products.

Food	Origin	Sample preparation	Extraction method	Quantification method	Typical concentrations and values						
					Total Se	Extracted Se	Extraction efficiency	Se species in extract	Se species concentration in extract	Fraction of Se species to total Se	
					($\mu\text{g Se g}^{-1}$)	($\mu\text{g Se g}^{-1}$)			($\mu\text{g Se g}^{-1}$)		
MARINE PRODUCTS											
Tuna	-	Lyophilised	Enzymatic	HPLC-ICP-MS	4.6 ± 0.1	4.4* ± 0.2	96%	SeMet	0.28 ± 0.02	6% a	Quijano, 2000
								TMSe+	0.51 ± 0.07	12% a	
Tuna	-	Lyophilised	Enzymatic	HPLC-ICP-MS	3.9 ± 0.2			SeMet	1.6*	41%* b	Moreno, 2004
Tuna	Portugal	Dried	Enzymatic	HPLC-ICP-MS	2.32 ± 0.03			SeMet	1.07 ± 0.03	46%* b	Cabañero, 2005
Canned tuna	USA	Freeze-dried	Enzymatic	HPLC-ICP-MS	5.6 ± 0.2	5.5 ± 0.2	98 ± 3%	SeMet	1.6 ± 0.1	29 ± 2% a	Reyes, 2009
Trout	-	Lyophilised	Enzymatic	HPLC-ICP-MS	2.9 ± 0.1			SeMet	0.76*	26%* b	Moreno, 2004
								TMSe+	0.083*	3%* b	
Shark	USA	Freeze-dried	Enzymatic	HPLC-ICP-MS	2.0 ± 0.1	0.9 ± 0.1	46 ± 5%	SeMet	0.5 ± 0.1	56 ± 11% a	Reyes, 2009
Marlin	USA	Freeze-dried	Enzymatic	HPLC-ICP-MS	3.9 ± 0.1	3.5 ± 0.1	89 ± 5%	SeMet	1.6 ± 0.1	46 ± 3% a	Reyes, 2009
Swordfish	Portugal	Dried	Enzymatic	HPLC-ICP-MS	2.09 ± 0.04			SeMet	1.95 ± 0.12	93%* b	Cabañero, 2005
Sardine	Portugal	Dried	Enzymatic	HPLC-ICP-MS	1.81 ± 0.02			SeMet	0.51 ± 0.02	28%* b	Cabañero, 2005
Oyster	IRMM (Belgium)	Lyophilised	Enzymatic	HPLC-ICP-MS	3.6 ± 0.3			SeMet	0.88*	24%* b	Moreno, 2004
								TMSe+	0.30*	8%* b	
Mussel	IRMM (Belgium)	Lyophilised	Enzymatic	HPLC-ICP-MS	1.7 ± 0.02			SeMet	0.48*	28%* b	Moreno, 2004
								TMSe+	0.13*	8%* b	
MEAT											
Chicken breast	-	Freeze-dried	Enzymatic	HPLC-ICP-MS	0.54 ± 0.03			SeMet	0.36 ± 0.09	67%* b	Bierla, 2008
								SeCys	0.11 ± 0.04	20%* b	
Chicken leg	-	Freeze-dried	Enzymatic	HPLC-ICP-MS	0.57 ± 0.09			SeMet	0.32 ± 0.05	56%* b	Bierla, 2008
								SeCys	0.18 ± 0.01	32%* b	
Lamb heart	-	Freeze-dried	Enzymatic	HPLC-ICP-MS	1.26 ± 0.12			SeMet	0.25 ± 0.07	20%* b	Bierla, 2008
								SeCys	0.75 ± 0.07	59%* b	
Lamb liver	-	Freeze-dried	Enzymatic	HPLC-ICP-MS	1.41 ± 0.1			SeMet	0.25 ± 0.07	18%* b	Bierla, 2008
								SeCys	0.85 ± 0.2	60%* b	
Lamb kidney	-	Freeze-dried	Enzymatic	HPLC-ICP-MS	4.53 ± 0.6			SeMet	0.40 ± 0.26	9%* b	Bierla, 2008
								SeCys	4.0 ± 0.07	88%* b	

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Table 6-1 (to be continued)

CEREALS											
Spring wheat grain	Austria	Powder	Enzymatic	HPLC-ICP-MS		0.0122 ± 0.0007		SeMet	~0.011	~90% a	Stadlober, 2001
Spring wheat grain	India	Freeze-dried	Enzymatic	HPLC-ICP-MS	83.1 ± 0.1	69.0*	83%	SeMet	55.1 ± 2.8	66% b	Cubadda, 2010
								MeSeCys	0.303 ± 0.026	<1% b	
								Selenate	1.18 ± 0.07	1% b	
Summer barley grain	Austria	Powder	Enzymatic	HPLC-ICP-MS		0.0065 ± 0.0011		SeMet	~0.005	~77% a	Stadlober, 2001
Durum wheat	Austria	Powder	Enzymatic	HPLC-ICP-MS		0.0241 ± 0.0032		SeMet	~0.015	~62% a	Stadlober, 2001
Winter wheat grain	India	Freeze-dried	Enzymatic	HPLC-ICP-MS	29.5 ± 0.2	24.5*	83%	SeMet	17.1 ± 0.8	58% b	Cubadda, 2010
								MeSeCys	0.041 ± 0.005	<1% b	
								Selenate	0.352 ± 0.041	1% b	
Selenate-enriched spring wheat grain	NA	Powder	Enzymatic	HPLC-ICP-MS		0.183 ± 0.014		SeMet	0.155 ± 0.008	85% a	Stadlober, 2001
								SeCys2	~0.016	~9% a	
Selenate-enriched summer barley grain	NA	Powder	Enzymatic	HPLC-ICP-MS		0.188 ± 0.008		SeMet	0.148 ± 0.004	79% a	Stadlober, 2001
								SeCys2	~0.016	~10% a	
Selenate-enriched durum wheat	NA	Powder	Enzymatic	HPLC-ICP-MS		0.218 ± 0.03		SeMet	0.187 ± 0.004	86% a	Stadlober, 2001
								SeCys2	~0.018	~9% a	
Selenate-enriched buckwheat seed	NA	Air-dried	Enzymatic	HPLC-UV-HG-AFS, HPLC-ICP-MS	3.58 ± 0.80	2.50		SeMet	2.42*	97% a	Vogrincic, 2009
Wheat flour	IRMM (Belgium)	Lyophilised	Enzymatic	HPLC-ICP-MS	0.69 ± 0.09			SeMet	0.36 ± 0.09	52% b	Moreno, 2004
								SeCys2	0.13 ± 0.01	19% b	
Basmati rice	India	Powder	Enzymatic	HPLC-ICP-MS	0.67*	0.543 ± 0.007	81 ± 5%	SeMet	0.507 ± 0.007	93% a	Guzman Mar, 2009
Jasmin rice	Thailand	Powder	Enzymatic	HPLC-ICP-MS	0.068*	0.048 ± 0.008	70 ± 7%	SeMet	0.046 ± 0.006	96% a	Guzman Mar, 2009
White rice	USA	Powder	Enzymatic	HPLC-ICP-MS	0.141*	0.124 ± 0.008	88 ± 8%	SeMet	0.116 ± 0.007	94% a	Guzman Mar, 2009
Rice-based cereal product	Italy	Powder	Enzymatic	HPLC-ICP-MS	0.062*	0.051 ± 0.009	82 ± 8%	SeMet	0.046 ± 0.008	90% a	Guzman Mar, 2009
Rice-based cereal product	USA	Powder	Enzymatic	HPLC-ICP-MS	0.323*	0.291 ± 0.01	90 ± 9%	SeMet	0.259 ± 0.007	89% a	Guzman Mar, 2009
Rice-based cereal product	Canada	Powder	Enzymatic	HPLC-ICP-MS	0.443*	0.39 ± 0.011	88 ± 10%	SeMet	0.341 ± 0.012	87% a	Guzman Mar, 2009

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Table 6-1

MUSCHROOMS											
Mushrooms <i>Agaricus</i>	Spain	Freeze-dried	Enzymatic	HPLC-ICP-MS	1.6 ± 0.1	0.99 ± 0.06	61 ± 3	SeMet	0.157 ± 0.007	16*% a	Diaz Huerta, 2006
Se-enriched mushrooms	NA	Lyophilised	Enzymatic	HPLC-ICP-MS	51.4 ± 0.8	29 ± 4	56 ± 7	SeMet	2.32 ± 0.04	8*% a	Diaz Huerta, 2006
Selenite-enriched mushrooms	NA	Powder	Enzymatic	HPLC-HHPN-AFS	110.2	83		SeCys2	27.7	33*% a	Stefanka, 2001
								Selenite	46.4	56*% a	
VEGETABLES											
Selenite-enriched onion leaves	NA	Dried powder	Methanol:chloroform: water (12:5:3)	HPLC-ICP-MS	154 ± 6			SeMet	0.5*	<1% b	Wrobel, 2004
								MeSeCys	6.2*	4% b	
Selenate-enriched onion leaves	NA	Dried powder	Methanol:chloroform: water (12:5:3)	HPLC-ICP-MS	601 ± 7			SeMet	1.2*	<1% b	Wrobel, 2004
								MeSeCys	11.4*	2% b	
Se-enriched garlic	NA	Mixed	Boiling water bath	HPLC-ICP-MS, ESI-MS/MS	136.0			Selenate	10.9*	8% a	Ogra, 2005
								MeSeCys	2.7*	2% a	
Se-enriched garlic	NA		Enzymatic	HPLC-ICP-MS	292		~100%	γ-glu-MeSeCys	122.4*	90% a	
								SeMet		13% b	Ip, Birringer, 2000
								γ-glu-MeSeCys		73% b	
Se-enriched shallot	NA	Mixed	Boiling water bath	HPLC-ICP-MS, ESI-MS/MS	226.8			Selenate	63.5*	28% a	Ogra, 2005
								MeSeCys	11.3*	5% a	
								γ-glu-MeSeCys	150.0*	66% a	
YEAST											
Se-enriched yeast	NA	Dried	Enzymatic	HPLC-ICP-MS	1214		>90%	SeMet		75% a	Yoshida, 2002
								SeCys2		10% a	
								Selenite		5% a	
Se-enriched yeast	NA	Lyophilised	Enzymatic	HPLC-ICP-MS	1180 ± 40			SeMet	672*	57*% b	Moreno, 2004
								Selenite	3.44*	<1*% b	
								Selenate	8.3*	<1*% b	
Se-enriched yeast	NA	Powder	Enzymatic	HPLC-ICP-MS	1922		80-90%	SeMet		85% b	Ip, Birringer, 2000

a: percentage of Se species compared to total extracted Se

b: percentage of Se species compared to total Se content of the sample

~ : approximate value

NA: not applicable

*: data calculated in the context of the present study on the basis of data provided in the original article

6.3. Selenium bioavailability

The present paragraph is a compilation of the relevant outcomes that have been published during the last decades concerning bioavailability of Se in food. This work was, to our knowledge, realised for the first time. Due to the multitude of definitions that have ever been associated with bioavailability, and because of the numerous methods, models and experimental designs that have been used to measure Se bioavailability, it is not easy to compare all the data that have been published. To allow anyway a comparison to a certain extent, we chose to not express the results as absolute values such as presented in the original articles, but the results were 'standardized' by calculation of the relative bioavailability of a Se species or a tested foodstuff compared to the reference selenocompound used in the study, usually selenite. The results were summarized in separate tables (tables 6-2, 6-3, 6-4 and 6-5) for each different kind of food matrix.

6.3.1. Selenium bioavailability of single species and of Se-enriched yeast

Many studies have aimed at investigating the degrees of bioavailability of individual organic and inorganic Se species and of Se-enriched yeast. Table 6-2 gives an overview of the results obtained in these studies by considering three types of parameters: Se apparent absorption (calculated as the difference between Se intake and Se excretion in faeces), Se concentration in various body locations, and induction of GPx activity. In some cases, a control was made with non-supplemented subjects. According to table 6-2, in comparison to controls, all reported Se species were able to affect positively the Se status of the subjects. In a general way, Se-rich yeast (known to contain mostly SeMet) increased Se status to a similar extent as SeMet (Wang & Lovell, 1997). Among the parameters considered, the apparent absorption was significantly higher in animals fed with feed supplemented with Se-rich yeast than with selenite (Rider *et al.*, 2010). Secondly, in each body location, Se concentrations were higher after SeMet supplementation than after supplementation with inorganic Se, and this with an order of magnitude increasing with the dose of administered Se (Whanger & Butler, 1988; Rider *et al.*, 2009). MeSeCys, similarly to selenite, was less retained by Caco-2 cells than SeMet (Zeng, Botnen & Johnson, 2008). Finally, regarding selenoenzyme induction, no big difference was observed between inorganic and organic Se species, except in Caco-2 cells where SeMet was considerably less efficient than selenite and MeSeCys in

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inducing GPx activity (Zeng, Botnen & Johnson, 2008). In studies where different doses of Se were administered, no effect of the dose was observed on the degree of enzymatic activity. This could reveal that Se requirements were already met by the basal diet (Rider *et al.*, 2009).

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Table 6-2 (to be continued): Selenium species bioavailability

Parameter of Se bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation	Relative bioavailability					
					Selenate	Selenite	SeMet	Se yeast	MeSeCys	Control
Se apparent absorption										
		Trout	10 weeks	~2 µgSe g ⁻¹ food		1 ^a		~1.2 ^b		~1.1 ^a Rider, 2010
Se concentration										
	Intestine ¹	Caco-2 cells	72h	15.6 nmol l ⁻¹		1 ^a	~0.6 ^{ab}	~0.8 ^a	~0.2 ^b	Zeng, 2008
				31.2 nmol l ⁻¹		1 ^a	~1 ^a	~0.8 ^a	~0.1 ^b	
				62.5 nmol l ⁻¹		1 ^a	~1.5 ^b	~0.9 ^a	~0.1 ^c	
				125 nmol l ⁻¹		1 ^a	~2.2 ^b	~1.1 ^a	~0.1 ^c	
	Whole body	Trout	10 weeks	~2 µgSe g ⁻¹ food		1 ^a		1.75 ^b		0.56 ^c Rider, 2009
				~4 µgSe g ⁻¹ food		1 ^a		1.71 ^b		0.32 ^c
				~8 µgSe g ⁻¹ food		1 ^a		1.34 ^b		0.15 ^c
	Whole blood	Rat	9 weeks	0.2 µgSe g ⁻¹ food		1*	1.2*			0.06* Whanger, 1988
				1 µgSe g ⁻¹ food		1*	1.75*			0.05*
				2 µgSe g ⁻¹ food		1*	2.47*			0.05*
				4 µgSe g ⁻¹ food		1*	4.13*			0.04*
		Human	17 weeks	100 µg day ⁻¹		1*	1.61*			Thompson, 1982
	Plasma	Trout	10 weeks	~2 µgSe g ⁻¹ food		1 ^a		1.12 ^b		0.82 ^c Rider, 2010
		Human	17 weeks	100 µg day ⁻¹		1*	1.5*			Thompson, 1982
		Human	11 weeks	200 µg day ⁻¹	1*		~1.5*			Levander, 1983
	RBC	Human	17 weeks	100 µg day ⁻¹		1*	1.7*			Thompson, 1982
		Human	11 weeks	200 µg day ⁻¹	1*		~1.8*			Levander, 1983

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Table 6-2 (to be continued)

Parameter of Se bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation	Relative bioavailability					
					Selenate	Selenite	SeMet	Se yeast	MeSeCys	Control
	Muscles	Channel catfish	9 weeks	0-0.4 $\mu\text{gSe g}^{-1}$ food	1*	4.78*	4.53*			Wang C., 1997
		Crucian carp	30 days	~0.5 $\mu\text{gSe g}^{-1}$ food	1 ^a	1.29 ^b			0.54 ^c	Wang Y., 2007
		Trout	10 weeks	~2 $\mu\text{gSe g}^{-1}$ food	1 ^a			1.61 ^b	0.91 ^a	Rider, 2010
		Rat	8 weeks	0.115 $\mu\text{gSe g}^{-1}$ food	1 ^a	1.49 ^b				Shi, 1994
		Broiler chicken	21 days	~0.2 $\mu\text{gSe g}^{-1}$ food	1 ^a			1.11 ^a	0.70 ^b	Wang Y., 2008
		Rat	9 weeks	0.2 $\mu\text{gSe g}^{-1}$ food	1*	2.80*			0.20*	Whanger, 1988
				1 $\mu\text{gSe g}^{-1}$ food	1*	9.36*			0.14*	
				2 $\mu\text{gSe g}^{-1}$ food	1*	16.80*			0.13*	
				4 $\mu\text{gSe g}^{-1}$ food	1*	26.41*			0.12*	
	Liver	Channel catfish	9 weeks	0-0.4 $\mu\text{gSe g}^{-1}$ food	1*	1.97*	1.84*			Wang C., 1997
		Trout	10 weeks	~2 $\mu\text{gSe g}^{-1}$ food	1 ^a			0.97 ^a	0.54 ^b	Rider, 2010
		Rat	8 weeks	0.115 $\mu\text{gSe g}^{-1}$ food	1 ^a	1.16 ^b				Shi, 1994
		Broiler chicken	21 days	~0.2 $\mu\text{gSe g}^{-1}$ food	1 ^a			1.26 ^b	0.63 ^c	Wang Y., 2008
		Rat	9 weeks	0.2 $\mu\text{gSe g}^{-1}$ food	1*	1.24*			0.03*	Whanger, 1988
				1 $\mu\text{gSe g}^{-1}$ food	1*	1.94*			0.02*	
				2 $\mu\text{gSe g}^{-1}$ food	1*	2.94*			0.02*	
				4 $\mu\text{gSe g}^{-1}$ food	1*	3.35*			0.01*	
	Kidneys	Broiler chicken	21 days	~0.2 $\mu\text{gSe g}^{-1}$ food	1 ^a			1.22 ^b	0.46 ^c	Wang Y., 2008
		Trout	10 weeks	~2 $\mu\text{gSe g}^{-1}$ food	1 ^a			1.08 ^b	0.91 ^c	Rider, 2010
		Rat	9 weeks	0.2 $\mu\text{gSe g}^{-1}$ food	1*	1.08*			0.21*	Whanger, 1988
				1 $\mu\text{gSe g}^{-1}$ food	1*	1.79*			0.14*	
				2 $\mu\text{gSe g}^{-1}$ food	1*	2.59*			0.13*	
				4 $\mu\text{gSe g}^{-1}$ food	1*	3.00*			0.10*	

Table 6-2 (to be continued)

Parameter of Se bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation	Relative bioavailability						
					Selenate	Selenite	SeMet	Se yeast	MeSeCys	Control	
GPx activity											
	Intestine ¹	Caco-2 cells	72h	15.6 nmol l ⁻¹		1 ^a	~0.2 ^b		~0.8 ^c	~0.1 ^b	Zeng, 2008
				31.2 nmol l ⁻¹		1 ^a	~0.2 ^b		~0.9 ^a	~0.1 ^c	
				62.5 nmol l ⁻¹		1 ^a	~0.5 ^b		~1 ^a	~0.1 ^c	
				125 nmol l ⁻¹		1 ^a	~0.7 ^b		~1.1 ^a	~0.1 ^c	
	Plasma	Channel catfish	9 weeks	0-0.4 µgSe g ⁻¹ food		1*	1.16*	1.16*			Wang C., 1997
		Crucian carp	30 days	~0.5 µgSe g ⁻¹ food		1 ^a	1.91 ^b			0.50 ^c	Wang Y., 2007
		Broiler chicken	21 days	~0.2 µgSe g ⁻¹ food		1 ^a		1.14 ^b		0.66 ^c	Wang Y., 2008
	Serum	Chick	9 days			1 ^a	0.78 ^b				Gabrielsen, 1980
		Human	17 weeks	100 µg day ⁻¹		1*	1.1*				Thompson, 1982
		Human	11 weeks	200 µg day ⁻¹	1*		~1*				Levander, 1983
	RBC	Rat	9 weeks	0.2 µgSe g ⁻¹ food		1*	0.96*			0.11*	Whanger, 1988
				1 µgSe g ⁻¹ food		1*	0.99*			0.09*	
				2 µgSe g ⁻¹ food		1*	1.04*			0.08*	
				4 µgSe g ⁻¹ food		1*	0.96*			0.08*	
	Muscles	Rat	9 weeks	0.2 µgSe g ⁻¹ food		1*	1.11*			0.10*	Whanger, 1988
				1 µgSe g ⁻¹ food		1*	1.20*			0.08*	
				2 µgSe g ⁻¹ food		1*	1.00*			0.08*	
				4 µgSe g ⁻¹ food		1*	1.14*			0.10*	
	Liver	Channel catfish	9 weeks	0-0.4 µgSe g ⁻¹ food		1*	1.47*	1.49*			Wang C., 1997
		Crucian carp	30 days	~0.5 µgSe g ⁻¹ food		1 ^a	~0.9 ^a			~0.7 ^b	Wang Y., 2007
		Trout	10 weeks	~2 µgSe g ⁻¹ food		1		1.02		0.88	Rider, 2010
		Trout	10 weeks	~2 µgSe g ⁻¹ food		1		0.81		0.94	Rider, 2009
				~4 µgSe g ⁻¹ food		1		1.01		1.09	
				~8 µgSe g ⁻¹ food		1		0.95		1.17	

Table 6-2

Parameter of Se bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation	Relative bioavailability									
					Selenate	Selenite	SeMet	Se yeast	MeSeCys		Control			
	Liver	Broiler chicken	21 days	~0.2 µgSe g ⁻¹ food		1 ^a		1.04 ^a		0.74 ^b	Wang Y., 2008			
		Rat	8 weeks	0.115 µgSe g ⁻¹ food		1 ^a	1.34 ^b				Shi, 1994			
		Rat	9 weeks	0.2 µgSe g ⁻¹ food		1 [*]	1.02 [*]			0.01 [*]	Whanger, 1988			
				1 µgSe g ⁻¹ food		1 [*]	0.81 [*]			0.01 [*]				
				2 µgSe g ⁻¹ food		1 [*]	1.03 [*]			0.01 [*]				
	Kidneys	Rat	9 weeks	4 µgSe g ⁻¹ food		1 [*]	0.98 [*]			0.01 [*]				
				0.2 µgSe g ⁻¹ food		1 [*]	1.05 [*]			0.05 [*]	Whanger, 1988			
				1 µgSe g ⁻¹ food		1 [*]	1.18 [*]			0.04 [*]				
				2 µgSe g ⁻¹ food		1 [*]	0.92 [*]			0.05 [*]				
				4 µgSe g ⁻¹ food		1 [*]	0.92 [*]			0.04 [*]				
				TRx activity										
				Liver	Trout	10 weeks	~2 µgSe g ⁻¹ food		1 ^{ab}		1.39 ^a		0.93 ^b	Rider, 2010
					Trout	10 weeks	~2 µgSe g ⁻¹ food		1 ^a		0.92 ^a		0.81 ^b	Rider, 2009
		~4 µgSe g ⁻¹ food			1 ^a		1.18 ^a		0.64 ^b					
		~8 µgSe g ⁻¹ food			1 ^a		1.03 ^a		0.66 ^b					

Results in italic have been calculated with the slope-ratio assay (cf. section 2.5.3. "Models for the study of Se bioavailability and bioactivity").

Letter superscript within a line indicates when difference between Se species is significant.

Number 1 superscript indicates that experiment has been conducted in vitro.

The sign * refers to results without indication on statistical significance.

6.3.2. Selenium bioavailability from fish

Selenium bioavailability from several species of fish is reported in table 6-3. As a matter of comparison, authors sometimes included a sample of wheat or Se-yeast in addition to the fish samples. Tuna is the species of fish that has been the most studied. Its ability to increase Se concentration in body tissues and in RBC was in general slightly lower than that of selenite and wheat (Douglas *et al.*, 1981; Alexander, Whanger & Miller, 1983; Wen *et al.*, 1997). Tuna-induced GPx activity was approximately half that of selenite, except in one study that reported greater GPx induction for the fish (Wen *et al.*, 1997). No impact of the dose was observed on relative bioavailability of Se from tuna compared to selenite (Alexander, Whanger & Miller, 1983). A previous study that performed *in vitro* gastro-intestinal digestions reported the bioaccessibility of Se from tuna to be low (about 50 %). This could be a cause of the relatively low Se bioavailability from this fish (Cabañero, Madrid & Camara, 2004). Regarding the other fish species, their responses to the different parameters of bioavailability were varied, either above or beneath selenite values. Fish might become a particularly interesting source of Se when it has previously been enriched with organic Se. Indeed, the study of Ornsrud & Lorentzen (2002) reported values for Se retention and GPx induction from SeMet-enriched salmon that were largely above those of selenite.

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Table 6-3 (to be continued): Selenium species bioavailability in fish

Parameter of bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation [µgSe g ⁻¹ food]	Relative bioavailability									
					Selenate	Selenite	Tuna	Trout	Capelin	Mackerel	Flounder	SeMet-enriched salmon	Wheat	Yeast
Se apparent absorption														
		Human	2 days		1 ^a			0.96 ^a					0.58 ^b	Fox, 2004
		Rat		0.1		1						1.0		Ornsrud, 2002
Se body retention														
		Human	2 days		1 ^a			1.44 ^b					0.95 ^{ab}	Fox, 2004
		Rat		0.1		1 ^a						1.84 ^b		Ornsrud, 2002
Se concentration														
	Whole blood	<i>Rat</i>	<i>4 weeks</i>	<i>0.05-0.15</i>		<i>1*</i>	<i>0.6*</i>						<i>0.8*</i>	Alexander, 1983
	Plasma	<i>Rat</i>	<i>30 days</i>	<i>0-0.2</i>		<i>1</i>						<i>1.25</i>		Ornsrud, 2002
	Young RBC	<i>Rat</i>	<i>4 weeks</i>	<i>0.2</i>		<i>1</i>	<i>1</i>						<i>1.14</i>	Douglass, 1981
	Muscles	<i>Rat</i>	<i>4 weeks</i>	<i>0.05-0.15</i>		<i>1*</i>	<i>1*</i>						<i>2*</i>	Alexander, 1983
		<i>Rat</i>	<i>9 weeks</i>	<i>0.8 ± 0.4</i>		<i>1</i>	<i>0.91</i>				<i>1.47</i>			Wen, 1997
		<i>Rat</i>	<i>30 days</i>	<i>0-0.2</i>		<i>1^a</i>						<i>2.25^b</i>		Ornsrud, 2002
	Liver	<i>Rat</i>	<i>4 weeks</i>	<i>0.2</i>		<i>1</i>	<i>0.69</i>						<i>0.79</i>	Douglass, 1981
		<i>Rat</i>	<i>4 weeks</i>	<i>0.05-0.15</i>		<i>1*</i>	<i>0.9*</i>						<i>1*</i>	Alexander, 1983
		<i>Rat</i>	<i>9 weeks</i>	<i>0.8 ± 0.4</i>		<i>1</i>	<i>1.09</i>				<i>1.21</i>			Wen, 1997
		<i>Rat</i>	<i>30 days</i>	<i>0-0.2</i>		<i>1</i>						<i>1.15</i>		Ornsrud, 2002
	Kidneys	<i>Rat</i>	<i>4 weeks</i>	<i>0.05-0.15</i>		<i>1*</i>	<i>0.75*</i>						<i>1*</i>	Alexander, 1983
	Femur	<i>Rat</i>	<i>30 days</i>	<i>0-0.2</i>		<i>1</i>						<i>1.13</i>		Ornsrud, 2002

Table 6-3

Parameter of bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation [µgSe g ⁻¹ food]	Relative bioavailability									
					Selenate	Selenite	Tuna	Trout	Capelin	Mackerel	Flounder	SeMet-enriched salmon	Wheat	Yeast
GPx activity														
	Whole blood	Rat	4 weeks	0.05-0.15		1*	0.44*						1.08*	Alexander, 1983
	Serum	Chick	9 days			1 ^a			0.48 ^b	0.34 ^c				Gabrielsen, 1980
		Rat	30 days	0-0.2		1						1.39		Ormsrud, 2002
	Young RBC	Rat	4 weeks	0.2		1 ^a	0.54 ^b						0.72 ^c	Douglass, 1981
	Liver	Rat	4 weeks	0.2		1 ^a	0.49 ^b						0.75 ^b	Douglass, 1981
		Rat	4 weeks	0.05-0.15		1*	0.38*						0.7*	Alexander, 1983
		Rat	9 weeks	0.8 ± 0.4		1	1.26				1.32			Wen, 1997
	Kidneys	Rat	4 weeks	0.05-0.15		1*	0.34*						1.33*	Alexander, 1983

Results in italic have been calculated with the slope-ratio assay (cf. section 2.5.3. "Models for the study of Se bioavailability and bioactivity").

Letter superscript within a line indicates when difference between Se species is significant.

The sign * refers to results without indication on statistical significance

6.3.3. Selenium bioavailability from meat

Most meat-related studies focused on beef since it is commonly eaten by western populations (table 6-4). Retention of Se and GPx induction associated with beef products were comparable to those of selenite and wheat. One study was particularly positive concerning Se from beef and reported it as “highly bioavailable” (Shi & Spallholz, 1994). Regarding pork meat, the only result presented in table 6-4 concerns its capacity of GPx induction which was similar to that of selenite (Wen *et al.*, 1997). In contrast, in a study unreported here (Bügel *et al.*, 2004), Se from pork meat had no impact on Se concentration nor on selenoenzymes activity, although Se had been highly absorbed and retained as was calculated on the basis of a low excretion rate. In that study, pure SeMet and selenite had on the other hand both increased the parameters of bioavailability, and this made the authors suggest that Se in pork meat should not be in the form of selenite or SeMet. Concerning the other kinds of meat, Se from veal and chicken was reported to induce GPx activity to a similar extent as selenite, while lamb meat had a lower effect (Wen *et al.*, 1997). Alternatively, it has been reported from an anterior study that Se from meat naturally rich in Se (with no detail concerning the kind of meat) was as available as Se from Se-rich bread, but no reference compound was used to calculate Se relative bioavailability (van der Torre *et al.*, 1991).

Table 6-4: Selenium species bioavailability in meat

Parameter of bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation [μgSe g ⁻¹ food]	Relative bioavailability									
					Selenite	Veal	Chicken	Pork	Lamb	Beef	Beef kidney	Beef liver	Beef striploin	Wheat
Se concentration														
	Young RBC	Rat	4 weeks	0.2	1						1			Douglass, 1981
	Muscles	Rat	8 weeks	~0.115	1							0.96	1.16	Shi, 1994
	Liver	Rat	4 weeks	0.2	1						0.74			Douglass, 1981
		Rat	8 weeks	~0.115	1							0.97	1.00	Shi, 1994
GPx activity														
	Young RBC	Rat	4 weeks	0.2	1 ^a						0.65 ^b			Douglass, 1981
	Liver	Rat	4 weeks	0.2	1						0.89			Douglass, 1981
		Rat	8 weeks	~0.115	1 ^a							1.19 ^b	1.16 ^b	Shi, 1994
		Rat	9 weeks	0.8 ± 0.4	1 ^{ab}	0.95 ^a	0.96 ^a	1.06 ^a	0.72 ^b	0.99 ^a				Wen, 1997

Results in *italic* have been calculated with the slope-ratio assay (cf. section 2.5.3. "Models for the study of Se bioavailability and bioactivity").

Letter superscript within a line indicates when difference between Se species is significant.

The sign * refers to results without indication on statistical significance.

6.3.4. Selenium bioavailability from plant products

Few studies have dealt with vegetables, probably because the low Se content of plants makes speciation analyses difficult or even impossible. Some studies could nevertheless be conducted on some products naturally rich in Se like mushrooms and Brazil nuts, or on Se-accumulator plants previously enriched with Se fertilizers. For example, Finley (1998) studied the ability of Se-enriched broccoli to restore Se and GPx levels in Se-depleted rats, in comparison with SeMet, selenite and selenate. As presented in table 6-5, the three single Se species produced greater responses than broccoli to these parameters. However, the responses induced by broccoli saturated less quickly with time. These results suggest that Se is present in broccoli in a different form than selenite, selenate or SeMet, and that this form participates less intensively in increasing Se status but on a longer period. With reference to the speciation analysis of *Brassica* plants (*cf.* table 6-1), this form is probably MeSeCys or γ -glut-MeSeCy. Those compounds have been reported to be more readily transformed into methylselenol (CH_3SeH) than into H_2Se , the precursor of selenoproteins synthesis. Thereby, they are rapidly excreted, and they induce less selenoenzymatic activity and are less retained than non-methylated species. Selenium bioavailability from Se-enriched broccoli was also investigated on an *in vitro* Caco-2 cells model (Zeng, Botnen & Johnson, 2008) after having been previously artificially digested *in vitro* with pepsin, pancreatin and bile extract (Glahn *et al.*, 1998). Pure MeSeCys and digested broccoli were compared regarding their ability to induce GPx activity at different levels of concentrations. After 72h of incubation, GPx enzymes were more induced by MeSeCys than by broccoli. Owing to the fact that broccoli contains the majority of its Se content as MeSeCys, it seems therefore that the complexity of the food matrix could impair Se bioavailability. The difference between MeSeCys and digested broccoli became however less significant with increasing doses of Se.

As for the few experiments that were conducted on naturally Se-rich plant products, Brazil nuts were able to increase Se content in the liver of rodents (Chansler *et al.*, 1986) and to enhance GPx activity in human blood (Thompson *et al.*, 2008). In contrast, mushrooms stimulated these parameters very little, which made authors suggest that mushrooms may contain slightly available Se species such as methylated compounds (Chansler *et al.*, 1986). With reference to tables 6-3 and 6-4, the behaviour of Se in wheat seemed close to that of

SeMet (Douglass *et al.*, 1981; Alexander, Whanger & Miller, 1983), which is not surprising as it was reported that SeMet can represent almost the totality of the Se content in wheat grain (Stadlober, Sager & Irgolic, 2001). Selenium from wheat has been reported to be particularly well retained by various body tissues in many studies (Levander *et al.*, 1983; Meltzer *et al.*, 1993), especially when it is milled in meal since this process disrupts the fibrous cell walls that can impede Se bioaccessibility (Reeves *et al.*, 2007). Selenium from wheat was also able to induce GPx activity but to a less significant extent than concerning Se retention. Finally, bioavailability of Se from soybean and corn gluten meal was found to be very low (Gabrielsen & Opstvedt, 1980).

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Table 6-5: Selenium species bioavailability in plant products

Parameter of Se bioavailability	Body location	Model of study	Duration of Se supplementation	Dose of Se supplementation	Relative bioavailability									
					MeSeCys	Selenite	Selenate	SeMet	Se-enriched broccoli	Mushroom	Brazil nut	Soybean	Corn gluten meal	Control
Se concentration														
	Plasma	Rat	9 weeks	0.1 µgSe g ⁻¹ food	1*	~0.93*	~1.07*	~0.82*						Finley, 1998
		Rat	4 weeks	0.1 µgSe g ⁻¹ food	1 ^a					0.21 ^b	1.06 ^a			Chansler, 1986
		Human	6 weeks	~100 µgSe day ⁻¹			1 ^a				0.97 ^a			0.62 ^b Thompson, 2008
	RBC	Rat	9 weeks	0.1 µgSe g ⁻¹ food	1*	~1*	~1*	~0.67*						Finley, 1998
	Muscles	Rat	9 weeks	0.1 µgSe g ⁻¹ food	1*	1*	~1.63*	~0.81						Finley, 1998
	Liver	Rat	9 weeks	0.1 µgSe g ⁻¹ food	1*	~1.27*	~1.35*	~0.9*						Finley, 1998
		Rat	4 weeks	0.1 µgSe g ⁻¹ food	1 ^a					0.52 ^b	1.36 ^c			Chansler, 1986
GPx activity														
	Intestine ¹	Caco-2 cells	72h	15.6 nmol l ⁻¹	1 ^a			~0.29 ^b						Zeng, 2008
				31.2 nmol l ⁻¹	1 ^a			~0.45 ^b						
				62.5 nmol l ⁻¹	1 ^a			~0.76 ^b						
				125 nmol l ⁻¹	1 ^a			~0.97 ^a						
	Plasma	Rat	4 weeks	0.1 µgSe g ⁻¹ food		1 ^a				0.11 ^b	0.96 ^a			Chansler, 1986
		Human	6 weeks	~100 µgSe day ⁻¹			1 ^a				0.92 ^a			0.89 ^b Thompson, 2008
		Chick	9 days			1 ^a						0.18 ^b	0.26 ^b	Gabrielsen, 1980
	Whole blood	Human	6 weeks	~100 µgSe day ⁻¹			1 ^a				1.20 ^b			1.06 ^a Thompson, 2008
	RBC	Rat	9 weeks	0.1 µgSe g ⁻¹ food	1*	0.94*	~1*	~0.69*						Finley, 1998
	Liver	Rat	9 weeks	0.1 µgSe g ⁻¹ food	1*	~1.25*	1.15*	~0.62*						Finley, 1998
		Rat	4 weeks	0.1 µgSe g ⁻¹ food	1 ^a					0.05 ^b	1.17 ^a			Chansler, 1986

Results in italic have been calculated with the slope-ratio assay (cf. section 2.5.3. "Models for the study of Se bioavailability and bioactivity").

Letter superscript within a line indicates when difference between Se species is significant.

Number 1 superscript indicates that experiment has been conducted in vitro.

The sign * refers to results without indication on statistical significance.

6.4. Discussion

According to the numerous and various studies that were reviewed in this article, the species-dependence of Se bioavailability seems evident. SeMet and organic Se from yeast were more available than inorganic Se when considering Se absorption (difference between Se intake and faecal excretion), Se retention (difference between Se intake and faecal + urinary excretion) and Se accumulation in blood and tissues. These observations were characteristic of SeMet rather than being a global characteristic of organic species. Indeed, MeSeCys generated lower responses than SeMet and Se-yeast in term of Se retention (Zeng, Botnen & Johnson, 2008). This capacity of accumulation of SeMet is explained by the fact that this species, in contrast with other organic and inorganic compounds, can be directly and largely incorporated into body proteins in place of methionine. But this important pool of SeMet is not automatically related to a greater bioactivity because in Se-containing proteins, the element is no longer recognized as Se and cannot be mobilized by the organism for selenoproteins synthesis. SeMet rather seems to be an efficient reserve of Se in case of long term shortage (Behne, Alber & Kyriakopoulos, 2009) given the retrieval of Se following the catabolization of SeMet during the natural protein turn-over (Fox *et al.*, 2004; Zeng, Botnen & Johnson, 2008). Once released as free SeMet, Se can be recognized and enter the selenoprotein synthesis pathway. The biological half-time of SeMet stored in tissues is dependent on metabolic factors of individual tissues, as well as on nutritional habits such as the dietary intake of methionine that competes with SeMet for integration into proteins (Waschulewski & Sunde, 1988).

Species-related differences among Se compounds were less obvious concerning the induction of GPx activity than they were for Se retention. The fact that even low quantities of Se are able to maintain at least some GPx activity explains partially the absence of clear trends (Thompson, 2004; Zeng, Botnen & Johnson, 2008; Rider *et al.*, 2009). In addition, different tissues do not equally respond to Se intake for GPx induction. For example, it was reported that GPx activity in platelets is a more sensitive indicator of Se bioactivity than GPx activities in plasma or in erythrocytes that rapidly reach a plateau (Thompson, 2004). By joining directly the pathway of selenoprotein synthesis, inorganic Se species are expected to be more bioactive than SeMet that is for a large part stored in proteins, and than methylated species that are widely excreted. However, in contrast with SeMet, the half-life of inorganic Se in the

body is much shorter since the excess is not stored and is directly excreted (Levander *et al.*, 1983).

On the basis of the above considerations, it seems clear that, in case of pure Se species, the speciation of Se is a major determinant of the element's bioavailability. In case of real food samples, the influence of speciation remains but the situation is complicated by the fact that several Se species with their proper degrees of bioavailability are present in food, and by the existence of a food matrix. Some general differences in Se bioavailability could nevertheless be perceived among different types of matrices. In marine products, bioavailability of Se has been reported to be relatively low, despite the considerable Se content of those products. This could be due to the presence in the food matrix of some metals like mercury and arsenic. Those elements can bind very tightly with Se, making Se insoluble and unable to be absorbed by the body (Meltzer *et al.*, 1993; Bügel, Sandström & Larsen, 2001). In addition, the presence in fish of methylated species such as TMSe^+ contributes to decrease Se overall bioavailability because these species are readily excreted (Quijano *et al.*, 2000; Moreno *et al.*, 2004). By way of contrast, Se from wheat and from Brazil nuts has been associated with high degrees of bioavailability in several studies, probably in relation with their important SeMet content. The total Se content of wheat was however highly dependent of its country of origin, which explains that, despite an important bioavailability, wheat-based products can contribute to various extents to the Se intake of a population, *i.e.* large contribution in the USA but much lower in Europe (Rayman, 2000; FAO/WHO, 2002; Waegeneers *et al.*, 2012). Bioavailability of Se from meat was reported to be good and comparable to that of wheat. The major sources of dietary Se of the general adult Belgian population have been recently identified as: meat & meat products > fish & shellfish > potatoes & pasta (Waegeneers *et al.*, 2012). In the three categories of food products, SeMet was the principal species identified. This means that, if there is no important limitation in bioaccessibility, Se from these products should be well absorbed and retained by the organism. In addition, people taken their Se through these kinds of food products should benefit from the essential selenoenzyme activity but to a limited extent (due to SeMet incorporation in Se-containing proteins), which should avoid toxicity. In that sense, those products seem to represent adequate sources of Se. Se-accumulator plants had relatively low Se bioavailability with respect to the parameters considered in this article but have been hypothesized to have anti-

cancer properties, which could make them particularly interesting. Unfortunately, in most studies about Se speciation, a large fraction of Se remained unidentified. This unidentified fraction of Se could contain Se species that are quantitatively minor but able to serve particular function in the human organism. The characterization of this unknown fraction of Se species is thus a challenge for future speciation studies, and may be of high importance to solve the scientific puzzle on Se bioavailability.

6.5. Conclusion

The fact that the total Se content of a kind of foodstuff is not sufficient to predict its effect on human health makes the study of Se speciation and Se bioavailability a challenging research subject. Based on the present article, it can be concluded with reasonable certainty that organic Se in the form of SeMet (abundant in cereals and Se-yeast) is on the long term the most efficient to prevent Se deficiency by maintaining high level of Se in body proteins. On the other hand, inorganic Se in the form of selenite (contained in selenite-enriched vegetables and food supplements) can respond more rapidly to an acute need of Se through induction of selenoproteins and -enzymes, but has been reported to be at higher risk to become toxic on the long term. Unfortunately, little is known about bioavailability of Se species other than SeMet and selenite. In addition, a large part of Se present in food products is still under unknown forms. When additional forms could be identified and characterized, this would open the door for additional bioavailability and bioactivity studies that may reveal some information that is still lacking today.

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Chapter 7

***In vitro* investigation on the
degree of bioavailability of
selenium species,
and on the mechanisms involved
for their intestinal absorption**

Chapter 7 – *In vitro* investigation on the degree of bioavailability of selenium species, and on the mechanisms involved for their intestinal absorption

Foreword

Based on numerous published studies, chapter 6 revealed that different Se species have their proper degree of Se bioavailability (*i.e.* capacity to elevate Se body content) and bioactivity (*i.e.* capacity to induce selenoenzymes activity). From chapter 6, it could be concluded that all the considered individual Se species were able to elevate the Se body content, and that SeMet was largely the most efficient for that purpose. Additional conclusions were however difficult to be drawn because of the diversity of study designs and methods having generated the reported results. In order to have a more accurate idea on the degree of bioavailability of distinct Se species, we quantified the bioavailable fractions of four species of Se with a unique model, *i.e.* a cellular (Caco-2) model mimicking the intestinal barrier. Such an *in vitro* model, due to its simplicity and its high degree of standardization, is particularly appropriate for the study of the mechanisms of absorption occurring at the intestinal step. Thereby, in addition to the determination of the degrees of bioavailability of Se species, the model was used for the investigation of the mechanistic processes mediating the species-dependent intestinal transport of Se.

This chapter is based on an article that was accepted for publication in the *British Journal of Nutrition* under the title “An *in vitro* investigation on the species-dependent intestinal transport of selenium and the impact of this process on selenium bioavailability” written by Celine Thiry^{1,2}, Ann Ruttens¹, Luc Pussemier¹ and Yves-Jacques Schneider² (2012).

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Abstract

A range of selenium species have been shown to occur in food. Depending on its speciation, Se is more or less bioavailable to humans. In this study, the role of speciation as a determinant of Se bioavailability was addressed with an investigation of species-specific mechanisms of transport at the intestinal level. This work focused on four distinct Se compounds (selenate, selenite, SeMet and MeSeCys), whose intestinal transport was mimicked through an *in vitro* bicameral model of enterocyte-like differentiated Caco-2 cells. Efficiency of Se absorption was shown to be species-dependent (SeMet>MeSeCys>selenate >selenite). In case of SeMet, MeSeCys and selenate, the highly polarized passage from apical to basolateral pole indicated that a substantial fraction of transport was transcellular, whilst results for selenite indicated paracellular diffusion. The passage of the organic Se species (SeMet and MeSeCys) became saturated after three hours, but no such effect was observed for the inorganic species. In addition, SeMet and MeSeCys transport was significantly inhibited by their respective sulfur analogues methionine and methionine, which suggests a common transport system for both kinds of compounds.

Keywords

Selenium, selenium species, intestinal absorption, transport mechanism

7.1. Introduction

Selenium enters the body almost exclusively through food. Depending on the type of food, both concentration and chemical form of Se can vary. These two factors (dose and speciation) critically influence Se bioavailability, bioactivity and potential toxicity (Meltzer *et al.*, 1990; Tiwary *et al.*, 2006). Several studies dealing with Se dietary enrichment of humans and animals have indicated that organic forms are more bioavailable and less toxic than inorganic ones (Whanger & Butler, 1988; Wang *et al.*, 2007; Wang & Xu, 2008). In rats, Se concentrations in blood and liver were reported to be up to four times higher following SeMet supplementation compared to selenite supplementation (Whanger & Butler, 1988). Similarly, in humans, plasmatic Se content was double as high after SeMet enrichment compared to selenite enrichment (Griffiths *et al.*, 1976; Thompson *et al.*, 1982). In crucian carps, the

selenoenzyme glutathione peroxidase was more expressed after SeMet intake than after comparable selenite intake (Wang *et al.*, 2007).

In the present study, a bicompartimented (apical vs basolateral) *in vitro* model of enterocyte-like Caco-2 cells mimicking the intestinal barrier was used for an in-depth investigation of bioavailability of four Se species (selenate (Se(VI)), selenite (Se(IV)), selenomethionine (SeMet) and methylselenocysteine (MeSeCys)). In this model, bioavailability -or the bioavailable fraction- of Se can be considered to be the fraction that is absorbed through the Caco-2-simulated intestinal membrane, from the apical to the basolateral compartment (figure7-1).

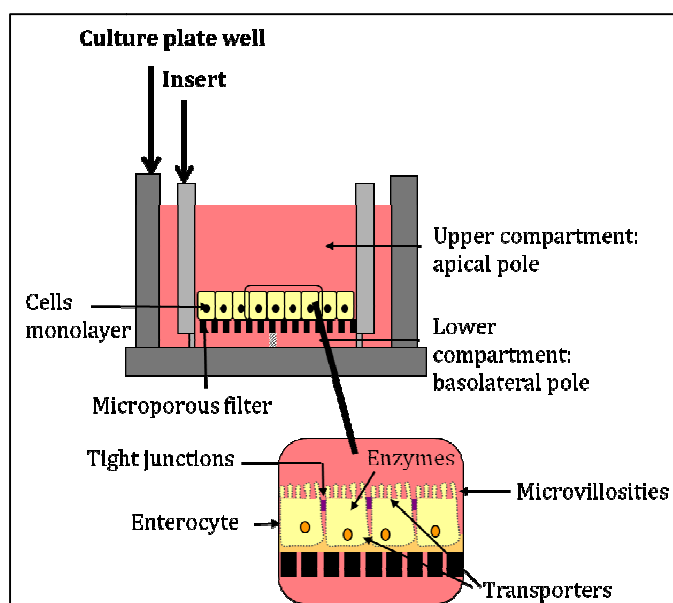


Figure 7-1: Schematic representation of the Caco-2 model of the intestinal barrier (illustration from Pr. Schneider, cours de technologie des cellules en culture, UCL, 2007).

Theoretically, the crossing of the intestinal membrane can be achieved by paracellular or transcellular pathways (figure 7-2), with both modes of transport making variable contributions to the total Se transported. During paracellular transport, nutrients simply diffuse through extracellular spaces along a concentration gradient. Because of numerous tight junctions that bind epithelial cells closely together, paracellular passage is limited to hydrophilic molecules of low molecular weight and low hydrodynamic dimension, which is the case of Se species under investigation here. On the other hand, when using a

more efficient transcellular pathway, nutrients enter the cells during their transport process. Since the phospholipid cell plasma membrane restricts the diffusion of hydrophilic Se compounds, passive or active membrane transporters are expected to be required for transcellular passage of Se (Knipp *et al.*, 1997; Tang & Goodenough, 2003).

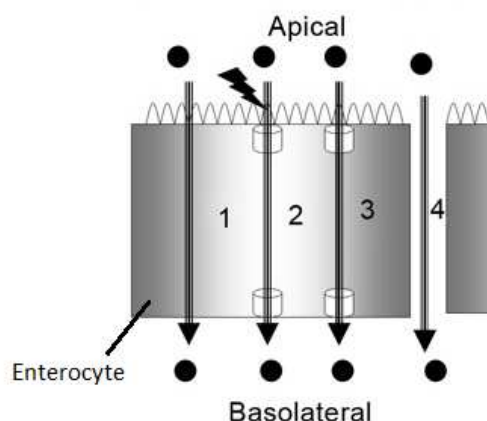


Figure 7-2: Possible mechanisms of Se transport across the intestinal epithelium.

1. Transcellular diffusion.
2. Active transcellular transport.
3. Facilitated transcellular diffusion.
4. Paracellular diffusion.

(illustration from Balimane, Han, & Chong, 2006).

In this way, this study aims at refining knowledge of the influence of the mode of transport of Se on its species-dependent bioavailability.

7.2. Materials and methods

7.2.1. Cell culture system

Caco-2 cells, originating from a human colorectal adenocarcinoma, were obtained from the American Type Culture Collection (ATCC HTB-37; Rockville, MD). Cells were stored in liquid nitrogen and aliquots were regularly defrosted for experiments. Caco-2 cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ (v/v) – 95% air in Dulbecco's modified Eagles medium (DMEM; Lonza, Verviers, BE) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone; Perbio-Sciences, Erembodegem, BE), 1% (v/v) non-essential amino acids (NEAA; Lonza) and 1% (v/v) L-glutamine 200 mM (Lonza). Cells were expanded in 175-cm² flasks and sub-cultured at 90% confluence with a trypsin-EDTA solution (Lonza). Each sub-cultivation incremented by one the passage number, and cells were used for experiments until passage number 60. After sub-cultivation, cells were seeded on permeable PE Transwell® cell culture inserts (24 mm diameter, 0.4 µm pore size; Corning;

Elscolab, Kruibeke, BE) at a density of 4×10^5 cells per insert. Inserts were placed in 6-well plates (Corning; Elscolab), delineating an upper (apical) and a lower (basolateral) compartments. Apical and basolateral compartments were filled respectively with 1.5 and 2.5 ml of medium of culture that was changed 3 times a week until full differentiation of cells (21 days post-seeding). Because serum contains traces of Se, the last medium change before starting the experiment used 0.5% FBS instead of 10%.

7.2.2. Monitoring of cell culture system

Several cell culture parameters were monitored to guarantee the suitability and reproducibility of the model. Integrity of the cell monolayer and epithelial tight junctions' effectiveness were verified prior to and after experiments by measuring the transepithelial electrical resistance (TEER; Endohm 24, World Precision Instruments, Sarasota, FL). The absence of Se influence on tight junctions functioning was assessed by measuring the permeability to $500 \mu\text{g ml}^{-1}$ lucifer yellow (LY; Sigma-Aldrich, St. Louis, MO) in the presence or absence of Se species, by fluorimetry at 485 nm (excitation) and 538 nm (emission) (Fluoroscanner Ascent FL, Thermo Fisher Scientific, Zellik, BE). As transport of LY occurs exclusively by extracellular diffusion, this compound was also used as a control for the degree of paracellular passage through the Caco-2 layer. Potentially induced cytotoxicity was estimated through the release of intracellular lactate dehydrogenase (LDH) into the extracellular medium. Activity of LDH was assayed by a cytotoxicity detection kit from Roche Diagnostics (Mannheim, DE) and compared to appropriate negative (absence of compound or condition to be tested) and positive (total cell lysis induced by 1% Triton, Sigma-Aldrich) controls.

7.2.3. Experimental design

7.2.3.1. Quantitative characterization of Se transport and influence of Se concentration

Selenium was added to the above-described cell culture system as a solution of individual Se species standards (Se(VI), Se(IV), SeMet and MeSeCys; Sigma-Aldrich) diluted in Hank's buffered salt solution (HBSS, Lonza) over a concentration range (25 - 50 - 100 and 200 ng Se ml^{-1}). Before addition of Se, cells were systematically pre-incubated for 20 min in Se-free HBSS. For each

treatment/replicate, a volume of 1.5 ml of the respective Se standards was added to the apical (AP) chamber, and 2.5 ml of HBSS containing 1 % (w/v) of bovine serum albumin (BSA; Sigma-Aldrich) was added to the corresponding basolateral (BL) chamber. Cells were maintained in these conditions at 37°C for 3 hours, a plausible duration for the passage of food through the small intestine. At the end of the 3h-period, both AP and BL culture media were directly collected in clear glass vials and appropriately diluted for Se quantification. Cells were doubly washed with Se-free phosphate buffered saline solution (PBS) and scrapped in 1 ml of this solution. One tenth of this quantity consistently served for protein determination via the Bicinchoninic Acid kit for Protein Determination (Sigma-Aldrich), and the rest was used for determination of cellular Se content. Cells in PBS were centrifuged at 13,000 rpm (13220 x g) for 4 min, supernatant was removed and an enzymatic extraction was applied to the pellet in order to liberate Se. Extraction was carried out with protease (protease XIV, Sigma) in Tris-HCl buffer 30 mM (Sigma-Aldrich) at pH 7.5 – 37°C with application of an ultrasonic probe (Vibra Cell, Bioblock Scientific, Illkirch, FR) for 1.5 min per sample. Certified reference materials (SELM-1 (selenized yeast) NRCC, Ottawa, CA ; BCR-185-R (beef muscle), IRMM, Geel, BE and NIST-2976 (mussel tissue), NIST, Gaithersburg, MD) were used to verify the efficacy of the extraction method. The preservation of Se species during extraction was verified by spiking experiments on SELM-1.

Methods for Se addition and sample collection were identical for all experiments in of this study and only specific differences in procedure for each experiment are further detailed.

7.2.3.2. Polarization of Se transport

Solutions of individual Se standards containing 100 ng Se ml⁻¹ were added either to the AP or BL compartment, and Se species were quantified in the opposite compartment after 3 hours. Lucifer yellow and radiolabelled ¹⁴C-mannitol (ARC, St. Louis, MO) were used as controls for paracellular passage. Mannitol was quantified using a liquid scintillation analyzer (Packard 1600 TR, Canberra, Meriden, CT) upon addition of scintillation solution (Pico-Fluor-15, Perkin-Elmer, Waltham, MA).

7.2.3.3. Influence of tight junction opening on Se transport

Solutions of Se standards (100 ng Se ml⁻¹) were added to the AP compartment for 3 hours. The opening of epithelial tight junctions was achieved by depriving them of calcium through the addition of ethylene glycol tetraacetic acid 2.5 mM (EGTA; Sigma-Aldrich), a chelator of Ca ions, to both AP and BL compartments.

7.2.3.4. Kinetics of Se transport

Solutions of Se species (100 ng Se ml⁻¹) were added to the AP compartment and aliquots from AP and BL media were sampled at 6 different times after Se addition (6 - 15 - 45 - 90 - 135 - 180 minutes). Volumes of collected aliquots corresponded to 5% of each chamber's volume, which represent 75 µl in the AP chamber and 125 µl in the BL chamber. Calculations of concentrations were adapted to remaining volumes. In this way, a constant ratio between the AP and BL volumes was maintained, preventing any effect of changes in solute concentration on Se transport.

7.2.3.5. Temperature-dependence of Se transport

Solutions of Se standards (100 ng Se ml⁻¹) were added to the AP compartment and transport took place at either 37°C or 4°C. For the 4°C-experiments, cells and solutions were pre-incubated for 20 min at this temperature in order to reduce enzymatic activity and to rigidify lipid plasma membranes, thus inactivating both active and passive carriers during Se transport. Lucifer yellow was added together with Se in order to compare the extent of paracellular transport at 37°C and 4°C. In contrast to other experiments, the duration of transport was for 2h instead of 3h in order to limit cold-induced cell damage.

7.2.3.6. Competition between selenium and sulfur analogues during transport

Caco-2 cells were treated with solutions containing Se species (100 ng Se ml⁻¹) in the presence or absence of their respective sulfur (S) analogues. The latter were added at concentrations 100-fold higher (10 µg S ml⁻¹) than the Se compounds. Respective analogues of Se(VI), Se(IV), MeSeCys and SeMet were

sulfate, sulfite, methylcysteine and methionine, respectively (all from Sigma-Aldrich). These compounds have identical molecular structures, but differ only by the presence of an S atom replacing a Se atom in the same position.

7.2.4. Chemical analysis of selenium

Selenium species were quantified in AP and BL culture media, as well as in scrapped cells. Selenium content of major medium components (DMEM, HBSS and FBS) was also determined. Speciation analyses were made with HPLC (Pro Star, Varian, Mulgrave, AU) coupled to ICP-MS (820-MS, Varian). The limit of quantification (LOQ) and the limit of detection (LOD) were calculated as respectively 6 times and 3 times the baseline standard deviation, and the LOQ was 0.5 ng Se ml⁻¹ for all Se species. Chromatographic separation of Se species was obtained with a reverse phase C8 Alltima column (250 mm x 4.6 mm id x 5 µm particle size) (Grace Davison Discovery Science, Lokeren, BE) using a mobile phase consisting of 0.2% (v/v) heptafluorobutyric acid (HFBA; Alfa Aesar, Karlsruhe, DE) in a gradient with 50% (v/v) methanol (HPLC grade; VWR, Leuven, BE). Total elution of all Se species was obtained within 10 min at a flow rate of 1 ml min⁻¹. The isotope ⁷⁸Se was used for measurement, with 90 ml min⁻¹ of H₂ as reaction gas to remove spectral polyatomic interferences on this mass. All dilutions were made with milliQ water (Millipore, Overijse, BE).

7.2.5. Statistical analysis

Results are given as means ± standard deviations (SD). Each experiment was repeated 3 times (N=3) and samples were produced in duplicate for each repetition (n=2). The effect of concentration on Se absorption was analyzed by one-way analysis of variance, followed by Tukey's HSD multiple comparison procedure in order to compare means of individual species. The effects of temperature and of the presence of EGTA and sulfur compounds were analyzed by a Student's t test in comparison to control conditions, *i.e.* respectively at 37°C and without EGTA or S. Software used was JMP 9.0.0 for Windows (SAS Institute, Cary, NC). Differences with a *p* value less than or equal to 0.05 were considered as being statistically significant (95% confidence level).

7.3. Results

7.3.1. Monitoring of cell culture system

Potential cytotoxicity was found to be less than 10% of the positive control for all experimental conditions. Transepithelial electrical resistance (TEER) values prior to the start of the experiment ranged from 1100 to 1400 Ω cm², which ensured the integrity of the cell monolayer (Biganzoli *et al.*, 1999). A decrease of about 10% of initial TEER values was systematically observed at the end of the experiment (except in the presence of EGTA, where there was a greater decrease). Average protein content of cells was 0.26 ± 0.08 mg per insert, corresponding to 56.7 μ g cm⁻². No Se was detected in either DMEM or HBSS, but a concentration of 20 ng Se ml⁻¹ was measured in pure FBS, mainly in the form of Se(IV) and SeMet. This means that 2 ng ml⁻¹ of Se was present in the 10% FBS nutritive medium. When 0.5% of FBS was used during the last two days of culture, Se background in media and cells was reduced to a value below the LOD. Transport of LY was similar (within 1 and 2 % of initial LY) in the presence or absence of Se compounds, with no difference between Se species, which indicated that Se had no effect on tight junctions opening.

7.3.2. Experimental results

7.3.2.1. Quantitative characterization of Se transport and influence of Se concentration

For all Se species and at all concentrations applied, Se species recovered in the BL compartment were identical to those that were initially added to the AP compartment, clearly indicating that no transformation of species occurred during transport. Within the applied concentration range (25 - 200 ng Se ml⁻¹), the degree of transport of Se(VI), Se(IV) and MeSeCys from the AP to BL compartment was proportional to the concentration applied (figure 7-3). Indeed, whatever the initial apical Se concentration, a constant fraction of Se (as a % of initial Se) was recovered in the BL compartment. In contrast, SeMet was recovered in the BL compartment in a smaller proportion when added at a concentration of 200 ng Se ml⁻¹ compared to the lower initial concentrations. Overall efficiencies of Se absorption were as follows: Se(IV) ($12 \pm 1\%$) < Se(VI) ($33 \pm 2\%$) < MeSeCys ($46 \pm 2\%$) < SeMet ($56 \pm 4\%$).

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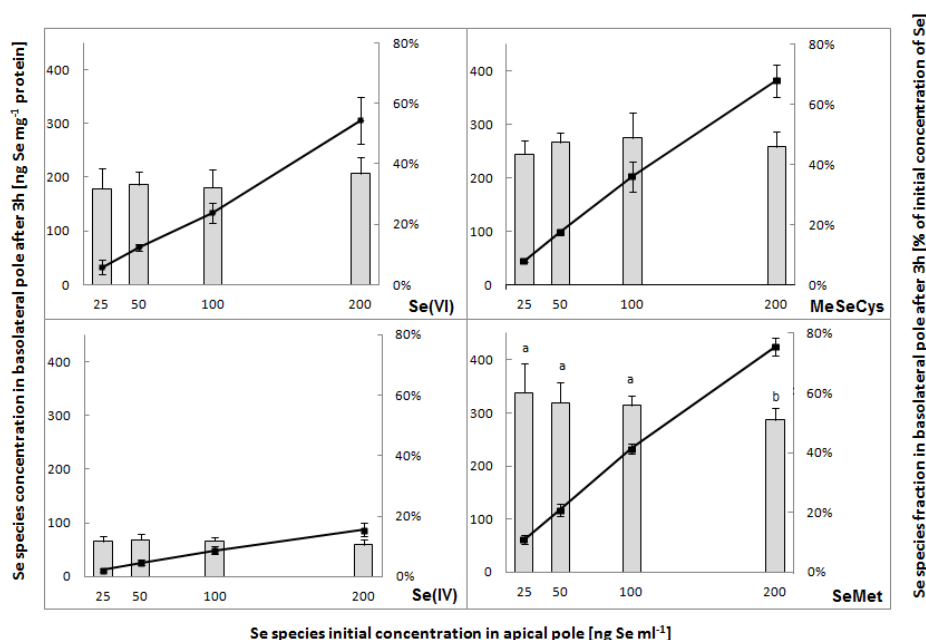


Figure 7-3: Concentrations and fractions of Se(VI), Se(IV), MeSeCys and SeMet in basolateral compartment 3h after having been added to apical compartment as individual standards at various concentrations (25 – 50 – 100 - 200 ng Se ml⁻¹). Lines show results expressed in [ng Se mg⁻¹protein] and columns show percentages [%] of initial added Se. For a given species, all Se values expressed in [ng Se mg⁻¹protein] were significantly different from each other while Se values expressed in [%] showing dissimilar letters were significantly different.

Figure 7-4 shows the distribution of Se species in different locations of the cell culture system 3h after the addition of Se species. Proteolytic extraction of cells revealed the presence of detectable amounts of Se following enrichment with both Se(IV) and SeMet. Intracellular Se(IV) and SeMet accounted respectively for 2% and 4% of initial Se.

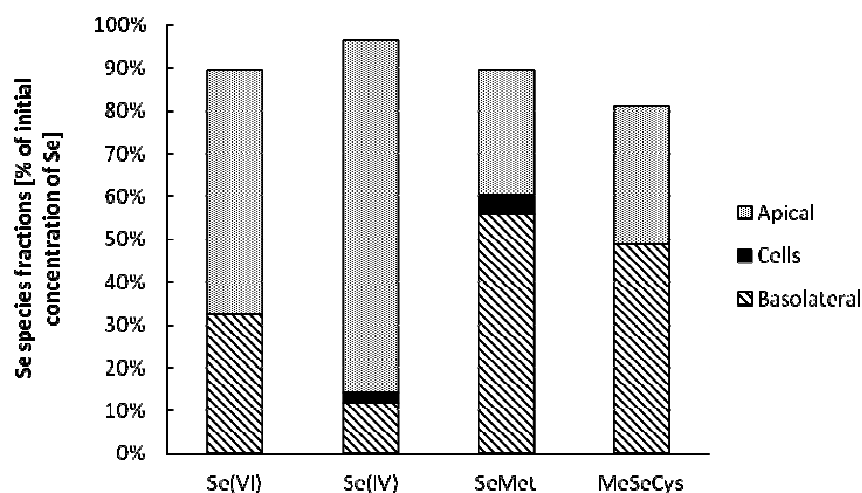


Figure 7-4: Distribution of Se(VI), Se(IV), SeMet and MeSeCys in different locations of the cell culture system 3h after having been added to apical compartment at 100 ng Se ml⁻¹.

7.3.2.2. Polarization of Se transport

To allow a better comparison between data obtained for Se, LY and mannitol, the results of this experiment were expressed as clearance values. These correspond to the volume of solution containing the compound of interest that passed across the cell monolayer per hour ($\mu\text{l h}^{-1}$) (Berger *et al.*, 2000). Data presented in table 7-1 shows that the BL to AP passage was low and very similar for all Se species and control substances (LY and mannitol). This suggests that all species were transported at the same low level, most probably by the paracellular route. Both LY and mannitol controls showed clearance ratios (*i.e.* clearance value in the AP-BL way compared to the same value for the BL-AP way) close to 1, which indicates a non-polarized transport. Among Se species, selenite also had a clearance ratio close to 1 (1.9), but the three other Se species showed considerably higher ratios, which means that their transport across the cell monolayer was importantly polarized in the AP-BL way. The accumulation of SeMet and Se(IV) in enterocytes was also polarized since no intracellular Se was detected following BL addition.

Chapter 7 - Degree of bioavailability of Se species and related mechanisms of intestinal transport

Table 7-1: Clearance values $\pm$ standard deviations and ratios for Se(VI), Se(IV), SeMet and MeSeCys (100 ng Se ml⁻¹) in comparison to mannitol and lucifer yellow (LY), from apical (AP) to basolateral (BL) pole and inversely, 3h after Se addition.

	Clearance [$\mu\text{l h}^{-1}$]		Clearance _{AP-BL} / Clearance _{BL-AP}
	AP-BL	BL-AP	
Se(VI)	101.4 $\pm$ 24.9	14.0 $\pm$ 4.3	7.3
Se(IV)	28.6 $\pm$ 15.0	15.2 $\pm$ 5.9	1.9
SeMet	233.9 $\pm$ 34.6	12.5 $\pm$ 1.9	18.7
MeSeCys	186.4 $\pm$ 6.4	13.9 $\pm$ 2.7	13.5
Mannitol	13.6 $\pm$ 1.7	12.9 $\pm$ 0.2	1.1
LY	9.3 $\pm$ 2.7	9.2 $\pm$ 0.8	1.0

7.3.2.3. Influence of tight junction opening on Se transport

The effect of EGTA on relaxing tight junctions and the concurrent increase in paracellular diffusion were verified after the 3h-treatment by TEER values that were reduced to 35% of their initial resistance, and by a 6-fold increase in LY clearance (from 9 to 54 $\mu\text{l h}^{-1}$). Selenite was the only species whose transport was amplified by EGTA (2.2-fold increase) (figure 7-5). All the other species had their passage lowered in the presence of EGTA.

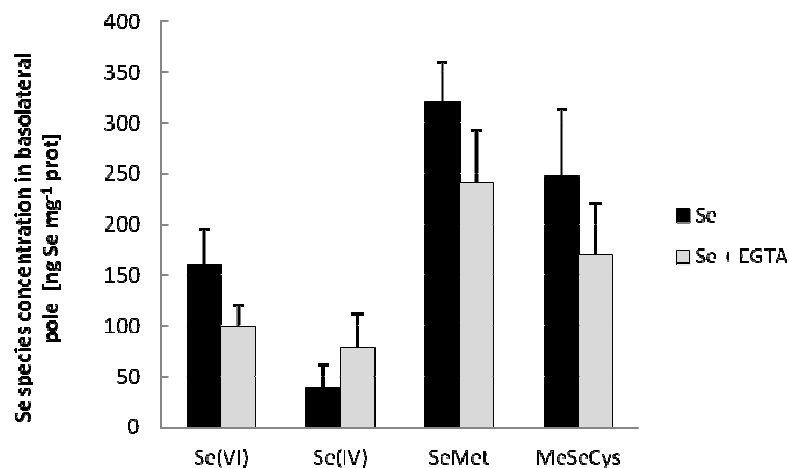


Figure 7-5: Concentrations of Se(VI), Se(IV), SeMet and MeSeCys in basolateral compartment 3h after having been added to apical compartment at 100 ng Se ml⁻¹ in the presence or the absence of EGTA. For each species, results in the presence or absence of EGTA were significantly different from each other.

7.3.2.4. Kinetics of Se transport

The concentration of Se in the BL chamber increased with the duration of exposure, regardless the species that was added to the AP compartment. However, organic and inorganic Se species showed distinct absorption profiles (figure 7-6):

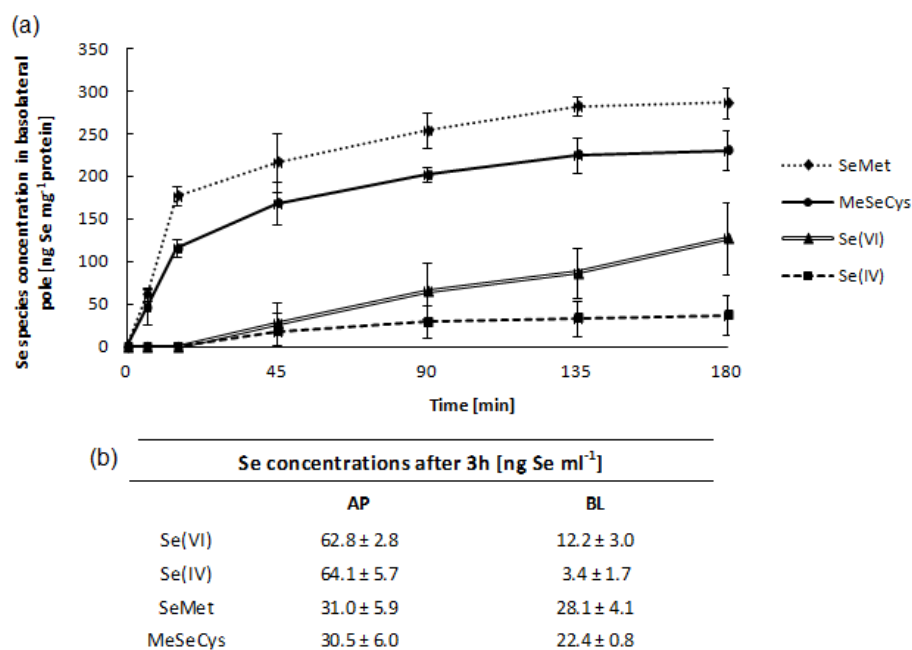


Figure 7-6: (a) Concentrations of Se(VI), Se(IV), SeMet and MeSeCys, per mg of protein, in basolateral compartment at 6 - 15 - 45 - 90 - 135 and 180 min after having been added to apical compartment (100 ng Se ml⁻¹). (b) Corresponding concentrations ± standard deviations of Se species per volume unit in AP and BL poles after 180 min.

- (i) Transport of SeMet and MeSeCys started very quickly after addition of Se to the AP compartment. After 15 min, Se concentration in the BL chamber was greater than 50% of the Se concentration measured after 3h. In contrast, inorganic Se species were only detected in the BL chamber 45 min after the start of exposure.
- (ii) While concentrations of Se(IV) and Se(VI) in the BL compartments were still increasing at the end of the 3h-experiment, absorption curves of SeMet and MeSeCys reached a plateau, suggesting that their transport was almost at its maximum. At that moment, final concentrations (per volume unit) were almost equal in both compartments for SeMet and MeSeCys. In contrast, final BL concentrations of the inorganic species were well below AP concentrations.

7.3.2.5. Temperature-dependence of Se transport

Selenite was the only species whose transport was not affected by low T°, similarly to LY (figure 7-7). At 4°C, there was no significant difference between final BL concentrations of Se species. In addition, the clearance values at this T° were very close to those found for LY. It is probable that, at 4°C, Se transport was only composed of the paracellular fraction.

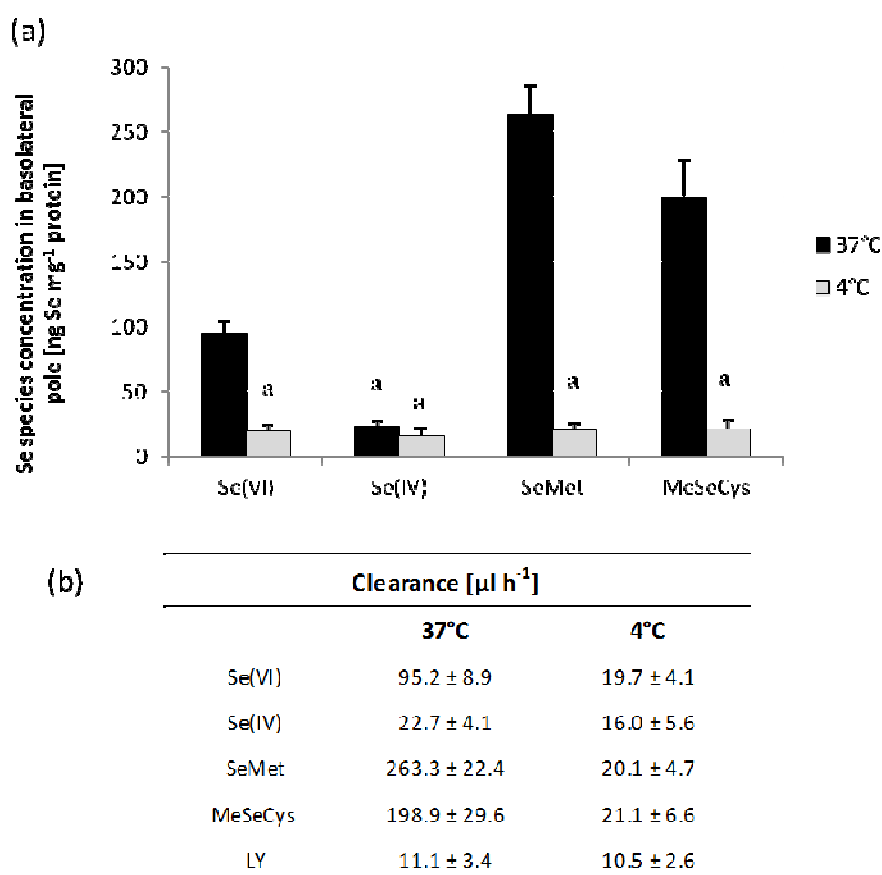


Figure 7-7: (a) Concentrations of Se(VI), Se(IV), SeMet and MeSeCys in basolateral compartment 2h after having been added to apical compartment at 100 ng Se ml⁻¹. Means showing a common letter are non-significantly different from each other. (b) Corresponding clearance values ± standard deviations for Se species and lucifer yellow (LY).

7.3.2.6. Competition for transport between selenium and sulfur analogues

Figure 7-8 shows that transport of SeMet and MeSeCys was significantly reduced in the presence of their respective S-analogues. The observed reductions were 62% and 60% respectively. Transport of inorganic Se species did not significantly decrease in the presence of sulfate or sulfite.

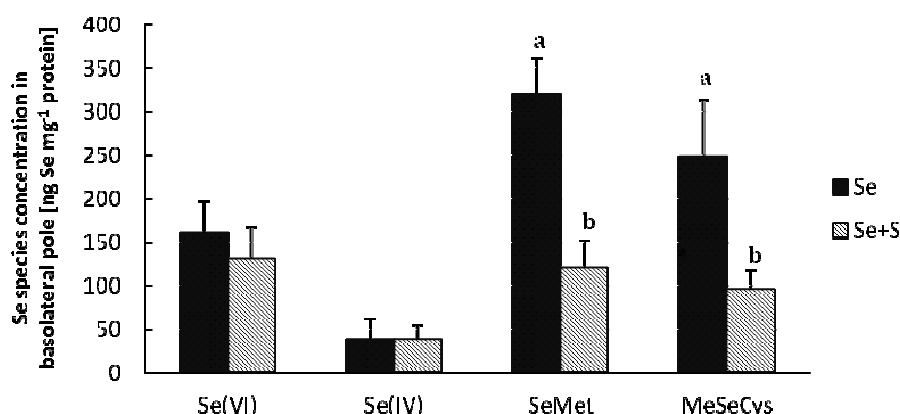


Figure 7-8: Concentrations of Se(VI), Se(IV), SeMet and MeSeCys in basolateral compartment 3h after having been added to apical compartment (100 ng Se ml⁻¹) in the presence or the absence of their sulfur analogue (10 µg Se ml⁻¹). For a given species, means showing different letters are significantly different from each other's.

7.4. Discussion

7.4.1. Cell culture system

Selenium bioavailability has been widely investigated using animal (Whanger & Butler, 1988; Wang & Lovell, 1977; Wang *et al.*, 2007; Wang & Xu, 2008; Rider *et al.*, 2010) and human (Thompson *et al.*, 1982; Levander, 1983) models, in which bioavailability was estimated by determination of Se content in blood or tissues after ingestion of specific Se species. Compared to *in vivo* models, recent *in vitro* cell culture systems allow more rapid and easy variations of some relevant parameters such as Se concentration, absorption duration, temperature or addition of certain components, which may help to obtain a deeper insight into mechanistic processes at the absorptive level. Caco-2 cells used in the present *in vitro* model originate from a human colon

adenocarcinoma and express, upon full differentiation, several morphological and biochemical characteristics of intestinal absorptive cells (enterocytes): cell polarization, presence of microvilli and tight junctions, and expression of membranous transporters (Sambuy *et al.*, 2005; Hidalgo & Li, 1996). In practice cells were cultivated on bicameral permeable inserts placed in culture wells in which the upper (apical) chamber mimicked the intestinal lumen while the lower (basolateral) chamber mimicked the bloodstream. Albumin, the principal plasma Se transporter (Suzuki *et al.*, 2009), was added to the basolateral compartment to simulate blood and extravascular fluids.

7.4.2. Selenium species and enrichment concentrations

Four Se species were tested: two inorganic (selenite (oxidation number IV) and selenate (VI)), and two organic (selenomethionine and methylselenocysteine). These species have been chosen because of their predominance in food and in food supplements usually consumed by the population, and because of their relative easiness to be obtained and analyzed. Working concentrations were determined on the basis of three criteria:

(i) The recommended daily Se intake. In Belgium, the recommended value for Se intake (RDA) was fixed at 60-70 $\mu\text{g day}^{-1}$ (SHC, 2009), a level supposed to guarantee maximal activity of selenoproteins (Monsen, 2000). It can be reasonably considered that a meal is dissolved in average into 1 to 3 liter(s) of liquid (drink water and digestive secretions). Considering a Se intake equivalent to Belgian RDA, Se concentrations reaching the intestinal duodenum should range from 24 to 60 ng Se ml^{-1} .

(ii) The Se-induced toxicity on Caco-2 cells. For concentrations ranging from 5 to 200 ng Se ml^{-1} , none of the considered Se species did generate cytotoxicity at level superior to 10% of positive control. Thereby it was considered that cultures were safe up to addition of 200 ng ml^{-1} of Se.

(iii) The limit of quantification (LOQ) of the ICP-MS. Analytical LOQ was stated for all species at 0.5 ng Se ml^{-1} , which implies that initial concentrations must be superior to 20 ng Se ml^{-1} in view of the dilution factor and the absorptive efficiency of Se through the cells monolayer.

Based on these three criteria, Se working concentrations were settled within 25 and 200 ng ml^{-1} for a first experiment, and the concentration of 100 ng ml^{-1}

was selected for the following tests. This concentration of 100 ng ml⁻¹ corresponds to a dose that is slightly superior to usual Se intake, but that remains in a reasonable order of magnitude and that allowed analytical response at early collection time-points of the kinetics experiment, or in the presence of sulfured Se inhibitors.

7.4.3. Efficiency of Se species transport

Selenium transport across the Caco-2 monolayer was clearly influenced by speciation. Organic species showed greater absorption than inorganic species over the 3 hours experimental exposure period. The extrapolation of our cellular model to the human intestinal tract would indicate that 56% of the SeMet released from the food matrix into the small intestine is absorbable through the intestinal barrier for potential further metabolic utilization, *i.e.* is bioavailable. Correspondingly, bioavailable fractions of MeSeCys, Se(VI) and Se(IV) are respectively 46%, 33% and 12%. In addition, Caco-2 cells were shown to accumulate significant quantities of Se(IV) and SeMet, exclusively from the apical pole. Neither Se(VI) nor MeSeCys were detected in cells. In a previous study, Caco-2 cells were reported to accumulate MeSeCys to a similar extent to Se(IV) (Zeng *et al.*, 2011). In that study, the duration of experiments was 72h versus 3h in the present study. It is therefore possible that Se(IV) accumulates more rapidly in cells compared to MeSeCys. Intracellular SeMet content detected in our study was higher than this of Se(IV). However, upon addition of high concentrations of Met, no SeMet was detected in cells. This illustrates the inability of cellular Met-tRNA to discriminate between SeMet and Met. Depending on the presence of SeMet or of Met in the extracellular environment, translational mechanisms permit the integration of one or the other compounds into proteins (Schrauzer, 2000). As long as SeMet supply is sufficient, it can accumulate in cells in substantial quantities (Swanson *et al.*, 1991). This ability of SeMet to be stored in body proteins represents an interesting mechanism that is not shared by other Se species having much shorter half-lives in the body.

7.4.4. Characterization of Se species transport

Selenium species transported via a transcellular route might undergo metabolic transformations inside cells prior to their release to the basolateral

side. In previous *in vitro* studies, bioavailable Se was systematically reported as total Se without reference to speciation (Leblondel *et al.*, 2011; Zeng *et al.*, 2011). In the present study, speciation analysis was performed by HPLC-ICP-MS on the products of the transport experiments. This showed that Se species remained in their original form after crossing the Caco-2 monolayer. Hence, Se species seem to cross the intestinal barrier without metabolic transformation.

The identification of para- or transcellular route was primarily based on the degree of polarization of Se species transport. A clearance ratio of 1 corresponds to a non-polarized transport and reveals that a compound should be exclusively using the paracellular route. Clearance ratios > 1 relate to compounds having an additional transcellular transport component whose importance is proportional to the size of this ratio. The use of EGTA was also expected to reveal the occurrence of para- or transcellular transport since this chelating agent has a particular affinity for calcium that is necessary to ensure epithelial tight junction integrity. The presence of EGTA entails a decrease in free Ca^{++} that causes loosening of the junctions and increases paracellular diffusion (Knipp *et al.*, 1997). As a consequence, in the presence of EGTA, Se species using the paracellular route should show enhanced transport in comparison with Se species transported transcellularly. Results obtained in this study were surprising in that sense that three of the four Se species had their transport lowered by EGTA rather than unchanged or slightly increased as it was expected considering that their paracellular fraction of transport had been amplified. An explanation could be the existence of a Ca-dependent transport process, which would have been inhibited by EGTA but, to our knowledge, such a mode of transport has never been reported for Se. Despite a lack of explanation for this decrease in Se transport, we could identify two distinct forms of behavior for Se species in combination with EGTA: either an increase or a decrease in Se passage. We propose that an EGTA-mediated increase in Se mobility should be indicative of a paracellular transport pathway, while a decrease suggests predominantly transcellular transport.

A second step in Se transport characterization was the detection of membrane-bound transporters. This was achieved by establishing kinetic profiles of Se species absorption. Carrier-mediated transport has typical enzyme-like kinetics *i.e.* rapid increasing absorption followed by a saturation plateau corresponding to a full occupation of transporters working at their maximal velocity (V_{max}). In contrast, paracellular diffusion has a profile of

absorption that is time-proportional, of low importance with saturation occurring after a much longer period, *i.e.* when concentrations are equal in both compartments. The existence of Se-transporters was also assessed by an investigation of the temperature-dependence of Se mobility. At low T°, most cellular enzymes are inhibited, which considerably slows down catabolic reactions providing ATP, and inactivates energy-dependent transporters. In addition, at 4°C, the plasma membrane becomes significantly more rigid so that membranous ion channels are expected to contract and get blocked or highly restricted. In this way, at 4°C, only non-carrier mediated transport is maintained.

Finally, concerning the identity of potential Se transporters, it is probable that mechanisms involved in S transport also play a role in transport of Se-compounds due to important chemical and morphological similarities between Se species and their S analogs (Johansson, Gafvelin & Arner, 2005). The existence of common transporters is expected to generate between Se and S a competition for absorption that is affected by the affinities of the transporters for one or the other type of compound.

7.4.4.1. Selenate (Se(VI))

In a previous *in vitro* study, Se(VI) was proposed to be transported by the paracellular route because a non-polarized transport had been observed (apparent permeability (P_{app}) ratio, *i.e.* parameter comparable to the clearance ratio, close to 1) (Leblondel *et al.*, 2011). However, our results do not support this hypothesis. Firstly, the clearance ratio was largely superior to 1 (7.3). Moreover, the presence of EGTA did not favor Se(VI) passage. Thirdly, despite the typically limited extent of paracellular diffusion, the efficiency of selenate transport reached 33%. Selenate passage was reduced to its paracellular fraction at low temperature which suggests a role for membrane transporters. We could not observe any significant competition between selenate and sulfate, despite a sulfate concentration 100-fold higher than selenate. A common transport mode for sulfate and selenate can however not be completely excluded because a slight inhibition was noticed, and because no saturation in selenate transport was observed after 3h. We can therefore hypothesize that in our experimental conditions, selenate/sulfate transporters were not saturated and allowed passage of both compounds. This hypothesis would support the conclusions of Wollfram and coworkers (1988) who identified, based on

experiments conducted on brush border membrane of pig jejunum, two active modes of transport (Na^+ - and H^+ -co-transporters) used by both sulfate and selenate.

7.4.4.2. Selenite (Se(IV))

Selenite had the lowest clearance ratio among Se species with a value of 1.9. This suggests that Se(IV) transport occurred mainly via a paracellular route. The absence of an effect of low T° and of the presence of sulfite, in combination with an increased transport in the presence of EGTA and a low and progressive absorption profile, were also all in favor of a paracellular mode of transport. These observations are in accordance with the conclusions of a previous study suggesting a passive diffusion of selenite based on a P_{app} ratio ~ 1 .

7.4.4.3. Selenomethionine (SeMet) and methylselenocysteine (MeSeCys)

Selenomethionine and methylselenocysteine behaved rather similarly under current experimental conditions. Their AP to BL transport was highly polarized, suggesting a dominant transcellular transport mode. In addition, SeMet and MeSeCys transport strongly decreased at low $T^\circ\text{C}$ and was partially inhibited by their respective S-analogues. Our results thus indicate that SeMet and MeSeCys are transported at least to a certain extent by an apical transport system shared with their S-analogue. Whereas there is little published data on MeSeCys absorption, the existence of a common transporter for SeMet and Met is better documented (Leblondel *et al.*, 2001; Bakke *et al.*, 2010). Two active mechanisms of SeMet transport were identified in differentiated Caco-2 cells, one of them being Na^+ -dependent (Vendeland *et al.*, 1994). These transporters were reported to have similar affinity for Se- and S-compounds (Nickel *et al.*, 2008). In our experiment, inhibition of Se transport by S compounds was not complete although Met and MeCys concentrations were 100-fold higher than those of the corresponding Se compounds. This suggests the existence of additional transport mechanisms.

Selenomethionine and methylselenocysteine both exhibited saturation kinetics of absorption following their addition at 100 ng ml^{-1} to the AP compartment. Saturation was concomitant with the diminution of the concentration gradient existing between the AP and BL chambers. Velocity of transport depended on the importance of the initial concentration gradient

applied to the cell culture system. Indeed, larger final Se concentrations were measured in BL pole after initial Se concentrations of 200 ng ml⁻¹ compared to 100 ng ml⁻¹, demonstrating a more rapid transport associated with the highest initial concentration of Se. Final BL concentration of MeSeCys was exactly 2-times more important when initial concentration was doubled. In case of SeMet, final BL concentration was slightly lower than the exact double, meaning that maximal velocity ($V_{\max}$) of SeMet transporters was closer to being reached compared to MeSeCys. The $V_{\max}$ values of Se transport seem thus to be species-dependent, with SeMet transporters reaching their $V_{\max}$ at slightly lower Se concentrations in comparison with MeSeCys transporters.

7.5. Conclusion

The existence of a species-dependent degree of bioavailability of Se is well-known. In this study, bioavailability of Se could be related to differences in efficiency of Se species absorption at the intestinal level. An explanation for these differences in Se absorption is the fact that Se species follow distinct transport routes through the intestinal barrier. Selenite crosses the intestinal epithelium almost exclusively by a passive paracellular pathway. In contrast, selenate transport is for a great part transcellular with a possible intervention of the sulfate transporter. Both SeMet and MeSeCys follow transcellular pathways and are able to use the same apical transporters as their S-analogues. In addition, SeMet can be stored in cellular proteins, which represents a Se supply in case of shortage. In line with their modes of transport, selenite turns out to be poorly bioavailable, while SeMet is the most available Se species, followed by MeSeCys.

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Chapter 8

Selenium bioaccessibility and bioavailability in Se-enriched food supplements

Chapter 8 - Selenium bioaccessibility and bioavailability in Se-enriched food supplements

Foreword

After having used Caco-2 cells to establish the degrees of bioavailability of individual standards of Se, we considered extending the model to real food products in order to quantify as absolute value their degree of Se bioavailability, in contrast with the relative values provided in chapter 6. In comparison to individual species, food can contain different species of Se and these species are entrapped in a matrix. Because of the matrix, an additional step of *in vitro* digestion must be conducted prior to measure the absorption through the Caco-2 monolayer. This step is expected to limit to a certain extent the final bioavailability of Se. In the present chapter, we focused on Se bioavailability from Se-rich food supplements. The choice of food supplements is related to the rather low Se intake of Belgium inhabitants who are thus expected to commonly recourse to such products. In addition, as food supplements consist in relatively simple matrices and Se profiles, this facilitated Se speciation analysis and thus permitted to test in a relatively comfortable way the methods for bioaccessibility and bioavailability determination. In Belgium, three types of food supplements, with different Se species profiles, are commonly distributed. Based on the previous chapters, we predict that the difference in Se speciation could result in different degrees of Se bioavailability among the food supplements. This chapter was thus intended to quantify the efficiency of absorption of Se from several food supplements having distinct Se profiles, and in this way estimate their actual capacity in improving the Se status of a potential consumer.

Chapter 8 - Selenium bioaccessibility and bioavailability in Se-rich food supplements

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Abstract

Most European people have a Se intake inferior to recommended values that are considered necessary to ensure the beneficial action of antioxidant selenoproteins. People could therefore tend to have recourse to Se-enriched food supplements (FS) aiming to increase their Se body level. On the Belgian market, three main types of Se-rich FS are available: Se-enriched yeast, selenate-based FS and selenite-based FS. In the present work, *in vitro* tests imitating gastro-intestinal digestion and intestinal absorption were used to determine the bioaccessible and bioavailable fractions of Se present in one specimen of each category of FS. The aim of the study was to verify to which extent the difference in Se speciation could influence the efficiency of FS for enhancing the human Se status. *In vitro* digestion was a two step process mediated firstly by pepsin at pH 2 and secondly by pancreatin and bile salts at neutral pH. *In vitro* absorption was realized by using a cellular model consisting in Caco-2 cells mimicking the intestinal barrier in a bicameral culture system. Results indicated that differences exist in both bioaccessibility and bioavailability between the three types of FS, and that these differences could be related, at least partially, to the Se species profile. Overall bioavailability of the three FS was low (maximum 14% of the original Se content). Among the three samples, the selenate-based FS produced the highest fraction of bioavailable Se, followed by Se-yeast, and finally by the selenite-based FS for which Se was almost not available at all. These results confirm the low availability of inorganic Se but were somewhat unexpected regarding the yeast-based FS since Se-rich yeasts are usually reported to contain an important fraction of available Se.

Keywords

Selenium, food supplements, *in vitro*, bioaccessibility, bioavailability

8.1. Introduction

Selenium is an oligo-element that exerts essential functions through the action of several types of selenoenzymes and -proteins (Gromer, 2005). The levels of Se intake recommended to benefit of Se action have been set to 55 $\mu\text{g day}^{-1}$ by the Scientific Committee on Food of the European Commission (SCF,

2000), and to a range of 60-70 $\mu\text{g day}^{-1}$ by the Superior Health Council in Belgium (SHC, 2009). In many European countries, people have a level of Se intake that is relatively low compared to these recommended values. In Belgium the average dietary intake of Se by the adult population was recently calculated to be 60 $\mu\text{g day}^{-1}$ (Waegeneers *et al.*, 2012), in France this value was 53.7 $\mu\text{g day}^{-1}$ (AFSSA, 2009), and in the UK, the range of Se intake has been set between 48-58 $\mu\text{g d}^{-1}$ (Rose *et al.*, 2010). In Finland, a global strategy of food Se-enrichment was carried out in 1984 in order to improve Se status of inhabitants. At that moment, application of selenized fertilization to practically all Finnish crops became obligatory. This measure resulted in a significant enhancement of the Se content of food (up to a 20-fold increase in spring wheat). Consequently the average Se intake by Finnish people increased from 38 $\mu\text{g d}^{-1}$ prior crops fortification to 80 $\mu\text{g d}^{-1}$ in 2001, with a concurrent enhancement of Se concentrations in plasma (Varo *et al.*, 1988; Rayman, 2008).

The Finnish example is however an isolated case of national action against Se deficiency and, in general, people recourse more readily to Se-enriched food supplements (FS) to improve their Se status. During the past decade, a variety of Se-enriched FS have emerged on the European market. The large majority of them can be classified in three distinct categories: Se-enriched yeast, selenate-based FS and selenite-based FS (communication from the Belgian Public Health Ministry). The last two are totally inorganic and contain either selenate or selenite usually as sodium salts surrounded by excipients. Yeast-based FS typically consist of Baker's yeast (*Saccharomyces cerevisiae*) appropriately cultivated in the presence of an inorganic source of Se (usually selenite) that yeast can accumulate and metabolize into organic Se species (Suhajda *et al.*, 2000), mostly SeMet non-specifically incorporated into peptide chains (Yoshida *et al.*, 2002; Reyes *et al.*, 2006). Hence, yeast represents a mean to obtain at low cost high quantities of Se under organic forms having been reported to be less toxic than inorganic Se (Ammar & Couri, 1981; Tiwary *et al.*, 2006). The exact composition of such yeast-based FS remains however uncertain because many metabolic intermediates, known or unknown, are present in variable proportions in addition to SeMet (Rayman, 2004). Recently up to 49 different selenometabolites could be identified in yeast samples by advanced hyphenated techniques of 2D chromatography coupled to Se-specific detectors ICP-MS and ESI-(multi)MS (Preud'homme *et al.*, 2012).

Chapter 8 - Selenium bioaccessibility and bioavailability in Se-rich food supplements

The efficiency of orally ingested Se in fulfilling its essential activity of selenoenzyme production is dependent on several previous steps determining Se bioaccessibility and bioavailability, which are in turn influenced by different factors such as the kind of food matrix entrapping Se, the presence of other food components that could interact with the element and the chemical form (speciation) of Se. The bioaccessible fraction of Se can be defined as the part of ingested Se that is released from the food matrix in the lumen of the intestine, and that can thereby possibly be absorbed through the intestinal mucosa (Ruby *et al.*, 1999). The bioavailable fraction is considered to be the part that is effectively absorbed through the intestinal epithelium, and is therefore a sub-fraction of the bioaccessible Se. From this bioavailable fraction, a subsequent bioactive fraction will finally lead to selenoprotein synthesis (Thiry *et al.*, 2012).

In this study we focussed on bioaccessibility and bioavailability of Se originating from different FS belonging to one of the three above-mentioned categories (*i.e.* Se enriched yeast, selenate-based FS or selenite-based FS). We selected one FS from each category and estimated the bioaccessible and bioavailable fractions of their Se content through *in vitro* methods. Bioaccessibility was measured through simulated gastro-intestinal digestion consisting in a first step of pepsin-mediated digestion at acidic pH, followed by a second intestinal step conducted at neutral pH with pancreatin and bile salts. Bioavailability was estimated through the use of a cellular (Caco-2) model mimicking the intestinal barrier. In this way, this study aimed to evaluate differences in Se efficiency that are suspected to exist between the three categories of most commonly taken Se-enriched FS.

8.2. Materials and methods

8.2.1. Sample collection, sample preparation and experimental approach

Food supplements were bought in a local pharmacy and an organic store. The three specimens of FS consisted in a Se-enriched yeast (LepiVits, Vincennes, FR), a multi-mineral and vitamin complex containing Se as sodium selenate (Bion 3, Merck, Dijon, FR) and a multi-mineral and vitamin complex containing Se as sodium selenite (Azinc Optimal, Arkopharma, Wavre, BE).

These samples are further referred to as yeast-FS, selenate-FS and selenite-FS, with reference to their type. According to the information provided by the producer, total Se concentrations of the samples were: yeast-FS, 167 $\mu\text{g Se g}^{-1}$; selenate-FS, 30 $\mu\text{g Se g}^{-1}$ and selenite-FS, 35 $\mu\text{g Se g}^{-1}$. Yeast-FS and selenate-FS were formulated as pills and were crushed in powder by pillar for homogenization. Selenite-FS was a capsule containing powder that was emptied. Homogenized samples were stored in the dark in polyethylene pots at room temperature prior to analysis. A schematic presentation of the experimental approach is presented in figure 8-1 and procedures are detailed in the following paragraphs.

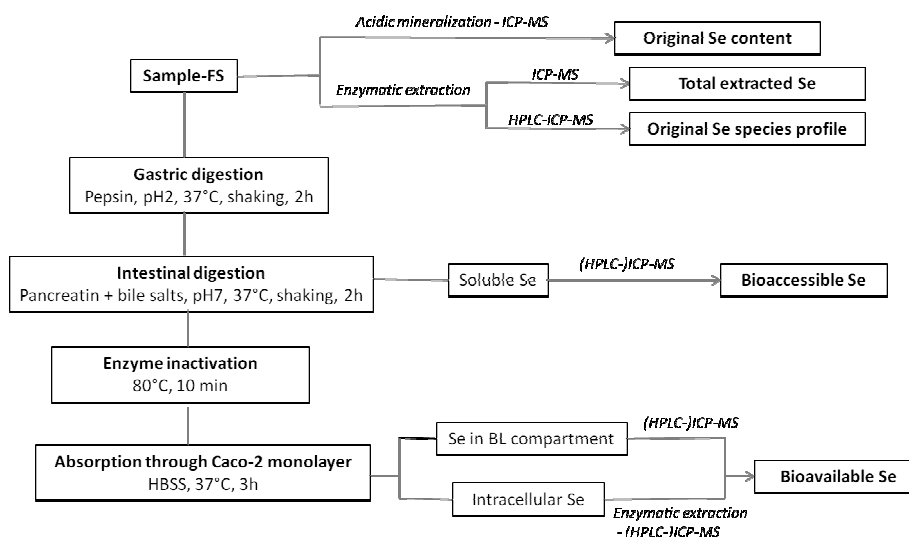


Figure 8-1: Schematic presentation of the experimental approach.

8.2.2. Selenium analysis

For verification of the information supplied by the producer, total Se concentration in the FS samples was measured by ICP-MS (820-MS, Varian) after acidic mineralization (50% nitric acid (v/v)) in microwave (X-PRESS; CEM Corporation, Matthews, NC, USA). Isotopes 77 and 78 of Se were measured with use of 90 ml min^{-1} of H_2 as reaction gas to remove interferences on these masses. Speciation analysis was realized by coupling HPLC (Pro Star, Varian, Mulgrave, AU) to ICP-MS. Chromatographic separation of Se species was obtained with a reverse phase C8 Alltima column (250 mm x 4.6 mm id x 5 μm particle size) (Grace Davison Discovery Science, Lokeren, BE) and Se was eluted

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with a mobile phase containing 2% (v/v) heptafluorobutyric acid (Alfa Aesar, Karlsruhe, GE) with a gradient of 50% (v/v) methanol (HPLC grade; VWR, Leuven, BE). Total elution was obtained within 10 min at a flow rate of 1 ml min⁻¹. All dilutions were made with milliQ water (Millipore, Overijse, BE). Identification of Se species was realized through matching of retention times with individual standards of corresponding Se species, and quantification occurred by external calibration with the same standards. Limits of quantification (LOQ) values were 0.12 µg per liter of measured solution for total Se, and 0.5 µg l⁻¹ for Se species.

8.2.3. Selenium extraction

Proteolytic extraction of Se was achieved in order to identify Se species in original FS samples and in Caco-2 cells after *in vitro* absorption experiments. This extraction was carried out with protease (protease XIV, Sigma-Aldrich, St. Louis, MO) in Tris-HCl buffer 30 mM (Sigma-Aldrich) at pH 7.5 – 37°C, with application of ultrasonic probe (Vibra Cell, Bioblock Scientific, Illkirch, FR). The process was repeated three times on FS samples in order to extract the maximum of Se. After that, samples were centrifuged and Se species were quantified in the liquid fraction. Details on the procedures applied to both types of samples are provided in table 8-1.

Table 8-1: Main characteristics of Se enzymatic extraction procedures applied to original FS samples and to post-absorption Caco-2 cells.

	FS samples	Cell samples
Protease	20 mg	5 mg
Tris	10 ml	2 ml
US probe	2 min	1 min
Centrifugation	30 min at 8000 rpm	-
Repetition	3 times	1 time

8.2.4. Bioaccessibility test - *in vitro* gastric and intestinal digestions

Bioaccessible fraction of Se from FS was considered to be the fraction of Se that was soluble in a digestive juice during the process of an *in vitro* gastrointestinal digestion:

$$\text{Bioaccessibility [\%]} = \frac{\text{Se in intestinal digest}}{\text{Se in original FS}} \times 100$$

The digestion protocol was inspired from the study of Gangloff and co-workers (1996) who investigated iron absorption on Caco-2 cells after having digested iron-containing samples *in vitro*. The digestion was sequenced in two successive steps, referred to as gastric and intestinal digestions (figure 8-1). The salivary digestion step was not addressed in this work owing to the fact that FS are usually directly swallowed. Quantities of sample used for digestion were dependent on the original Se content of FS and were: selenite-FS - 280 mg, selenate-FS - 320 mg and yeast-FS - 58 mg. Such quantities were intended to permit Se concentration in final digested juices of approximately 200 ng Se ml⁻¹. Samples were weighted in 100 ml polycarbonate erlenmeyers (Corning; Elscolab, Kruibeke, BE) and diluted in 40 ml of Hank's buffered salt solution (HBSS, Lonza, Verviers, BE) at pH 2.0. For the gastric digestion, 2 ml of a solution of porcine pepsin from gastric mucosa (40 g l⁻¹ in HCl 0.1M) were added to the samples. Erlenmeyers were then saturated with nitrogen to create an anaerobic environment and they were placed for 2 hours in an agitating (350 rpm) water bath at 37°C. After the gastric digestion, 1 ml of the digest was taken and reserved for further Se analysis. For the intestinal digestion, pH was elevated up to 5.3 with NaHCO₃ 1M, and 6 ml of a solution of porcine bile extract mixture and pancreatin from porcine pancreas (both at 3.33 g l⁻¹ in NaHCO₃ 0.1M) (Sigma-Aldrich) were added to the samples. Pancreatin contains several enzymes including amylase, lipase, ribonuclease, trypsin and protease. The pH was then re-adjusted up to pH 7.0, erlenmeyers were saturated with nitrogen, and the intestinal digestion occurred for 2 h in an agitating (350 rpm) water bath at 37°C. At the end of the digestion, digestive enzymes were inactivated by placing erlenmeyers for 10 min at 80°C. Finally, samples were centrifuged for 15 min at 3000 rpm (1510 x g) and the supernatant was kept for Se analysis and further study of bioavailability.

8.2.5. Bioavailability test - *in vitro* intestinal absorption

The bioavailable fraction of Se from FS was considered to be the sum of, firstly, the fraction of soluble Se that was transported through a monolayer of Caco-2 cells in a bicameral culture system and, secondly, the fraction that was retained in cells during the process of absorption (figure 8-1):

$$\text{Bioavailability [\%]} = \frac{\text{Se in basolateral pole} + \text{Se in cells}}{\text{Se in original FS}} \times 100$$

Caco-2 cells were cultured on microporous inserts according to the procedure described in chapter 7, section 7.2.1. "Cell culture system". For the transport experiments, solutions of bioaccessible Se obtained from the *in vitro* digestion were diluted 1:1 (vol/vol) in Hank's buffered salt solution (HBSS; Lonza, Verviers, BE) in order to limit cytotoxicity induced by bile salts, and they were added to apical compartment while HBSS containing 1% (w/v) albumin (BSA; Sigma-Aldrich) was added to basolateral chamber. Absorption process lasted 3 hours, a probable duration for the passage of food in the small intestine. After that, basolateral media were collected, as well as cells that were scrapped out, and both were analyzed for their Se content. Potential cytotoxicity of the substances used was estimated through the importance of cell lyses, which were determined by the release of intracellular enzyme lactate deshydrogenase (LDH) into extracellular medium (Cytotoxicity detection kit, Roche Diagnostics, Mannheim, GE). The integrity of the Caco-2 monolayer was verified by measuring the TEER prior to and after the passage experiments. TEER values ranged between 1300 and 1500 Ω cm² after 21 before the experiment and were systematically about 10% lower at the end of the 3h-transport. Cells were used within passage numbers 45 and 53.

8.3. Results

8.3.1. Concentration of Se and its speciation in original FS

The acid mineralization conducted on FS permitted to recover 100% of the Se content reported on the package, which confirmed the information provided by the producer. The triple proteolytic extraction released the totality of Se from inorganic Se-FS and 86% of Se from yeast-FS (table 8-2). Regarding yeast, the first extraction released about 90% of extractable Se and the second extraction released the 10% remaining. The third extraction did not release

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significant quantities of Se, which ensured that extraction efficiency was at its maximum.

Table 8-2: Total Se and Se species concentrations [ng Se mg⁻¹], ± standards deviations, in original food supplements determined by (HPLC-)ICP-MS after US-enzymatic extraction.

	Original Se content	Total extracted Se	Se(VI)	Se(IV)	SeMet	MeSeCys
Yeast-FS	167	144 ± 3	ND	29 ± 9	56 ± 6	6 ± 4
Selenate-FS	30	31 ± 2	30 ± 1	ND	ND	ND
Selenite-FS	35	35 ± 1	ND	32 ± 2	ND	ND

(N=3, n=3), ND: not detected.

Selenate-FS and selenite-FS contained exclusively those species but Se yeast generated a variety of peaks (figure 8-2). Among them, three were identified as SeMet (the main species), Se(IV) and MeSeCys. The other detected peaks were unknown species, probably selenopeptides that could not be quantified due to the lack of appropriate standards of calibration.

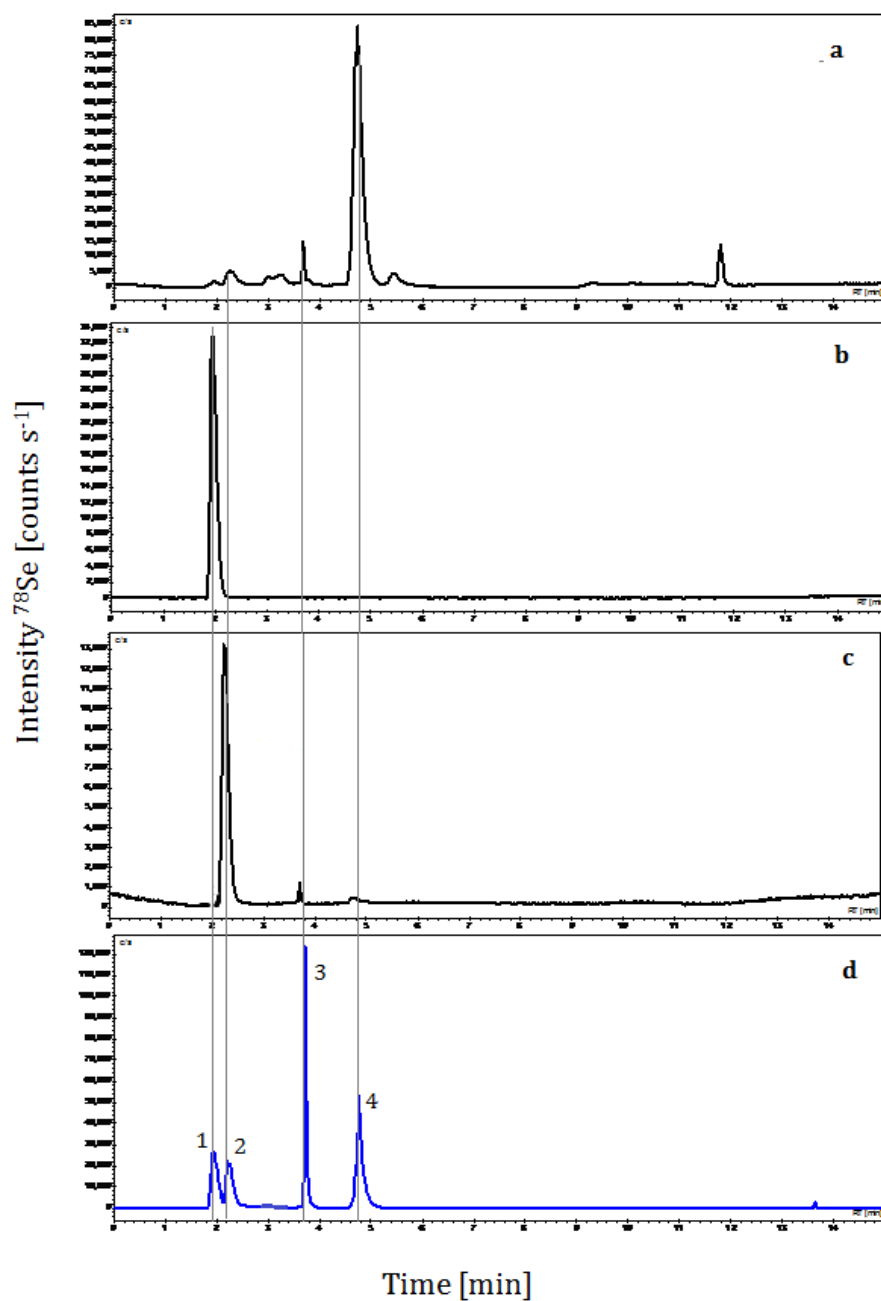


Figure 8-2: Reversed-phase HPLC-ICP-MS chromatograms of the three original food supplements samples under investigation, in comparison with Se species standards.

a yeast-FS, **b** selenate-FS, **c** selenite-FS, **d** Se species standards (10 ppb)

1 Se(VI), 2 Se(IV), 3 MeSeCys, 4 SeMet

8.3.2. Bioaccessible and bioavailable fractions of total Se from FS

A 1:1 dilution of the final FS digests in HBSS permitted to limit cytotoxicity induced by bile salts to values lower than 10% of the positive control (LDH release test), thereby guarantying the safety of the cells (results not illustrated).

Figure 8-3 shows the bioaccessible and bioavailable fractions of Se as percentages of the original Se content of FS. Neither the *in vitro* digestion nor the *in vitro* absorption did modify the original speciation of Se. For the three FS, the totality of Se released during the *in vitro* digestion was liberated during the gastric step, and no additional Se was released during the intestinal step.

8.3.2.1. Yeast-FS

Only 57% of Se from yeast-FS was liberated through the *in vitro* gastrointestinal digestion and was thus considered as bioaccessible. During the phase of absorption, some Se from yeast-FS accumulated in Caco-2 cells and the bioavailable fraction of Se corresponded therefore to the sum of Se content in basolateral compartment and in cells (respectively 6% and 1% of original Se content). Speciation of Se in the different fractions will be addressed in paragraph 7.3.3.

8.3.2.2. Selenate-FS

The totality of Se present in selenate-FS was bioaccessible. Fourteen percents of this quantity were absorbed through the Caco-2 monolayer and corresponded to the bioavailable fraction of Se. No Se was detected in cells upon selenate-FS presence.

8.3.2.3. Selenite-FS

The bioaccessible fraction of Se in selenite-FS represented 80% of the original Se content, while its bioavailable fraction was barely of 1%. No Se was detected in cells.

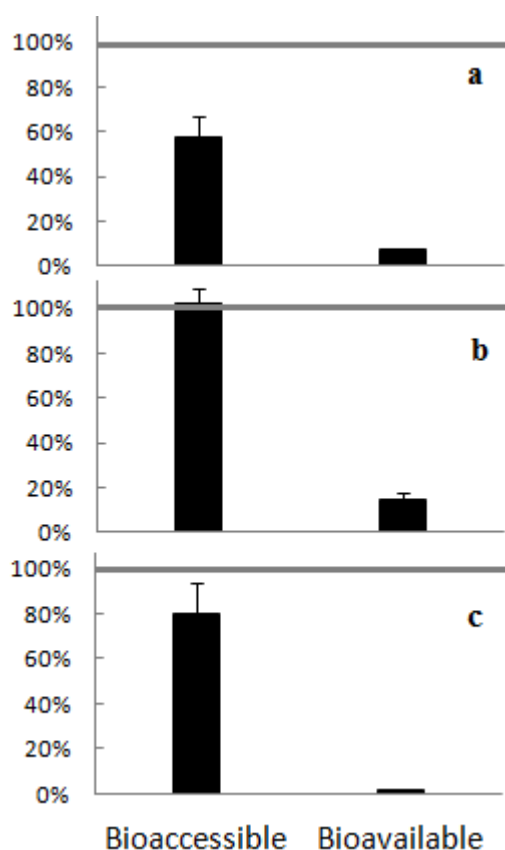


Figure 8-3: Percentage of original Se from **a** yeast-FS, **b** selenate-FS and **c** selenite-FS that was released in gastro-intestinal juice (i.e. bioaccessible Se), and absorbed through Caco-2 monolayer or accumulated in cells during a 3h period (i.e. bioavailable Se). Results are given as means $\pm$ standard deviations ($N=3$, $n=3$).

8.3.3. Bioaccessible and bioavailable fractions of Se species from yeast-FS

Figure 8-4 presents the Se species profiles in bioaccessible and bioavailable fractions of Se in yeast-FS. On the one hand, SeMet is shown to be less accessible (70% of the original SeMet content) than Se(IV) and MeSeCys for which the totality of the original content was retrieved in the digested solution. On the other hand, SeMet was the only species detected in the basolateral compartment and in cells after *in vitro* absorption. The bioavailable fraction of SeMet accounted for 24% of the original SeMet content, and of about 35% of bioaccessible SeMet.

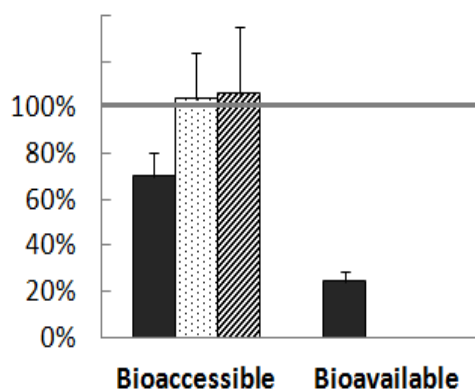


Figure 8-4: Percentage of original Se species from yeast-FS that were released in gastrointestinal juice (i.e. bioaccessible Se), and absorbed through Caco-2 monolayer or accumulated in cells during a 3h period (i.e. bioavailable Se). ■ SeMet (selenomethionine), ▨ Se(IV) (selenite) and ▩ MeSeCys (methylselenocysteine). Results are given as means $\pm$ standard deviations ($N=3$, $n=3$).

8.4. Discussion

Because Se intake of Belgian people is lower than recommended values, and considering that the majority of local food products have low Se content (Rayman, 2000), the use of Se-enriched FS is an evident option in order to increase Se dietary intake. In this study, the degrees of Se bioaccessibility and bioavailability from three different kinds of FS were investigated through *in vitro* methods that imitated the human digestive tract from the entry of the FS in the stomach, to the intestinal absorption and delivery of its Se content in the bloodstream. These *in vitro* approximations present the advantages to be easier, faster, cheaper and more controllable than if having monitored human subjects.

8.4.1. Bioaccessibility

A common observation for the three investigated FS was that the totality of the bioaccessible Se was liberated at the gastric stage of the *in vitro* digestion. The gastric phase consists in the first proteolytic attack of the digestion and occurs through the action of pepsin/HCl, while the further intestinal phase, mediated by bile and pancreatin (mixture of proteases, amylase and lipase), permits a second peptidic hydrolysis and the breakdown of other categories of

food components (Jackson & McLaughlin, 2009). The rapid release of selenite and selenate from the FS matrix was awaited considering the high degree of solubility of inorganic Se species. In yeast-FS, our model seems to indicate that the pepsin-mediated proteolysis would be sufficient to release the maximum of soluble Se present in yeast. This could illustrate the fact that SeMet, *i.e.* the major species present in yeast, occurs entrapped into proteins that are surrounded by a relatively simple matrix. In this scheme, Se compounds remaining in the matrix after the *in vitro* digestion should be insoluble or linked to insoluble fractions of the yeast. Significant amounts of Se have notably been detected in the cell-wall constituents of Se-enriched yeasts (Polatajko *et al.*, 2003).

The three FS were not equal in terms of Se bioaccessibility. The difference was especially marked between FS containing inorganic Se (selenate or selenite), and Se-rich yeast. The totality of Se contained in selenate-FS was bioaccessible and this value was 80% in selenite-FS. In contrast, the liberation of Se from yeast-FS was only 57%. Different factors could explain the low bioaccessibility of Se from yeast-FS. Firstly, contrary to inorganic-based FS, yeasts constitute a matrix, though being relatively simple, from which Se must be extracted. Secondly, Se-rich yeast contains a variety of unknown species potentially non-bioaccessible (*e.g.* insoluble or volatile) which can decrease the overall bioaccessibility of Se. Thirdly, the major Se species in yeast-FS is SeMet that, unlike other Se-species, is stored for a large part in the proteins of the food matrix. As a result, the majority of SeMet is more fixed into the yeast matrix than other species and is thus more difficult to extract (Schrauzer, 2006). In that sense, the importance of SeMet content in yeast may be determinant to assess its Se bioaccessibility: the more SeMet is present, the less bioaccessible the total Se content likely is.

In two previous studies where *in vitro* digestions were performed on yeast for 8h, the bioaccessible fractions of Se were 89% (Reyes *et al.*, 2006) and 80% (Yoshida *et al.*, 2002). Based on these results, the authors concluded that Se from yeast was highly bioaccessible. However, in the study of Yoshida and coworkers, the liberation of Se was time-dependent, with the release of Se being only 35% after 2h and 45% after 4h (Yoshida *et al.*, 2002). Those are more realistic durations for digestion processes and do correspond to the values found in the present study. These findings suggest that the degree of Se

bioaccessibility from yeast is influenced by the rapidity of its transit in the digestive tract.

8.4.2. Bioavailability

The degree of Se absorption through the Caco-2 monolayer was strongly species-dependent. The bioavailability of Se(IV) was the lowest: Se(IV) present in selenite-FS was hardly absorbed through the Caco-2 monolayer (~1% of the original Se(IV) content) (figure 8-3), and none of the Se(IV) present in yeast-FS was detected in the absorbed fraction (figure 8-4). In comparison, Se(VI) from selenate-FS was much more available (14% of the original Se(VI)) (figure 8-3). Regarding yeast-FS, the presence of SeMet was crucial as it was the only species detected in the absorbed fraction of Se and in cells. The percentage of SeMet that was absorbed from the yeast digest represented 35% of the bioaccessible SeMet and only 7% of the total Se content of the yeast-FS. In a previous investigation conducted with the similar Caco-2 absorptive model, we had determined the degrees of bioavailability of pure individual Se species to be Se(IV) ($12 \pm 1\%$) < Se(VI) ($33 \pm 2\%$) < MeSeCys ($46 \pm 2\%$) < SeMet ($56 \pm 4\%$) (Thiry *et al.*, 2012). These values are higher than those observed here for the Se species present in the bioaccessible fraction of the yeast-FS. This suggests that the presence of some additional components of the intestinal *bolus* (digested matrix and digestive enzymes) could impede or slow down the absorption of Se species from FS. It should also be noted that the degree of bioavailability of Se from yeast-FS is likely to vary widely in function of the specimen of Se-rich yeast considered because the Se species composition - and especially the proportion of SeMet - can differ importantly from one specimen to another (Suhajda *et al.*, 2000; Rayman, 2004; Schrauzer, 2006).

In the context of Se diet enrichment, several *in vitro* and *in vivo* studies have reported that SeMet was more efficiently absorbed and retained by the organism than other species (Whanger & Butler, 1988; Wang *et al.*, 2007; Zeng, Botnen & Johnson, 2008) because of its capacity of integration to proteins (Schrauzer, 2000). Selenium can therefore be progressively released into the organism during the regular turn-over of proteins, and continuously meet the Se body requirements. In addition, SeMet has been showed to be less toxic than inorganic species (Ammar & Couri, 1981; Tiwary *et al.*, 2006). Hence, despite the fact that our results did not show a very high release of bioavailable Se from the yeast-FS, this kind of dietary supplement seems to represent an interesting

source of Se for long-term supplementation. Contrary to SeMet, selenate was not retained in cells and it is expected to be more readily excreted as reflected by the rapid decrease in Se plasma concentrations observed in humans at the end of period of selenate diet supplementation compared to Se-rich yeast supplementation (Levander *et al.*, 1983). This makes us suggest that selenate-FS, which here showed the highest bioavailable fraction of Se, would be more appropriate to respond to an acute and occasional need of Se, while Se-yeast would be convenient for a long-term supplementation.

Despite it was not highlighted in this work, it is strongly believed on the basis of recent research about cancer (Ramoutar & Brumaghim, 2007; Lippman *et al.*, 2009) that Se species other than SeMet, and likely present in small quantities, are available from yeast. In 1996, anti-cancer effects were reported for Se that was administered as yeast-based FS to patients with history of skin cancer (Clark *et al.*, 1996). Another study conducted on Se-deficient patients in China confirmed a protective effect of Se-rich yeast face to liver cancer. However, a decade later, the vast intervention trial SELECT failed to demonstrate any anti-cancer effect related to pure SeMet dietary enrichment (Lippman *et al.*, 2009). This indicates that SeMet was probably not the species at the origin of the anti-cancer effect and that other species should have been taken from yeast and used by the organism for that purpose. In this frame, there is a need for in depth identification of minor Se compounds present in Se-yeast and for the study of their bioavailability. However this requires materials able to identify and to measure a large variety of Se compounds at very low concentrations, which remains a complicated task despite the progress that has been made with recent advanced instrumentation and state-of-the-art techniques (Rayman, 2004).

8.5. Conclusion

Within the limits of prediction allowed by an *in vitro* model, our results indicate that Se from the three investigated specimens of Se-enriched FS is in general poorly available (maximum 14% of original Se content was released from the matrix and crossed the Caco-2 monolayer). The major determinant of Se bioaccessibility and bioavailability in food supplements seems to be their speciation, which sounds coherent considering their simple matrix characteristics, especially for FS based on inorganic Se. Among the FS under

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investigation, selenate-FS produced the highest fraction of bioavailable Se, followed by yeast-FS. Selenite-FS appeared to be of very low utility as only 1% of its Se content was bioavailable. In yeast-FS, the fraction of SeMet may be crucial as it affects both bioaccessibility and bioavailability, but in opposite ways. Compared to selenite and methylselenocysteine, SeMet was less bioaccessible, but more bioavailable. Only Se from yeast accumulated in cells, suggesting a potential long term beneficial effect of yeast-FS compared to selenate-FS. Certainly it became clear that different kinds of food supplements will not have the same efficiency in enhancing human Se status.

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Chapter 9

**General discussion, conclusions
& perspectives**

Chapter 9 - General discussion, conclusions and perspectives

9.1. General discussion

9.1.1. Main outcomes of this work

The general aim of this work was to improve the current knowledge about the influence of the nature of Se species on the degree of Se bioavailability, in the context of the Se status and food habits of the Belgian population. At the end of this work, we have collected appreciable information concerning several aspects of the species-dependent bioavailability of Se in food. We can also provide a value for the average Se intake of Belgian people, and present a method for Se speciation analysis. The specific outcomes of the successive chapters are summarized below:

The purpose of chapter 4 was to present an analytical method of Se speciation in cellular material and in food products.

Chapter 4 provides a tutorial for Se species quantification in cellular material and in Se-enriched FS by IP-RP-HPLC-ICP-MS, with a US-mediated enzymatic extraction of Se. This chapter also gives a critical description of the analytical devices and of the methods involved in Se species determination in the context of the present thesis.

Chapter 5 aimed at estimating the current Se status of Belgian people and at identifying the major food sources of Se.

The work realized in chapter 5 permitted to lay the foundations of the present thesis by determining that the average Se intake of the adult general Belgian population is 60 µg Se per day. This reveals that the Se intake of Belgian people is low, but remains in the range of recommended values for Se intake. Chapter 5 also revealed that the major sources of Se are, by order of importance, meat and meat products, fish and shellfish, and pasta and potatoes. The highest Se concentrations were found in food products that are not eaten in abundance by Belgian people, like offal and Brazil nuts.

The objectives of chapter 6 were to establish Se species profiles in food and to evaluate the influence of the chemical form on Se status.

The Se species profile was investigated throughout a survey of the literature for a variety of food products commonly eaten by Western populations. Due to the complexity of Se speciation analyses, each study focused on a single or a few number of Se species, with the result that the Se profiles of food products were rarely complete and a considerable fraction of Se remained under unknown form. Nevertheless, it was observed that all types of food contained SeMet, usually as an important fraction of the total Se content. The methylated species MeSeCys and γ -glu-MeSeCys, precursors of the probable anti-cancer compound methylselenol, were identified at high concentrations in Se-enriched *Allium* plants (Ip, Birringer *et al.*, 2000; Ogra *et al.*, 2005). Inorganic forms of Se were exclusively detected in Se-enriched plants, at low concentrations (Ogra *et al.*, 2005; Cubadda *et al.*, 2010).

Chapter 6 also highlighted the influence of speciation on Se bioaccessibility, -availability and -activity. The impact of various food products on different biomarkers of Se status was reported relatively to selenite. It appeared that SeMet and Se from Se-rich yeast (containing a majority of SeMet) were better retained by the organism and permitted to elevate to a larger extent the Se content of blood (Whanger & Butler, 1988), plasma (Thompson *et al.*, 1982, Levander *et al.*, 1983 ; Rider *et al.*, 2010) and of various body tissues and organs (Whanger & Butler, 1988; Wang & Lovell, 1997; Wang *et al.*, 2007; Rider *et al.*, 2010). In contrast, the impact of SeMet on selenoenzyme induction was comparable to that of selenite (Whanger & Butler, 1988; Wang & Lovell, 1997; Wang *et al.*, 2007; Rider *et al.*, 2009, 2010). Unfortunately, few data were reported for species other than SeMet and selenite. Nevertheless, it could be observed on the basis of one study, that although MeSeCys is an organic species, it generated responses more similar to those of selenite than of those SeMet (Zeng *et al.*, 2008). Regarding food, the products rich in SeMet like wheat (Douglass *et al.*, 1981; Alexander, Whanger & Miller, 1983) and, to a lesser extent, fish (Douglass *et al.*, 1981; Alexander, Whanger & Miller, 1983; Wen *et al.*, 1997; Ornsrud and Lorentzen, 2002) and meat (Douglass *et al.*, 1981; Shi & Spallholz, 1994; Wen *et al.*, 1997) appeared to have the most bioavailable Se contents. In a general way, vegetables were reported to contain less available Se, at least in regard to the considered parameters of bioavailability (Gabrielsen & Opstvedt, 1982; Chansler *et al.*, 1986; Finley, 1998).

In parallel, the metabolic pathways followed by distinct Se species were reviewed. This permitted us to explain the capacity of SeMet accumulation in body tissues by its unspecific integration to Se-containing proteins. It also revealed that methylated species follow a different pathway than other species, which explains that they can build up in Se-accumulator plants like *Allium* without causing toxicity.

The aims of chapter 7 were to determine the precise degree of bioavailability of several Se species commonly present in food, and to investigate the mechanisms mediating the species-dependent bioavailability of Se.

The bioavailable fractions of pure SeMet, MeSeCys, selenate and selenite were determined with a bicameral *in vitro* enterocyte-like Caco-2 model of the intestinal barrier for a dose of Se corresponding to an intake slightly superior to recommended values. The degrees of Se species absorption through the Caco-2 monolayers (in percentage of initial Se added to the apical compartment) were 56% for SeMet, 46% for MeSeCys, 33% for selenate and 12% for selenite. The fractions of initial Se that were retained in Caco-2 cells were 4% for SeMet, 2% for selenite, and were under the detection limit for selenate and MeSeCys.

The insight into the mechanistic processes of Se species intestinal absorption revealed that selenite is absorbed mainly by paracellular pathway while the three other species have a large fraction of their transport occurring by transcellular way. In addition, it was observed that all species, except selenite, are transported by mechanisms that require energy, and that SeMet and MeSeCys share, at least partially, active mechanisms of transport with their sulfur analogues methionine and methionine.

Chapter 8 aimed at determining the degrees of bioaccessibility and bioavailability of Se in Se-enriched food supplements.

Three specimens of Se-enriched food supplements typically found in Belgium (selenite, selenate and Se-rich yeast) were investigated by means of *in vitro* tests that simulated the gastro-intestinal digestion and the intestinal absorption. It came out that Se from all three FS was, in general, poorly available: 1% (of the total Se content) for selenite-based FS ; 7% for yeast-based FS and 14% for selenate-based FS. Selenium content of inorganic Se-

based FS's was highly bioaccessible, but its bioavailability was limited, especially for the selenite-based FS. In Se-rich yeast, SeMet was poorly accessible due to its integration to the yeast protein structure, but was the only species absorbed through the Caco-2 monolayer. Hence, the fraction of SeMet in yeast could be crucial for the determination of its Se bioavailability.

Unfortunately, the conclusions of this chapter are limited to the three specimens of FS investigated and cannot be extended to a whole category of FS. Nevertheless, this gives a first idea of the differences in efficiency that are expected to exist between different FS.

9.1.2. Discussion about the outcomes of this work

9.1.2.1. The question of Se status in Belgium

The question of Se status of Belgian people had been raised with regards to the low Se intake and status reported for people of several European countries (Rayman, 2000; FAO/WHO, 2002), and the expectation that this could also be the case in Belgium considering the low concentrations of Se in soils (Waegeneers *et al.*, 2012). Selenium status, *i.e.* Se concentrations in various body locations and activity of selenoenzymes, was not directly measured but was estimated through the level of Se dietary intake of the general adult Belgian population. The fact that Se exists in food under different chemical forms and that these forms have distinct degrees of bioavailability complicates the situation. We therefore had to consider, in addition to the total Se intake, the identity and the concentration of Se species present in foodstuffs contributing to the Se intake. Our concern was that Se status of Belgian people would be unable to provide beneficial action of Se, and that it could require a Se enrichment of the diet. It was shown in chapter 5 that the general adult population in Belgium has a level of Se intake that is comparable to the values that are recommended to ensure the maximum activity of glutathione peroxidases. It also appeared that the food products that most contribute to the total Se intake (meat, fish and pasta) contain important fractions of SeMet (*cf.* chapter 6), a species that is better absorbed and retained by the body (*cf.* chapters 7), and less toxic than other Se-species (Ammar & Couri, 1981; Tiwary *et al.*, 2006). On this basis, it seems that the general adult population in Belgium should meet its Se requirements.

Nevertheless the relevancy of the level of recommended Se intake should be questioned. Indeed, the value that is the most often mentioned (*i.e.* 55 $\mu\text{g day}^{-1}$) was established for the particular case of a single Se species (SeMet) and a single biomarker of Se status (plasma GPx) (Thompson, 2004). However, considering the diversity of Se species and of the potential health effects of Se, the range of optimal Se intake could actually be wider. On the one hand, there is still debate whether maximal level of enzymes activity is necessary for optimal functioning of Se. In this case, the RDA would be lower than 55 $\mu\text{g day}^{-1}$ (WHO/FAO/IAEA, 1996). On the other hand, the maximization of the activity of certain selenoenzymes could require higher Se intakes than those guaranteeing full expression of GPx. It is notably the case of selenoprotein P (Xia *et al.*, 2005). Furthermore, it has been hypothesized that Se taken in larger quantities than the recommended intake ($\sim 200 \mu\text{g day}^{-1}$) could increase immune defenses and help to prevent diseases that are not directly related to Se deficiency such as cancer and cardiovascular diseases (Thompson, 2004). In addition, it was suggested that low molecular weight metabolic intermediates could have equal or more beneficial effects than the major Se compounds, as it is the case with methylselenol that has a preventive action against cancer (Ip *et al.*, 1991; Ip, Thompson *et al.*, 2000). In this case, high level of total Se intake would be required in order to produce such metabolites in quantities allowing them to impact on health (Gromer *et al.*, 2005; Rayman, 2008). Ideally, the optimal level of Se intake should therefore be adapted to the Se species and to the desired metabolic action. However, in practice, considering the complexity of Se speciation in food, the misidentification of a large fraction of Se food content and the lack of knowledge about Se biochemical functions other than GPx activity, it is probable that an estimation of the optimal Se intake through the maximization of GPx activity remains the best alternative. But it is important to be aware of the limitations of such values.

9.1.2.2. Selenium enrichment of the diet

Although Se requirements seem to be met for a majority of Belgian people, Se enrichment of the diet is not uncommon. The contexts of Se supplementation can be varied:

- people having too low Se intake because they follow a specific diet without or with low levels of meat, fish and/or pasta ;

- people having Se requirements larger than recommended such as *e.g.* feed-breasting women (SHC, 2009), people undergoing high level of oxidative stress (Thompson, 2004), or persons with impaired liver and/or kidneys functions compromising the selenoprotein synthesis (Rayman, 2008) ;
- people who intentionally aim at reaching supranutritional level of intake in order to enhance their immune system, to benefit from Se protection against cancer, chronic disease and aging, or simply for cosmetic purpose.

The means used for Se enrichment are multiple as well. A global Se enrichment of the diet can be achieved by fortifying agronomic productions through selenized fertilization of crops, genetic selection of plants species with enhanced Se-accumulation characteristics, or Se-supplementation of cattle feed. Alternatively, individuals can enhance their Se intake by consumption of food products naturally rich in Se such as Brazil nuts, offal or shellfish, or by the addition of food supplements to their ordinary diet.

i. Selenium bioavailability in Se-enriched food supplements

The consumption of food supplements is particularly attractive for the population since it is easy to carry out and it is rapidly effective. In Belgium, it has been reported that 37% of a random sample of 167 persons regularly take dietary supplements from their own initiative (Mormont *et al.*, 2009). As for Se, three types of Se-enriched FS are generally freely available in pharmacies, organic stores or supermarkets under the form of selenite, selenate or as Se-containing yeast (communication from the Public Health Ministry; Rayman, 2004). The results obtained in chapter 8 suggest that Se bioavailability of those three types of FS remains low (from 1 to 14% of the total Se content). Unfortunately, only one specimen of each type of FS has been examined, which limits the extent of the conclusions drawn from this experiment. Regarding the sample of selenite-based FS, the results obtained in chapter 8 suggest that, despite an important Se bioaccessibility, selenite-based FS's efficiency in increasing Se status of individuals should largely be limited by the low absorption of selenite through the intestinal mucosae. With reference to chapter 7, we can say that this low bioavailability is a consequence of the slow paracellular mode of intestinal transport of selenite. In comparison, selenate, MeSeCys and SeMet, at least in the form of pure individual species, should be better absorbed through the intestinal membrane because they mainly use transcellular pathways. In the case of Se-rich yeast sample analyzed in chapter

8, the fraction of bioavailable Se was 7%. This is unexpectedly low considering the important ability of yeast, reported in several *in vivo* studies, to elevate plasma and tissue Se concentration (*cf.* chapter 6). However, what remains a disadvantage of yeast as a source of Se is that its profile in Se species can vary significantly from one manufacturer to another and, to lower extent, from one specimen to another, despite conditions of culture as standardized as possible (Suhajda *et al.*, 2000; Rayman, 2004; Schrauzer, 2006). In the sample used in this work, the fraction of SeMet was small (40% of total extracted Se) compared with those usually reported for yeast, *i.e.* 54-74% (Rayman, 2004) or 80-90% (Schrauzer, 2006). It is probable that in specimens of yeast containing more SeMet, the fraction of bioavailable Se would have been higher than that observed in our experiment. Therefore, food supplements containing exclusively pure SeMet could be an alternative to further elevate the fraction of bioavailable Se. An additional advantage of SeMet over Se-yeast is the absence of matrix that can retain a considerable fraction of SeMet. Such SeMet supplements exist, especially available on the internet, but yeast-FS are much more frequent, probably because they represent the cheapest way to obtain SeMet (Rayman, 2004; Schrauzer, 2006).

Another outcome of chapter 8 deserving attention concerns the degrees of bioavailability of Se species contained in the digested solution of FS with regards to the values measured in chapter 7 for pure individual species. It appeared that Se species were less absorbed through the Caco-2 monolayer when they were in the presence of the digested solution of FS than when they were simply diluted in HBSS (table 9-1).

Table 9-1: Comparison of the fractions of Se absorbed through the Caco-2 monolayer during a 3h-period when Se is present as pure individual species or as a digested solution of FS.

	Se bioavailability (in % of initial Se)	
	Pure individual species	Species present in FS digest
Selenite (Se(IV))	12 ± 1%	< 1%
Selenate (Se(VI))	33 ± 2%	14 ± 3%
Methylselenocysteine (MeSeCys)	46 ± 2%	ND
Selenomethionine (SeMet)	56 ± 4%	35 ± 7%

In both cases, initial Se concentrations were about 100 ng Se ml⁻¹.

The digested juice contains, in addition to Se, the enzymes of the digestion, some bile salts and various compounds originating from the digested FS matrix. It seems that the presence of these components impeded or slowed down the absorption of Se species in our cell culture model. The limitation of Se absorption could occur through the binding of Se species with a compound present in the digest, which could impair Se recognition by a membranous transporter (in case of transcellular transport), or which could lead to the formation of a compound too voluminous to diffuse through extracellular spaces (in case of paracellular transport). Regarding yeast, the limitation of SeMet transport might also come from the presence of methionine that can compete with Se for the transporter sites. Furthermore, the influence of the model itself cannot be excluded as the cells for one or the other experiment originated from two different batches of frozen cells. It is possible that, for whatever reason, the second batch of cells had shown lower capacity of absorption than the first pool. In order to prevent as much as possible the misinterpretation of our results because of the model, the absorption of pure Se species was tested on the second batch of cells before beginning the experiments with FS. This enabled us to verify that similar degrees of absorption were obtained with the two batches of cells. In addition, similar protein contents and TEER values were observed for the cells of the two batches. But those tests were not sufficient to guarantee the suitability of the cells at the higher passages that were used for FS experiments.

ii. Potential toxicity of Se-enriched food supplements

Although it is an essential element at low to moderate doses of intake, Se remains toxic at higher doses. In Belgium, the risk of Se intoxication in the context of a normal diet is reduced due to the low Se concentrations of local food products, and the fact that almost the totality of the food content is under organic forms. However, the danger could arise from an important consumption of Se-enriched FS. Indeed, dietary supplements represent a highly concentrated dose of Se, often under inorganic form. When it comes to food supplements, the risk also comes with the typical unconscious behaviour of FS consumers. Since FS are freely available in shops, it is natural to believe that, unlike drugs and medicines, FS are safe and can be consumed without risk, regardless of the quantity.

The potential toxicity of Se species was not experimentally tested in the context of this thesis. Our only concern was to guarantee that Caco-2 cells did not suffer from Se-induced toxicity. Using a LDH release test, we noticed that cells were considered as safe for experiments up to a concentration of 200 ng Se ml⁻¹, with no difference among species. According to the literature, no clinical signs of selenosis can be observed under a level of daily intake of ~1000 µg Se (SCF, 2000). It is improbable that such a level of intake would be reached only by the consumption of FS. However, in view of the literature surveyed for this thesis, we expect that, at lower supranutritional level of Se intake (200-300 µg day⁻¹), selenite is able to induce oxidative damage to a limited extent, not necessarily generating clinical symptoms but contributing to oxidative stress promotion (Letavayova, Vlckova & Brozmanova, 2006; Brozmanova *et al.*, 2010). Indeed, at this level of intake, the selenoprotein synthesis should have reached its maximal level, which favors the occurrence of free Se compounds that can thereby exert an oxidant action.

As for organic forms of Se, the Scientific Committee of Food of the European Commission expressed their concern regarding a possible long-term toxicity. It was feared that an accumulation of SeMet into proteins could lead to an excessive release of Se during protein turnover and to a deficit in methionine, both able to induce toxicity (SCF, 2000). The poor information about Se species characterization in yeast had also been pointed out by the European Commission. However, according to Rayman (2004, 2008), there is no evidence for organic Se toxicity since long term studies on Se supplementation with

important doses of Se yeast (up to 800 µg Se day⁻¹) did not reveal any toxic symptoms. In addition, during the three decades of its world-wide use as a source of supplemental Se, no cases of Se poisoning due to dosage or formulation errors have occurred (Schrauzer, 2006). For these reasons, Se-enriched yeast was finally allowed by the European Commission as a nutritional supplement in 2009 (regulation 1170/2009 of Novembre 30th 2009, modifying appendix II of the directive 2002/46).

Finally, for any type of FS, the risk of intoxication might also be limited by their low level of Se bioavailability, as observed in the three samples investigated in chapter 8.

iii. Preliminary recommendations about the most appropriate form of Se for diet enrichment

Within the limits of the predictions allowed by the model, the methods and the experimental designs used in this work, the findings of the present thesis enable us to formulate an opinion about the suitability of distinct forms of Se in the context of a Se enrichment of the diet. Those considerations are formulated in a context of public health and could be subject to adaptations in particular cases where individual solutions should be envisaged. In a general way, it is our opinion that a varied diet including different types of Se-rich products is the best way to reach an optimal status in Se. Indeed, in comparison with artificial food supplements that usually contain one or a few Se species at concentrated doses, “normal” food allows a wider panel of Se species to be progressively brought into the organism. For example, animal products like meat, fish and offal contain large fractions of SeMet, that is very profitable because it serves as a stock of Se in the body proteins, but also SeCys, contributing to selenoprotein synthesis, and other minor organic species. Se-enriched accumulator plants like *Allium* products can contain small amounts of inorganic Se as well as large fractions of methylated species that are involved in the protection against cancer. Moreover, in addition to the species that could be identified, other species present in food that remain unidentified until now might also play an active role in the people’s health.

In the event that it is necessary to have recourse to FS, we would give the preference to organic forms of Se because of their higher degree of safety. Brazil nuts could be regarded as FS in view of their important Se concentration.

It was reported that the daily consumption of two nuts is as effective in increasing Se status and GPx activity as 100 µg of SeMet (Thompson *et al.*, 2008). Moreover, nuts contain additionally to Se other compounds of nutritional interest such as magnesium, phosphorus, zinc or vitamins E and B1 (Anses, 2012). However, in order to prevent any risk of Se intoxication, and because Brazil nuts are also very rich in lipids - notably in saturated fatty acids (Anses, 2012) - and contain traces of barium (IRSN, 2002), their consumption should be restricted to no more than one or two nuts a day. Regarding strictly speaking FS, Se-rich yeast is particularly interesting because of its reasonable price, but on the condition that the fraction of SeMet should be important enough in order to limit the risk of toxicity and to provide acceptable level of Se bioavailability. As was previously mentioned, pure SeMet should represent another valuable alternative, but such FS were not tested in this work because they are not found as easily in pharmacies and stores as the other types of FS. As for inorganic Se FS, their advantages lie in the facts that they are still cheaper than Se yeast, their Se is highly bioaccessible and their composition is totally known. Intuitively, however, the consumption of inorganic Se FS raises concern because the ingestion of high amounts of inorganic Se is totally unnatural in regards with the Se content of natural food that is almost exclusively organic. In addition, the demonstrated pro-oxidant properties of inorganic forms of Se, especially selenite, lead us to not recommend inorganic Se FS for Se enrichment purpose, especially for a long period. The fact that pro-oxidant Se compounds have been shown to be more effective against cancer is beyond the scope of this research.

Finally, at the question if Se should be taken preventively at supranutritional level ($\sim 200 \mu\text{g day}^{-1}$) to benefit from an enhanced immunity and an increased protection against viral infection and cancer, we respond that probably not in a general way. Although several positive events have been reported for such level of Se intake (Kiremidijan-Schumacher *et al.*, 1994; Clark *et al.*, 1996; Yu, Zhu & Li, 1997), those have also been associated with superoxide-mediated pro-oxidant activity and inhibition of cell growth (*cf.* chapter 2) (Letavayova, Vlckova & Brozmanova, 2006). In the context of the current knowledge, we think therefore that supranutritional intakes of Se should be restricted to particular cases of individuals for whom the potential benefits have been balanced against the possible risks with respects to the person's proper health and Se backgrounds.

9.2. Conclusions

This study was a multi-thematic work that allowed us to bring to light several outcomes regarding (i) the Se status of Belgian people, (ii) the species-dependence of Se bioavailability and (iii) the elemental analysis of Se.

- (i) Considering first that the average level of Se intake of the general adult Belgian population is 60 µg Se per day, secondly that SeMet contributes largely to the speciation profile of the main food sources of Se, and thirdly that SeMet is characterized by high bioavailability and low toxicity, we can conclude that the general adult Belgian population should not be at risk of any deficiency or excess of Se. However, some specific categories of the population could be.
- (ii) The bioavailability of Se is species-dependent: SeMet > MeSeCys > selenate > selenite. These degrees of absorption can be related to species-specific modes of intestinal transport, *i.e.* paracellular route for selenite and mainly transcellular route for SeMet, MeSeCys and selenate. In contrast with selenate, SeMet and MeSeCys share to some extent membrane transporters with their sulfur analogues.
- (iii) The identification and quantification of Se species by HPLC-ICP-MS is a complex task that must be continuously adapted to several parameters including the type of matrix, the identity, quantity and concentrations of Se species to be detected, the performances of the analytical device and the environmental conditions. We have developed an analytical process that is able to quantify SeMet, MeSeCys, selenate and selenite in the cellular material generated by our experiments and in several Se-enriched food supplements. This method includes a (multistep) proteolytic extraction of Se with ultrasonic probe, a chromatographic separation of Se species in ion-pairing reverse-phase partition mode, and the detection of the isotope 78 of Se with 90 ml min⁻¹ of hydrogen as a reaction gas.

9.3. Perspectives

With these findings, advances have been made in the knowledge of Se in living organisms. However this has also opened the door to further research. Some of the most critical questions to be addressed in the future are listed below:

Perspectives related to the investigation of Se-enriched FS:

It would be interesting on the short term to continue the investigation about Se-enriched food supplement. Several aspects could be addressed in order to reinforce the conclusions drawn in chapter 8:

- Chapter 8 was above all a preliminary research that was conducted on three specimens of FS chosen randomly in pharmacies and organic stores, and aiming at giving a first idea of the differences in bioavailability that could exist between distinct categories of FS. It would be necessary to extend the conclusions of chapter 8 to additional specimens of each type of FS in order to reveal trends related to whole categories of FS rather than to single specimens. In the case of Se-yeast, the analysis of various samples provided by different manufacturers - and therefore with different Se species profiles and SeMet contents - would permit us to draw more detailed conclusions concerning the influence of SeMet on Se bioaccessibility and bioavailability. In addition, FS that contain pure SeMet should be investigated in the same manner as other FS since they could appear as particularly valuable candidates for Se enrichment. With such additional information, we could formulate more accurate recommendations regarding the appropriateness of distinct types of FS for Se dietary enrichment.
- The specimen of Se-rich yeast that was analyzed in chapter 8 contained only 40% of its extractable Se content in the form of SeMet and an important fraction of selenite. This does not correspond to an optimal Se profile for a Se-enriched yeast. In yeasts of good quality, Se content must be almost exclusively under organic form, mainly as SeMet (Rayman, 2004; Schrauzer, 2006). It would be profitable to validate an analytical method for SeMet determination in yeast-FS in order to provide a tool that could guarantee the quality of these commercially available FS. The validation of our current

method of analysis would require several improvements in order to make it sufficiently efficient and reproducible:

- The current extraction method was tested on several specimens of yeast (not all reported in this thesis) and gave variable extraction efficiency depending on the sample. In order to be validated, the extraction method must guarantee minimal extraction efficiency for all types of yeast samples. For that purpose it is necessary to optimize the nature and quantity of enzymes as well as the duration, frequency and power of sonication.
 - The duration of sample validity and the condition of storage of the samples must be fixed to prevent sample evaporation or SeMet oxidation that could lead to a misinterpretation of the results.
 - The environmental conditions of analysis should be standardized since they can increase importantly the variability of the results. In our opinion, there are critical needs for a clean room for sample preparation, for air-conditioning in the local of analysis and cool system, and for a gas extraction device guaranteeing constant extraction rate from the ICP area.
- After 3h, the degree of absorption of Se species present in the digested solution of FS (*cf.* chapter 8) was low compared to the absorption of the same Se species as pure standards (*cf.* chapter 7). It would be interesting to identify the factors contributing to limit Se absorption from the digested solution. Several tests could be considered:
- Testing in the same experiment (thus on the same batch of cells and for the same number of passages) the absorption of digested solution of FS and of pure species. This could confirm the existence of the difference in Se absorption observed in chapters 7 and 8, as well as the extent of this difference.
 - Extending the duration of absorption over 3h. This would highlight whether the absorption is decreased or only slowed down in the presence of the digested solution of FS.

- Mimicking the composition of Se species profiles of FS with individual standards placed in the mixture of digestive enzymes, and measuring their absorption. If the absorption is not decreased, this would reveal that some compounds of the matrix present in the digested solution impede Se absorption through the Caco-2 layer. If the absorption is still decreased, it is probable that digestive enzymes limit the absorption of Se.

Perspectives related to Se bioavailability:

- In this study, our investigation on Se bioaccessibility and bioavailability focused exclusively on FS. After having explored different aspects related to FS, the method of determination of bioaccessible and bioavailable Se fractions could be applied to other food products. The determination, in standardized experimental conditions, of exact percentages of Se bioavailability for various food products would make it possible to classify them in term of Se bioavailability. This would appear more clear and accurate than by comparing studies that use various models and experimental designs as it was done in chapter 6.

Perspectives related to Se species determination:

- With reference to the previous paragraph, the method of Se species identification and quantification should be adapted to food products. An attempt was made in the context of this work to carry out Se speciation on various food products as it was made in our laboratory for arsenic. However, the strategy consisting in developing a single method for all types of food matrices proved to be unsuccessful for Se and the methods tested for that purpose did not produce convincing results. In order to succeed in the Se speciation analysis in food products, we believe that matrices should be considered one at a time. Furthermore, each relevant parameters of the analytical process (extraction - separation - detection) should be successively optimized. Among most critical parameters to be considered are the choice of enzymes for extraction, the mode(s) of Se species separation, and the Se elution process.
- A more thorough understanding of Se could also be achieved by identifying species other than those for which a standard is commercially available.

Indeed, we noticed peaks on our chromatograms that did not correspond to any of our standards. The destructive ionization mode of the ICP-MS makes it impossible to identify these species. However, by using a softer ionization mode like ESI-MS, we could determine the molecular weight of those compounds and broaden our knowledge on the Se spectrum of food products, like *e.g.* Se-rich yeast.

Perspectives related to Se bioactivity:

- In this study, we focused our approach on the bioavailability of Se species. This informed us about the capacity of Se species to penetrate into the organism. However, at this stage, we still ignore to what extent Se species can exert an effective action through Se metabolites such as selenoenzymes and -proteins. On a longer term, it would thus be interesting to assess the species-dependent bioactivity of Se. For this purpose, testing kits assaying the activity of GPx selenoenzymes are commercially available and easy to use. Nevertheless, we did not measure GPx activity in the present work because of an extra difficulty related to our cell culture system. Indeed, the low concentrations of Se present in the serum are sufficient to maintain a considerable level of GPx activity in Caco-2 cells, even with the reduction of the serum concentration to 0.5% during the last two days of culture. The replacement of the usual 10%-DMEM medium by a serum-free synthetic medium also routinely used in the laboratory (basal defined medium, BDM) (Halleux & Schneider, 1991) was not appropriate either because BDM analysis by ICP-MS revealed the presence of low concentrations of Se. Alternatively, a team suggested a method for total depletion of Se in Caco-2 cells by progressively reducing serum concentrations in DMEM-based medium during several successive passages. This method should permit us to generate Caco-2 cells with minimal GPx activity, making it possible to assess the bioactivity of distinct Se species. Thereby, to have information on Se bioactivity, -availability and -activity from food products and food supplements would provide a complete view on their impact on the Se status.

Perspectives related to the Se status of the Belgian population:

In parallel of the experimental part, it would be interesting to address some particular cases of the population that could be at particular risk of Se deficiency or toxicity:

- We established that the general Belgian population should not suffer from Se deficiency. Vegetarian people however could be at risk since it was established in chapter 5 that the major food sources of Se are meat and fish. Vegetarians represent approximately 2 % of the Belgian inhabitants and this fraction is increasing following the example of other European countries like the Netherlands (4.3 % vegetarians) or Germany (9 % vegetarians) (EVU, 2007). The Belgian vegetarians are thus a population of slightly more than 200 000 persons, which is worth considering. The average Se intake of vegetarians should be calculated in the same way as for the general population. For this purpose, a consumption survey should be realized among this category of consumers and the Se content of specific vegetarian food products like tofu, soy, etc. should be determined. In addition, information should be obtained regarding the Se bioavailability of these products.
- We stated that high-level adult consumers of Se-rich products like meat and pasta were not at risk of Se excess. However, the exposure to Se has been reported to decrease with the age and, children could actually have too high Se intake. A report of the EFSA has recently estimated that European young children could have a Se intake close to the tolerable upper intake value, but this work did not detail the Se intake per country. In this context, it would be relevant to estimate the mean dietary intake of the Belgian population under 15 years in a similar way that it has been accomplished for adults. A food consumption survey specifically related to children is currently ongoing at the Belgian Public Health Ministry and will certainly be useful for that purpose.

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Appendix

Appendix: Review of Se speciation in various food products

(Rayman, M. (2008). Food-chain selenium and human health: spotlight on speciation. *British Journal of Nutrition* 100, 238-253)

Table 1. Species and concentrations of selenium in foods and supplements (concentrations of selenium species are expressed in terms of concentration of elemental selenium) and the methodology used for species identification

Food or source	Growth conditions	Species	Typical concentration	Identification methodology	References
Supplements (also see Se-yeast)	Not applicable	Selenite/selenate Selenomethionine Se-methyl-selenocysteine	30–100 µg/tablet 60–200 µg/tablet Total Se: 1200–2200 µg/g		Whanger (1989, 2003) ^(4,5) Whanger (2003) ⁽⁶⁾ Rayman (2003) ⁽¹²⁾ Korabel (2007) ⁽²³⁾ Uden (2003) ⁽²⁴⁾ Larsen (2004) ⁽²⁵⁾ Goeraga Infante (2005) ^(27,28) Mestor (2006) ⁽²⁹⁾
Se-yeast (in supplements; as a food additive; in functional foods)	Medium enriched with inorganic Se	Selenomethionine Selenocysteine Selenite	60–84 % 3–5 % <1 % total extracted Se	ESI-MS/MS Retention time	
		Se-methyl-selenocysteine γ-Glutamyl-Se-methyl-selenocysteine Se-adenosyl-selenomethionine Se-cystathionine Selenomethionine Selenocysteine Selenomethionine-selenoxide/hydrate Sulfide-Asr-Ala-Gly-Arg Selenodiglutathione and the mixed selenodiglutathione and the mixed selenodiglutathione and cysteinylglycine	0.5 % 0.5 % 0.5–5 % 0.5–1 % 0.5 % 0.5 % 0.5 % – –	ESI-MS/MS Retention time ESI-MS/MS Retention time ESI-MS/MS Retention time ESI-MS/MS Retention time ESI-MS/MS Retention time	Enchiar (2003) ⁽³⁰⁾ Lindemann (2002) ⁽³¹⁾ Enchiar (2003) ⁽³²⁾ Goenaga Infante (2006) ⁽³³⁾
Cereals		Se-containing proteins (SIP18 and HSP12) S-selenomethyl-glutathione Glutathione-S-selenoglutathione	0.1–30 µg/g (Western USA) 0.016–0.021 µg/g (Denmark) 0.007–0.011 µg/g (Finland, Sweden, Norway) ≤ 30 µg/g	ESI-MS/MS ESI-MS/MS	Whanger (1989) ⁽⁴⁾ Gissel-Nielsen (1984) ⁽³⁴⁾ Gissel-Nielsen (1984) ⁽³⁴⁾ Whanger (2002) ⁽⁶⁾
Wheat, maize, barley, oats, rye	North and South Dakota, Nebraska, Kansas, Colorado Other parts of USA	–	0.1 µg/g 12–19 % total eluted Se	Retention time	Whanger (2002) ⁽⁶⁾
Wheat grain		Selenate (includes selenite, and selenocysteic acid) Selenomethionine Selenocysteine Se-methyl-selenocysteine Selenate	56–83 % total eluted Se 4–12 % total eluted Se 1–4 % total eluted Se ≤ 40–50 % total Se at ≤ 100 µg/g dry weight	Retention time Retention time Retention time Retention time	Untschinger (2000) ⁽²⁶⁾
Wheat sprouts		Selenomethionine	50.4–81.4 % of total Se	Retention time	Yang (1997) ⁽³⁵⁾
Wheat flour	China USA	Selenomethionine Selenate	Total: 439 ng/g 5.5 ng/g 8.7 (range 0.5–44.0) µg/g (selenosis)	ESI-MS/MS Retention time	Warburton (2007) ⁽²⁷⁾
Maize	Seleniferous soil High-Se area of China	Selenomethionine Selenocysteine Selenomethionine	18 µg/g 61–64 % of total eluted Se 15–16 % of total eluted Se 0.01–19.01 µg/g representing 46–82 % of the total Se ≤ 0.11 µg/g 4.0 (0.3–20.2) µg/g (selenosis)	Retention time Retention time Retention time	Whanger (1989) ⁽³⁶⁾ Beisstein (1991) ⁽³⁹⁾ Yang (1997) ⁽³⁸⁾ Whanger (1989) ⁽⁴⁾ Whanger (1989) ⁽⁴⁾
Barley Rice	China, grain Denmark Seleniferous soil	– – –	– – –	– – –	– – –

Table 1. *Continued*

Food or source	Growth conditions	Species	Typical concentration	Identification methodology	References
	High-Se area of China	–	3.6 µg/g Se	Retention time	Whanger (1996) ⁽³⁸⁾
		Selenomethionine	68–81 % of total eluted Se	Retention time	
		Selenocysteine	6–10 % of total eluted Se	Retention time	
		Selenite	5–13 % of total eluted Se	Retention time	
		Selenate	1–3 % of total eluted Se	Retention time	Yang (1987) ⁽³⁹⁾
Soya beans	China, grain	Selenomethionine	54.9–66.5 % of total Se	Retention time	Whanger (1999) ⁽⁴¹⁾
		Selenomethionine	>80 % of eluted Se	Retention time	Yang (1987) ⁽³⁹⁾
	China	Selenomethionine	62.9–71.8 % of total Se	Retention time	Whanger (1999) ⁽⁴¹⁾
Lima beans		Se-methyl-selenocysteine	–	Retention time	
		γ-Glutamyl-Se-methyl-selenocysteine	–	Retention time	
		Se-cystathionine	–	Retention time	
	Natural	–	2.54 µg/g	Retention time	Barceloux (1999) ⁽⁴⁰⁾
Brazil nuts	Acres – Rondonia, Brazil (shelled)	–	3.06 (range 0.03–31.7) µg/g fresh weight	Retention time	Chang (1995) ⁽⁴¹⁾
	Manaus – Belém, Brazil (unshelled)	–	38.0 (range 1.25–512.0) µg/g fresh weight	Retention time	
	Natural (UK purchased)	–	2.54 (range 0.85–6.86) µg/g	Retention time	Barclay (1995) ⁽⁴²⁾
	Natural (unshelled)	–	22 µg/g	Retention time	Lisk (1988) ⁽⁴³⁾
	Natural	Selenomethionine	≤100 µg/g	Retention time	Palmer (1982) ⁽⁴⁴⁾
	Purchased with shells	Selenomethionine	–	Retention time	Wrobel (2003) ⁽⁴⁵⁾
		Selenomethionine	Total Se: 35.1 µg/g	Retention time	Kannamkumath (2002) ⁽⁴⁶⁾
		Selenomethionine	Major Se species, 25 % total Se	Retention time	
		Weakly protein-bound Se	12 % total Se	Retention time	
		Low molecular-weight compounds	3.1 % total Se	Retention time	
		–	Total Se: 8.3 µg/g	Retention time	
	Purchased without shells	–	–	Retention time	
		Selenomethionine	Major Se species, 21 % total Se	Retention time	
		Weakly protein-bound Se	12.0 % total Se	Retention time	
		Low molecular-weight compounds	5.0 % total Se	Retention time	
		Fifteen Se-containing peptides (fourteen selenomethionine-containing peptides + one Se-cysteine-containing peptide)	Total Se: 62.9 µg/g	ESI-MS/MS	Danovics (2007) ⁽⁴⁷⁾
Walnuts	Black and white	–	–	Retention time	Kannamkumath (2002) ⁽⁴⁸⁾
		Selenomethionine	Total Se: 0.38 and 0.20 µg/g respectively	Retention time	
		Low molecular-weight compounds	19 % and 23 % respectively of total Se	Retention time	
		–	15 % and 10 % respectively of total Se	Retention time	
		–	Total Se: 0.031 (0.012–0.060) µg/g	Retention time	Barclay (1995) ⁽⁴²⁾
Cashew nuts	Unspecified	–	Total Se: 0.27 µg/g	Retention time	Kannamkumath (2002) ⁽⁴⁸⁾
	Natural	Selenomethionine	22 % of total Se	Retention time	
		Low molecular-weight compounds	12 % of total Se	Retention time	Barclay (1995) ⁽⁴²⁾
	Natural	–	Total Se: 0.27 (range 0.17–0.39) µg/g	Retention time	Kannamkumath (2002) ⁽⁴⁸⁾
		–	Total Se: 0.10 µg/g	Retention time	
Pecan nuts	Natural	Selenomethionine	25 % of total Se	Retention time	Danovics (2007) ⁽⁴⁸⁾
		Low molecular-weight compounds	Not detected	Retention time	
Monkeypot nuts	Natural	–	Total Se: 4.5 mg/g	Retention time	

Table 1. Continued

Food or source	Growth conditions	Species	Typical concentration	Identification methodology	References
		Selenocystathionine Isoforms of γ -glutamyl-selenocystathionine	—	ESI-MS/MS	
Pumpkin seeds	Enriched by leaf spraying	Selenomethionine	$81 \pm 8\%$ total Se	Retention time	Smrkal (2005) ⁽⁴⁹⁾
Vegetables Vegetables: rutabagas, cabbage, peas, beans, carrots, tomatoes, beets, potatoes, cucumbers	Natural Seleniferous soil	Selenate	$<0.01\ \mu\text{g/g}$ $\leq 6\ \mu\text{g/g}$ total Se	Retention time	Barceloux (1999) ⁽⁴⁰⁾ Whanger (1989) ⁽⁴¹⁾
Vegetables	Sludge-amended soil	—	—	—	Whanger (1989) ⁽⁴¹⁾
Asparagus	UK	Selenate	Up to 50% of total Se $0.001\text{--}0.054\ \mu\text{g/g}$ $11\ \mu\text{g/g}$ Total Se: $345\ \mu\text{g/g}$ Ratio 2:1	Retention time	Barclay (1995) ⁽⁴²⁾ Whanger (1989) ⁽⁴¹⁾ Cai (1995) ⁽⁴³⁾
Broccoli	Seleniferous soil Enriched	Se-methyl-selenocysteine: selenocystathionine Selenomethionine Selenate	Minor amounts: $\leq 44\%$ and $\leq 38\%$ total Se respectively Total Se: $62.3 \pm 0.6\ \mu\text{g/g}$ dry weight	Retention time Retention time Retention time	Whanger (2002) ⁽⁹⁾ Firley (2001) ⁽⁵¹⁾
Broccoli florets and leaves	Enriched	—	—	Retention time	—
Broccoli sprouts	Enriched	Se-methyl-selenocysteine Selenate	45% 20% 12% 3%	Retention time Retention time Retention time Retention time	Pedrero (2007) ⁽⁵²⁾
Broccoli roots	Grown in hydroponic culture with Na_2SeO_3	Adenosyl-selenohomocysteine Selenomethionine	Major species	Retention time	—
Broccoli florets	Enriched	Se-methyl-selenocysteine Se-methyl-selenocystathionine-selenoxide	Major species $\leq 21.5\%$ of total extractable Se	Retention time Retention time	Hamilton (1975) ⁽⁵³⁾
Cabbage (Brassica oleracea capitata)	Enriched	Selenate	$\leq 40\%$ of total Se Total: $94\ \mu\text{g/g}$	Retention time	Whanger (2002) ⁽⁹⁾ H Geonaga, infante, G O'Connor and MP Rayman (unpublished results)
Cabbage	Sludge-amended soil Enriched	—	—	Retention time Retention time Retention time	Pedrero (2006) ⁽⁵⁴⁾
Radish (Raphanus sativus)	Hydroponic culture with (i) Na_2SeO_3 (ii) Na_2SeO_4	Se-methyl-selenocysteine Selenomethionine Selenate	(i) Total: $112 \pm 7\ \mu\text{g/g}$ (ii) Total: $120 \pm 6\ \mu\text{g/g}$ 49% fresh weight (i) 6 ± 1 (ii) 19 ± 2 83 ± 7 7 ± 1 18 ± 1 20 ± 1 1 ± 0 68 ± 5 Total Se dry weight: <i>Agaricus bisporus</i> $0.77\ \mu\text{g/g}$; <i>Lentinula edodes</i> $0.043\ \mu\text{g/g}$	Retention times	—
Mushrooms (<i>Agaricus bisporus</i> and <i>Lentinula edodes</i>)	Enriched	Selenocystathionine	—	Retention times	Gargely (2006) ⁽⁵⁵⁾

Table 1. Continued

Food or source	Growth conditions	Species	Typical concentration	Identification methodology	References
<i>Lentinula edodes</i> (Shiitake mushrooms) <i>Agaricus bisporus</i>	Enriched	Selenomethionine Se-methyl-selenocysteine Inorganic Se	—	ESI-MS/MS	Ogra (2004) ⁽⁶⁹⁾
	Enriched	Selenomethionine	40.6% of the total water-soluble Se Total Se: 69 µg/g	—	H Geenaga, Infante, G O'Connor and MP Rayman (unpublished results), Rayman (2007) ⁽⁵⁷⁾
	—	—	—	—	—
Onions (<i>Allium cepa</i>)	Natural (USA)	Se-methyl-selenocysteine Selenomethionine Selenate	114 ng/g 17 µg/g 61 parts per billion	ESI-MS/MS ESI-MS/MS Retention time	Korabai (2000) ⁽²³⁾
Onions (<i>Allium cepa</i>)	Seleniferous soil Enriched	— Selenate — Selenocysteine Se-methyl-selenocysteine	Total Se: <0.5 µg/g 100% of total enzymatic extract of Se 17 µg/g Total Se: 96 µg/g; equal amounts of both compounds Total Se: 96 140 µg/g of which % distribution as follows: 35 63 % 10 5 % 5 1 % 33 10 % 4 0.5 % 1 % Sum 92 96 % 30-5 µg Se/g plant tissue	Retention time Retention time Retention time Retention time Retention time Retention times	Whuriger (1989) ⁽⁴⁾ Cai (1995) ⁽⁵⁸⁾ Korabai (2000) ⁽²³⁾
Garlic (<i>Allium sativum</i>)	Enriched with 15 µg/g Se IV added to growth medium	— γ-Glutamyl-Se-methyl-selenocysteine Selenomethionine Se-methyl-selenocysteine Selenate Se-cystathionine Selenocysteine	—	—	Shah (2004) ⁽⁶⁸⁾
	—	—	—	—	—
	—	—	—	—	—
Garlic (<i>Allium sativum</i>)	Natural Natural	Selenocysteine Se-methyl-selenocysteine SeMet γ-Glutamyl-Se-methyl-selenocysteine Inorganic Se Selenocysteine — γ-Glutamyl-Se-methyl-selenocysteine Selenomethionine Se-methyl-selenocysteine Selenate	— — — — Total Se: 0.02 µg/g Total Se: <0.5 µg/g; % distribution: 31 % 53 % 12 % 4 % Total Se: <0.5 µg/g; % distribution: 48 % 28 % 14 % 10 % Total Se: 68 µg/g Total Se: 135 µg/g	Retention time Retention time Retention time Retention time Retention time Retention times Retention times	Ca (1995) ⁽⁶⁰⁾ Korabai (2000) ⁽²³⁾ Korabai (2000) ⁽²³⁾
Garlic (<i>Allium sativum</i>)	Supplement	— γ-Glutamyl-Se-methyl-selenocysteine Selenomethionine Se-methyl-selenocysteine Selenate	— — — —	ESI-MS ESI-MS ESI-MS Retention time	Cai (1995) ⁽⁵⁸⁾ Cai (1995) ⁽⁵⁸⁾
Garlic (<i>Allium sativum</i>)	Enriched Enriched	— Se-methyl-selenocysteine Selenocysteine Selenomethionine	— — — Total Se: 1355 and 235 µg/g	Retention times	Blid (1997) ⁽⁵³⁾ , Whuriger (2002) ⁽⁸⁾
Enriched	Enriched	—	—	—	—

Table 1. Continued

Food or source	Growth conditions	Species	Predominant form	Typical concentration	Identification methodology	References
Ramps (wild leeks, <i>Allium tricoccum</i>)	Enriched	Se-methyl-selenocysteine (γ-glutamyl-Semethyl-selenocysteine)	—	—	Retention times	Ip (2000) ⁽⁶²⁾
		Selenocystine	—	—	—	
		Selenomethionine	—	—	—	
		Se-propyl-selenocysteine	—	—	—	
		Selenite	—	—	—	
		—	206 µg/g Se	—	ESI-MS	
		γ-Glutamyl-Se-methyl-selenocysteine	73% of total eluted Se	—	ESI-MS	
		Selenomethionine	13% of total eluted Se	—	Retention time	
		γ-Glutamyl-selenomethionine	4% of total eluted Se	—	ESI-MS	
		Se-methyl-selenocysteine	3% of total eluted Se	—	Retention time	
		Selenate	2% of total eluted Se	—	Retention time	
		Selenocystine	0.5% of total eluted Se	—	Retention time	
		Selenocystathionine	0.5% of total eluted Se	—	Retention time	
		—	28.3 µg/g wet weight, 99 µg/g lyophilised	—	—	
Enriched	Enriched	γ-Glutamyl-Se-methyl-selenocysteine	49.7% Se in hot water extract	—	ESI-MS/MS	Dumont (2006) ⁽⁶¹⁾
		Se-methyl-selenocysteine	28.8% Se in hot water extract	—	ESI-MS/MS	
		Selenomethionine	15.5% Se in hot water extract	—	ESI-MS/MS	
		Selenocystine	6.0% Se in hot water extract	—	Retention time	
		—	Total Se, 68 112 235 296 1355 µg/g, of which % distribution as follows:	—	—	
		—	68 52 70 73 8%	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	18 28 17 13 13%	—	—	
		Selenomethionine	25 30 30 60 60%	—	—	
		Se-methyl-selenocysteine	10 0.1 1.5 2.0 4.0%	—	—	
		Selenate	0.5 1.5 0.5 0.5 1.5%	—	—	
Enriched	Enriched	Se-cystathionine	0.5 1.0 0.5 0.5 —%	—	—	Korrebai (2000) ⁽²³⁾
		Selenocystine	Sum 53 93 95 96 87%	—	—	
		—	Total, 205 µg/g	—	—	
		—	> 78% of total Se	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	Total, 1 960 µg/g	—	—	
		—	496 ng/g	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	35 ng/g	—	—	
		Se-methyl-selenocysteine	Total Se, 48 77*230 252 405 524 µg/g, of which % distribution as follows:	—	—	
		—	3 1 1 1 1.5%	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	10 21 8 8 5 5%	—	—	
Enriched	Enriched	Selenomethionine	35 50 50 44 44%	—	—	McSweeney (2000) ⁽⁶²⁾
		Se-methyl-selenocysteine	35 50 50 44 44%	—	—	
		Selenate	3 1 2 0.5 1.5%	—	—	
		Se-cystathionine	3 1 2 0.5 1.5%	—	—	
		—	Sum 73 60 76 76 74%	—	—	
		Se-methyl-selenocysteine	35–50% total Se (at 48–524 µg/g)	—	—	
		Selenate	15–25%	—	—	
		Se-cystathionine	0.5–3%	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	1–2%	—	—	
		—	—	—	Retention times	
Enriched	Enriched	γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	Whanger (2000) ⁽⁶³⁾
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
Enriched	Enriched	γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	H. Goenaga Infanta, G. O'Connor and MP Rayman (unpublished results)
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
Enriched	Enriched	γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	Korrebai (2000) ⁽²³⁾
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	
		γ-Glutamyl-Se-methyl-selenocysteine	—	—	—	
		—	—	—	—	

Table 1. Continued

Food or source	Growth conditions	Species	Typical concentration	Identification methodology	References
Chives (<i>Allium schoenoprasum</i>) leaves	Enriched with Se IV	–	Total: 222 µg/g	Retention times	Kápolna (2007) ⁽⁶⁴⁾
		Selenocystine	49% of total Se		
		Se-methyl-selenocystine	38% of total Se		
		Selenomethionine	5% of total Se		
		Inorganic Se (Se(IV) + Se(VI))	8% of total Se		
	Enriched with Se VI	–	Total: 613 µg/g		
		Selenocystine	24% of total Se		
		Se-methyl-selenocystine	22% of total Se		
		Se(VI)	>50% of total Se		
	Enriched with SeMet	–	Total: 265 µg/g		
		Selenocystine	44% of total Se		
		Se-methyl-selenocystine	52% of total Se		
		Selenomethionine	3% of total Se		
Horseradish	Enriched	Selenosinigrin (a glucosinolate)	–	Unknown	Stewart (1974) ⁽⁶⁵⁾
Meat and poultry		Selenocystine, selenomethionine, seleno-trisulfide, selenopersulfide, metallic selenide	Selenocystine is predominant	Retention times	Burk (1976) ⁽¹⁰⁾ , Behne (1998) ⁽⁶⁶⁾ , Whanger (2002) ⁽⁹⁾
Meats	USA	–	0.3 µg/g		Barceloux (1995) ⁽⁴⁰⁾
Beef, pork, lamb	UK	–	0.03–0.15 µg/g		Barclay (1995) ⁽⁴²⁾
Turkey (raw)	UK	–	0.1–0.2 µg/g		
Liver, kidney (raw)	UK	–	0.2–2.0 µg/g		Barceloux (1995) ⁽⁴⁰⁾
Fish (whole)	109 US stations	–	0.42 µg/g		Barceloux (1995) ⁽⁴⁰⁾
Fish: black marlin	USA	–	0.4–4.3 µg/g		
blue marlin		–	2.5–4.2 µg/g		
Fish: mackerel, octopus	Spain/Portugal	–	0.26 and 0.13 µg/g respectively	Retention time	Cabanero (2005) ⁽⁶⁷⁾
Fish: sardine, swordfish, tuna	Spain/Portugal	Selenomethionine	Total Se: 0.43, 0.47 and 0.92 µg/g respectively	Retention time	Cabanero (2005) ⁽⁶⁷⁾
Fish: tuna	Canned	Selenate	7.6–44.8% of total Se (at 0.36–1.33 µg/g)	Retention time	Cappon (1982) ⁽⁶⁸⁾
Several fish, mollusks, crustaceans and pods	Canned, edible tissue	Se(II), Se(IV)	4–47% of total Se (at 0.15–4.15 µg/g)	Retention time	Cappon (1982) ⁽⁶⁸⁾
Several species of marine and freshwater fish	Muscle	Se(II), Se(IV)	14–36% of total Se (at 0.14–0.83 µg/g)	Retention time	Cappon (1981) ⁽⁷⁰⁾
Cod	Cooked	Se(II), Se(IV)	12% of total Se at 1.5 µg/g	Retention time	Crews (1996) ⁽⁷¹⁾
Cod muscle	Dried and powdered	Selenomethionine	70% of the total Se	Retention time	Diaz Huerla (2004) ⁽⁷²⁾

ESI-MS, electrospray ionisation MS; ESI-MS/MS, electrospray ionisation MS with fragmentation of the precursor/molecular ion.

*For ramps, sample from 2nd year of growth.