SELENIUM SUPPLEMENTATION OF CEREAL CROPS

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DIFFERENT APPROACHES TO ENHANCING SELENIUM LEVELS IN WHEAT CULTIVARS

Proefschrift

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To my mum, dad and brother

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"Enrichment factors and transfer coefficients from soil to rye plants by INAA"
Galinha C, Freitas MC & Pacheco AMG (2010)
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"Neutron activation analysis of wheat samples"
Galinha C, Anawar HM, Freitas MC, Pacheco AMG, Almeida-Silva M, Coutinho J, Maçãs B & Almeida AS (2011)
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&
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1.1 Motivation

Back in the early 1980s, sodium selenite was already being used in moderate dosage at animal-breeding farms, for prophylactic and therapeutic purposes (Bem 1981). Strategies to increase selenium (Se) status were implemented in Australia, New Zealand and also in Europe, where this methodology was adopted to protect animal health and improve production, which were both noticeably dwindling as a result of an extensive Se deficiency (Arthur 2003). More recently, Finland and United Kingdom have addressed the issue of Se supplementation to crops as well (Eurola *et al.* 1991, Broadley *et al.* 2006).

Due to climatic and geochemical features, Se availability from agricultural soils in Finland was remarkably low, and, therefore, Se contents of domestic agricultural products were also minimal (Eurola *et al.* 1990). In the mid 1970s, the average daily Se intake in Finland was as low as 25 μg (Pietinen *et al.* 2010), well below the recommended dietary intake (RDA) for adults (50-200 μg per day) defined, at that time, by the U.S. National Academy of Science's National Research Council (Eurola *et al.* 1990). Such low intakes, plus an ever-growing evidence that Se could be essential for human health, and the common occurrence of Se-responsive disorders in farm livestock, led

Finnish authorities to start a programme to boost Se status (Arthur 2003). An official decision was taken in 1984 to supplement compound fertilisers with Se, with the purpose of improving animal health, the quality of Finnish food, and the Se intake of the population (Pietinen *et al.* 2010).

The strategy adopted by the Finnish authorities was to add Se – as sodium selenate – to the main multinutrient fertilisers used for both grain and forage production (Eurola 1990, Arthur 2003). From 1984 to 1991, the Se levels in fertilisers were 16 mg kg^{-1} and 6 mg kg^{-1} for cereal and feed/hay production, respectively (Kantola and Vartiainen 2001). After the beginning of the Se-supplementation programme, both the Se content of foodstuff and the mean daily Se intake reached their peak within two years: the latter was between 110 μg and 120 μg (Kantola and Vartiainen 2001, Pietinen *et al.* 2010). There were still further (minor) increases, but, since 1991, the Se dosage in fertilisers has been decreased to 6 mg kg^{-1} (Arthur 2003). After that cutback, the daily intake of Finnish people has levelled around 70-80 μg of Se through the 2000s (Pietinen *et al.* 2010). Even if successful, the Finnish experience cannot be transferred and applied directly (in)to Portugal – or any other country, for that matter: there are a few major differences between mainland Portugal and Finland, especially in what concerns climatic features, farming practices and common cultivars.

The Portuguese situation is difficult to assess due to scarce-to-null information (Oldfield 1999) and lack of consistent research on the subject, save for two limited cohort studies on the potential Se intake based on actual diets (Reis *et al.* 1990, Ventura 2008). It should not be that different from much of Europe, though, where falls in Se intake – and corresponding drops in blood indicators of human Se status – have long been raising a widespread concern (Rayman 2002). Considering the essentiality of Se for human health and the low levels of Se in Portuguese soils, this work has been designed and performed to assess agronomical ways of improving Se contents in wheat. Since breads and wheat derivatives make up a sizeable share of Portuguese diets, an increase in Se intake through Se-biofortified wheat may contribute to an upgrade in the Se status of the whole population (Galinha *et al.* 2013a).

1.2 Selenium

Selenium is a naturally-occurring metalloid element with an atomic number of 34, atomic mass of approximately 79 and six natural isotopes, five of which are stable: ^{74}Se, ^{76}Se, ^{77}Se, ^{78}Se and ^{80}Se (Broadley *et al.* 2006). This element is one of the most peculiar chemical elements in the geo- and biosphere, which occurs in nature in a number of inorganic forms, including selenide-, selenite-, and selenate-containing minerals. It is usually found quite impurely, replacing part of the sulphur in sulphide ores of many metals (Kabata-Pendias 1998). In living systems, it can be found in the aminoacids selenomethionine, selenocysteine and methylselenocysteine, where it plays a role similar to that of sulphur (Wessjohann *et al.* 2007).

Selenium is an essential micronutrient for humans, animals and certain lower plants, and its supply in global food systems is greatly uneven (Lyons 2010). This element enters the food chain through plants and, consequently, it is highly dependent upon its bioavailability in soils (Ducsay and Ložek 2006). The increasing attention paid to the role of Se and selenoproteins in human health stems primarily from a similarly growing body of evidence about not only their actual (general) importance for a healthy immune system, but also for their protective (specific) effects against cardiovascular disorders, asthma, male sterility, and, especially, certain forms of cancer (Clark *et al.* 1991, Clark *et al.* 1996, Clark *et al.* 1998, Combs 2001, Rayman 2002, Whanger 2004, Combs 2005, Stranges *et al.* 2006). These are not scattered observations or random effects. From about 100 selenoproteins that may exist in mammalian systems (Burk and Hill 1993), more than 30 have been positively identified for Se through radiotracing (Evenson and Sunde 1988), and at least 15 have been deemed essential in what concerns their biological function and physiological significance for major metabolic pathways (Arthur 1999, Brown and Arthur 2001, Rayman 2000, Lyons *et al.* 2003, Van Cauwenbergh *et al.* 2004, Navarro-Alarcón and Cabrera-Vique 2008). The state of knowledge, with an emphasis on major medical endpoints, has been recently reviewed by Rayman (2012) and Roman *et al.* (2014).

Chronic Se deficiency is rare in humans, but can be found in certain areas of China with extremely low basal levels of Se as Keshan disease, an endemic cardiomyopathy, and as Kashin-Beck disease, an endemic osteoarthritis (Thomson 2004). Results from China indicate that Keshan disease does not take place in areas where Se intakes are 20 μg per day or above. From those results, the World Health Organization (WHO) calculated the necessary Se intake to prevent pathologically and clinically relevant signs of dietary inadequacy (minimum Se requirement), and came to the value of 21 μg per day and 16 μg per day for men and women, respectively (Thomson 2004).

Even if most aspects of the Se conundrum (Stapleton 2000) have yet to attain scientific closure, its seemingly pivotal role in human health has long been recognised by both global institutions – such as the Scientific Committee on Food (SCF) of the European Commission, the Food and Agriculture Organization (FAO) of the United Nations, the International Atomic Energy Agency (IAEA) and the World Health Organization (WHO) – and several countries, leading to a range of dietary recommendations (Table 1.1) for Se intake that currently averages 60 μg per day and 53 μg per day for adult men and women, respectively (Thomson 2004). Of course, between the minimum dietary intake for preventing severe conditions, such as the Keshan disease, and a few higher normatives in countries like Australia, New Zealand or the United Kingdom, there is still a wide scope of debate on how to achieve optimal plasma or serum Se concentrations for, say, cancer prevention (Thomson 2004).

As mentioned before, the Portuguese situation is difficult to assess due to scarce information (Viegas-Crespo *et al.* 2000, Pavão *et al.* 2003, Lopes *et al.* 2004) and lack of consistent studies on this subject; there are no national guidelines or reference values either. However, it should not be that different from much of Western Europe (Van Cauwenbergh *et al.* 2004), where falls in Se intake – and corresponding drops in the blood indicators of Se status – have long been a matter of concern (Rayman 1997, Combs 2001, Rayman 2002).

Table 1.1 Recommended dietary intakes (μg day^{-1}) of Se for adults (Thomson 2004).

Country/Organisation	Men	Women
AUSTRALIA (Recommended dietary intake)	85	70
EUROPE (Population reference intake)	55	55
GERMANY, AUSTRIA, SWITZERLAND (Reference nutrient intake)	30-70	30-70
UNITED KINGDOM (Reference nutrient intake)	75	60
USA, CANADA (Recommended dietary allowance)	55	55
WORLD HEALTH ORGANIZATION (Normative requirement estimate)	40	30

1.3 Cereals

Cereals are extremely important food items for human nutrition, owing to their content in carbohydrates, proteins, dietary fibres and some minerals, such as calcium, iron, selenium and zinc. One of the problems of diets based predominantly on cereals is that the intake of some essential elements can be unsatisfactory, because, generally, the cultivation soils become exhausted due to decades, or even, centuries of farming. Some countries had been successful in correcting some of those micronutrients' deficiencies, with the implementation of programs that may include fertilisation, education and supplementation (Galinha *et al.* 2011a).

Although protein-rich foods usually contain higher levels of Se than other food categories (Marzec *et al.* 2002, McNaughton and Marks 2002, Klapec *et al.* 2004, Sirichakwal *et al.* 2005, Pappa *et al.* 2006, Navarro-Alarcón and Cabrera-Vique 2008), to the point that unbalanced diets (vegetarian, vegan, ethnic) may lead to nutritional Se deficiency (Srikumar *et al.* 1992, Donovan

et al. 1992), cereals – and, notably, among them, wheat – remain one of the most important dietary sources of Se (Lyons *et al.* 2003, Lyons *et al.* 2005). There is a wide variation of Se levels in cereals (Dumont *et al.* 2006), and given that cereals are the backbone of human diets worldwide (Lorenz *et al.* 1977, Shewry and Halford 2002), it is no wonder that they appear as obvious candidates for biofortification strategies that may help enhance the Se status of an entire population (Lyons *et al.* 2004, Welch and Graham 2004, White and Broadley 2005, Graham *et al.* 2007, White and Broadley 2009, Hawkesford and Zhao 2007). Portugal is no exception to such pattern, since cereals and their derivatives (breads, breakfast blends, pastas, etc) do make up a sizeable share of the Portuguese diets, as aforesaid.

1.3.1 Survey of cereals and cultivation soils

Soil is a complex, heterogeneous mixture of organic and inorganic matter, with different components that determine its physical, chemical and biological properties. Not less than 68 trace elements in soils represent only about 0.6 % of their total composition, while 12 minor and major elements – Si, Al, O, Ca, Fe, K, Ti, Mg, Mn, Na, Cr, Ni – account for the remainder (Baize 1997). Significant local or regional imbalances (relative to gross pedological averages) may occur in soil composition, due to, for instance, volcanic events (Davies 1980, Shoji *et al.* 1994). However, it is the problem of recurrent, widespread mineral deficiency in soils – often translating into recurrent, widespread mineral malnutrition in humans up the food chain – that has caused major concern and prompted an array of remedial strategies to meet human wellbeing requirements in essential elements (Welch and Graham 2004, White and Broadley 2005, Graham *et al.* 2007, White and Broadley 2009).

Of course, mineral composition (of soils) is one thing, whereas mineral bioavailability (in soils) is quite another. Elemental uptake by all plants – and, therefore, by agricultural plants and staple crops – is contingent upon specific chemical forms (Marschner 1995). Still, it is obvious that soil type and properties are absolutely germane to devising any agronomic procedure that may aim at improving the mineral-nutrient quality of some cultivar

(Bisbjerg and Gissel-Nielsen 1969, Shuman 1998, Frossard *et al.* 2000). Agronomic solutions for the supplementation (biofortification) of edible crops are sometimes viewed as short-term alternatives, albeit effective, to longer-term genetic improvement (Lyons *et al.* 2004, Hawkesford and Zhao 2007, Cakmak 2008).

For an eventual nutrient-supplementation purpose, an extensive investigation of elemental levels in home-grown cereals and their cultivation soils has been carried out across the main production areas of mainland Portugal, under the framework of a research contract by the Portuguese Foundation for the Science and the Technology (PTDC/QUI/65618/2006; FCT, Portugal). That investigation has resulted in sampling of rice (*Oryza sativa* L.) (Galinha *et al.* 2011b), rye (*Secale cereale* L.) (Galinha *et al.* 2010), barley (*Hordeum vulgare* L.) (Galinha *et al.* 2011c), bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* L.) (Galinha *et al.* 2013b), from the 2009 harvest campaign. Cereal and soil samples from distinct areas that feature significant productions for each cereal were collected through the summer of 2009.

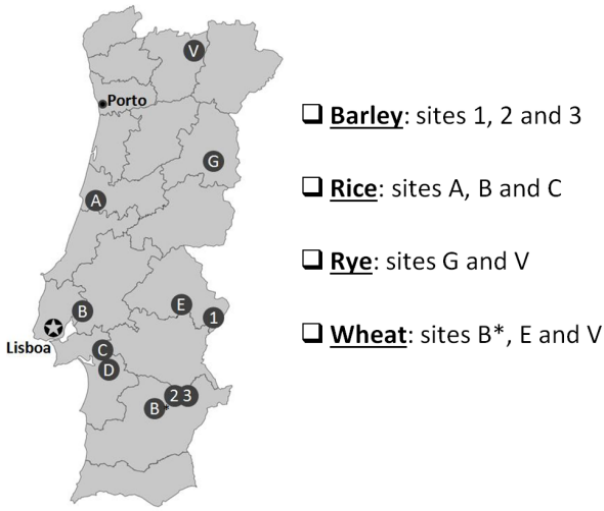

Figure 1.1 Outline of mainland Portugal, showing the approximate locations of the agricultural fields for plant and soil sampling.

1.3.1.1 Rice, rye and barley

As a cereal grain, rice is the most important staple food for a large part of the world's human population: rice ranks second among all cereals' worldwide production, after maize (corn) (FAOSTAT 2014). Since a large portion of maize crops are grown for purposes other than (direct) human consumption, rice is the most important grain with regard to nutrition and caloric intake, providing more than one fifth of the calories consumed worldwide by the human species (Smith 1998).

Rye is second only to wheat among the edible grains most commonly used in bread making, and is also important for production of mixed animal feeds (Bushuk 2001). Because it is extremely winter-hardy and able to grow in sandy, low-fertility soils, rye can be cultivated in areas that are generally not suitable for other cereal crops (Bushuk 2001).

Barley is an ancient cereal and a relevant (founder) crop, and it ranks fourth among all crops as dry-matter tonnage in the world today (FAOSTAT 2014). It is a cereal of great importance, mainly used in livestock fodder (about two-thirds) and for the brewing industry (about one-third); only a residual amount (about 2 %) is used directly for human food (Rodrigo *et al.* 2013).

1.3.1.2 Wheat

Wheat is one of the most important cereals in the world, with a production of more than 670 millions of tons – about 26 % of the cereals' production worldwide – only surpassed by rice and maize that represent 28 % and 34 %, respectively (FAOSTAT 2014). However, wheat remains unrivalled as to overall area and latitudinal range of cultivation, nutritional relevance, and civilisational significance at large. Wheat has a wide range of diversity and can be sown in quite different climates and terrains, including high-altitude regions in the tropics and sub-tropics (Shewry 2009). Current production is split between an overwhelming majority of hexaploid bread wheat (about 95 %), and a remaining share of (mostly) tetraploid durum wheat plus small amounts of hulled-grain species (einkorn, emmer, spelt) (Peng *et al.* 2011, Brouns *et al.* 2013).

In Portugal, wheat represents more than 85 % of the human consumption of cereals: within this apportionment, 87 % is bread wheat and 13 % is durum wheat (INE 2013). The former figures, together with the fact of wheat being a major dietary source of Se (Lyons *et al.* 2004), clearly point out this cereal as an obvious candidate for Se-improvement strategies that can help upgrade the Se-status indicators for the Portuguese population.

Overall, the cereal survey resulted in a screening of several elements, with Se among them (Galinha *et al.* 2010, Galinha *et al.* 2011b, Galinha *et al.* 2011c, Galinha *et al.* 2013b). However, quantitative determination of total Se concentrations in cereal and soil samples has not been feasible, because they invariably fell below the detection limit of the analytical methodology. This finding has made an even stronger case for looking into the possibility of supplementing Portuguese cereals – especially, wheat – with Se, through an experimental assessment of different biofortification procedures in actual field conditions.

1.4 Supplementation methods

Selenium concentrations in food and feed crops can usually be improved by addition of Se to soil-crop systems, a practice that is known as agronomic biofortification (Hawkesford and Zhao 2007). The most common methods of supplementation with a specific element are soil and foliar applications; the use of Se-enriched seeds is another option, even if much less common than the former ones.

1.4.1 Soil application

Soil application occurs when an element-containing vehicle or an element-enriched fertiliser is directly applied to the soil, and, usually, prior to sowing. This method presents several advantages, such as early treatment and easy operation, since it can be combined with a regular fertilisation program. A few drawbacks may be identified as well, mainly related to: i) application schedule – bigger investment at the beginning of the crop cycle; ii) soil

physics and chemistry – temperature, pH or chemical interactions may limit the bioavailability of elements (WolfTrax 2014).

Although studies involving Se addition to soil have begun in the 1970s, this remains a popular topic of research, with ongoing programs in experimental fields and greenhouses around the world, and focusing in a wide variety of plants. Some examples of studies worldwide are New Zealand (Reilly 1996), Europe (Broadley *et al.* 2006, Seppänen *et al.* 2010), Australia (Lyons *et al.* 2004, Lyons *et al.* 2005a, Lyons *et al.* 2005b), USA (Carvalho *et al.* 2003), Canada (Gupta and Macleod 1994, Grant *et al.* 2007), Brazil (Fernandes *et al.* 2014). As for soil-addition routines already implemented, the benchmark example is the already mentioned Finnish case (Eurola *et al.* 1990, Arthur 2003, Pietinen *et al.* 2010).

1.4.2 Foliar application

Foliar application takes place when an element-vehicle solution is applied to the plants' foliage. Foliar applications become soil applications when excess solution is used, or when rain falls shortly after the procedure. This results in an inadequate absorption of the nutrient through the foliage, thus in a loss of chemicals, time, machinery-use and labour. However, it is not a complete waste, as some nutrients that may get to the soil will be taken up by the root system (McCall 1980).

This method avoids soil interactions and potential nutrient tie-up; elements are generally absorbed more rapidly than when added to soil; rates and times of application are more precise; aerial application can be combined with existing spray programs; and smaller quantities of the active material are required than when applying to soil. On the other hand: if nutrient deficiency is severe, the timing of the application may be too late to solve the problem before the end of the cycle (harvest); and if nutrient requirement is high, this can be an impractical method of supply. Also, the chemical vehicle of the element must be water-soluble, and it provides supplementation only for that years' crop, as it has no soil build. Last yet by no means least, bad weather may delay or even prevent applications (McCall 1980, WolfTrax 2014).

Foliar application of Se-containing solutions for biofortification purposes is also an issue of studies worldwide, namely in Australia (Lyons *et al.* 2005a, Lyons *et al.* 2005b), Canada (Gupta and Macleod 1994, Grant *et al.* 2007), China (Fang *et al.* 2009), USA (Kopsell *et al.* 2009), and several European countries (Milovac *et al.* 1998, Galinha *et al.* 2013a, Poblaciones *et al.* 2014, Giacosa *et al.* 2014).

1.4.3 Selenium-enriched seeds

An alternative to both soil- and foliar-supplementation approaches to crop biofortification may be envisaged: the seed enrichment prior to sowing. This method consists in soaking seeds in a Se solution off-site, i.e. at a laboratory, before sowing them in a non-supplemented field (Smrkolj *et al.* 2007). The topic of Se-enriched seeds is much less studied than the other methods, and the available literature is scarce. Smrkolj *et al.* (2007) used Se-enriched bean seeds that produced beans with increased Se contents, while Ožbolt *et al.* (2008) and Štrekelj *et al.* (2014) also succeeded in improving the Se contents of buckwheat. Barley and wheat have been the subject matter of akin studies as well (Liu *et al.* 2011, Davydenko and Mayurnikova 2014). So far, though, the seed-enrichment approach has yet to move from the realm of bench or greenhouse experimentation into open testing in actual field conditions.

1.5 Plant breeding

Plant breeding can be summed up as informed selection and accomplished propagation of distinctive variants from a population. The two major keys to successful breeding are ‘variation’ and ‘selection’. In short, all that a breeder really needs is some degree of genetic variation between the individuals in a given population, together with a way of identifying and selecting the most appropriate variants. These variants are then mated or crossed with each other, in order to yield a population that is now composed almost exclusively of the newly-selected, genetic variety (Murphy 2007).

Within cereal-crop varieties, there exists a substantial variability for iron, zinc and other micronutrients, and this may also be the case for Se; however

little research has been done as yet. Long-term genetic improvements (plant breeding ≡ genetic biofortification) and short-term agricultural procedures (soil/plant fertilisation ≡ agronomic biofortification) are viewed as the most attractive and effective methods to increase the Se-status indicators of a whole population (Lyons *et al.* 2005b).

1.5.1 Archival wheat collections

Wheat germplasm was among the first to be stored in archival collections and seed banks, despite early technical difficulties in preserving genetic resources as germplasm holdings (Sachs 2009, Dierig *et al.* 2014). Wheat (*Triticum* genus) also tops the list of the largest worldwide germplasm and *ex situ* collections by crop (Börner 2006, Kilian and Graner 2012, Carvalho *et al.* 2013).

Cereal-plant germplasm, including cereal landraces, is often used for trait evaluation, breeding or pre-breeding, basic research and assemblage of core collections. Archival collections are especially important for the preservation of landrace resources, in order to prevent their disappearance as they are often underestimated sources of new crop traits (Carvalho *et al.* 2013).

Following the first morphological and taxonomic inventory of Portuguese wheat landraces and old cultivars (Vasconcelos 1933), an archival collection of representative varieties has been maintained, replanted and documented by the National Institute of Agricultural and Veterinary Research (INIAV), specifically by its division formerly known as the National Station for Plant Improvement (ENMP-Elvas, now INIAV-Elvas). The INIAV-Elvas wheat collection has always been an invaluable asset in studies of agronomic and/or genetic development of wheat lines, as well as providing a reference frame for the nutritional evolution of Portuguese wheat crops (Carvalho *et al.* 2012, Santos *et al.* 2012). Despite a relatively limited extent, the INIAV-Elvas collection has also been regularly present in international surveys and core-collection studies (Asins and Carbonell 1989, Balfourier *et al.* 2007).

1.6 Thesis outline

The main aim of this thesis has been to study – implement and evaluate – different methods to enhance Se contents in wheat, using some of the most important Portuguese varieties of bread and durum wheat, under Portuguese weather conditions, and through cultivation practices used by the Portuguese farmers. One of the prime concerns within this framework was to carry out most trials in actual field conditions, thus avoiding indoor experiments in laboratories or greenhouses.

The primary impact of this work is to provide real field knowledge about the most efficient methodologies of Se supplementation in wheat, with a view to upgrading the Se-status indicators of the Portuguese population.

Chapter 2 is focused on bread wheat and durum wheat from the 2009 harvest campaign, collected at Trás-os-Montes, Alto Alentejo and Baixo Alentejo, the main Portuguese production areas. The purpose is to make an extensive investigation of the levels of several elements in wheat and their agricultural soils, looking to a possible nutrient supplementation.

Chapter 3 comprises the Se status of cereals and their cultivation soils. From the 2009 cereal survey, it became apparent that Se could not be determined due to very low levels of this element in samples, making it necessary to find an analytical technique that was capable of quantitatively assessing Se.

Chapter 4 addresses the tentative enrichment of bread-wheat seeds with Se, while optimising both their soaking time and washing time through detection of a Se radiotracer (^{75}Se).

Chapter 5 deals with the ability of bread and durum wheat to accumulate Se after supplementation via alternative application procedures: the first part of the chapter is focused on foliar addition and the second part on soil addition. Representative Portuguese bread- and durum-wheat cultivars were selected for Se-supplementation trials, that were conducted in actual field conditions. Experimental field design was devised to account for the following attributes germane to either procedure: wheat cultivar, growth stage (foliar application only), Se matrix and field replication.

Chapter 6 evaluates and discusses the potential of wheat plants to assimilate and biotransform Se, and to accumulate organically-bound Se in their mature grains after supplementation. Inedible wheat by-products (straw, chaff) can be used as livestock fodder, and, in principle, could as well convey Se up the food chain. However, what really matters in terms of major human Se intake via wheat is the Se concentration and ,especially, the chemical form of Se in post-harvest, pre-milling grains.

Chapter 7 addresses the current status of Se in a pool of 46 accessions of bread wheat from a Portuguese archival collection.

Chapter 8 provides an overview and a discussion of the achieved results, and some suggestions for future research.

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2 Characterisation of bread and durum wheat and their cultivation soils

Based on article:
"Elemental characterization of bread and durum wheat by instrumental neutron activation analysis"
Galinha C, Freitas MC & Pacheco AMG (2013)
Journal of Radioanalytical and Nuclear Chemistry 297:221-226

2.1 Abstract

Cereals are by far the most significant agricultural crops, not only due to the sheer amount of their gross-tonnage production and prevalence in human diets worldwide, but also as food vehicles of important items for human nutrition and wellness at large– proteins, dietary fibres and oligoelements, such as selenium, calcium, zinc and iron, to name just a few. Still, some micronutrients feature an uneven distribution in the upper continental crust, and thus in cultivation soils deriving therefrom. Whether soils have always been poor in an essential element, or have just become deprived of it by intensive farming, the result is the same: insufficient soil-plant transfer, feeble-to-nonexistent plant uptake, and, therefore, unsatisfactory dietary distribution of that element up the food chain.

Countries that have implemented corrective measures, or programs of crop biofortification and consumer education, have been successful in dealing with some micronutrients' deficiencies as well. Given their relative weight in Portuguese diets, cereals are obvious candidates for crop supplementation strategies that may contribute to an upgrade in the health status of the whole population. A good knowledge of element-baseline data for major cereal varieties (plants) and main production areas (soils) is a prerequisite though. The present work was aimed at an elemental characterisation of cereals and soils from relevant wheat-producing areas of mainland Portugal.

This paper is focused on wheat samples – bread and durum wheats; *Triticum aestivum* L. (Farak and Jordão cultivars) and *Triticum durum* Desf. (Don Duro and Simeto cultivars), respectively – from the 2009 campaign,

collected at Trás-os-Montes, Alto Alentejo and Baixo Alentejo (inland regions). Elemental concentrations were determined by instrumental neutron activation analysis (INAA; k_0-variant), and assessed with the k_0-IAEA software. Quality control was asserted through the analysis of NIST-SRM® 1567a (Wheat Flour), NIST-SRM® 1568a (Rice Flour) and GBW 07404 (Limy-yellow Soil). Results are discussed and compared to available data from abroad.

2.2 Introduction

Wheat is one of the most important agricultural food and feed crops worldwide. Its production ranks second among all cereals, with an annual production of almost 686 million tonne and an area of 226 million ha, that is about 27 % of the total cereal production (2009 data) (FAOSTAT 2011). The humankind directly consumes more than 60 % of that production. Other than its caloric value, wheat is the most important source of plant protein in the human diet. Wheat supplies about 20 % of the energy and about 25 % of the protein requirements of the world population (Sahrawat *et al.* 2003, Högy and Fangmeier 2008).

Portugal is no exception to such pattern, accounting for the relative weight of cereals in Portuguese food consumption (Ventura *et al.* 2007, Ventura 2008). Actually, cereals (especially wheat) and their derivatives (breads, breakfast blends, pastas, etc.) represent a significant portion of the Portuguese diet, thus increasing the bioavailability of essential elements through wheat biofortification can help upgrade the health status of the entire population (Galinha *et al.* 2010). Looking to a possible nutrient supplementation, an extensive investigation on the levels of several elements in wheat cultivars and their agricultural soils was carried out across the main production areas of mainland Portugal, under the research contract PTDC/QUI/65618/2006 (FCT; Portugal). This paper is focused on two wheat species – bread and durum wheats; *Triticum aestivum* L. (Farak and Jordão cultivars) and *Triticum durum* Desf. (Don Duro and Simeto cultivars), respectively – from the 2009 campaign, collected at the inland regions of Trás-os-Montes (site V), Alto Alentejo (sites E) and Baixo Alentejo (sites B).

2.3 Experimental

Three different regions of Portugal were selected to sample cereal plants of *T. aestivum* L. and/or *T. durum* Desf. Those cultivars were sampled in July 2009. Figure 2.1 shows the locations (in mainland Portugal) where the samples were collected. Table 2.1 summarizes the types of wheat collected in each sampling site, as well as their variety and farming procedure.

Topsoils were collected from every site at depths down to 15 cm. Each collection batch (around 4 kg per site) was processed in the laboratory to make a homogeneous sample: soils were allowed to dry at room temperature, mixed up, homogenised, sieved through a 1-mm mesh screen for removal of coarser materials, and ground to a fine powder using a porcelain mortar and pestle. Site-representative samples of about 150 mg each were then put into ultrapure polyethylene containers for further analysis (three replicates per sample).

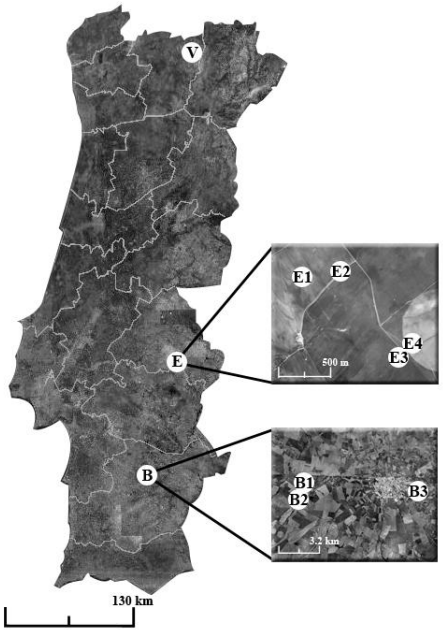

Figure 2.1 Outline of mainland Portugal, showing the location of the wheat fields for soil and plant sampling (see Table 2.1 for coding).

Cereal grains were collected from spike kernels and then weighed, washed for 10-15 s with distilled water, and frozen-stored within polyethylene bags. Prior to elemental analysis, grain samples were lyophilised with an Edwards Modulyo® freeze-dryer (plate at -40 ºC; vacuum of 0.4 atm), ground to a fine powder in a Waring® blender HGB50E2 for about 2-5 min, and then put into ultrapure polyethylene capsules (three replicates per sample; 250-300 mg each).

Table 2.1 Characteristics of sampling sites and collected cereals in three different regions of mainland Portugal: Trás-os-Montes (TM), Alto Alentejo (AA) and Baixo Alentejo (BA).

Site	**Cereal**	**Variety**	**Location (region)**	**Farming**
V	Bread wheat	Sacho	Alvarelhos, Valpaços (TM)	Rainfed
E1	Durum wheat	Simeto	Herdade dos Ledos, Fronteira (AA)	Irrigation
E2	Bread wheat	Jordão	Herdade dos Ledos, Fronteira (AA)	Rainfed
E3	Durum wheat	Simeto	Herdade dos Ledos, Fronteira (AA)	Irrigation
E4	Bread wheat	Jordão	Herdade dos Ledos, Fronteira (AA)	Irrigation
B1	Durum wheat	Don Duro	Herdade da Misericórdia, Pisões, Beja (BA)	Irrigation
B2	Durum wheat	Don Duro	Herdade da Misericórdia, Pisões, Beja (BA)	Irrigation
B3	Bread wheat	Farak	Beja (BA)	Rainfed

All samples were irradiated at the Portuguese Research Reactor (RPI; CTN-IST, Sacavém) for 1 h (soil) and 5 h (grain), at a thermal-neutron fluence rate of 2.25×10^{12} cm^{-2} s^{-1}, together with disks (thickness:125 mm; diameter: 5 mm) of an Al-0.1%Au alloy as comparators. Gamma spectra were acquired with a liquid N_2-cooled, high-purity Ge detector (1.85 keV resolution at 1.33 MeV; 30 % relative efficiency). Samples were measured after 2-3 days and 3-4 weeks of decay time, and comparators after one week. Element concentrations were assessed through k_0-standardised, instrumental neutron activation analysis (k_0-INAA), and calculations were done with the current version of the k_0-IAEA software (version 5.22).

Quality control was performed through concurrent analysis of three certified reference materials: NIST-SRM®1567a (Wheat Flour), NIST-SRM® 1568a (Rice Flour) and GBW 07404 (Limy-yellow Soil). Reference materials were analysed in the same way as the corresponding field samples (pedological or biological), and irradiated simultaneously with them. Moisture contents of reference materials have been measured as well.

2.4 Results and discussion

2.4.1 Quality control

Quality control of the present analytical procedure is shown in Figure 2.2, which depicts the ratios between results obtained in this work for the reference materials and their certified values. Uncertainty intervals include both uncertainties on results and certified values. Overall, the ratio data seem acceptable, even if there are a few elements in the Chinese soil standard (GBW 07404) and Wheat Flour (NIST-SRM® 1567a) that are appearing below the expected values. The weight amount of NIST-SRM® 1567a for k_0-INAA was much less than what was recommended in the corresponding certificate of analysis (150 mg instead of 500 mg). Still, the agreement may be considered fair, taking into account the total uncertainties (at the 67 % confidence level), and the possibility of some heterogeneous distribution of molybdenum in the bulk of that reference material (NIST-SRM® 1567a). Accounting for the uncertainties in the ratio data for NIST-SRM® 1568a, which feature a 95 % confidence level, all such ratios may be viewed as pointing to a good agreement.

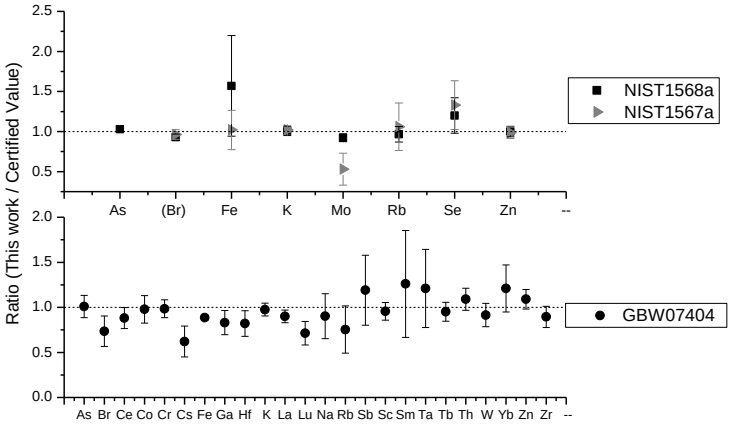

Figure 2.2 Ratios between the concentration results obtained in this work for the NIST-SRM® 1567a, NIST-SRM® 1568a and GBW 07404 reference materials and their certified values, with uncertainties at the 67 % confidence level for NIST-SRM®1567a and GBW 07404, and at the 95 % confidence level for NIST-SRM® 1568a, respectively (chemical elements in brackets are informative only).

2.4.2 Soil samples

Elemental concentrations in soil samples from agricultural fields that were supporting the wheat crops are given in Figure 2.3 The concentrations can be seen to increase in the order Br < Sc < Co < As < Zn < Cr < Rb < Na < K < Fe < Ca. For soils from bread-wheat fields, it was not possible to determine the amount of calcium. According to Kabata-Pendias and Pendias (2001), the abundance of bromine in the earth's crust varies in the range of 0.2 to 10 mg kg^{-1}; within this work, the lowest value of 2.2 ± 0.3 mg kg^{-1} and the highest value of 7.3 ± 0.3 mg kg^{-1} were found for sites B2 and E1, respectively. Soil from the northern part of Portugal (site V) shows the lower value of scandium, that increases as we go down south. All concentrations for cobalt were below the interim criterion (40 mg kg^{-1}) by the Canadian Soil Quality Guidelines (CSQG 2011); the highest value was for site B1 (35 ± 1 mg kg^{-1}). Concentration of zinc ranges from 50 to 85 mg kg^{-1}, that is

in accordance with literature data, and far below the maximum tolerable limit of 300 mg kg^{-1} (Dias *et al.* 2007).

Chromium results are slightly above the reported values except for site V, whose result is quite similar (FOREGS 2011); results are above the CSQG (2011) guideline for agricultural land as well (64 mg kg^{-1}), again with the exception of site V. Conversely, site V has the highest value of rubidium (360 ± 20 mg kg^{-1}), which represents three times the average of the other values; still, all concentrations obtained for this element are within the range found in the literature (FOREGS 2011). Rubidium could not be determined for sites B, for being below the detection limit of 30 mg kg^{-1}. Sodium concentrations are significantly lower in sites E, while the distribution of potassium shows exactly the opposite trend, with sites E featuring higher values than sites B: this may be likely due to fertilisers based on (enriched in) potassium (sites E) or sodium (sites B).

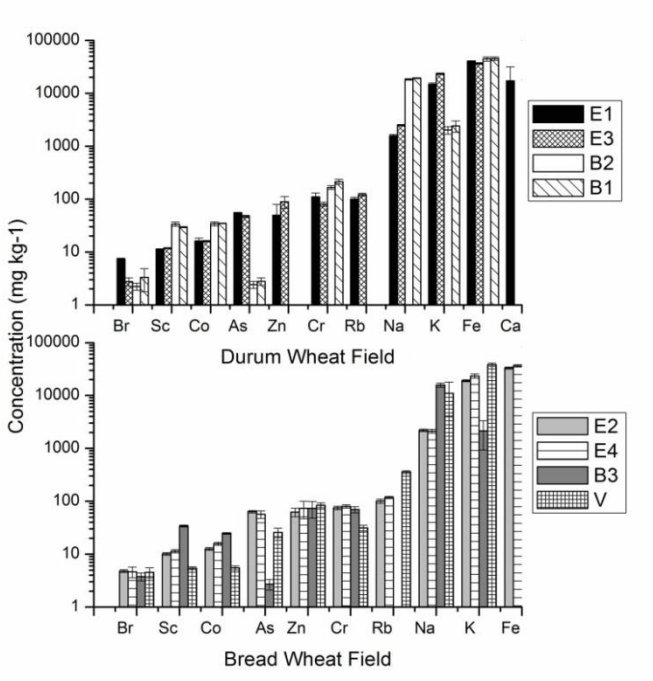

Figure 2.3 Elemental concentrations (in mg kg^{-1}) in samples of cultivation soils from fields of bread and durum wheat.

Total content of iron in soil is generally about 38 g kg^{-1} (Pais and Benton Jones 1997), and our samples have a mean value of 43 g kg^{-1}. Samples from sites E and V show results for arsenic higher than those reported in the literature: Kabata-Pendias and Pendias (2001) state that a grand mean for arsenic in soil should be put around 9 mg kg^{-1}, while Pais and Benton Jones (1997) indicate the range 3.6-8.8 mg kg^{-1}. According to the CSQG (2011), arsenic should not exceed 12 mg kg^{-1}, but other authors have put forward a recommended limit of 20 mg kg^{-1} for agricultural land use and a permissible limit of 40 mg kg^{-1}(Seiler *et al.* 1994). Our results show that sites B and V comply with the recommended value of 20 mg kg^{-1}, but sites E are slightly above that permissible limit, even if they still keep in agreement with baseline data by the Forum of European Geological Surveys (FOREGS 2011). In the near past, arsenic-containing compounds, such as pesticides and herbicides, had been widely used, which most likely explains the present arsenic levels in some of our agricultural soils.

2.4.3 Cereal samples

Elemental concentrations in mature grains of bread and durum wheat were found to increase in the order Sc < Co < Mo < Cr < Br < Rb < Na < Zn < Fe < Ca < K, as shown in Figure 2.4. Arsenic was detected in samples from site E4 and site V, with concentrations of 11 ± 5 and 21 ± 5 μg kg^{-1}, respectively. All samples from the other sites were below the detection limit (around 10 μg kg^{-1}). Although the arsenic contents in soil samples from sites E were higher than in samples from other sites, this trend does not occur here, which means that wheat does not have a tendency to accumulate arsenic in the grain. The acceptable daily intake (ADI) of arsenic for a human weight of 70 kg is 0.14 mg (Seiler *et al.* 1994): considering that, as an average, each Portuguese ingests around 60 g of wheat bread per day (Galinha *et al.* 2012), the higher arsenic content in the present grain samples would correspond to just 1 % of such an ADI.

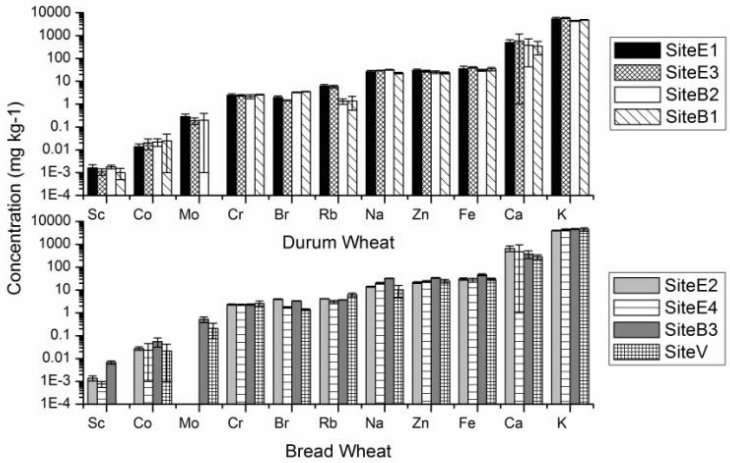

Figure 2.4 Elemental concentrations (in mg kg^{-1}; dry weight) in grain samples of bread and durum wheat.

Data about scandium in plants are scarce in the literature; however, an indicative value of 0.02 mg kg^{-1} for rye grains (Galinha *et al.* 2010) and a range of 0.002-0.1 mg kg^{-1} for several plant foods (Kabata-Pendias and Pendias 2001) have been reported, which is consistent with our results. Also, the contents of cobalt in wheat-grain samples were near to the normal level of this element in wheat (0.05 mg kg^{-1}) (Al-Gahri and Almussali 2008), thus standing very far behind the toxicity threshold of 500 mg day^{-1} (Pais and Benton Jones 1997).

Kabata-Pendias and Pendias (2001) have reported a range of 0.18-0.42 mg kg^{-1} for molybdenum in wheat from the Czech Republic, and our results appear very similar (0.19-0.52 mg kg^{-1}). Toxic intake of chromium starts at 200 mg day^{-1} (Pais and Benton Jones 1997), so there is a very small contribution of chromium (less than 0.1 %) to the dietary intake of an adult from eating bread made of wheat. The value ranges for bromine, rubidium, zinc and iron are 2-4, 3-6, 20-30 and 30-45 mg kg^{-1}, respectively, which seems consistent with the literature data as well (Pais and Benton Jones 1997, Kabata-Pendias and Pendias 2001).

2.5 Conclusions

The elemental concentrations in wheat-cultivation soils of mainland Portugal were found to increase in the order Br < Sc < Co < As < Zn < Cr < Rb < Na < K < Fe < Ca, whereas for mature wheat grains the order was Sc < Co < Mo < Cr < Br < Rb < Na < Zn < Fe < Ca < K. Arsenic contents in soils from sites E (Alto Alentejo) are slightly above the tolerable limit for agricultural land; however, wheat grains do not show significant levels of this element, much less any risk for human intake. For almost all elements discussed here, and for both soil and cereal samples, concentration results are in generally fair agreement with the corresponding values reported in the literature.

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3 Analytical methodologies to access low levels of total selenium in cereal samples

Based on article:
"Selenium determination in cereal plants and cultivation soils by radiochemical neutron activation analysis"
Galinha C, Freitas MC, Pacheco AMG, Kameník J, Kučera J, Anawar HM, Coutinho J, Maçãs B & Almeida AS (2012)
Journal of Radioanalytical and Nuclear Chemistry 294:349-354

3.1 Abstract

Selenium (Se) is an essential micronutrient for human health but it is deficient in at least 1 billion people around the globe. Cereals are by far the most significant agricultural crops, not only on a gross tonnage basis, but also by what they represent in terms of energy supply and dietary intake for human nutrition worldwide. Portugal is no exception to such pattern. The Portuguese situation is difficult to assess though, due to scarce information and lack of consistent studies on the subject. In these terms, the Se status of major cereals and their cultivation soils are dealt with herein. Two species of wheat – bread and durum wheat – were sown in the end of November 2009, and then sampled at different growth stages. Rye was collected during harvest season, and cultivation soils were analysed as well. Se results were within the range of: 100-225 ng g^{-1} for soils; 3-55 ng g^{-1} for durum wheat; 6-80 ng g^{-1} for bread wheat; and 4-30 ng g^{-1} for rye. Accuracy of the RNAA procedure was proved by analysis of reference materials NIST-SRM® 1515 and NIST-SRM® 8433.

3.2 Introduction

An increase in the bioavailability of essential elements through cereal-crop biofortification may contribute to an upgrade in the health status of the Portuguese population. Selenium (Se) is of utmost importance for a healthy individual, for its protective (specific) effects against the cardiovascular disease, asthma, male sterility, and, especially, certain forms of cancer.

Minimum Se intakes of 40 and 30 μg per day for adult males and females, respectively, are internationally-suggested average requirements. Portuguese studies on Se are scarce, yet the available data indicate that current daily intakes fail to meet the above requirements (Ventura *et al.* 2005, Ventura *et al.* 2007, Ventura 2008); hence, a Se-supplementation project targeting common cereals is currently under way (Galinha *et al.* 2010).

Back in the early 1980s, sodium selenite was already being used in moderate dosage at animal-breeding farms, for prophylactic and therapeutic purposes (Bem 1981). More recently, Finland and the UK have also addressed the issue of Se supplementation to crops (Eurola *et al.* 1991, Broadley *et al.* 2006). Concerning our own program, the Se levels in Portuguese cereals and their cultivation soils should be known prior to any supplementation trials. Still, the first attempts to quantify Se in wheat and rye samples through instrumental neutron activation analysis (INAA) at the Technological and Nuclear Institute (ITN) were unsuccessful, regardless using the long-lived ^{75}Se or the short-lived ^{77m}Se via cyclic INAA.

The main reason was a high content of elements, mainly Al, Hf and Ta, which form interfering radionuclides. A high activity of ^{28}Al, resulting either from the ^{27}Al(n,γ)^{28}Al reaction with thermal neutrons or from the ^{31}P(n,p)^{28}Al reaction with fast neutrons, adversely affects the Se detection limit when the short-lived ^{77m}Se is measured, due to an increased background below the 161.9 keV photopeak of ^{77m}Se. On the other hand, when INAA is used for low-level determinations via long-lived ^{75}Se, the most intense gamma-lines of this radioisotope – 121.2, 136.0, 264.7, 279.5 keV – are interfered by the gamma-lines of^{152}Eu (121.8 keV), ^{181}Hf (136.2 keV), ^{182}Ta (264.1 keV) and ^{203}Hg (279.2 keV), respectively.

In this work, two approaches of NAA were employed: (1) INAA, optimised for maximum ^{75}Se activation, using an extended irradiation at the highest neutron fluence rate available at ITN, while minimising ^{181}Hf interference by prolonging the decay time; and (2) a slightly modified radiochemical neutron activation analysis (RNAA) procedure, based on the separation of ^{75}Se (Kučera and Soukal 1993). This paper is focused on the latter methodology,

reporting the Se status of wheat in various stages of plant growth and grain formation, as well as the Se contents in seeds and soil. Se levels in mature (harvested) rye plants will also be reported herein. The accuracy of Se determination by INAA is assessed by comparing RNAA and INAA results.

3.3 Experimental

Table 3.1 lists the characteristics of the soil, wheat and rye samples, together with their coding (abbreviations) for this work: the generic string [A-B-C-D-nr] stands for, consecutively, the cereal type (wheat: W; rye: R), the cultivation site (Elvas: E; Guarda: G; Valpaços: V), the wheat variety (Jordão: J; Marialva: M), the sample type (soil: So; seed: Se; root: Ro; straw: St; spike: Sp), and the growth stage for sampling (wheat cultivation period: 1; tillering: 2; booting: 3; grain filling: 4; rye harvesting: 5). Zero is used whenever an attribute does not apply.

3.3.1 Sampling

Two wheat varieties – bread wheat (*Triticum aestivum* L.; Jordão cultivar, J) and durum wheat (*Triticum durum* Desf.; Marialva cultivar, M) – were sown at the end of November 2009 (period 1), and then sampled in January 2010 (period 2), at the end of March/beginning of April 2010 (period 3), and in May 2010 (period 4). Cultivation took place at Herdade da Comenda (Elvas; Alentejo province). Soil and seed samples (both varieties) were taken at the sowing time. Rye samples were collected in July 2009 (period 5), when the cereal was about to be harvested, at Ribeira dos Carinhos (Guarda; Beira Alta province) and Santiago-Alvarelhos (Valpaços; Trás-os-Montes province). Cultivation soils were sampled at the same (harvest) time.

3.3.2 Preparation of samples at ITN

Topsoil samples (0-E-0-So-1, 0-G-0-So-5, 0-V-0-So-5) were collected at depths down to 15 cm, allowed to dry at room temperature, sieved through a 1-mm mesh screen for removal of coarser materials, and ground to a fine powder using a porcelain mortar and pestle. For this work, not all samples

from the three sampling campaigns were dealt with: a selection was made to show the capabilities of RNAA for Se determination at the ng g^{-1} level.

The wheat plants were split into roots (W-E-J-Ro-2, W-E-M-Ro-2), straws (W-E-J-St-2, W-E-J-St-3, W-E-MSt-2, W-E-M-St-3, W-E-M-St-4) and spikes (W-E-M-Sp-3, W-E-M-Sp-4). After removing the bulk of soil, roots were washed with a solution of HCl 0.1 M for 15-20 s, dried in open air, cut into small pieces, weighed and frozen. Seeds, straws and spikes were also cut into small pieces, weighed, washed for 10-15 s with bidistilled water and frozen. Prior to elemental analysis, all samples were lyophilised in an Edwards Modulyo® freeze-dryer (plate at -40 ºC; vacuum of 40 kPa). Roots were ground to a fine powder in a Braun® Mikro-Dismembrator U ball mill with Teflon™ capsules, at 1500 rpm for about 9 min. Seeds (W-E-J-Se-1, W-E-M-Se-1), straws and spikes were milled using a Waring® blender HGB50E2.

The rye plants were also split into roots (R-G-0-Ro-5, R-V-0-Ro-5), straws (R-G-0-St-5, R-V-0-St-5) and seeds (R-G-0-Se-5, R-V-0-Se-5), followed by sample-preparation procedures as above. Water losses during the freeze-drying of all biological samples were assessed, in order to enable the conversion of dry-mass results to a fresh-mass basis.

3.3.3 Analysis of samples at ITN

For initial experiments, soil and plant samples were irradiated at the Portuguese Research Reactor (RPI-ITN; Sacavém) for 1 and 5 h, respectively, at a thermal-neutron fluence rate of 2.25×10^{12} cm^{-2} s^{-1}, together with one disc (thickness: 125 μm; diameter: 5 mm) of an Al-0.1%Au alloy as comparator. Gamma spectra were acquired with an HPGe detector (1.85 keV resolution at 1.33 MeV; 30% relative efficiency). The samples were measured after decaying for 3-4 weeks; the comparator was measured after 1 week. Elemental concentrations were assessed through k_0-standardised, INAA (k_0-INAA) (De Corte 1987), and calculations were done with the k_0-IAEA software (version 3.21).

Two field samples (cultivation soil from Elvas and rye root from Valpaços; ~200 mg each) and one reference material (GBW 07406 'Yellow–red Soil'; ~200 mg) were irradiated for 12 h at a thermal-neutron fluence rate of 5×10^{13} cm^{-2} s^{-1}, and then counted for 7 h after a decay time of 5 months, with a low-energy (planar) HPGe detector. Elemental concentrations were assessed through the relative method, using the reference material as the standard.

3.3.4 Analysis of samples at NPI

Sample aliquots of about 150 mg were weighed into silica ampoules, which were sealed. All ampoules were cleansed prior to use by leaching in dilute HF (1:6) for 24 h, leaching in aqua regia for 3 days, and by rinsing with deionised water several times. Reference materials NIST-SRM® 1515 'Apple Leaves' and NIST-SRM® 8433 'Corn Bran' were prepared in the same way as samples. A Se standard solution was prepared from 23.15 mg of elemental Se (99.995%; Fluka®), dissolved in approximately 5 mL of dilute HNO_3 (1:1) under reflux, and made up to 25 mL with deionised water in a measuring flask. For irradiation, 100 μL of the former solution containing 92.96 ± 0.45 μg of Se was pipetted and weighed into the Si ampoule, that was subsequently sealed.

Samples, reference materials, blank ampoules and standards were irradiated in the LVR-15 light-water reactor for 20 h, at a thermal neutron fluence rate of 6×10^{13} cm^{-2} s^{-1}. Thirteen test ampoules were accommodated in one Al irradiation container. Each ampoule carried an iron wire at half-height of the sample, for monitoring of the axial neutron flux gradient. The irradiated samples were allowed to cool for 1 month. The ampoules were cleaned on their surfaces by leaching in aqua regia and washing with distilled water. Then, the ampoules were cooled in liquid nitrogen, wrapped in paper tissue, put into a polyethylene bag, and crushed.

Table 3.1 Selenium results (total and fractional) for pedological and biological samples by RNAA (see Experimental for sample coding).

Sample	Code	Origin	Variety	Part	Sampling date	Organic fraction[a], ng g^{-1}	Mineral fraction[a], ng g^{-1}	Total Se[a], ng g^{-1} (dry mass)	Total Se[a], ng g^{-1} (fresh mass)
Soil	0-E-0-So-1	Elvas	–	< 1 mm	End of 11/2009	115 ± 6	3.1 ± 0.3	118 ± 6	118 ± 6
Wheat	W-E-J-Se-1	Elvas	Jordão	Seed	End of 11/2009	57 ± 3	< 0.3	57 ± 3	54 ± 3
Wheat	W-E-J-Ro-2	Elvas	Jordão	Root	19/01/2010	39 ± 2	< 0.3	39 ± 2	6.1 ± 0.3
Wheat	W-E-J-St-2	Elvas	Jordão	Straw	19/01/2010	22 ± 1	< 0.5	22 ± 1	3.1 ± 0.1
Wheat	W-E-J-St-3	Elvas	Jordão	Straw	13/04/2010	26 ± 1	0.46 ± 0.05	26 ± 1	24 ± 1
Wheat	W-E-M-Se-1	Elvas	Marialva	Seed	End of 11/2009	41 ± 2	< 0.3	41 ± 2	38 ± 2
Wheat	W-E-M-Ro-2	Elvas	Marialva	Root	19/01/2010	35 ± 2	0.39 ± 0.05	35 ± 2	31 ± 2
Wheat	W-E-M-St-2	Elvas	Marialva	Straw	19/01/2010	64 ± 3	0.62 ± 0.04	65 ± 3	55 ± 3
Wheat	W-E-M-St-3	Elvas	Marialva	Straw	30/03/2010	83 ± 4	< 0.3	83 ± 4	78 ± 4
Wheat	W-E-M-St-4	Elvas	Marialva	Straw	05/05/2010	53 ± 3	< 0.3	53 ± 3	9.4 ± 0.5
Wheat	W-E-M-Sp-3	Elvas	Marialva	Spike	30/03/2010	32 ± 2	< 0.3	32 ± 2	5.6 ± 0.4
Wheat	W-E-M-Sp-4	Elvas	Marialva	Spike	05/05/2010	25 ± 1	< 0.3	25 ± 1	22 ± 1
Soil	0-G-0-So-5	Guarda	–	< 1 mm	14/07/2009	97 ± 5	0.20 ± 0.03	97 ± 5	97 ± 5
Rye	R-G-0-Se-5	Guarda	–	Seed	14/07/2009	29 ± 2	0.51 ± 0.06	30 ± 2	23 ± 2
Rye	R-G-0-Ro-5	Guarda	–	Root	14/07/2009	3.3 ± 0.2	0.92 ± 0.06	4.3 ± 0.3	4.2 ± 0.3
Rye	R-G-0-St-5	Guarda	–	Straw	14/07/2009	7.0 ± 0.4	< 0.3	7.0 ± 0.4	6.7 ± 0.4
Soil	0-V-0-So-5	Valpaços	–	< 1 mm	14/07/2009	210 ± 10	15.1 ± 0.8	225 ± 11	225 ± 11
Rye	R-V-0-Se-5	Valpaços	–	Seed	14/07/2009	39 ± 2	< 0.3	39 ± 2	31 ± 2
Rye	R-V-0-Ro-5	Valpaços	–	Root	14/07/2009	5.0 ± 0.3	< 0.3	5.0 ± 0.3	4.9 ± 0.3
Rye	R-V-0-St-5	Valpaços	–	Straw	14/07/2009	7.2 ± 0.5	< 0.3	7.2 ± 0.5	6.8 ± 0.5

[a]combined uncertainties (coverage factor $k = 1$)

The samples, together with silica splinters, were transferred into a Hostaflon™ flask, to which 5-8 mL of conc. HNO_3 and 1 mL of a Se-carrier solution (5 mg mL^{-1}) were added. A pressurised, microwave-assisted, digestion system ERTEC® Magnum II (Poland) was employed for sample decomposition. The silica splinters were removed from the flask, and its content was washed out with several mL of conc. HNO_3, and filtered through glass fibre to separate the possibly present mineral fraction. The filtrate was put into a quartz Kjehldahl flask with 3 mL of conc. $HClO_4$, and heated over the flame of a gas burner until copious fumes of $HClO_4$ appeared. Then, 5 mL of dilute HCl (1:1) and 1 mL of saturated solution of $MgCl_2$ were added. About 250 mg of ascorbic acid were added to precipitate elemental Se; the precipitate was left overnight to coagulate properly.

The precipitate was filtered off with a Pragopor™ (Pragochema®; Czech Republic) nitrocellulose membrane (pore size: 1.5 μm; diameter: 35 mm), using a Sartorius® vacuum-filtration unit. After drying, the filter with the precipitate was sealed into a polyethylene bag for counting with a well-type HPGe detector (active volume: 150 cm^3; well diameter: 16 mm; well depth: 40 mm; FWHM resolution: 2.02 keV for the 1,332.5 keV photons of ^{60}Co), for 2 h. This separated fraction, obtained by sample decomposition in conc. HNO_3, is further denoted as ‘‘organic Se fraction’’.

The glass-fibre filter, with the possibly-present mineral fraction, was digested in a mixture of 5 mL of conc. HNO_3 and 1-2 mL of conc. HF in the microwave system, and the resulting solution was repeatedly evaporated in a Teflon™ beaker almost to dryness, to get rid of HF. Then, the former procedure for Se separation was followed. This fraction was counted with the well-type HPGe detector for 8 h, and is further denoted as ‘‘mineral Se fraction’’. The Se separation yield was determined through reactivation of the added carrier by short-time irradiation (30 s) in a pneumatic facility, and counting ^{77m}Se with a coaxial HPGe detector (relative efficiency: 20.8 %). For Se quantification, the irradiated liquid ^{75}Se standard was washed out the ampoule into a 5 mL measuring flask, and 25 μL of it were deposited onto a filter paper of the same size as the above membrane filters for counting.

3.4 Results and discussion

3.4.1 Gamma-ray spectrometry

Several detector types were tested in preliminary experiments at NPI, for counting of separated fractions with ^{75}Se. Figure 3.1 shows a comparison of counting for the most intense gamma-lines of three coaxial HPGe detectors with relative efficiencies of 20.8, 52.9 and 78.0 % (FWHM resolution in the range of 1.75-1.85 keV for the 1332.5 keV photons of ^{60}Co), the above-mentioned well-type HPGe detector, and a planar HPGe detector (active area: 500 mm^2; thickness: 15 mm; FWHM resolution: 550 eV for the 122.1 keV photons of ^{57}Co). The highest sensitivity of measurement (in counts s^{-1}) for the same amount of ^{75}Se on top of the coaxial and planar HPGe detectors and inside the well HPGe detector was obtained for the sum peak of 400.66 keV. Hence, measurement of this peak was used for quantification of Se in RNAA at NPI.

In INAA, selection of the most suitable detector is not that straightforward, since the most intense gamma lines of 121.2, 136.0, 264.7 and 279.5 keV are interfered. When a well-type HPGe detector is not available, the highest sensitivity of ^{75}Se measurement is achieved using the 136.0 keV gamma-line, which is interfered with a minor 136.17 keV gamma-line (intensity: 5.8 %) of ^{181}Hf ($t_{1/2}$ = 42.39 day). This interference can be minimised by an extended decay time. Hence, the 136.17 keV gamma-line was selected for quantifying Se in the INAA procedure at ITN, and counting with the above-described planar HPGe detector was carried out after 5 months of decay.

Counting with a planar HPGe detector significantly reduces the background below the 136.17 keV photopeak, compared to a coaxial HPGe detector: the result for the soil sample (0-E-0-So-1) was 150 ± 40 ng g^{-1}, and for the root sample (R-V-0-Ro-5) was 27 ± 18 ng g^{-1} (fresh mass). Still, the combined uncertainties (k = 1) are considerable high, suggesting that INAA is not a suitable analytical technique to determine Se contents in these samples. When INAA was used after 3-4 weeks of decay, only the detection limits of

around 2000 ng g^{-1} for soil, 450 ng g^{-1} for roots and straws, 250 ng g^{-1} for spikes and 150 ng g^{-1} for seeds were obtained.

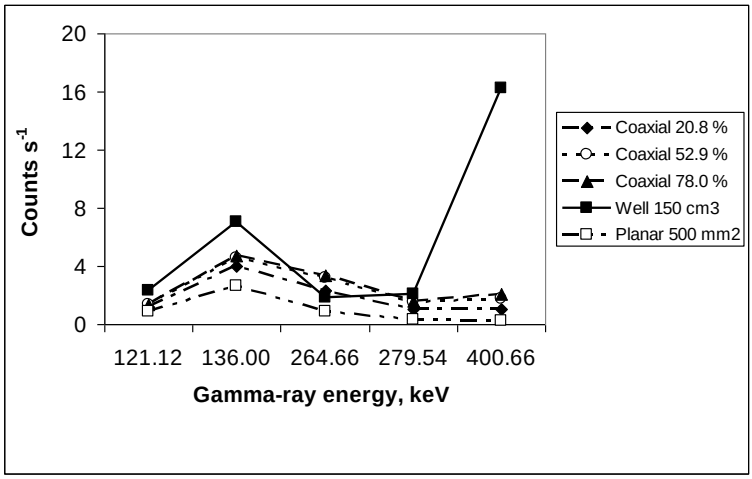

Figure 3.1 Comparison of detection efficiency for counting the same amounts of ^{75}Se with various HPGe detectors.

3.4.2 Radiochemical separation

The yield of chemical separation of Se in the RNAA procedure was very high and well reproducible (mean: 95.0 %; standard deviation: 3.8 %; *N*: 52). Still, a correction for the actual yield was employed to achieve the lowest uncertainty in each determination. In what concerns the radiochemical purity of the separated fractions, an unexpected co-precipitation of ^{233}Pa was found in most of the analysed wheat and rye samples. This is likely due to an appreciable content of Th, that resulted in a doublet of the 398.66 keV peak of ^{233}Pa and the 400.66 keV sum peak of ^{75}Se. Such interference was resolved by the interactive peak-analysis procedure of the CANBERRA® Genie™ 2000 software. No occurrence of the 398.66 keV peak was observed in the NIST-SRMs. No detectable amount of Se was found in the blank ampoules, which were processed as the samples. The detection limit of 0.3 ng g^{-1} for Se was achieved with the RNAA procedure, after counting the separated fractions for 8 h.

The accuracy of the RNAA procedure was asserted by analysis of low-level reference materials NIST-SRM® 1515 'Apple Leaves' and NIST-SRM® 8433 'Corn Bran'. The results (mean ± standard deviation, in ng g^{-1}; dry mass) were: for NIST-SRM® 1515, 43 ± 4 (this work; 6-fold replication) and 50 ± 9 (NIST data); and for NIST-SRM® 8433, 44 ± 3 (this work; 4-fold replication) and 45 ± 8 (NIST data). Uncertainty of the NIST values is the combination of a 95 % confidence limit with an allowance for systematic error between the methods used for certification.

3.4.3 Results for Se in plants and soils, and observed Se patterns

Selenium contents in various parts of wheat and rye plants, and in cultivation soils determined by RNAA are given in Table 3.1. While most Se was contained in the organic fractions, the mineral fractions featured non-detectable Se amounts, or only 1-1.8 % of those in organic fractions of most plant samples. Rather low Se amounts (0.2-7.2 %) were also found in mineral fractions of soils, compared to organic fractions. An apparently high Se value in the mineral fraction of rye straw from Guarda area (27.9 % of that in the organic fraction), was not seen in rye straw from Valpaços area.

The baseline Se concentration of wheat grain used in UK breads has been analysed in samples from 1982, 1992 and 1998, and a minimal difference in mean concentrations between samples has been found: 0.025, 0.033 and 0.025 mg Se per kg, respectively, with interquartile ranges from 0.015 to 0.019 mg Se per kg (Adams *et al.*2002). Worldwide, the Se content of wheat grain may vary from 0.001 to 30 ng g^{-1}, even if most values are between 0.02 and 0.60 ng g^{-1}(Lyons *et al.* 2005).

3.4.4 Transfer coefficients (TC_{Soil}) relative to soil

TC_{Soil} from soil to root, straw, spike and seed of wheat and rye are shown in Figure 3.2. These coefficients were obtained by assessing the ratios between the Se concentrations in plants' parts (root, straw, spike or seed, on a fresh-weight basis; $[Se]_{plant}$) and in soil ($[Se]_{soil}$): $TC_{Soil} = \frac{[Se]_{Plant}}{[Se]_{Soil}}$

The coefficients increase as the wheat plant goes through the growth stages (tillering < booting <grain filling), and Jordão variety features lower values than Marialva. The TC_{Soil} values for rye were lower than the corresponding ones for wheat in all plants' parts, with the exception of roots.

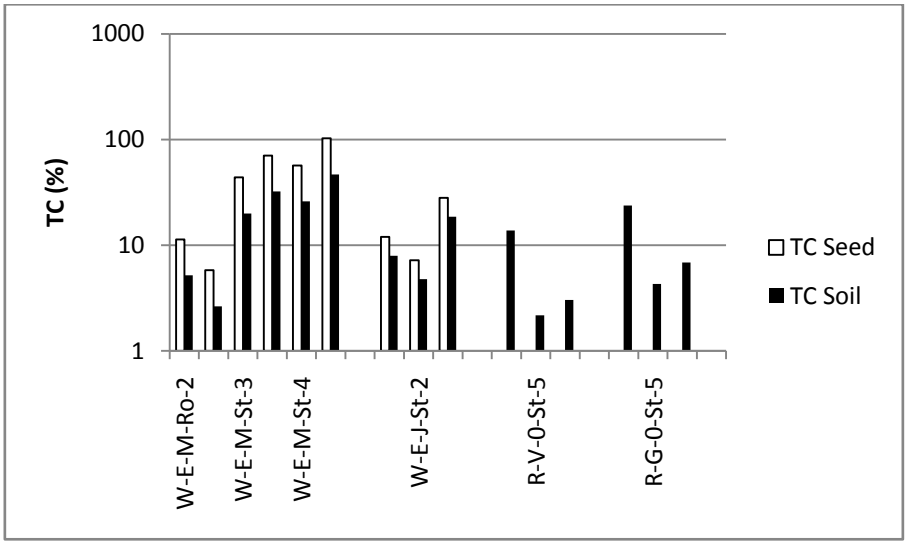

Figure 3.2 Transfer coefficients from soil (TC_{Soil}) and seed (TC_{Seed}) to wheat- and rye-plant parts (fresh mass).

3.4.5 Transfer coefficients (TC_{Seed}) relative to seed

TC_{Seed} from seed to root, straw and spike of wheat are also shown in Figure 3.2. These coefficients were obtained by assessing the ratios between the Se concentrations in plants' parts (root, straw or spike, on a fresh weight basis; $[Se]_{plant}$) and in the sowing seeds ($[Se]_{seed}$): $TC_{Seed} = \frac{[Se]_{Plant}}{[Se]_{Seed}}$

The trend of TC_{Seed} is similar to TC_{Soil}, that is an increase along the growth stages (tillering < booting < grain filling), with the Jordão variety featuring lower values than Marialva.

3.5 Conclusions

The present study has shown that a Se detection limit as low as 0.3 ng g^{-1} (a 3-σ criterion) can be achieved for plant and soil materials by RNAA, due to a very high yield and selectivity of the Se chemical separation. On the contrary, INAA was not capable of determining Se in those materials. Due to the interferences caused by non-negligible concentrations of Al, Hf and Ta in the samples. Accuracy of the RNAA procedure was asserted by analysis of low-level reference materials NIST-SRM® 1515 and NIST-SRM® 8433, pointing to an agreement between results of this work and NIST values within reasonable uncertainty margins. This study has also shown that Se transfer from soil or seed to wheat plants increases as the plants go through the growing stages (tillering < booting < grain filling), and depends on the wheat variety (Jordão < Marialva). Overall, transfer coefficients from soils were lower for rye than for wheat in almost all plants' parts.

3.6 References

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4 Optimisation of seed enrichment using radiotracers

Based on article:
"Radiotracing selenium in bread-wheat seeds for a Se-biofortification program: an optimization study in seed enrichment"
Galinha C, Freitas MC, Pacheco AMG, Coutinho J, Maçãs B & Almeida AS (2012)
Journal of Radioanalytical and Nuclear Chemistry 291:193-195

4.1 Abstract

Selenium (Se) is an essential micronutrient for human health, but its deficiency may affect at least one billion people worldwide. Plants and plant-derived products transfer the soil-uptaken Se to humans through the food chain, which is hardly enough when soils have been always poor or already exhausted in bioavailable Se species. Other than agronomic methods for enhancing Se levels in cereals, such as soil and foliar supplements, seed enrichment may be viewed as an alternative Se-biofortification technique.

This study addresses the protocol for preparing Se-enriched wheat seeds, with the specific purpose of optimising the administration of Se to the seeds prior to sowing. The first step was to soak an amount of bread-wheat seeds in an active Se solution, made with irradiated [Na_2O_4Se], and then monitoring ^{75}Se in periodically-retrieved samples from that original amount. To avoid losing Se to soil (after sowing), and, especially, to ensure that Se gets really absorbed into the seeds – and not just adsorbed onto them – the washing time of the seeds should be optimised as well. This was carried out by washing Se-treated seeds several times, until no significant amount of the radiotracer could be detected in the washing water. In what concerns the full optimisation procedure, the overall results of the present study point to an optimum time of 48 h for soaking and 24 h for washing.

4.2 Introduction

Even though protein-rich foods usually contain higher levels of selenium (Se) than other food categories (Marzec *et al.* 2002, McNaughton and Marks 2002, Klapec *et al.* 2004, Sirichakwal *et al.* 2004, Pappa *et al.* 2006, Navarro-Alarcón and Cabrera-Vique 2008), to the point that unbalanced diets (vegetarian, vegan, ethnic) may lead to a nutritional Se deficiency (Srikumar *et al.* 1992, Donovan *et al.* 1992), cereals – and, notably, among them, wheat – remain one of the most important dietary sources of Se (Lyons *et al.* 2003, Lyons *et al.* 2005). Of course, there is a wide variation of Se levels in cereals (Dumont *et al.* 2006), and given that cereals are the backbone of human diets worldwide (Lorenz *et al.* 1977, Shewry and Halford 2002), it is no wonder that they appear as obvious candidates for biofortification strategies that may help enhance the Se status of an entire population (Lyons *et al.* 2004, Welch and Graham 2004, White and Broadley 2005, Graham *et al.* 2007, Hawkesford and Zhao 2007, White and Broadley 2009).

Agronomic solutions for the supplementation (biofortification) of cereal crops are sometimes viewed as short-term alternatives, albeit effective, to longer-term genetic improvement, that is exploiting the genetic variability in crop plants or breeding new crop varieties to achieve higher Se concentrations in edible parts – grains for human food or other parts for animal feed. Another alternative or, say, an ab initio approach, may be envisaged though: seed enrichment prior to actual sowing.

Within the framework of a Se-supplementation program of representative wheat varieties, the present paper addresses such an approach for enriching seeds of bread wheat (*Triticum aestivum* L.; Jordão cultivar), while optimising both their soaking time (in an active Se solution) and washing time (in bidistilled water) through detection of a Se radiotracer (^{75}Se). The two-step procedure aims at ensuring an optimal absorption of Se (into the seeds) without noticeable adsorption of the element (onto the seeds).

4.3 Experimental

To prepare an active Se solution for the radiotracing experiments, 12.94 mg of sodium selenate [Na_2O_4Se; analytical grade] were irradiated for 5 h at a thermal-neutron fluence rate of about 10^{12} cm^{-2} s^{-1}, in the Portuguese Research Reactor (RPI-ITN), and then allowed to decay for 7 days. Certified seeds of *Triticum aestivum* L. (bread wheat; Jordão cultivar, one of the most representative varieties in the country) were soaked into 0.5 L of the former solution, prepared with the irradiated sample. The Se concentration for these bench trials is equivalent to an area concentration of 100 g of Se per hectare, and was chosen to match the top supplementation rate that has been used in the factorial design of our Se-biofortification program for wheat, in actual field conditions (Galinha *et al.* 2012). Periodically (from 1 h to 80 h), a small portion of seeds (6-8 g), was removed from the soaking solution, dried at room temperature, and the ^{75}Se activity was measured.

To optimize the washing time as well, seed samples of about 7 g – previously soaked in the same solution – were processed as follows: seeds were put into 50 mL of bidistilled water for a certain period of time, then the liquid was decanted and replaced by another 50 mL of fresh bidistilled water. This procedure was repeated several times, until a total washing time of about 35 h. The whole optimisation routine (soaking + washing) was carried out inside a hood prepared for handling radioactive materials.

Both types of samples (seeds and waters) were measured for 2 h each, to determine the full-energy peak areas of ^{75}Se, 136 keV gamma line. For that purpose, gamma spectra of seeds and waters were respectively acquired with an ORTEC® and a CANBERRA® liquid-N_2-cooled, high purity-Ge detectors (1.85 keV resolution at 1.33 MeV, both; 30 and 25 % relative efficiency, respectively), connected to a 4096 multi-channel analyzer.

4.4 Results and discussion

Supplementation of specific micronutrients to crops can be done through agronomic procedures, such as foliar application or soil addition (Lyons *et al.* 2005), and, in much scarcer cases, by seed enrichment (Smrkolj *et al.*

2007). Still, the latter approach generally appears associated to bench and/or greenhouse experiments, without a corresponding field practice, and, to the best of our knowledge, has never been performed upon cereal seeds. Therefore, the study herein should be viewed just as the first phase (optimisation) of an alternative supplementation technique using Se-enriched seeds, inasmuch as such seeds are already being tested in actual field conditions (seed planting: November 2010; crop harvesting: July 2011).

Figure 4.1 shows the accumulation of ^{75}Se in Jordão wheat seeds along time, with data normalised to seed mass. There is an increase of ^{75}Se activity with an increase in the soaking time, which seems to stabilize around the 48-h exposure to the radioactive solution. The upward trend apparently recurs after around 72 h, yet, for all practical purposes and accounting for the mean accumulation rate (next), an optimum soaking time of 48 h has been selected.

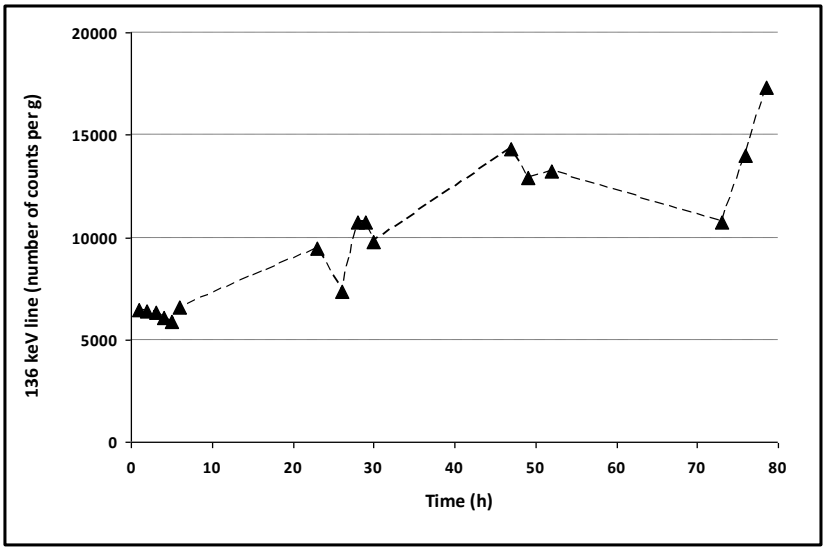

Figure 4.1 Net peak area of the 136 keV gamma-energy band for seed samples (^{75}Se; counting-statistics error: < 1 %), per gram of seeds and for different times of soaking. Lines between markers are just a guide to the eye.

Figure 4.2 shows the accumulation rate of ^{75}Se in Jordão wheat seeds per hour, with data normalised to seed mass. There is a sharp drop from the onset of the experiment – when the accumulation rate is very high – meaning that the mean absorption and/or adsorption of ^{75}Se into and/or onto the seeds decreases with the time of contact with the soaking solution. After that steep fall, the graph flattens out gradually as, seemingly, seeds become saturated in radioactive selenium.

For an effective enrichment, one must make sure that Se gets (and stays) inside the wheat seeds, instead of just being adsorbed or otherwise bound to their surfaces. Outer Se could easily be leached into the cultivation soil, and would certainly be devoid of any significant physiological role in the plant metabolism and development. In these terms, a washing procedure was applied to a sample of wheat seeds, featuring 2600 counts per gram and hour of exposure to radioactive Se solution. Figure 4.3 shows that the ^{75}Se activity in the washing waters decreases with an increase of the washing time. Again, there is a sharp drop in the first 4 h, followed by a much smoother decrease up to around 24 h, and a near plateau beyond that time. Therefore, after a 24-h period, outer (adsorbed) ^{75}Se is considered to have been washed out, and only the inner (absorbed) ^{75}Se remains enriched into the seeds. The two-step procedure for enriching bread-wheat (Jordão) seeds with Se thus comprises soaking them for 48 h and washing them for 24 h.

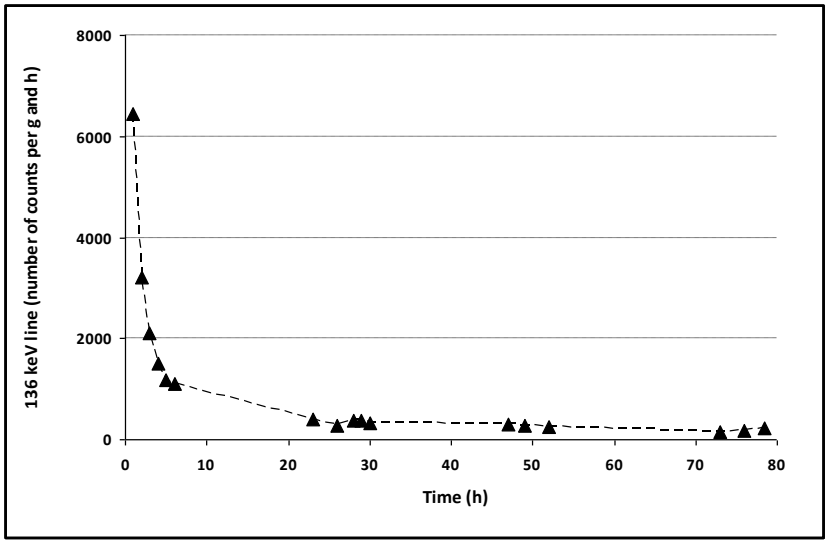

Figure 4.2 Net peak area of the 136 keV gamma-energy band for seed samples (^{75}Se; counting-statistics error: < 1 %), per gram of seeds and per hour of soaking. Lines between markers are just a guide to the eye.

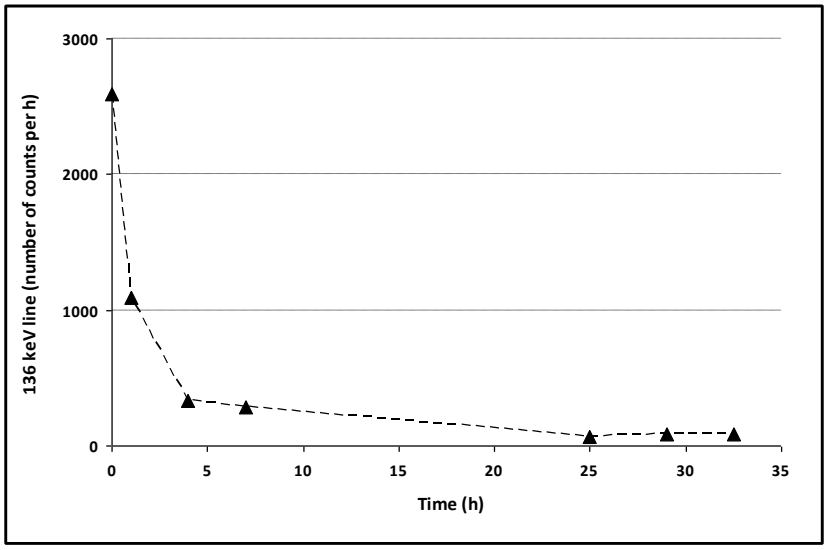

Figure 4.3 Net peak area of the 136 keV gamma-energy band for water samples (^{75}Se; counting-statistics error: < 5 %), per hour of washing. Lines between markers are just a guide to the eye.

4.5 Conclusions

Seed enrichment may be viewed as an alternative to classical biofortification strategies, such as soil amendments or foliar treatments. This study has dealt with the first phase of such an alternative, that is with the preparation of Se-enriched seeds of bread wheat (*Triticum aestivum* L.; Jordão cultivar) by optimising both their soaking time (in an active Se solution) and washing time (in bidistilled water), through detection of a Se radiotracer (^{75}Se). The study has been designed to conform to realistic Se-supplementation rates and to have an extension in actual field trials, that are already under way. The optimised times for administering Se to seeds (soaking time) and ensuring inner-seed levels only (washing time) are 48 and 24 h, respectively. The use of radiotracing proved to be very useful in the present laboratory conditions.

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5 Methods of selenium supplementation

5.1 Selenium supplementation of Portuguese wheat cultivars through foliar treatment in actual field conditions

Based on article of same title:
Galinha C, Freitas MC, Pacheco AMG,
Coutinho J, Maçãs B & Almeida AS (2013)
Journal of Radioanalytical and Nuclear Chemistry 297:227-231

5.1.1 Abstract

Selenium (Se) is a trace element essential to the well-being and health quality of humankind. Plant-derived foodstuffs, namely cereals, are the major dietary sources of Se in most countries throughout the world, even if Se contents are strongly dependent upon the corresponding levels in cereal-growing soils. Therefore, wheat is one of the staple crops that appears as an obvious candidate for Se biofortification, considering its gross-tonnage production and nutritional relevance worldwide.

The present paper focuses on the ability of bread and durum wheat – *Triticum aestivum* L. and *Triticum durum* Desf., respectively – to accumulate Se after supplementation via a foliar-addition procedure. Two of the most representative wheat cultivars in Portugal – Jordão (bread) and Marialva (durum) – have been selected for supplementation trials, following the same agronomic practices and field schedules as the regular (non-supplemented) crops of those varieties (sowing: November 2010; harvesting: July 2011). Foliar additions were performed at the booting and grain-filling stages, using sodium selenate and sodium selenite solutions at three different Se concentrations – equivalent to field supplementation rates of 4, 20 and 100 g of Se per ha – with and without potassium iodide. Selenium contents in wheat grains obtained under foliar application are compared to data from

regular wheat samples (field blanks) grown at the same soil/season, yet devoid of Se supplementation. Total Se in all field samples was determined by cyclic neutron activation analysis (CNAA), via the short-lived nuclide ^{77m}Se ($t_{1/2}$: 17.5 s). Quality control of the analytical procedure was asserted through concurrent analyses of NIST-SRM® 1567a (Wheat Flour). Results show that foliar additions can increase Se contents in mature grains up to 15 and 40 times for Marialva and Jordão, respectively, when compared to non-supplemented crops. Jordão and Marialva varieties responded differently to the stage of application.

5.1.2 Introduction

Wheat is one of the most important cereal crops in the world, with a production of more than 650 millions of tons – almost 30 % of the cereals' production worldwide – only surpassed by maize that represents 35 % (FAOSTAT 2012). Wheat has a wide range of diversity and can be sown in quite different climates, from Scandinavia and Russia to Argentina, including high-altitude regions in the tropics and sub-tropics. Wheat plays an important role in human dietary intake, being considered a staple crop and one of the major sources of nutrients and energy in many parts of the world (Shewry 2009). In Portugal, wheat represents more than 75 % of the human consumption of cereals: within this, 85 % is bread wheat and 15 % is durum wheat (Eurostat 2012).

Selenium (Se) is an essential micronutrient for mammals, where it is present as selenocysteine in several enzymes (Shewry 2009). This element enters the food chain through plants and, consequently, it is highly dependent upon its bioavailability in soils (Ducsay and Ložek 2006). Deficiency in selenium can cause several diseases, namely cardiovascular disorders, asthma, male sterility and certain forms of cancer (Brown and Arthur 2001). A minimum intake of 40 and 30 μg of selenium per day, respectively for males and females, has been advised (Levander and Whanger 1996), and the current recommended dietary allowance (RDA) for adult men and women regardless of age, by the Food and Nutrition Board of the Institute of Medicine (Washington DC, USA), is 55 μg per day.

Considering the essentiality and importance of selenium in human health and that the selenium concentrations of the Portuguese soils are quite low, there is an ongoing project to study several ways of improving selenium levels in wheat (PTDC/QUI/65618/2006; Fundação para a Ciência e a Tecnologia – FCT; Portugal). Breads and wheat derivatives (breakfast blends, pastas, etc.) make up a sizeable share of Portuguese diets, so an increase in selenium intake through Se-biofortified, wheat crops may contribute to an upgrade in the health status of the general population. The present work will focus on the results of selenium supplementation to major wheat cultivars of the bread and durum varieties, as performed in two different stages of the growth cycle of the wheat plants: booting and grain filling (Galinha *et al*. 2012).

5.1.3 Experimental

Triticum aestivum L. (Jordão cultivar) and *Triticum durum* Desf. (Marialva cultivar), two of the most representative varieties of bread and durum wheats in the country, were selected for selenium supplementation through a foliar application procedure. These cultivars were sown at Herdade da Comenda, Caia (Elvas), Portugal, in the end of November 2009, and 3 × 12 field plots (about 1.5 × 0.5 m each) were prepared to apply 12 different combinations of selenium supplements, in a threefold replication to ensure the significance of field results.

Those combinations were done using: (i) two different selenium compounds: sodium selenate (Na_2SeO_4; Sigma-Aldrich®, purum p.a. ≥ 98.0 %) and sodium selenite (Na_2SeO_3; Sigma-Aldrich®, 99.0 %); (ii) supplementation in two different growth stages: booting and grain filling; and (iii) three selenium concentrations: 4, 20 and 100 g ha^{-1} (equivalent to 0.2, 1 and 5 mg L^{-1}, respectively; 1 ha = 10000 m^2). Foliar application was carried out at the beginning of April 2010 (booting stage) and in June 2010 (grain filling stage). For every application, 0.5 L of selenium solution was quantitatively – and evenly – added to the wheat plants in each plot with a manual sprayer of 1-L capacity. Sprayers were previously – and individually – prepared in the laboratory, one device per plot.

Within the framework of the research contract that supports this work (PTDC/QUI/65618/2006), there is a side study concerning the potential influence of iodine (I) on selenium uptake and retention by wheat plants, with a view to a possible joint supplementation of both elements. For that, and to take advantage of the present field experiments, their factorial design was conceived to include wheat plots where selenate or selenite supplements have been applied jointly with potassium iodide (KI; Merck®, 99.5 %) as an additive: in such cases, a flat rate of 10 μM (as I^-) has been used. Even if higher rates might be envisaged (Blasco *et al.* 2008), results have shown that [I^-] higher than 10 μM could have a detrimental effect on plant growth and biomass yields (Mackowiak and Grossl 1999, Zhu *et al.* 2003).

Both Jordão and Marialva were sampled during the harvest period, in July 2010. The wheat plants were cut with the help of pruning shears, and the spikes were collected from each plot. The grains were separated from the spikes using a Hege™16 laboratory thresher, available at INIAV-Elvas. Prior to elemental analysis, whole grains were cleaned, weighed and stored in a dry room. All grain samples were ground to a fine powder in a Waring® blender HGB50E2, heat-sealed in polyethylene vials (1.2 mL), and placed in medium-size irradiation vials (7 mL). The analyses were performed using three replicates per sample, around 800 mg each.

All samples were irradiated on the fast pneumatic system of the Portuguese Research Reactor (RPI; CTN-IST, Sacavém), at a thermal-neutron fluence rate of 1.7×10^{12} cm^{-2} s^{-1}. Gamma spectra were acquired with a liquid-N_2 cooled, high-purity Ge, coaxial detector (1.85 keV resolution at 1.33 MeV; relative efficiency: 25 %), and an advanced digital gamma-ray spectrometer DSPEC Pro from ORTEC®, to correct for dead-time losses. Samples were put through cyclic neutron activation analysis (CNAA; 10 cycles); in each cycle, irradiation and counting times were 20 s, and the decay time was 5 s. Elemental concentrations were assessed by the relative method using NIST-SRM® 1568a (Rice Flour). Quality control was performed with NIST-SRM® 1567a (Wheat Flour), resulting in 1.2 ± 0.3 mg Se kg^{-1}, which is in good agreement with the certified value of 1.1 ± 0.2 mg Se kg^{-1}, at a 95 % confidence level.

5.1.4 Results and discussion

Selenium concentration in soil of the experimental fields prior to any supplementation was 118 ± 6 μg kg^{-1} (Galinha *et al.* 2012b). Total selenium in the wheat seeds used for setting up the field trials has been previously analysed, and values of 54 ± 3 and 38 ± 2 μg kg^{-1} have been reported for Jordão and Marialva, respectively (Galinha *et al.* 2012b). Selenium content in samples of bread and durum wheat without supplementation was 59 ± 10 (Galinha *et al.* 2012a) and 160 ± 50 μg kg^{-1}, respectively (95 % confidence level). Although seeds of Marialva feature a lower concentration of selenium, their grains grown under a no-supplementation regime show a higher content in this element. The value of selenium for soil falls within the standard range by Kabata-Pendias and Pendias (2001) for several European countries (50-200 μg kg^{-1}). According to Gupta and Gupta (2002), though, the soil of the experimental fields might be considered deficient to grow crops that meet the needs in Se of animals and humans, since it contains less than 600 μg Se kg^{-1}.

Selenium in wheat grains without supplementation is also in agreement with other European values found in the literature: 33 μg kg^{-1} for Norway, 36 μg kg^{-1} for France, 100-170 μg kg^{-1} for Finland, 21 μg kg^{-1} for Denmark, and 200 μg kg^{-1} for Germany (unspecified wheat types) (Kabata-Pendias and Pendias 2001). Ducsay and Ložek (2006) report an average of 45 μg $kg^{-1}$for winter (bread) wheat grown in the Slovak Republic. From southern Australia, a value of 65 μg kg^{-1} has been reported for bread wheat (Lyons *et al.* 2005a), while durum wheat grown in Canadian soils ranged 195-532 μg kg^{-1}, which is much higher than most published data and, in particular, than the concentration herein.

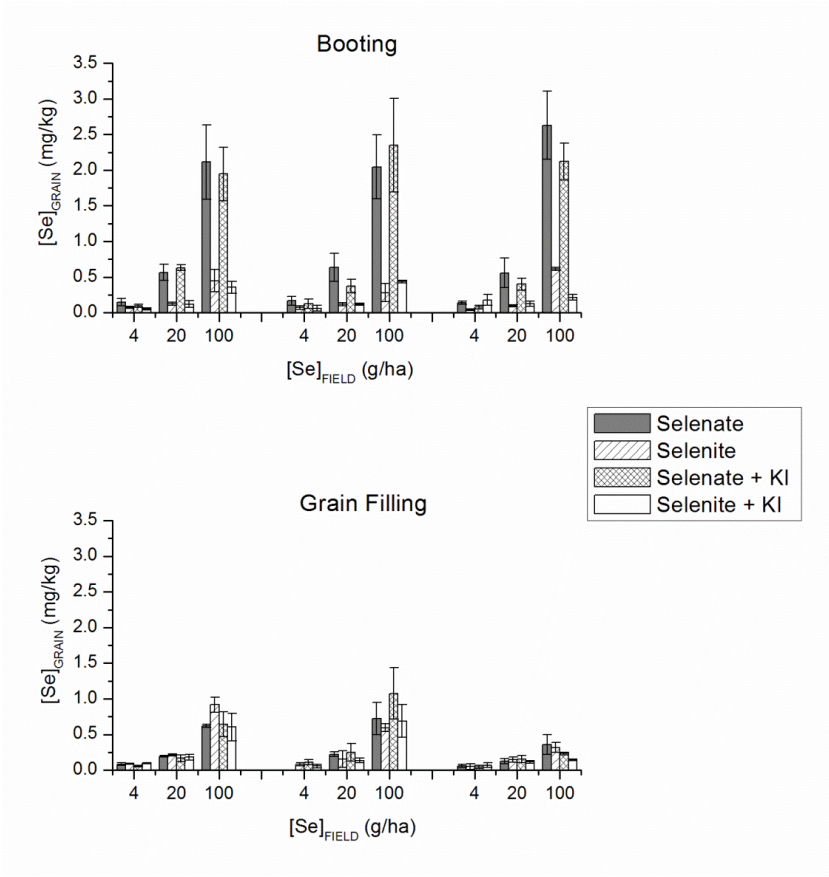

Figure 5.1 Selenium concentration (fresh weigh) in Jordão grains supplemented with different concentrations of selenium, as selenate and selenite forms, with and without potassium iodide, at two different growth stages: booting and grain filling (three-fold field replication).

Figures 5.1 and 5.2 show that both bread and durum wheat grains accumulate as much selenium as it is supplemented, thus increasing in the following order: 4 < 20 < 100 g ha^{-1}. Such an increase occurs regardless of Se being applied during booting or grain-filling stages. The presence of KI in Se-supplementation solutions does not seem to influence the accumulation of Se in grains, for both wheat varieties and for both growth stages.

All field replicates show similar results for selenium concentration in the wheat grains, with the exception of the third replicate of Jordão, which presents slightly lower concentrations than the other two. Local evidence suggests that a soil-borne disease has impaired the normal development of plants in several plots of that field replicate, which, in turn, may have affected the assimilation of selenium by those plants.

Marialva grains show greater accumulation of selenium when the foliar-supplementation procedure is done during the grain-filling stage, and when a concentration of100 g ha^{-1} is used. For lower supplementation rates, the accumulation seems to be very similar between growth stages. On the contrary, Jordão appears much more efficient in accumulating selenium when it is applied during the booting stage, and this is also more apparent for the concentration of 100 g ha^{-1}. At this stage, Jordão is more efficient in accumulating selenium when it is supplemented in the form of selenate, with or without potassium iodide; at the grain-filling stage, this difference is not relevant, with all chemical forms giving similar results. The results also show that, for Marialva at any stage of growth, the assimilation of selenium by the plant and the corresponding accumulation in the grains are independent of the chemical form used in the foliar supplementation.

When foliar supplementation is carried out at the grain-filling stage, Marialva accumulates higher amounts of selenium than Jordão. Still, it should be noticed that the growth cycles of Jordão and Marialva are not identical, and, consequently, the supplementation has not been applied in the same day. As a result of this time lag, local weather conditions may have added to the inherent field variance. More field trials, in different calendar years, are required to abate such variance, and to clarify the effect of weather in the selenium supplementation through foliar applications.

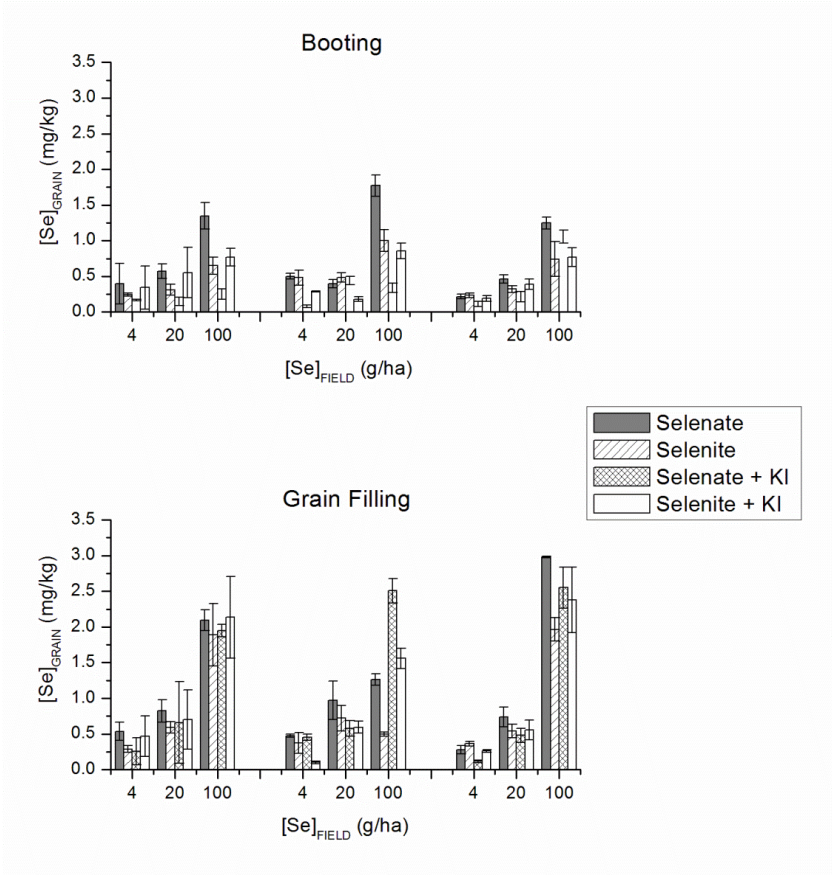

Figure 5.2 Selenium concentration (fresh weigh) in Marialva grains supplemented with different concentrations of selenium, as selenate and selenite forms, with and without potassium iodide, at two different growth stages: booting and grain filling (three-fold field replication).

When comparing Marialva and Jordão cultivars supplemented at the booting stage with rates of 4 g ha^{-1} and 20 g ha^{-1}, the results are quite similar. Grain concentrations for Marialva are slightly higher, but one must take into account that its basal values (without supplementation) are already higher than Jordão's, so higher selenium contents in supplemented Marialva grains could easily be expected. Overall, when a selenate solution equivalent to a field supplementation rate of 100 g Se ha^{-1} is used for foliar application, Jordão appears as the wheat variety that accumulates Se more efficiently.

Selenium supplementation of durum wheat through foliar application has also been done by Grant *et al.* in Canada (Grant *et al.* 2007). The authors used a supplementation rate of 20 g ha^{-1} in three different soils, to get selenium concentrations of 853, 859 and 1008 μg kg^{-1} in mature grains. These numbers are way beyond the data from this work, but, then again, the average selenium in Canadian soils is much higher than in the Portuguese ones. Lyons *et al.* (2005a, 2005b) did similar experiments with bread wheat in two distinct soils of Australia. Results for these two soils, and for field supplementation rates of 4, 20 and 100 g Se ha^{-1}, were 100 and 700 μg kg^{-1}, 200 and 1000 μg kg^{-1}, and 600 and 1800 μg kg^{-1}, respectively (Lyons *et al.* 2005b). Our results are consistent with the values for the second type of soil.

Assuming, for the sake of argument, that an average Portuguese person consumes 43 g of durum wheat per day (Eurostat 2012), the contribution of this foodstuff to the intake of selenium would be around 10 % of the RDA, whereas such contribution could be raised to 30, 65 and 160 % for supplementation rates of 4, 20 and 100 g Se ha^{-1}, respectively, in the light of the present results. In the case of bread wheat, and assuming an average consumption of one piece of bread (60 g) per day (INE 2010), the contribution in Se would be about 6 %. Should the plain (non-supplemented) wheat be replaced by the Se-biofortified one, the dietary intake of selenium would increase to 15, 65 and 250 % for supplementation rates of 4, 20 and 100 g Se ha^{-1}, respectively, again in the light of the present results.

5.1.5 Conclusions

The accumulation of Se in mature grains of Jordão (bread) and Marialva (durum) wheat cultivars after foliar supplementation increases in the order of the field rates 4 < 20 < 100 g Se ha^{-1}, for both booting and grain-filling stage applications. The presence of KI as an additive to supplementation does not seem to affect the Se accumulation in mature grains in either case (cultivar, stage). Marialva shows higher accumulation when the procedure is carried out at the grain-filling stage; accumulation of Se in Jordão is more efficient at the booting stage.

Accumulation of Se in Marialva is independent of the chemical form used in foliar supplementation; Jordão accumulates more Se when a selenate matrix is used at the booting stage. Judging only from the present results, and regardless of efficiency considerations, a foliar application equivalent to a field supplementation rate of 100 g Se ha^{-1} would not be advisable for either cultivar, since the recommended dietary intake of Se could be seriously exceeded.

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5.2 Selenium in bread and durum wheats grown under a soil-supplementation regime in actual field conditions, determined by cyclic and radiochemical neutron activation analysis

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5.2.1 Abstract

Even if selenium (Se) is often regarded as remarkably suited to agronomic biofortification of food crops, such an efficiency must be carefully weighed against the fact that Se itself is a non-renewable, hardly-recyclable, relatively-scarce resource. Therefore, it seems advisable to screen potential crop candidates prior to implementing any biofortification routine. This work focuses on the ability of bread and durum wheat – *Triticum aestivum* L. and *Triticum durum* Desf., respectively – to accumulate Se through a soil-supplementation procedure. Four representative wheat cultivars – Jordão and Roxo (bread); Marialva and Celta (durum) – have been selected for biofortification trials, following the same agronomic practices and field schedules as their regular (unsupplemented) crops. Soil additions were performed at sowing time, using sodium selenate and sodium selenite solutions equivalent to field supplementation rates of 4, 20 and 100 g of Se per hectare (ha). Total Se in grain samples was determined by cyclic neutron activation analysis via the short-lived nuclide ^{77m}Se, and by radiochemical neutron activation analysis via the long-lived nuclide ^{75}Se. Results show that supplementation at the top rate (100 g Se ha^{-1}) can increase Se in mature grains up to 2, 16, 18 and 20 times for Jordão, Roxo, Marialva and Celta, respectively. The present findings are also matched with former data from an alternative method (foliar application), with a view to discussing an eventual trade-off between agrochemical costs, field logistics and Se recovery.

5.2.2 Introduction

Given its biochemical background (Rotruck 1973), no wonder that the practical effects of selenium (Se) – or lack thereof – in human health and well-being have been thoroughly researched, spawning a wealth of studies about the potential (beneficial) roles of Se in a plethora of life-threatening issues (cardiovascular disease, critical illness and, especially, cancer) or, at least, life-deteriorating (cognitive, metabolic, muscular, reproductive) conditions. Some evidence may be inconclusive or even inconsistent, yet the relevance of Se and selenoproteins to health seems unquestionable (Letavayová *et al.* 2006, Steinbrenner *et al.* 2009, Ferguson *et al.* 2012, Weeks *et al.* 2012). The current state of knowledge, with an emphasis on major medical endpoints, has been recently reviewed by Rayman (2012) and Roman *et al.* (2014).

Even if most aspects of the Se conundrum (Stapleton 2000) have yet to attain scientific closure, its seemingly pivotal role in human health has long been recognised by both global organisations (EC Scientific Committee on Food, FAO, IAEA, WHO) and several countries, leading to a range of dietary references for Se intake that currently averages 60 μg and 53 μg per day for the adult male and female, respectively (Thomson 2004). The Portuguese situation is difficult to assess due to scarce information (Viegas-Crespo *et al.* 2000, Pavão *et al.* 2003, Lopes *et al.* 2004) and lack of consistent studies on this subject; there are no national guidelines or reference values either. Still, it should not be much different from Western Europe (Van Cauwenbergh *et al.* 2004), where falls in Se intake – and corresponding drops in the blood indicators of Se status – have long been a matter of concern (Rayman 1997, Combs 2001a, Rayman 2002).

Cereals are far from being the main sources of Se on a content basis, but they are likely the major contributors to intake on a dietary basis (Finley 2006, Hawkesford and Zhao 2007, Navarro-Alarcón and Cabrera-Vique 2008). Breads and cereal derivatives (breakfast blends, flours, pastas, etc.) make up a considerable share of Portuguese diets, so an increase in the Se intake by biofortified cereals may contribute to an upgrade in the Se status of

the whole population through a normal food matrix, without the need for concentrated dietary supplements (Mulholland and Benford 2007). In general, Se seems especially suited to agronomic biofortification of food crops (Lyons and Cakmak 2012), and, in particular, the availability of Se from wheat has been reported to be high and quite unaffected by post-harvest processing (Alexander *et al.* 1983, Van der Torre *et al.* 1991, Lyons *et al.* 2005a). Still, such an efficiency must be carefully weighed against the fact that Se is a non-renewable, hardly-recyclable, relatively-scarce resource (Haug *et al.* 2007, Lenz and Lens 2009), with a narrow safe-intake range – and an even narrower homeostatic range – which calls for a balance between actual (nutritional) needs, overall (field) costs and potential (health) benefits (Combs 2001b, Burk 2002, Arthur 2003). It thus seems advisable to screen potential crop candidates prior to implementing any biofortification routine. The present paper focuses on the ability of bread wheat (*Triticum aestivum* L.) and durum wheat (*Triticum durum* Desf.) to accumulate Se on the grain after supplementation via a soil-addition procedure.

5.2.3 Experimental

Four of the most representative wheat cultivars in the country – Jordão and Roxo (*Triticum aestivum* L.; bread wheat); Marialva and Celta (*Triticum durum* Desf.; durum wheat) – have been selected for supplementation trials, following the same agronomic practices and field schedules as the regular (unsupplemented) crops of those varieties. Wheat crops were sown in the experimental fields of the National Institute of Agricultural and Veterinary Research (INIAV-Elvas; Portugal).

The full factorial for Se-supplementation trials has been designed to account for the following attributes. (i) Field procedures: foliar application and soil application; (ii) Growth stages (foliar application only): booting and grain filling; (iii) Chemical matrices: sodium selenate (Na_2SeO_4; Sigma-Aldrich®, purum p.a. ≥ 98.0 %) and sodium selenite (Na_2SeO_3; Sigma-Aldrich®, 99.0 %); (iv) Supplementation rates: 4 g ha^{-1}, 20 g ha^{-1} and 100 g ha^{-1} (in elemental Se); (v) Field replication: 3-fold.

The present work deals with grain samples from field plots pertaining to soil application. Soil additions were performed just prior to sowing. Each plot (with an area of about 1.5 × 0.5 m) was manually sprayed with 0.5 L of the corresponding solution – as evenly as possible – using dispenser bottles of 1 L (nominal volume). Wheat plants were cut off with the help of pruning shears. All spikes and a representative share of straws were collected from each plot. Grains were separated from spikes using a Hege^{TM}16 laboratory thresher.

Total Se in grains was determined by cyclic neutron activation analysis (CNAA) via the short-lived nuclide ^{77m}Se ($t_{1/2}$: 17.5 s) at the Technological and Nuclear Campus (CTN-IST; Sacavém, Portugal), and by radiochemical neutron activation analysis (RNAA) via the long-lived nuclide ^{75}Se ($t_{1/2}$: 120.4 d) at the Nuclear Physics Institute ASCR (ÚJF; Husinec-Řež, Czech Republic). The need for complementing CNAA with RNAA (higher sensitivity) was due to the very low Se levels in some grain samples.

For CNAA (10 cycles), samples were prepared according to Galinha *et al.*(2013), and irradiated in the fast pneumatic system of the Portuguese Research Reactor (1-MW, pool-type reactor) at a thermal-neutron fluence rate of 1.7×10^{12} cm^{-2} s^{-1}. Gamma spectra were acquired with a HPGe coaxial detector (1.85 keV FWHM resolution at 1.33 MeV; relative efficiency 25 %), interfaced with a digital gamma-ray spectrometer DSPEC Pro (ORTEC®) with built-in dead-time and loss-free counting correction modules. In each cycle, irradiation and counting times were 20 s, and decay time 5 s. Elemental concentrations were assessed by the relative method, using NIST-SRM® 1568a Rice Flour as a calibrator. Quality control was pursued by analysis of NIST-SRM® 1567a Wheat Flour.

For RNAA, sample aliquots of about 150 mg were weighed into high-purity quartz-glass ampoules (Suprasil® 310, Heraeus Quarzglas), following Kučera and Soukal (1993); quality-control samples NIST-SRM® 1515 Apple Leaves and NIST-RM® 8433 Corn Bran were prepared in the same way. Samples, reference materials, blank ampoules and standards were irradiated in the LVR-15 research reactor of the Research Center Řež, Ltd. for 20 h, at a thermal-neutron fluence rate of 6×10^{13} cm^{-2} s^{-1} within the CANAM

infrastructure (MŠMT Project no. 2011019), and allowed to cool for one month. The irradiated samples were then handled according to a procedure already described (Kučera and Soukal 1993, Galinha *et al.* 2012), and went for counting for 2 h in a well-type, HPGe detector (active volume: 150 cm^3; well diameter: 16 mm; well depth: 40 mm; FWHM resolution: 2.02 keV for 1332.5 keV photons of ^{60}Co).

The Se-separation yield was determined through reactivation of the carrier by short-time irradiation (30 s) in a pneumatic facility, and by counting the ^{77m}Se radioisotope with a coaxial HPGe detector (relative efficiency: 20.8 %). The yields were in the range of 91-102 %. For quantification of the Se contents in samples, standards were used: they were prepared by pipetting 100-µL aliquots (gravimetrically-calibrated pipette) from a stock solution of high purity Se (Fluka®, 99.995 %).

Nonparametric *U* tests (Mann-Whitney) were run to probe the pairwise effect of soil-Se applications in grain-Se concentrations, that is whether a statistically significant difference had arisen between grain samples from control (blank) and treated (Se-supplemented) plots. The choice of the *U* statistic was based on the samples' dimension (9 variates), on non-normality assumptions – all distributions are fairly skewed and/or non-mesokurtic – and on its power advantage over corresponding parametric tests (e.g. Student's *t* for independent samples) when samples are drawn from non-normal populations. In the following discussion, statistical significance was asserted by one-sided tests, keeping the probability of occurrence of false positives (type I errors) under 1 %. For practical (biofortification) purposes, only significant increases have been considered.

Table 5.1 Total Se concentrations in mature grains from wheat crops grown under Se-supplementation via a soil-addition procedure, at nominal field rates of 4, 20 and 100 g Se ha^{-1} as sodium selenate (Sa) or sodium selenite (Si); blanks correspond to wheat grains from unsupplemented plants.

	Mean (mg kg^{-1})	**Median (mg kg^{-1})**	**SD (mg kg^{-1})**	**CV (%)**
Durum				
		Marialva		
Blank	0.043	0.044	0.005	11
4Sa	0.018	0.019	0.004	24
20Sa	0.036	0.037	0.013	34
100Sa	0.768	0.712	0.234	30
4Si	0.025	0.026	0.004	18
20Si	0.021	0.022	0.006	26
100Si	0.087	0.096	0.020	23
		Celta		
Blank	0.038	0.037	0.007	20
4Sa	0.022	0.021	0.006	29
20Sa	0.040	0.040	0.013	32
100Sa	0.770	0.676	0.360	47
4Si	0.020	0.023	0.005	26
20Si	0.019	0.017	0.006	31
100Si	0.042	0.040	0.008	20
Bread Wheat				
		Jordão		
Blank	0.048	0.044	0.010	22
4Sa	0.018	0.017	0.006	33
20Sa	0.048	0.048	0.026	54
100Sa	0.079	0.093	0.040	51
4Si	0.022	0.024	0.008	34
20Si	0.027	0.020	0.015	56
100Si	0.046	0.048	0.006	14
		Roxo		
Blank	0.072	0.076	0.013	18
4Sa	0.019	0.018	0.006	32
20Sa	0.034	0.027	0.017	49
100Sa	1.141	0.935	0.758	66
4Si	0.017	0.016	0.006	37
20Si	0.021	0.020	0.006	28
100Si	0.068	0.074	0.015	23

SD and CV stand for standard deviation and coefficient of variation; all central-tendency and variability descriptors are based on 9 variates (3 × 3 field replicates).

Table 5.2 Results for total Se concentration in quality-control samples (mg kg^{-1}).

Reference material	**NAA mode**	**This work[a]**	**NIST value**
NIST-SRM® 1567a Wheat Flour	CNAA	1.2 ± 0.3	1.1 ± 0.2
NIST-SRM® 1515 Apple Leaves	RNAA	0.046 ± 0.002	0.050 ± 0.009
NIST-RM® 8433 Corn Bran	RNAA	0.041 ± 0.002	0.045 ± 0.008

[a]mean value ± expanded uncertainty (coverage factor $k = 2$)

5.2.4 Results and discussion

Results for total Se in mature grains from the soil-supplementation trials are listed in Table 5.1. Their accuracy can be inferred from Table 5.2, in which our results for NIST-SRMs/RMs are compared with NIST data. The background levels of Se in soil can vary at a metre-to-metre scale (Lyons and Cakmak 2012), and this may be an explanation for some of the high coefficients of variation found here, meaning that the basal level of Se differs considerably through the field layout. Soil samples were randomly collected across the trial plots and then fully mixed, yielding an average of 92±3 μg Se kg^{-1} for the whole experimental field (3 × 28 plots). Without further amendment, this soil would be deficient to grow crops that meet the needs in Se of animals and humans, since it contains less than 600 μg Se kg^{-1} (Gupta and Gupta 2002). Still, the uneven spatial distribution of soil-Se levels likely explains why several supplemented samples actually ended up with grain-Se levels lower than blanks: these may have grown in plots with a nugget-like effect in what concerns local soil-Se levels.

When compared to unsupplemented crops (blanks), there are only a few cases in which the Se contents of wheat grains show a significant increase after biofortification. Although three different rates of soil supplementation have been used, only the highest one (100 g Se ha^{-1}) has resulted in mature grains with more Se than blanks. Durum wheat appears to have better potential for Se biofortification: Se contents went up 18 and 20 times for Marialva and Celta, respectively, while bread -wheat increases were 2 times

for Jordão and 16 times for Roxo. Since Se is incorporated into proteins as selenoaminoacids, a superior ability of durum-wheat cultivars to accumulate Se may be linked to their higher protein content (Barclay and MacPherson 1992). Experiments in Australia on bread wheat with soil addition of Se as selenate gave much higher results than those here: grain concentrations of 2125 μg Se kg^{-1} for a supplementation rate of 40 g Se ha^{-1} and blanks with 57 μg Se kg^{-1} (Lyons *et al.* 2005b). Even accounting for distinct soil types, wheat varieties and climate conditions, a major divergence with the present results is apparent.

With the exception of Marialva at the top field rate (100 g Se ha^{-1}), adding selenite to soil seems ineffective for increasing Se in mature grains; none of the other wheat varieties – at any supplementation rate of Se as selenite – shows significant increases either. Previous studies with the non-hyperaccumulating *Chlorophytum comosum* (Thunb.) Jacques (spider plant) reported that sequestration of Se in roots does occur; however, the translocation rate of the element from roots to leaves is higher when Se is supplemented as selenate instead of selenite (Afton *et al.* 2009). In soils, selenates are highly soluble and easily uptaken by plants (Lyons *et al.* 2005c). When using selenate-containing fertilisers, Se is readily absorbed by roots whereas, in the case of selenite, a combination of direct and passive absorption takes place, due to partial oxidation of Se^{IV} to Se^{VI} in the soil chemistry (Afton *et al.* 2009).

Related Se-supplementation experiments with foliar addition instead of soil addition had already been carried out with Marialva and Jordão cultivars, using the same Se matrices (Na_2SeO_4 and Na_2SeO_3) and Se supplementation rates (4, 20 and 100 g Se ha^{-1}) (Galinha *et al.* 2013). Overall, judging from our results, foliar addition is much more efficient than soil addition: in similar conditions, 17-fold and 29-fold ratios for Marialva and Jordão, respectively, not to mention a much lower Se-supplementation threshold for significant gains in grain-Se concentrations. A trade-off between plain efficiency and field logistics should be considered, though: the latter might be easier even if supplements would be costlier (higher amounts). And in the absence of aerial irrigation systems to assist in the foliar application(s) – as

in the case of rainfed agriculture – farmers may as well be constrained to apply Se supplements via soil procedures.

In what concerns the response to soil supplementation by wheat type (bread, durum), differences in assimilation and accumulation of Se by bread wheats (Jordão, Roxo) are much more noticeable than in durum wheats (Marialva, Celta). There is a substantial variation between Se-grain concentrations in Jordão and Roxo cultivars: the latter seems much more effective an accumulator than the former. From foliar studies (Galinha *et al.* 2013), Jordão appears to be quite sensitive to the timing of Se application, so maybe it could benefit from a later dressing, further into the springtime. It is quite possible that distinct varieties can react in a different way to environmental conditions, even if there are no conclusive studies to support this hypothesis. Lyons *et al.* (2005c) assert that grain-Se contents seem overwhelmingly determined by soil-Se availability and not by genotypic variation, but such findings were based on soils devoid of any supplementation (basal levels). More field research – and probably an enhanced array of genotypes – is needed to ascertain which Portuguese wheat cultivar, especially of the bread type, can be better suited to soil supplementation.

5.2.5 Conclusions

Usually viewed as short-term procedures, as opposed to long-term genetic improvements, agronomic strategies for element/nutrient supplementation (biofortification) of food crops must rely on an effective uptake by their root systems, followed by an equally effective translocation to (and accumulation in) their edible parts. Generally speaking, Se contents of mature wheat grains showed a significant increase after soil supplementation only when selenate was used at an equivalent field rate of 100 g Se ha^{-1}. Regardless of rate, selenite was mostly ineffective, and one cultivar (Jordão) stood far behind the others in terms of grain-Se accumulation. Soil supplementation may pale by comparison with foliar procedures (efficiency-wise), and may actually need some tune-up as to its timing, yet may be logistically advisable or even inescapable when automated irrigation (sprinkler-type) is not available.

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5.3 General discussion of the chapter

Selenium concentrations in food and forage crops can be improved by the addition of Se supplements to soil-crop systems, a practice that is known as agronomic biofortification (Hawkesford and Zhao 2007). The most common supplementation methods are the soil and foliar applications. Although much less common than the former procedures, another supplementation method available is the use of Se-enriched seeds. All those three methodologies were evaluated within this work.

From the foliar-application studies, it became apparent that the presence of potassium iodide as an iodine additive to the Se supplementation of wheat does not seem to affect the Se accumulation in mature grains, regardless of the wheat type – bread or durum wheat – and the growth stage – booting or grain-filling stage. The inclusion of such an additive was thus discontinued in further field experiments.

Selenium-enriched seeds were also used as a supplementation methodology, by optimising both their soaking time (in an active Se solution) and washing time (in bidistilled water), through detection of a Se radiotracer (^{75}Se) in the laboratory. The optimised times for administering Se to seeds (soaking time) and ensuring inner-seed levels only (washing time) were 48 h and 24 h, respectively (Galinha *et al.* 2012). The two-step procedure aimed at ensuring an optimal absorption of Se (into seeds) without noticeable adsorption of the element (onto seeds), before sowing them in the agricultural test fields. The experimental-plot design was set up to account for: (i) Wheat varieties: bread wheat (Jordão cultivar) and durum wheat (Marialva cultivar) (ii) Chemical matrices: sodium selenate and sodium selenite; (iii) Supplementation rates: 4, 20 and 100 g ha^{-1} (in elemental Se); (iv) Field replication: 3-fold.

The former approach to Se-supplementation using Se-enriched seeds proved ineffective for all the studied attributes, though: mature grains collected from wheat plants grown from Se-enriched seeds did not present any significant increase of this element, even for the top supplementation rate that had been used in the factorial design (equivalent to 100 g of Se per hectare). These results are in agreement with those of Davydenko and Mayurnikova (2014),

who found that wheat-seed soaking in a sodium-selenite solution before planting led to an insignificant increase of selenium in mature grains.

Overall, judging from our results, foliar addition is much more efficient than soil addition. A trade-off between plain efficiency and field logistics should be considered, though: the latter might be easier even if supplements would be costlier (higher amounts). And in the absence of aerial irrigation systems to assist in the foliar application(s) – as in the case of rainfed agriculture – farmers may as well be forced to apply Se supplements via soil procedures.

5.3.1 References

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6 Speciation of selenium in cereal samples

Based on article:
"Characterization of selenium-enriched wheat by agronomic biofortification"
Galinha C, Sánchez-Martínez M, Pacheco AMG, Freitas MC, Coutinho J, Maçãs B, Almeida AS, Pérez-Corona MT, Madrid Y, Wolterbeek HT (2014)
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6.1 Abstract

Agronomic biofortification of staple feed and food crops is an effective way to enhance their contents in essential nutrients up the food chain, with a view to correcting for their deficiencies in animal and/or human status. Selenium (Se) is one such case, for its uneven distribution in the continental crust and, therefore, in agricultural lands easily translates into substantial variation in nutritional intakes.

Cereals are far from being the main sources of Se on a content basis, but they are likely the major contributors to intake on a dietary basis. To assess their potential to assimilate and biotransform Se, bread and durum wheat were enriched with Se through foliar- and soil-addition procedures, at an equivalent field supplementation rate of 100 g of Se per hectare (ha), using sodium selenate and sodium selenite as Se-supplementation matrices. All wheat plants were grown outdoors in real field conditions. Biotransformation of inorganic Se was evaluated by HPLC–ICP-MS after enzymatic hydrolysis for Se-species extraction in the resulting mature wheat grains.

Selenomethionine and Se^{VI} were identified and quantified: the former was the predominant species, representing 70-100 % of the total Se in samples; the maximum amount of inorganic Se was below 5 %. These results were similar for both supplementation methods and for both wheat varieties. Judging from the present results, one can conclude that agronomic biofortification of wheat may improve the nutritional quality of wheat grains with significant amounts of selenomethionine, which is an attractive option for increasing the Se status in human diets through Se-enriched, wheat-based foodstuff.

6.2 Introduction

Although selenium (Se) has been initially known for its toxic characteristics (Davidson 1940, Moxon and Rhian 1943, Busby 1957, Oldfield 1987, Prince *et al.* 2007), it is also an essential trace element of utmost relevance to human health (Navarro-Alarcón and López-Martínez 2000, BNF 2001, SACN-UK 2013). Selenium was firstly – and explicitly – ascribed an essential role in mammalian nutrition by Schwarz and Foltz (1957), even if it still took another one and a half decade for, independently yet almost simultaneously, glutathione peroxidase (GSHPx) to be recognised as a selenoenzyme (Flohé *et al.* 1973), and Se itself as a key biochemical regulator for the GSHPx antioxidant activity (Rotruck *et al.* 1973). After those major breakthroughs, and in one of the more dramatic twists in modern science, selenophobia (Frost 1972) quickly turned into selenophilia (Casey 1988), to the point that, by 1996, this new face of Se had already been the subject of over 100000 technical papers (Reilly 1996).

The former research trend has not changed ever since, and interest in the biochemistry of such an "essential toxin" (Lenz and Lens 2009) has all but faded away (Flohé 2009). The mechanisms through which Se plays a beneficial role in humans may not be fully understood as yet, but they primarily stem from the involvement of selenoproteins in the redox regulation of intracellular signalling, redox homeostasis and thyroid hormone metabolism, and, ultimately, in the maintenance of DNA integrity and genomic stability (Letavayová *et al.* 2006, Ferguson *et al.* 2012). Of course, Se retains its dual face, hence most clinical puzzles aren't finished yet, to borrow from an editorial by Richman and Chan (2012). The current state of knowledge linking Se and selenoproteins to human health, with an emphasis on major medical endpoints, has recently been reviewed by Rayman (2012) and Roman *et al.* (2014).

Relevant sources of Se in human diets are fish/seafood, meat/offal, eggs (especially yolk), cereals, nuts, leafy vegetables and roots/tubers (Morris and Levander 1970, Combs 2001, Navarro-Alarcón and Cabrera-Vique 2008). Still, it is currently accepted that Se bioavailability, intake and metabolism

are heavily dependent on its chemical forms (Finley 2006, Thiry *et al.* 2012, Kieliszek and Błażejak 2013): organic forms, such as selenoaminoacids, Se-methyled forms and complex selenoproteins, or inorganic forms, such as selenite (Se^{IV}) and selenate (Se^{VI}) (Sager 2006, Pedrero and Madrid 2009).

Selenium contents in grazing animals and plants are highly correlated with the available Se in soils (Durán *et al.* 2013), therefore many countries feature an average Se intake that is insufficient to achieve an adequate activity of protective selenoenzymes. Such deficit has led to an increasing interest in the development of Se-enriched food items and nutritional supplements (White and Broadley 2005, Broadley *et al.* 2006, Welch and Graham 2012).

One approach for enhancing the concentration of Se and other micronutrients in food is through agronomic biofortification of crops, especially cereals (Hawkesford and Zhao 2007). Cereals are far from being the main sources of Se on a content basis (Combs 2001), but they are likely the major contributors to worldwide intake on a dietary basis (Haug *et al.* 2007). Generally speaking, though, micronutrient levels can be very low, a situation more acute for grains in which micronutrients are less biologically available to monogastric animals (Wright and Bell 1966, Hidiroglou *et al.* 1968) – which, of course, include man – due to high amounts of anti-nutrients such as phytate and various phenolic compounds likely present (Swain and Hillis 1959, Reddy *et al.* 1989, García-Estepa *et al.* 1999, Valencia *et al.* 1999, Lestienne *et al.* 2005, ElMaki *et al.* 2007).

In what concerns Se, some plants show a high tolerance and are able to transform inorganic forms in selenoaminoacids. The threshold concentration for toxicity depends, among other factors, on the vegetal organism and the chemical form through which Se is conveyed. In general, selenate is readily absorbed and transported within plants due to its similarity with sulphate; on the other hand, selenite is faster transformed into selenoaminoacids (Pedrero and Madrid 2009). Some of the Se-organic compounds identified in plant tissues are: selenomethionine, selenocysteine, selenomethylselenocysteine, selenocystathione, selenomethylselenomethionine, selenohomocysteine and gamma-glutamyl-selenomethylselenocysteine (Terry *et al.* 2000, Sors *et al.* 2005). Selenocysteine and selenomethionine can replace their sulphur

analogues into the proteins, which may lead to phytotoxicity. However, selenomethylselenocysteine is a non-proteinogenic selenoaminoacid, which has been identified in plants that exhibit quite a tolerance to selenium (Pedrero and Madrid 2009).

Given the above, it is thus crucial to know how Se occurs in foodstuff samples and not just its total concentration. Several actions need to be performed in speciation studies: sample treatment, species separation and species identification. The first step – sample treatment – is intended to quantitatively extract species preventing their interconversion (Pedrero and Madrid 2009), that is obviously a major drawback in speciation at large. In the particular case of Se, enzymatic hydrolysis (using non-specific proteases) is the preferred method, its main advantages being mild conditions and selectivity (Seppänen *et al.* 2010). The combined use of enzymatic hydrolysis and ultrasonic probe sonication is an effective way to assist the breakdown of selenoproteins into selenoaminoacids, and to allow for quantitatively extracting Se species in a short time (Cabañero *et al.* 2005).

Once species have been isolated from the matrix, the resulting extract is processed for species separation, detection and quantification, mainly by using HPLC–ICP-MS. The lack of Se standards is one of the major problems associated with its speciation, since it increases the difficulty of identifying Se species (Seppänen *et al.* 2010). Another issue is that selenocompounds can be poorly retained and easily co-eluted from chromatographic columns, leading to incorrect assignments (Pedrero and Madrid 2009).

The present work was aimed at studying the potential of wheat plants to assimilate and biotransform Se, and to accumulate organic Se in their mature grains, which constitute the ingredient for preparing Se-enriched, wheat-based food. For this purpose, selenium speciation was carried out on mature grains of bread and durum wheat, cultivated under two field supplementation regimes: foliar addition at different growth stages (Galinha *et al.* 2012a) and soil addition at sowing time. All plants were grown from certified seeds of major Portuguese cultivars, in actual field conditions, and Se was supplied as sodium-selenate and sodium-selenite solutions.

6.3 Experimental

6.3.1 Selenium supplementation

Two of the most representative varieties of wheat in the country – Portuguese cultivars certified by the National Institute of Agricultural and Veterinary Research (INIAV) – were selected for Se-supplementation trials: Jordão (*Triticum aestivum* L.; bread wheat) and Marialva (*Triticum durum* Desf.; durum wheat). Wheat crops were sown in the experimental fields of the INIAV, the first campaign (for foliar application) at Herdade da Comenda (Galinha *et al.* 2012a), and the second one (for soil application) at fields adjoining the INIAV main campus (Elvas, Portugal). There are no significant agro-meteorological differences between the two locations, and their soil-Se contents are 118±6 μg kg^{-1} (Galinha *et al.* 2012b) and 92±3 μg kg^{-1}, respectively.

The full factorial for the Se-supplementation trials has been designed to account for the following attributes. (i) Field procedures: foliar application and soil application; (ii) Wheat species: Jordão and Marialva; (iii) Growth stages (foliar application only): booting and grain filling; (iv) Chemical forms: sodium selenate (Na_2SeO_4; active form: Se^{VI}), sodium selenite (Na_2SeO_3; active form: Se^{IV}), sodium selenate with potassium iodide, and sodium selenite with potassium iodide (KI); (v) Supplementation rates: 4 g ha^{-1}, 20 g ha^{-1} and 100 g ha^{-1} (in elemental Se; plus 10 μM of KI per plot, where applicable); (vi) Field replication: 3-fold.

As for the above-mentioned growth stages for foliar application, booting goes from the onset of flag leaf sheath extending until the first awns are visible, if any (Feekes scale: 10.0-10.1; Zadoks scale: 40-49), and grain filling from post-anthesis to physiological maturity or, on practical grounds, from medium milk to hard dough (Feekes scale: 11.1-11.4; Zadoks scale: 75-92) (Feekes 1941, Large 1954, Zadoks *et al.* 1974). Our actual treatments were carried out at mid booting and early grain-filling stages. It should also be noted that KI is only germane to an independent side study within the whole Se-supplementation program, which is not addressed per se here for

being out of scope. Therefore, the present work deals with samples (mature wheat kernels) from field plots treated at an equivalent field rate of 100 g of Se per ha, regardless of the KI presence in the Na_2SeO_4 or Na_2SeO_3 base solutions.

For foliar supplementation, wheat plants, in each plot, were manually sprayed with 0.5 L of the corresponding solutions (per plot) – as evenly as possible – using dispenser bottles of 1 L (nominal volume). For soil supplementation, and just prior to sowing, each plot (with an area of about 1.5 x 0.5 m) was manually sprayed with 0.5 L of the corresponding solution, again using a dispenser bottle of 1 L per plot. In either case, wheat plants were cut off with the help of pruning shears. All spikes and a representative share of straws were collected from each plot.

6.3.2 Instrumentation

Wheat grains were separated from their spikes with the help of a Hege™16 laboratory thresher (Wintersteiger AG; Austria), available at the INIAV. All grain samples were ground to a fine powder using a Waring® blender HGB50E2, a Sartorius® Mikro-Dismembrator U ball mill at 1500 rpm, and Teflon™ capsules.

For total Se determination by cyclic neutron activation analysis (CNAA), wheat-flour samples were irradiated on the fast pneumatic system of the Portuguese Research Reactor (1-MW, pool-type reactor; CCTN-IST; Sacavém, Portugal), at a thermal-neutron flux density of $1.7x10^{12}$ n cm^{-2} s^{-1}. Gamma spectra were acquired with a liquid-N_2 cooled, high-purity Ge, coaxial detector (1.85 keV resolution at 1.33 MeV; relative efficiency: 25 %), and an advanced digital gamma-ray spectrometer DSPEC Pro™ from ORTEC®, to correct for dead-time losses.

For total Se determination by inductively coupled plasma mass spectrometry (ICP-MS), wheat-flour samples were first digested in double-walled, advanced-composite vessels, using a 1000 W microwave sample preparation system from CEM Corporation (Matthews NC, USA). Total Se was then measured through an Agilent® 7700 Series ICP-MS (Agilent Technologies;

Santa Clara CA, USA), fitted with a MEINHARD® nebulizer and a Peltier cooled sample introduction system (PerkinElmer Inc.; Waltham MA, USA).

Enzymatic hydrolysis of the samples was carried out in a SONOPULS™ HD 2200 ultrasonic homogenizer (BANDELIN®; Berlin, Germany), with a 3-mm diameter, titanium microtip. Extracts were obtained with an Eppendorf® 5804, fixed-angle rotor F-34-6-38 centrifuge (Hamburg, Germany), and cleared through 0.22-µm, Nylon filters (Scharlab S.L.; Sentmenat-Barcelona, Spain).

Chromatographic separation of Se species was done with a high performance liquid chromatography (HPLC) system coupled to the ICP-MS, featuring a JASCO® PU-2089 compact HPLC quaternary low pressure gradient pump (JASCO Corporation; Tokyo, Japan) fitted with a six-port, sample-injection valve (Model 7725i; Rheodyne®; Rohnert Park CA, USA) and a 100-µL injection loop. Selenium species separation was based on a Hamilton® PRP-X100 anion-exchange column and an Agilent® Zorbax™ Rx-C8 reversed-phase column. The instrumental (optimal) parameters for HPLC–ICP-MS are listed in Table 6.1.

6.3.3 Reagents and materials

Reagents for making Se-supplementation solutions (field work) were sodium selenate (Na_2SeO_4 purum p.a. ≥ 98.0 %; Sigma-Aldrich®) and sodium selenite (Na_2SeO_3 99.0 %; Sigma-Aldrich®). All remaining chemicals (laboratory work) were of analytical grade, and solutions were prepared with deionised water (18 MΩ.cm) from a Milli-Q® water purification system (EMD Millipore Corporation; Billerica MA, USA). Selenomethionine (SeMet), selenomethylselenocysteine (SeMetSeCys) and selenocysteine ($SeCys_2$) from Sigma-Aldrich® were dissolved in 3 % (v/v) hydrochloric acid (HCl fuming 37 %; Merck®) to prepare standard stock solutions of 1000 mg L^{-1}.

Solutions of inorganic Se were prepared by dissolving Na_2SeO_4 and Na_2SeO_3 in 2 % (v/v) nitric acid (HNO_3 60 %; Scharlab S.L.). Stock solutions of 1000 mg L^{-1} were stored at 4 °C, and working solutions were

prepared daily by dilution. Selenomethionine-Se-oxide (SeMetO) was obtained by oxidation of SeMet with hydrogen peroxide (H_2O_2 35 %; Panreac Química S.L.U.), following the procedure by Sánchez-Martínez *et al.* (2012).

Acid digestion was carried out using HNO_3 and H_2O_2; enzymatic hydrolysis was achieved with a non-specific enzyme, Protease XIV (Sigma-Aldrich®), dissolved in 30 mM TRIS Buffer (Fluka®) and adjusted to pH = 7.5 with HCl. Selenium-species separation by anionic-exchange column was performed by 10 mM citric acid ($C_6H_8O_7$; Sigma-Aldrich®) in 2 % (v/v) methanol (MeOH HPLC grade; Scharlab S.L.), adjusted to pH = 5.0 with ammonium hydroxide (NH_4OH; Fluka®), as mobile phase. For reversed-phase, chromatographic separation, a solution of 0.1 % trifluoroacetic acid (TFA; Sigma-Aldrich®) in 2 % MeOH was employed.

6.3.4 Total selenium determination

6.3.4.1 Cyclic neutron activation analysis (CNAA)

Wheat-flour samples were heat-sealed in polyethylene vials (1.2 mL), and then placed in mid-size irradiation vials (7 mL). Analyses were performed using 3 replicates per sample, around 800 mg each. Samples were put through cyclic neutron activation analysis (CNAA; 10 cycles): in each cycle, irradiation and counting times were 20 s, and the decay time was 5 s. Elemental concentrations (total Se) were assessed by the relative method, using NIST-SRM® 1568a (Rice Flour). Quality control was performed with NIST-SRM® 1567a (Wheat Flour), resulting in 1.2 ± 0.3 mg Se kg^{-1}, which is in good agreement with the certified value of 1.1 ± 0.2 mg Se kg^{-1}, at a 95 % confidence level.

6.3.4.2 Inductively coupled plasma mass spectrometry (ICP-MS)

For validation purposes, several wheat-flour samples were also analysed for total Se by ICP-MS. Despite good storage conditions and absence of visible signs of deterioration, a few samples were randomly chosen for reanalysis by

ICP-MS to ascertain whether Se content and sample homogeneity had been preserved since the CNAA work. About 300 mg of each sample (three replicates, whenever possible) were digested with 5 mL of HNO_3 and 1 mL of H_2O_2 in a microwave oven at 130ºC for 15 min, then cooled until room temperature, and finally diluted with deionised water to a final volume of 50 mL. Total Se was determined using standard addition and external calibrations. The operational conditions for ICP-MS were given in Table 6.1.

6.3.5 Selenium speciation

The extraction of Se species from samples of approximately 0.1 g was carried out by applying 2 min of sonication (power: 40 W; frequency: 20 kHz), after adding 3 mL of TRIS-HCl buffer and 0.020 mg of Protease XIV, followed by high-speed centrifugation (9000 rpm) for 20 min at 4 °C and filtration using 0.22µm Nylon filters. The supernatants were analysed by anion-exchange or reversed-phase HPLC coupled to ICP-MS; the operational conditions for HPLC were given in Table 6.1.

After separation, Se species were identified by comparing their retention times with those of the standards, by spiking experiments, and finally determined by monitoring ^{76}Se, ^{77}Se ^{78}Se, and ^{80}Se isotopes and H_2 as reaction gas (ICP-MS). Selenium quantification was performed by standard addition and external calibrations; an evaluation of the Se species in Protease XIV was made to check for impurities.

Table 6.1 Operating conditions for selenium determination by HPLC–ICP-MS.

ICP-MS parameters	
Forward power	1550 W
Plasma gas flow rate	15.0 L min^{-1}
Auxiliary gas flow rate	1.26 L min^{-1}
Carrier gas flow rate	1.1 L min^{-1}
Nebulizer type	Meinhard®
Spray chamber type	Scott double-pass
Monitored isotope	^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se, ^{79}Br
Reaction gas flow (H_2)	4.5 mL min^{-1}
HPLC parameters	
Analytical column	Hamilton® PXP-X100 (250 x 4.1 mm; 10 μm)
Mobile phase	10 mM ammonium citrate, 2 % MeOH; pH = 5
Flow rate	1 mL min^{-1}
Injection volume	100 μL
Elution program	Isocratic
Run Time	15 minutes
Analytical column	Zorbax™ Rx-C8 (250 x 4.6 mm; 5μm)
Mobile phase	0.1 % TFA, 2% MeOH
Flow rate	1 mL min^{-1}
Injection volume	100 μL
Elution program	Isocratic
Run time	20 minutes

6.4 Results and discussion

6.4.1 Selenium accumulation

An overview of the response of wheat cultivars to supplementation is given in Table 6.2, in terms of total Se by CNAA (Galinha *et al.* 2013) and ICP-MS (this work). Other than an appreciable increment in Se levels of the supplemented crops, there is an excellent agreement between values by both techniques, wherever that comparison is possible. Because of such an agreement not all samples were analysed by ICP-MS, as mentioned before.

ICP-MS presents the advantages of multielemental analysis, low detection limits and capability to measure isotopic ratios. However, Se determination by ICP-MS is affected by polyatomic interferences in the plasma which overlap the most abundant isotopes, $^{78}Se^{+}$ ($^{40}Ar^{38}Ar^{+}$) and $^{80}Se^{+}$ ($^{40}Ar^{2+}$) (Zhang and Combs 1996). The use of collision/reaction cells is a way to remove polyatomic interferences, which allows ^{78}Se and ^{80}Se analysis free from interferences (Feldmann *et al.* 1999). Nevertheless, other interferences like $^{79}Br^{1}H^{+}$ (m/z = 80) (Zhang and Combs 1996) could appear when using H_2 as reaction gas, so Se determination was carried out by monitoring ^{78}Se isotope in the cell mode to avoid all interferences.

Total Se contents of wheat grains show an important increase after biofortification, with respect to the initial concentrations of Se in unsupplemented plants (blanks): without supplementation, only traces of Se could be detected. Wheat grains from plants exposed to (treated with) Se^{VI} present higher concentrations of total Se, as a likely consequence of selenate using the sulphate path through plants (Zhu *et al.* 2009), other than its generally higher uptake/retention and translocation efficiencies (Keskinen *et al.* 2013, Hopper and Parker 1999). These findings concur with results by Poblaciones *et al.* (2014), which show selenate to be much more effective than selenite for increasing the Se accumulation in grains. On the other hand, Se levels in durum wheat (Marialva) are higher than in bread wheat (Jordão), regardless of the chemical vehicle of Se used in the field supplementation – selenite or selenate – and the supplementation procedure itself (foliar or soil addition). Durum (hard) wheats have been known to contain more Se than bread (soft) wheats (Lorenz 1978), a feature that has been linked to the corresponding protein content (Barclay and MacPherson 1992).

Field trials in Australia have shown that Se applied to soil as sodium selenate at seeding was more effective than foliar application (Lyons *et al.* 2004), whereas, for instance, Yläranta (1984a,b) has long made a case for foliar application instead. In quantitative terms and for the Portuguese cultivars and soils herein, foliar application appears to be much more efficient than soil addition, even if other aspects should be considered for biofortification purposes, such as field logistics and, of course, overall costs (see Chapter 5).

Table 6.2 Concentrations of total Se in mature grains from wheat crops grown under Se-supplementation regimes (foliar addition, soil addition) in actual field conditions, by CNAA and ICP-MS. Selenium supplements: 100 g Se ha^{-1} as sodium selenite (Se^{IV}) or sodium selenate (Se^{VI}), plus 10 μM of KI per plot where applicable. Results are expressed as mean ± standard deviation (n = 3); blanks correspond to wheat grains from unsupplemented plants

	$[Se]_{CNAA}$ (mg kg^{-1})	$[Se]_{ICP-MS}$ (mg kg^{-1})
Durum wheat		
Foliar addition		
Blank	0.15±0.01	0.17±0.08
	Booting	
Na_2SeO_3	1.00±0.06	–
Na_2SeO_3+KI	0.85±0.04	0.87±0.01
Na_2SeO_4	1.77±0.06	2.09±0.04
Na_2SeO_4+KI	1.06±0.04	–
	Grain filling	
Na_2SeO_3	1.97±0.07	2.0±0.2
Na_2SeO_3+KI	2.38±0.19	–
Na_2SeO_4	2.98±0.01	3.0±0.2
Na_2SeO_4+KI	2.55±0.12	–
Soil addition		
Blank	a	0.07±0.01
Na_2SeO_4	1.064±0.003	–
Na_2SeO_4+KI	1.51±0.04	–
Bread wheat		
Foliar addition		
Blank	0.06±0.01	–
	Booting	
Na_2SeO_3	0.61±0.01	–
Na_2SeO_4	2.7±0.2	–
	Grain filling	
Na_2SeO_3	0.92±0.04	–
Na_2SeO_4	0.72±0.09	–
Soil addition		
Blank	a	0.060±0.003
Na_2SeO_4+KI	0.76±0.01	–

a: below detection limit

6.4.2 Selenium speciation

In the present study, a methodology based on HPLC–ICP-MS coupling was optimised to release Se presumably bound to proteins; enzymatic hydrolysis assisted by ultrasonic probe sonication was performed first. Similar Se concentrations were obtained from both proteolytic and acid digestion, with efficiency values ranging between 80-100 %, which suggests that enzymatic hydrolysis was effective in catalysing the breakdown of selenoproteins into smaller fractions.

The combined use of enzymatic hydrolysis and ultrasonic probe sonication allowed us to quantitatively extract Se species in a short period of 2 minutes. To optimise the Se species separation, both an anion-exchange column and a reverse-phase column were tested. Figure 6.1 presents the profile of the chromatograms obtained for samples analysed by the latter, which featured several peaks and overlapping for low retention times, which could turn the identification of the selenocompounds into a much more complex procedure; therefore, most samples have been put through the anion-exchange column.

Figure 6.2 illustrates the chromatographic profile of Se species in a standard solution containing 10 $\mu g\, L^{-1}$ of each, after elution through an anion-exchange column, and Figures 6.3a and 6.3b stand for the typical profiles of the chromatograms obtained for almost all field samples – blank and supplemented ones, respectively. Regardless of scale magnitude, SeMet was invariably the major Se species found in the actual field samples, and was identified by comparing its retention time and by spiking experiments.

Other minor Se species were found by anion-exchange HPLC–ICP-MS as well. The first peak (around 2 min) may correspond to $SeCys_2$ or selenomethionine Se-oxide (SeMetO), or to a combination of both (Pedrero and Madrid 2009). Pedrero *et al.* (2007) have reported the oxidation of selenomethionine during sample treatment as one of the main problems associated with an accurate determination of this species, for yielding a peak that appears very close to the void-volume signal (~ 2.1 min). Given such an uncertainty, the first peak cannot be unambiguously assigned to $SeCys_2$.

The last Se species found in the samples was Se^{VI}, that corresponds to the forth peak around 9 min (Figures 6.3a and 6.3b). Spiking experiments also identified this species; its quantification has been precluded by very low amounts, though.

The concentrations of SeMet and Se^{VI} after supplementation and a mass balance are listed in Table 6.3. Samples did not present matrix effects, so external calibration was elected as the method for Se-species determination (instead of standard addition). Speciation analysis of Protease XIV, used for enzymatic hydrolysis, was also performed to check for impurities. Results show the presence of SeMet, which has been taken into account for determining SeMet in field samples.

The speciation procedure has been validated through analyses of a Se-enriched yeast, certified reference material (SELM-1) from the National Research Council Canada (NRC; Ottawa, Canada), using separation by anionic exchange column: certified and experimental values for SeMet in SELM-1 were 3448 ± 146 mg kg^{-1} and 3215 ± 122 mg kg^{-1}, respectively. Since no significant differences were found between certified and experimental values (at a confidence level of 95 %), it was deemed accurate for total Se and SeMet determinations.

As shown in Table 6.3, SeMet was the major Se species found in all samples, which is in agreement with Cubadda *et al.* (2010) and Hart *et al.* (2011), who have stated that about 75 % and 60 %, respectively, of the total Se present in wheat flour is in the form of SeMet. Even if the matrix material of our samples cannot be strictly viewed as typical (commercial) wheat flour, 70 % to 100 % of their total Se is in the form of SeMet.

The present results also show that, regardless of the chemical form of inorganic Se used in the supplementation procedure (Se^{IV} or Se^{VI}) and the supplementation procedure itself (foliar or soil), conversion to SeMet was almost complete. This is important for conveying Se through the food chain, since SeMet can be unspecifically incorporated into proteins instead of methionine, that, in turn, is known for an elevated incorporation into proteins and enzymes (Pedrero *et al.* 2007).

Hart *et al.* (2011) have also reported that SeMet might account for 65-87 % of total extractable species in Se-enriched flour, after supplementing *Triticum aestivum* L. (bread wheat) with 100 g of Se per ha (as sodium selenate), just prior to booting stage. Even if the growth stage is not exactly the same, the corresponding values herein for booting stage (63 ± 8 %) are in agreement with the former ones. In any case, to fully understand the bioaccessibility of Se, it seems reasonable that a determination of Se contents and the distribution of Se species should be completed by also analysing the samples' extracts after a simulated gastrointestinal digestion (Pedrero *et al.* 2006).

The highest value of Se^{VI} (11 %; Table 6.3) was determined in Marialva (durum wheat) flour samples, from crops supplemented through foliar application at grain-filling stage, and at an equivalent field rate of 100 g of Se per ha in the form of selenate. These samples were the ones with top levels of total Se as well (Table 6.2), suggesting that Se supplementation to wheat plants at very high rates can result in higher concentrations of inorganic Se in the form of Se^{VI}. Similar evidence for wheat grown in seleniferous soil, that is Se^{VI} increasing with total Se contents, was also found by Cubadda *et al.* (2010). No significant differences in Se-species distribution could be ascribed to distinct wheat varieties (bread wheat, durum wheat) and supplementation procedures (foliar addition, soil addition): in either case, SeMet is consistently the major species.

Wheat flours from grains devoid of any type of supplementation were also analysed. When compared to supplemented ones, these samples show much lower concentrations of total Se (Table 6.2) and also have SeMet as the major species (Table 6.3), pointing to a most likely association between total Se and SeMet. Blanks from both wheat varieties and supplementation procedures have shown the same distribution profile of selenocompounds already depicted in Figure 6.3a. The presence of KI as an iodine additive to Se-supplementation does not seem to affect either the total accumulation of Se in mature grains, or the distribution of selenocompounds.

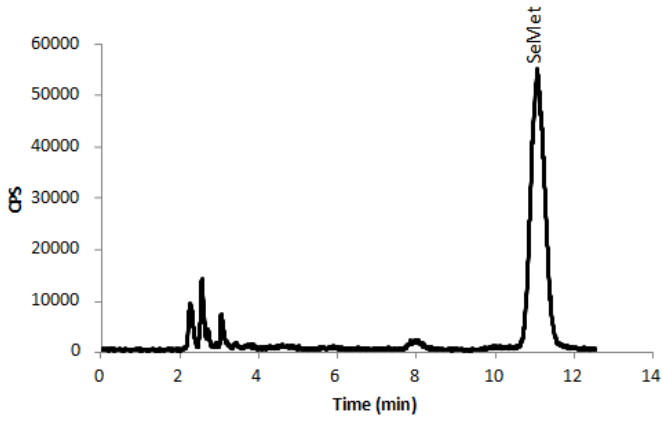


Figure 6.1 Representative HPLC–ICP-MS (reversed-phase column) chromatogram of mature-grain flour from durum wheat (*Triticum durum* Desf.; Marialva cultivar) with selenium supplementation (100 g Se ha^{-1}, as selenate, at the grain-filling stage), from the foliar-addition campaign. CPS ≡ counts per second; monitored isotope: ^{78}Se.

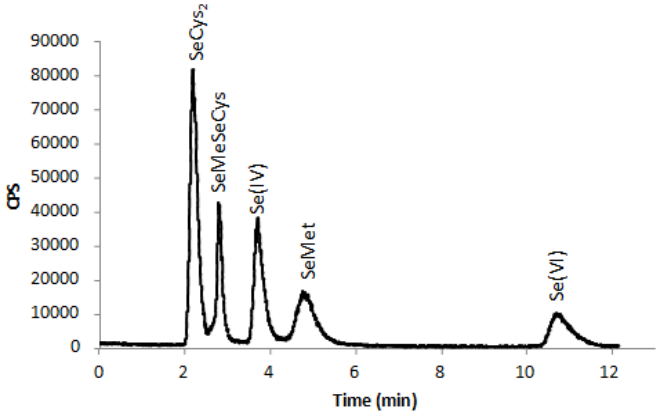
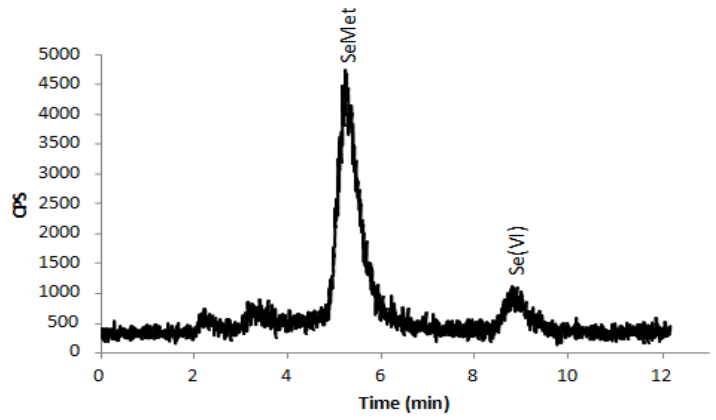
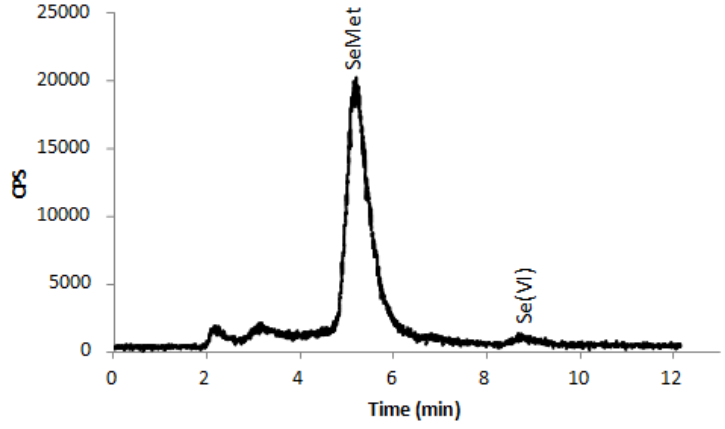


Figure 6.2 Chromatographic profile of a standard solution containing 10 μg L^{-1} of each inorganic (Se^{IV}, Se^{VI}) and organic (SeMet, SeMeSeCys, $SeCys_2$) selenium species, by anion-exchange HPLC–ICP-MS. CPS ≡ counts per second; monitored isotope: ^{78}Se.



a



b

Figure 6.3 Representative HPLC–ICP-MS (anion-exchange column) chromatograms of mature-grain flour from bread wheat (*Triticum aestivum* L.; Jordão cultivar), from the foliar-addition campaign: **a)** without selenium supplementation (blank sample); **b)** with selenium supplementation (100 g Se ha^{-1}, as selenite, at the grain-filling stage). CPS ≡ counts per second; monitored isotope: ^{78}Se.

Table 6.3 Concentrations of Se species in mature grains from wheat crops grown under Se-supplementation regimes (foliar addition, soil addition) in actual field conditions, by ICP-MS. Selenium supplements: 100 g Se ha^{-1} as sodium selenite (Se^{IV}) or sodium selenate (Se^{IV}), plus 10 μM of KI per plot where applicable. Results are expressed as mean ± standard deviation (for *n* determinations); Se-species' percentages refer to total Se; blanks correspond to wheat grains from unsupplemented plants

	n	**SeMet (mg kg^{-1})**	**SeMet (%)**	**Se^{VI} (mg kg^{-1})**	**Se^{VI} (%)**
Durum wheat					
Foliar application					
Blank	3	0.122±0.002	73±1	0.004±0.002	2.5±1.1
		Booting			
Na_2SeO_3	3	0.6±0.2	58±20	0.04±0.01	3.6±1.1
Na_2SeO_3+KI	1	0.995	100	0.020	2
Na_2SeO_4	3	1.0±0.1	60±8	0.064±0.019	3.6±1.1
Na_2SeO_4+KI	3	0.6±0.04	54±4	0.02±0.013	1.8±1.2
		Grain filling			
Na_2SeO_3	2	1.34±0.03	69±2	0.09±0.04	5.1±2.1
Na_2SeO_3+KI	3	1.50±0.05	63±2	–	–
Na_2SeO_4	2	2.0±0.3	68±11	0.333±0.003	11.1±0.1
Na_2SeO_4+KI	3	1.7±0.2	65±6	0.13±0.02	5.0±0.6
Soil addition					
Blank	2	0.07±0.01	100	0.002±0.003	3.4±4.9
Na_2SeO_4	2	1.0±0.2	95±17	0.006±0.004	0.5±0.4
Na_2SeO_4+KI	2	1.1±0.2	72±16	0.014±0.002	0.9±0.1
Bread Wheat					
Foliar application					
Blank	3	0.061±0.007	75±8	–	–
		Booting			
Na_2SeO_3	3	0.36±0.06	86±13	–	–
Na_2SeO_4	3	1.5±0.1	61±4	0.018±0.004	3.2±0.7
		Grain filling			
Na_2SeO_3	3	0.6±0.1	63±9	–	–
Na_2SeO_4	3	0.36±0.05	63±8	0.010±0.004	0.4±0.1
Soil addition					
Blank	1	0.039	65	0.002	3.1
Na_2SeO_4+KI	3	0.65±0.03	86±4	0.008±0.002	1.1±0.3

6.5 Conclusions

The field experiments supporting this study show that an agronomic biofortification of wheat crops with Se has a positive effect not only on grain concentrations of total Se, but also on specific levels of valuable Se-organic compounds. Regardless of the chemical vehicle of Se used in the field supplementation – selenite or selenate, that is Se^{IV} or Se^{VI} as active forms – and of the supplementation procedure itself, the major species in all samples was SeMet, meaning that the conversion of inorganic Se to SeMet was almost complete. Up to 100 % of the total Se was assimilated as SeMet; inorganic Se, in the form of Se^{VI}, scored below 5 % in almost all flour samples from mature wheat grains. Higher values of total Se concentration resulted in higher SeMet concentrations, suggesting that both wheat varieties are likely to assimilate SeMet in mature grains proportionally to the corresponding field supplementation rate. Judging from the present results, either supplementation procedure fits the objective of biofortifying wheat with Se, with no noticeable impact on the distribution of Se species. Hence, other aspects must be considered for a decision on an eventual field routine. The results also show that an agronomic biofortification of wheat crops with Se can improve the nutritional quality of mature wheat grains, by boosting their levels of SeMet, and thus providing an attractive option for enhancing the Se status in populations with Se-deprived diets, through Se-enriched, wheat-based foodstuff.

6.6 References

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7 Selenium characterisation of the Portuguese bread-wheat archival collection

Based on article of same title:
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7.1 Abstract

Following the first morphological and taxonomic inventory of Portuguese wheat (1933), an archival collection of representative varieties has been maintained, replanted and documented by the National Institute of Agricultural and Veterinary Research (INIAV), specifically by its division formerly known as the National Station for Plant Improvement (ENMP-Elvas, now INIAV-Elvas). The INIAV-Elvas wheat collection has always been an invaluable asset in studies of agronomic and/or genetic development of wheat lines, as well as providing a reference frame for the nutritional evolution of Portuguese wheat crops. This work addresses the current status of selenium (Se) in a pool of 46 accessions of bread wheat. Special attention is paid to the (low) levels of Se, for which wheat acts as an important source in human diets, with a view to curbing its deficiency in Portuguese cultivars through biofortification strategies. All grain samples were irradiated at the Portuguese Research Reactor, and total Se was determined through cyclic neutron activation analysis (CNAA); quality control was carried out by concurrent analysis of NIST-SRM® 1567a (Wheat Flour). Our results imply that the best candidates for an improvement of Se contents in their mature grains are the 'Ideal', 'Ribeiro (b)' and 'Ribeiro (a)' cultivars, and the worst candidates are the 'Restauração', 'Galego Rapado' and 'Rieti' cultivars.

7.2 Introduction

More than being just one of the "big three" cereal crops (with maize and rice), wheat is *the* staple food of humankind, with a history that is closely intertwined with humanity's own. Despite ongoing arguments about core

areas and/or time scales (Zohary 1999; Willcox 2005; Tanno and Willcox 2006; Allaby *et al.* 2008; Abbo *et al.* 2010; Fuller *et al.* 2011; Purugganan and Fuller 2011; Fuller *et al.* 2012), early agricultural beginnings – the tangled roots of agriculture, in the colourful expression by Balter (2010) – are invariably traced back to cereal domestication, i.e. einkorn and emmer wheats (and barley) (Araus *et al.* 2007), foremost among seven Pre-Pottery Neolithic founder food crops (Lev-Yadun *et al.*2000).

Domestication of diploid wild einkorn and tetraploid wild emmer is arguably thought to have begun some 10000 years before present, across the vast and geographically diverse expanses of the Fertile Crescent in southwest Asia and the Middle East (Özkan *et al.* 2002; Araus *et al.* 2007; Brown *et al.* 2009; Matsuoka 2011; Gepts *et al.* 2012), even if opportunistic gathering and incipient cultivation of autochthonous landraces may have started before that, in the early Holocene (Moore *et al.* 2000; Salamini *et al.* 2002; Tanno and Willcox 2006; Weiss *et al.* 2006; Willcox *et al.* 2008; Gepts *et al.* 2012). Although, again, wide estimates are expected, the first domesticated forms of einkorn and emmer apparently showed up in southern Levant and southeast Turkey ca. 9600-9000 years before present (Luo *et al.* 2007), with the latter (emmer) en route to the modern, free-threshing forms of tetraploid durum wheat (*Triticum durum*) and hexaploid bread wheat (*Triticum aestivum*) (Nesbitt and Samuel 1996).

Over the course of human history, up to 100000 plant species have been used – on a regular or occasional basis – since prehistoric times to meet the various needs (food, clothing, shelter, health, ritual) of an ever growing population (Ceccarelli 2009), with nutrition alone accounting for more than 7000 foraged and farmed species (Gepts 2006). Among them, wheat and its wild ancestors were at the locus of an amazing evolutionary step – the so-called Neolithic Revolution (Childe 1936) – that turned hunters/gatherers into settlers and, eventually, farmers (Weisdorf 2005).

The status of wheat as the universal crop of early farming has been kept through this day. Tonnage (though not acreage) of wheat grown worldwide

may have been overtaken by both maize's and rice's shortly before the turn of last century. However, wheat remains unrivalled as to overall area and latitudinal range of cultivation, nutritional relevance, and civilisational significance at large (Shewry 2009). Current production is split between an overwhelming majority of hexaploid bread wheat (~ 95 %), and a remaining share of (mostly) tetraploid durum wheat plus small amounts of hulled-grain species (einkorn, emmer, spelt) (Peng *et al.* 2011; Brouns *et al.* 2013).

Given such a background, no wonder that wheat germplasm was among the first to be stored in archival collections and seed banks, despite early technical difficulties in preserving genetic resources as germplasm holdings (Sachs 2009; Dierig *et al.* 2014). Wheat (*Triticum* genus) also tops the list of the largest worldwide germplasm and *ex situ* collections by crop (Börner 2006; Kilian and Graner 2012; Carvalho *et al.* 2013). Besides, it is only fitting that wheat was the founding subject of one of the longest-running experiments in the history of science (1843-): the Broadbalk experiment at Rothamsted, UK (Fan *et al.* 2008a, 2008b) – over 170 years and counting!

Following the first morphological and taxonomic inventory of Portuguese wheat landraces and old cultivars (Vasconcelos 1933), an archival collection of representative varieties has been maintained, replanted and documented by the National Institute of Agricultural and Veterinary Research (INIAV), specifically by its division formerly known as the National Station for Plant Improvement (ENMP-Elvas, now INIAV-Elvas). The INIAV-Elvas collection has always been an invaluable asset in studies of agronomic and/or genetic development of wheat lines, as well as providing a reference frame for the nutritional evolution of Portuguese wheat crops (Carvalho *et al.* 2012; Santos *et al.* 2012). Despite its relatively limited extent, the collection has been regularly present in international surveys and core-collection studies as well (Asins and Carbonell 1989; Balfourier *et al.* 2007).

This work addresses the current status of selenium (Se) in a pool of 46 accessions of bread wheat. Special attention is paid to the (low) levels of Se, for which wheat acts as an important source in human diets (Finley 2006; Hawkesford and Zhao 2007; Navarro-Alarcón and Cabrera-Vique 2008),

with a view to curbing its deficiency in Portuguese cultivars given the unique role of Se and selenoproteins in human health (Rayman 2012; Roman *et al.* 2014).

7.3 Materials and methods

7.3.1 Field work

The INIAV-Elvas wheat collection (the collection, hereinafter) consists of a total of 99 wheat accessions: 48 bread wheat cultivars (*Triticum aestivum* L.) and 51 durum wheat cultivars (*Triticum durum* Desf. and *Triticum turgidum* L.). The present work has been focused on grain samples from the 2011-2012 maintenance campaign (see below), and pertaining only to bread-wheat cultivars. Other than the former bread-wheat accessions, a control variety – 'Pirana', also a bread-wheat cultivar – was analysed as well. 'Pirana' was sown in 5 different plots evenly distributed across the experimental field, together with the bulk of the collection.

All bread-wheat cultivars were sown in Herdade da Comenda (Caia-Elvas, Portugal), in mid-December 2011, and sampled in mid-July 2012 during the harvest season. Field schedule and agricultural details were as follows: i) Soil preparation by cross ploughing plus one single run of vibrocultivator, and, just prior to sowing, a basal dressing with 200 kg ha^{-1} of NPK (15-15-15) fertiliser; ii) Top dressing with 150 kg ha^{-1} of urea (NPK 46-0-0) in mid-March 2012; iii) 2 applications (3 L ha^{-1}) of 5C Cycocel® (chlormequat chloride 57 % w/w; BASF), a plant-growth regulator, in the beginning and middle of April 2012; iv) Pre-emergence herbicide treatment with 3 L ha^{-1} of Trigonil® (Bayer CropScience) plus 1.5 L ha^{-1} of Buggy® XTG (Sipcam-Oxon) in mid-December 2011; and v) Post-emergence herbicide treatment with 350 g ha^{-1} of Atlantis® OD (Bayer CropScience) plus 1 L ha^{-1} of Genapol® T-150 (Aventis CropScience) in the beginning of February 2012.

After threshing, the whole grains were cleansed, weighed and stored in a dry room. All grain samples were then ground to a fine powder in a Waring® blender HGB50E2, and kept in polyethylene vials until further analysis.

7.3.2 Selenium determination

Total Se concentration in the wheat grains was determined by cyclic neutron activation analysis (CNAA) via the short-lived nuclide ^{77m}Se ($t_{1/2}$: 17.5 s), at the Technological and Nuclear Campus (CTN-IST; Bobadela, Portugal). Samples were prepared according to Galinha *et al.* (2013a), and irradiated in the fast pneumatic system of the Portuguese Research Reactor (pool-type reactor; 1-MW nominal power) at a thermal-neutron fluence rate of 1.7×10^{12} cm^{-2} s^{-1}. Gamma spectra were acquired with a liquid N_2-cooled, high-purity Ge coaxial detector (1.85 keV FWHM resolution at 1.33 MeV; 25 % relative efficiency), linked to a digital gamma-ray spectrometer DSPEC Pro (ORTEC®) with built-in dead-time and loss-free counting correction modules.

Analyses were performed upon 9 replicates per sample, around 900 mg each, and using 10 cycles. In each cycle, irradiation and counting times were 20 s, and decay time 5 s. Elemental concentrations were assessed through the relative method, using NIST-SRM® 1568a (Rice Flour) as a standard calibrator. Quality control was carried out by analysis of NIST-SRM® 1567a (Wheat Flour).

7.4 Results

Table 7.1 lists all the analysed cultivars from the collection's bread-wheat subset, with the exception of 'Eborense' cultivar that belongs to the botanical variety *Triticum aestivum* L. var. *milturum* (Alef.) Velican, or, in abbreviated notation, just *Triticum aestivum* var. *milturum*. To avoid making the text somewhat cumbersome, the latter notation will be used onwards. Field and passport data have been provided by the INIAV-Elvas, while biological status has been checked through the wheat database of the N.I. Vavilov Research Institute of Plant Industry (St. Petersburg, Russia). A few cases of homonymy among cultivars in Table 7.1 go referenced by (a) or (b) next to their corresponding name.

Although 'Eborense' is not part of the wheat collection maintained by the INIAV-Elvas, it is a traditional Portuguese cultivar and, as such, properly stored in the Portuguese Plant Germplasm Bank (Banco Português de Germoplasma Vegetal; Braga, Portugal). The bread-wheat cultivar 'Serrano (b)' (plot code: 136; Table 7.1) could not be analysed due to sample loss during its preparation. The analysis of 'Magueija' (plot code: 148; Table 7.1) was carried out with 6 replicates only, due to the low sample mass available. The traditional cultivar 'Sacho' was also found in small-scale (subsistence) farming in northern mainland Portugal, during a cross-country cereal survey (Galinha *et al.* 2013b).

Results for total Se concentrations in mature grains from experiments with the bread-wheat collection and 'Pirana' trials are illustrated by Figures 7.1 and 7.2, respectively. Quality control of the results for Se was performed using NIST-SRM® 1567a (Wheat Flour), resulting in 1.2 ± 0.3 mg Se kg^{-1}, which is in good agreement with the certified value of 1.1 ± 0.2 mg Se kg^{-1}, both at a 95 % confidence level.

Table 7.1 Field and passport data for the analysed bread-wheat cultivars.

Plot code	Botanical variety	Cultivar name	Biological status
	Triticum aestivum aestivum		
101		Restauração	Advanced/Improved
104		Anafil Claro	n.a.
107		Egípcio	n.a.
108		Fronteiriço	n.a.
113		Guaditano	n.a.
125		Mole Algarvio	Other
126		Português	n.a.
131		Rieti	n.a.
132		Sacho	Traditional
138		Tremês Preto	n.a.
142		Transmontano (a)	n.a.
148		Magueija	Traditional
	Triticum aestivum milturum		
102		Alentejano	n.a.
123		Mocho de Espiga Ruivo	n.a.
146		Galego Rapado	Traditional
	Triticum aestivum lutescens		
103		Almadense	Traditional
119		Mocho Cabeçudo	Traditional
122		Mocho de Espiga Branca	Traditional
124		Mocho Rapado	n.a.
135		Santareno	Advanced/Improved
147		Gentil Grosso	n.a.
	Triticum aestivum ferrugineum		
105		Ardito	n.a.
106		Belém	Traditional
109		Funchal	n.a.
111		Galego Barbado	Traditional
114		Ideal	n.a.
115		Liz	Advanced/Improved
116		Manzanares	n.a.
117		Mestiço	Advanced/Improved
118		Mirandês	Traditional
127		Precoce	Traditional
133		Saloio (a)	n.a.
134		Saloio (b)	n.a.
136		Serrano (b)	Advanced/Improved
139		Tremês Ruivo	n.a.
141		Temporão de Coruche	Traditional
143		Transtagano	n.a.
149		Ruivo	n.a.
151		Serrano (a)	Advanced/Improved

Different cultivars with an identical name are denoted by (a) or (b); n.a. ≡ not available.

Table 7.1 (cont.) Field and passport data for the analysed bread-wheat cultivars.

Plot code	Botanical variety	Cultivar name	Biological status
	Triticum aestivum graecum		
112		Grécia	n.a.
	Triticum aestivum creticum		
121		Mocho de Espiga	Traditional
	Triticum aestivum		
128		Ribeiro (a)	Traditional
129		Ribeiro (b)	Traditional
144		Trigo da Maia	n.a.
152		Transmontano (b)	n.a.
	Triticum aestivum		
137		Tremês Branco	n.a.
	Triticum aestivum hostianum		
145		Viloso Mole	n.a.

Different cultivars with an identical name are denoted by (a) or (b); n.a. ≡ not available.

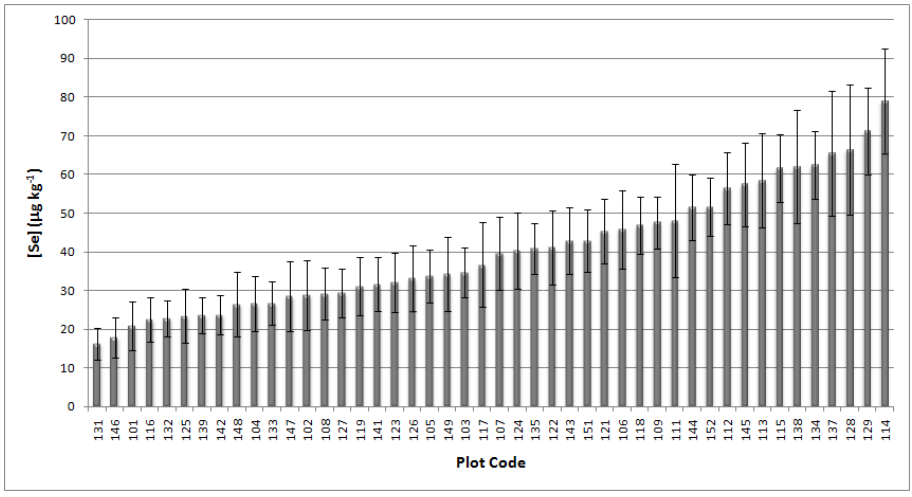

Figure 7.1 Selenium concentration (fresh-weight basis) in 46 bread-wheat cultivars from the Portuguese wheat collection (see Table 7.1 for plot coding). Uncertainties are at the 95 % confidence level.

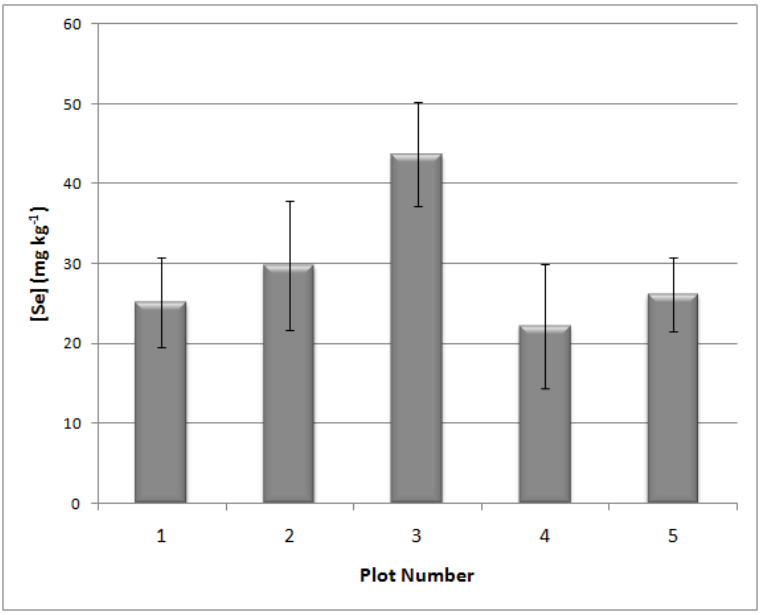

Figure 7.2 Selenium concentration (fresh-weight basis) in field replicates of extra-collection 'Pirana' cultivar (corresponding to five plots sown, 1-5). Uncertainties are at 95 % confidence level.

7.5 Discussion

To protect and improve autochthonous taxa, and varieties that are naturally adapted to local and regional conditions while threatened by genetic erosion (Hammer and Teklu 2008; Wouw *et al.* 2009), the Portuguese Government (2009) has taken measures to include some of those so-called conservation varieties in the yearly National Catalogue of Varieties (MAM/DGAV 2014), available to all agricultural partners. 'Pirana' is one of those varieties, and, as such, inscribed in the Catalogue since 2011. Other than its conservation status, 'Pirana' has been chosen as the control cultivar for presenting fair yields and an acceptable resistance to the characteristic dry-weather conditions of the major wheat-producing region of mainland Portugal (southern interior).

Figure 7.1 summarises the results attained for total Se in mature grains from bread-wheat cultivars belonging to the collection. To minimise the results' uncertainty, and to cope with the (expected) very low amounts of Se in near all analysed grain samples, the number of analytical replicates by field sample was stepped up to nine. The results show that total Se concentrations in 46 analysed cultivars were found between 16 μg kg^{-1} and 79 μg kg^{-1}, with: i) 2 cultivars, 'Rieti' and 'Galego Rapado', below 20 μg kg^{-1}; ii) 28 % of cultivars within 20-30 μg kg^{-1}; iii) 20 % of cultivars within 30-40 μg kg^{-1}; iv) 22 % of cultivars within 40-50 μg kg^{-1}; v) 11 % of cultivars within 50-60 μg kg^{-1}; vi) 11 % of cultivars within 60-70 μg kg^{-1}; and vii) 2 cultivars, 'Ribeiro (b)' and 'Ideal', above 70 μg kg^{-1}.

Even accounting for a non-homogeneous distribution of Se in the cultivation soil, it is apparent that such heterogeneity could hardly be the only reason for the variability of Se contents among mature grains of the bread-wheat cultivars. Apart from the variability imparted by the soil-Se distribution, not only the ability of the wheat plant to uptake, transfer and assimilate Se, but also, and perhaps especially, the chemical forms of Se (bio)available in soil play an important role as well. Some authoritative sources claim that grain-Se contents appear overwhelmingly determined by soil-Se availability and not by genotypic variation (Lyons *et al.* 2005). Still, the present results seem to indicate that bread-wheat cultivars can have a very distinct accumulation of Se in their mature grains, not necessarily related to soil features only.

In terms of experimental field design, the control variety 'Pirana' was sown in five separate plots, each one of them after an array of ten plots allocated to the collection cultivars. Plots number 1, 2, 4 and 5, as per their field codes, yielded mature grains with total Se levels ranging from 22 μg kg^{-1} to 30 μg kg^{-1} (Figure 7.2), which is quite consistent taking into account all uncertainties. Wheat grains from plot number 3 presented a significantly higher Se concentration (44 ± 7 μg kg^{-1}), which may indicate that the assimilation of Se by this single (control) cultivar was not that uniform through the field layout, most likely due to nugget-like effects on the basal levels of Se. Besides, it is well-known that background levels of Se in soil

can vary at a metre-to-metre scale (Lyons and Cakmak 2012). However, the spatial variation gauged by the control variety does not seem enough to explain *per se* the whole extent of grain-Se variability in the collection cultivars, as mentioned above.

Based on morphological characterisation, the 48 bread-wheat cultivars of the collection (Table 7.1) can be classified into 9 different botanical varieties, that are, in descending order of representativeness: *Triticum aestivum* vars. *aestivum, ferrugineum, lutescens, plenoerythrospermum, milturum, graecum, creticum, nigroerythrospermum* and *hostianum* (Carvalho *et al.* 2009). The *ferrugineum* variety comprises 37 % of the bread-wheat cultivars, with a wide range of Se concentrations (23-79 μg kg^{-1}; Figure 7.1): 52 % of them above 40 μg kg^{-1} and 18 % above 60 μg kg^{-1}; the top Se cultivar ('Ideal') belongs to this variety as well.

All four cultivars of *T. aestivum* var. *plenoerythrospermum* presented grain-Se values above 50 μg kg^{-1}, with two cultivars –'Ribeiro (b)' and 'Ribeiro (a)' – scoring second and third highest concentrations, respectively. On the other side of the Se spectrum, the varieties *milturum* and *aestivum* are the ones that feature the lowest amounts of total Se, with all cultivars of *milturum* and 83 % of cultivars of *aestivum* below 40 μg kg^{-1}. Thus, judging only from the present results, the bread-wheat cultivars that seem most unfit to further improvement concerning their Se levels are, in descending order, 'Restauração', 'Galego Rapado' and 'Rieti'.

On an end note, it is worth emphasising that *ex* situ (archival) preservation and *in situ* (field) maintenance of this type of germplasm resources seems crucial not only for genetic research, breeding programs and assemblage of core collections, but also for assessing the specific ability of some cultivars to assimilate essential elements from soils. This is particularly important in the case of Se: its relevance in human nutrition must be carefully weighed against the fact that it is also a non-renewable, hardly-recyclable, relatively-scarce resource (Haug *et al.* 2007). With sufficient genotypic variation in Se accumulation, it may be possible to use or breed varieties with enhanced Se contents and thus minimise the need for Se fertilisers (Broadley *et al.* 2006).

7.6 Conclusions

The present elemental snapshot of an old Portuguese wheat collection – focused on total Se contents of its bread-wheat accessions, so to speak – shows significant differences in Se accumulation between mature grains from a recent maintenance campaign (2011-2012). *Triticum aestivum* L. cultivars of the *ferrugineum* and *plenoerythrospermum* varieties yielded grains with the highest Se concentrations, while the lowest concentrations were found in grains from vars. *aestivum* and *milturum*. Genotypic variation is not likely the only source of grain-Se variability, though: significant differences in Se assimilation by the control cultivar 'Pirana' indicate that the basal level of Se can fluctuate considerably through the field layout. Still, in what concerns the possibility of enhancing Se contents through plant breeding, major candidates appear to be the cultivars 'Ideal', 'Ribeiro (b)' and 'Ribeiro (a)', whereas 'Rieti', 'Galego Rapado' and 'Restauração' seem unfit for such purpose.

7.7 References

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8 General discussion

This work has been primarily concerned with providing knowledge about the most efficient methods to enhance Se contents in wheat, with a view to an increase in the Se status of the Portuguese population. Those methodologies were applied/tested using some of the most important Portuguese bread- and durum-wheat cultivars, in open-field, real-weather conditions, and through agricultural practices and crop schedules that are customary to Portuguese farmers.

8.1 Overview

Prior to Se-supplementation studies, an extensive investigation of elemental levels in home-grown cereals and their cultivation soils has been carried out across the main production areas of mainland Portugal, in order to assess their baseline levels in relevant elements – contaminants and micronutrients, especially Se. Chapter 2 is thus focused on an elemental characterisation of cereals and soils collected at major producing areas of Trás-os-Montes, Alto Alentejo and Baixo Alentejo (inland regions), in the summer of 2009, during the corresponding harvest campaigns. Quantitative determination of Se was not possible, though, due to (very low) levels of Se, below the detection limit of the analytical technique.

For Se supplementation proper, wheat was chosen as the target cereal because it supplies about 20 % of the energy and about 25 % of the protein requirements of the world population (Högy and Fangmeier 2008, Sahrawat *et al.* 2003). In Portugal, wheat represents more than 85 % of the human consumption of cereals (INE 2013). This market share, together with the fact that wheat is a major dietary source of Se (Lyons *et al.* 2004), makes it an obvious candidate for biofortification strategies that may help curb nutrient deficiencies in the general population. Of course, this might not be the case of Se in Portuguese wheat – or in another Portuguese cereal, for that matter. However, as we shall see next, the Se levels in Portuguese cereals do appear to be somewhat low, thus prompting an intervention on the soil-plant system that may bring them to acceptable contents in terms of human dietary intake.

Chapter 3 addresses the Se levels of major cereals and their cultivation soils. The general survey during the 2009 cereal-harvest campaign resulted in field samples for which Se could not be quantitatively determined due to very low levels of this element. There were instrumental shortcomings for that as well. The main reason was a high content of some elements in samples – mainly Al, Hf and Ta – which form interfering radionuclides. A high activity of ^{28}Al seriously affects the detection of Se when the short-lived ^{77m}Se is measured. On the other hand, when INAA is used for low-level determinations via the long-lived ^{75}Se, the most intense gamma-lines of this radioisotope are interfered by the gamma-lines of ^{181}Hf and ^{182}Ta. To solve the problem of determining Se in such samples, RNAA was used: a Se detection limit as low as 0.3 ng g^{-1} could be achieved for plant and soil materials, due to a very high yield and selectivity of the Se chemical separation.

Chapter 4 describes the protocol for preparing Se-enriched wheat seeds, with the specific purpose of optimising the administration of Se to seeds prior to sowing. A two-step procedure was devised: first, soaking bread-wheat seeds in an active Se solution; then, monitoring ^{75}Se in the soaked seeds. To avoid losing (leaching) Se to soil after sowing, and, especially, to ensure that Se really gets into wheat seeds – and not just onto them – the washing time of the seeds was adjusted as well. The results of this optimisation study in seed enrichment point to an optimal cycle of 48 h for soaking + 24 h for washing.

Chapter 5 deals with the ability of bread and durum wheat to accumulate Se in their edible parts (mature grains) after supplementation via soil and foliar applications, and also through Se-enriched seeds. The latter procedure was thoroughly ineffective, though: mature grains from wheat plants grown from Se-enriched seeds did not present any significant increase of this element, as compared to blanks (unsupplemented samples). Overall, foliar application seems much more efficient than soil application. Nevertheless, in terms of practical implementation, some trade-off between plain efficiency and field logistics should be considered: soil addition might be easier, yet supplements would be costlier (higher Se amounts). And in the absence of aerial irrigation systems to assist in the foliar application – as in rainfed agriculture – farmers may as well be constrained to apply Se supplements via soil procedures.

Chapter 6 is concerned with the Se speciation in cereal samples, after being biofortified with Se through foliar or soil additions. Selenium bioavailability, intake and metabolism are heavily dependent on its chemical forms (Finley 2006, Thiry *et al.* 2012, Kieliszek and Błażejak 2013), which can be organic or inorganic (Sager 2006, Pedrero and Madrid 2009). Regardless of the chemical matrix of Se used in the field experiments (selenate or selenite), the wheat varieties (bread or durum wheat) and the supplementation procedure itself (foliar or soil addition), the major species in all samples was SeMet, meaning that the conversion of the supplemented inorganic Se to SeMet was almost complete. This is vital for conveying Se through the food chain, since SeMet can be unspecifically incorporated into proteins instead of methionine (Pedrero *et al.* 2007). In practical terms, an agronomic biofortification of wheat crops with Se can improve the nutritional quality of wheat grains, by boosting their levels of SeMet to an extent that may help curb Se defficiency in population groups with Se-deprived diets.

From the soil-addition studies, we noticed that differences in the assimilation and accumulation of Se by bread wheat are much more apparent than in the case of durum wheat, as they translate into wider variation between grain-Se concentrations among bread-wheat cultivars. On the other hand, from foliar-addition studies, the bread-wheat cultivar 'Jordão' appeared quite sensitive to the time of Se application. It is thus quite possible that distinct genotypes react differently to environmental conditions.

As so, Chapter 7 looks into the current status of Se in a pool of 46 accessions of bread wheat from an archival (official), Portuguese wheat collection. Long-term strategies of plant breeding (genetic biofortification) have also been viewed as technically attractive and potentially helpful to increase the efficiency of Se uptake (Lyons *et al.* 2005). The argument is straightforward: if sufficient genetic variation exists in Se accumulation, and if such variation is inheritable, traditional breeding programs could be developed. That would provide an alternative to agronomic biofortification, and thus minimise the need for Se-enriched fertilisers (Broadley *et al.* 2006). From our work with the collection, and for enhancing Se contents through plant breeding, the best candidates are the 'Ideal', 'Ribeiro (b)' and 'Ribeiro (a)' cultivars.

8.2 Future research

First and foremost, the effect of weather conditions on the relative efficiency (sensu lato) of field supplementation procedures for wheat with Se should be clarified through additional field trials in different calendar years. Rainfall, in particular, may have a serious, if dissimilar impact on the outcome of Se biofortification through either procedure.

In case of intensive implementation of any field procedure dicussed herein, it would be advisable to implement an environmental-monitoring program that could prevent agricultural Se from straying away unnoticed from farming fields, leading to an undesirable Se-enrichment of water bodies and riparian habitats (Hawkesford and Zhao 2007). On another research level, a public-health program that could follow up the evolution of human-Se indicators might be quite informative as well.

Ensuring adequate concentrations of Se at the base of the food chain – here, through cereal enrichment – is necessary but not quite sufficient to cope with the complex human biochemistry. To fully understand the bioaccessibility of Se – and modulate its mean dietary requirements – it seems reasonable that the determination of Se contents and the distribution of Se species should be completed by analysing the extracts of biofortified cereal samples too, after a simulated gastrointestinal digestion (Pedrero *et al.* 2006).

Long-term genetic biofortification, as a complement to short-term agronomic biofortification, is also an attractive issue. Germplasm resources in archival collections and gene banks can be extremely useful to investigate why some landraces and cultivars seem more efficient in assimilating Se from the soil, and translocating it to their edible parts (mature grains). Should genetic and breeding research be able to identify and/or develop wheat varieties that are more capable of accumulating Se, the global Se budget would greatly benefit because Se is a non-renewable, hardly-recyclable, relatively-scarce resource (Haug *et al.* 2007, Lenz and Lens 2009). This calls for a subtle equilibrium between actual (nutritional) needs, overall (field) costs and potential (health) benefits (Combs 2001, Burk 2002, Arthur 2003), prior to implementing any wheat-biofortification routine.

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List of abbreviations

Chemical elements

Al	Aluminium	Eu	Europium	Mn	Manganese	Se	Selenium
As	Arsenic	Fe	Iron	Mo	Molybdenium	Si	Silicium
Au	Gold	Ge	Germanium	Na	Sodium	Ta	Tantalum
Ar	Argon	H	Hydrogen	Ni	Nickel	Ti	Titanium
Br	Bromine	Hf	Hafnium	O	Oxygen	Th	Thorium
Ca	Calcium	Hg	Mercury	Pa	Protactinium	Zn	Zinc
Co	Cobalt	K	Potassium	Rb	Rubidium		
Cr	Chromium	Mg	Magnesium	Sc	Scandium		

Chapter 1

ENMP	National Station for Plant Improvement
FAO	Food and Agriculture Organization
IAEA	International Atomic Energy Agency
INIAV	National Institute of Agricultural and Veterinary Research
RDA	Recommended dietary intake
WHO	World Health Organization

Chapter 2

AA	Alto Alentejo
ADI	Acceptable daily intake
BA	Baixo Alentejo
CSQG	Canadian Soil Quality Guidelines
FCT	Fundação para a Ciência e a Tecnologia
FOREGS	Forum of European Geological Surveys
INAA	Instrumental neutron activation analysis
HPGe	High-purity germanium
k_0-INAA	Instrumental neutron activation analysis by k_0 method
RPI-ITN	Portuguese Research Reactor
NIST-SRM	Standard reference material from NIST – National Institute of Standards and Technology, USA
TM	Trás-os-Montes

Chapter 3

E	Elvas
FWHM	Full width at half maximum
G	Guarda
HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HF	Hydrofluoric acid
HNO_3	Nitric acid
HPGe	High-purity germanium
INAA	Instrumental neutron activation analysis
ITN	Technological and Nuclear Institute
J	Jordão
k_0-INAA	Instrumental neutron activation analysis by k_0 method
M	Marialva
$MgCl_2$	Magnesium chloride
NAA	Neutron activation analysis
NIST	National Institute of Standards and Technology, USA
NIST-SRM	Standard reference material from NIST – National Institute of Standards and Technology, USA
NPI	Nuclear Physics Institute of the ASCR
RNAA	Radiochemical neutron activation analysis
Ro	Root
Se	Seed
So	Soil
Sp	Spike
TC_{Seed}	Transfer coefficient (relative to seed)
TC_{Soil}	Transfer coefficient (relative to soil)
UK	United Kingdom
V	Valpaços
W	Wheat

Chapter 4

Na_2O_4Se	Sodium selenate
RPI-ITN	Portuguese Research Reactor

Chapter 5

CNAA	Cyclic neutron activation analysis
CTN-IST	Technological and Nuclear Institute
CV	Coefficient of variation
FAO	Food and Agriculture Organization of the United Nations
FCT	Fundação para a Ciência e a Tecnologia
FWHM	Full width at half maximum
HPGe	High-purity germanium
I^-	Iodide
IAEA	International Atomic Energy Agency
INIAV-Elvas	Instituto Nacional de Investigação Agrária e Veterinária, Elvas
KI	Potassium iodide
Na_2SeO_4	Sodium selenate
Na_2SeO_3	Sodium selenite
NIST-RM	Reference material from NIST – National Institute of Standards and Technology, USA
NIST-SRM	Standard reference material from NIST – National Institute of Standards and Technology, USA
RDA	Recommended dietary intake
RM	Reference material
RNAA	Radiochemical neutron activation analysis
RPI	Portuguese Research Reactor
SD	Standard deviation
ÚJF	Nuclear Physics Institute of the ASCR
WHO	World Health Organization

Chapter 6

$C_6H_8O_7$	Citric acid
CCTN-IST	Campus Tecnológico e Nuclear, Instituto Superior Técnico
CNAA	Cyclic neutron activation analysis
DNA	Deoxyribonucleic acid
GSHPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HNO_3	Nitric acid
HPLC	High-performance liquid chromatography
HPLC-ICP-MS	High-performance liquid chromatography with inductively-coupled plasma mass spectrometry
ICP-MS	Inductively-coupled plasma mass spectrometry
INIAV	Instituto Nacional de Investigação Agrária e Veterinária
KI	Potassium iodide
MeOH	Methanol
Na_2SeO_4	Sodium selenate
Na_2SeO_3	Sodium selenite
NH_4OH	Ammonium hydroxide

NIST-SRM	Standard reference material from NIST – National Institute of Standards and Technology, USA
NRC	National Research Council, Canada
Se^{IV}	Selenite
Se^{VI}	Selenate
$SeCys_2$	Selenocysteine
SeMet	Selenomethionine
SeMetO	Selenomethionine-Se-oxide
SeMetSeCys	Selenomethylselenocysteine
TFA	Trifluoroacetic acid

Chapter 7

CNAA	Cyclic neutron activation analysis
CTN-IST	Technological and Nuclear Campus
ENMP-Elvas	National Station for Plant Improvement, Elvas
INIAV	National Institute of Agricultural and Veterinary Research
INIAV-Elvas	National Institute of Agricultural and Veterinary Research, Elvas

Chapter 8

INAA	Instrumental neutron activation analysis
RNAA	Radiochemical neutron activation analysis
SeMet	Selenomethionine

Summary

The main endeavour of this thesis, as outlined in Chapter 1, was to study – implement, evaluate and discuss – different methods to enhance Se contents in wheat, using some of the most important Portuguese varieties of bread and durum wheat, under Portuguese weather conditions, and through cultivation practices used by Portuguese farmers. Supplementation trials were carried out in the open, in actual field conditions, thus avoiding indoor experiments in laboratories or greenhouses.

Chapter 2 is focused on the elemental characterisation of cereals (and their cultivation soils) from the 2009 campaign, collected at the main Portuguese production areas. Basal levels of Se were invariably low, below the detection limit of the analytical technique (INAA).

Chapter 3 addresses the way the former issue was worked out, by resorting to RNAA instead. A detection limit as low as 0.3 μg Se kg^{-1} (a 3-σ criterion) could be achieved for plant and soil materials, due to a very high yield and selectivity of the Se chemical separation.

Chapter 4 describes the tentative enrichment of bread-wheat seeds with Se, while optimising both their soaking time and washing time through detection of a Se radiotracer (^{75}Se). An optimal cycle of 48 h for soaking plus 24 h for washing could be established. These laboratory results were not matched by the outcome of field trials, though: seed enrichment did not prove effective as a Se-supplementation procedure.

Chapter 5 deals with the ability of bread and durum wheat to accumulate Se after supplementation through soil- and foliar-addition procedures. The latter appeared much more efficient, yet field logistics and cost effectiveness may weigh in an eventual biofortification routine.

Chapter 6 is concerned with the Se speciation in cereal samples, after being biofortified with Se via foliar or soil additions. Regardless of experimental attributes, the major species in all samples was selenomethionine (SeMet), which means an almost full conversion of the supplemented inorganic Se.

Chapter 7 looks into the current status of Se in an array of 46 accessions of bread wheat from an archival wheat collection, with a view to discriminate between potential candidates for Se improvement through plant breeding. Among traditional landraces and advanced cultivars, best candidates to fit the purpose appear to be the 'Ideal', 'Ribeiro (b)' and 'Ribeiro (a)' varieties.

Chapter 8 provides an overview and a discussion of the achieved results, and some suggestions for future research.

Samenvatting

Het hoofddoel van dit proefschrift, zoals aangegeven in hoofdstuk 1, was de bestudering, de implementatie, de evaluatie en de discussie van verschillende methoden om te komen tot verhoging van de selenium (Se) status in tarwe, daarbij gebruikmakend van enige van de meest belangrijke Portugese variëteiten van brood- en harde tarwe, onder Portugese weer-omstandigheden, via de cultivatiepraktijk in gebruik bij Portugese boeren, dit alles onder actuele veldomstandigheden, dus vrijwel zonder experimenten in laboratoria of kassen.

Hoofdstuk 2 is gericht op de element-karakterisering van granen (en hun cultivatie-grond) geoogst in de 2009 campagne, verzameld in de voornaamste Portugese produktiegebieden. Basale Se niveaus waren onveranderlijk laag, lager dan de detektielimieten van de gebruikte analytische methode (INAA = Instrumentele NeutronenActiverings Analyse).

Hoofdstuk 3 is gericht op de verdere uitwerking van deze eerdere issues, waarbij werd overgestapt op RNAA (Radiochemische Neutronen Activerings Analyse). Een detektielimiet van 0.3 μg Se kg^{-1} (3 σ kriterium) werd bereikt voor planten- en grondmateriaal, gebaseerd op een zeer hoge opbrengst en selectiviteit van de Se chemische scheiding.

Hoofdstuk 4 omschrijft de tentatieve verrijking van brood-tarwe zaden met Se, met optimalisatie van hun inweektijd en wastijd, via gebruik en detectie van een Se radiotracer (^{75}Se). Een optimale cyclus van 48 h voor inweken en 24 h voor wassen kon worden vastgesteld. Deze laboratoriumresultaten konden echter niet worden gematched in veldproeven: zaadverrijking bleek niet effectief als een Se-supplementatie procedure.

Hoofdstuk 5 omschrijft de mogelijkheden voor brood- en harde tarwe om Se te accumuleren via supplementatie door bodem- en bladadditie procedures. De laatste procedure leek het meest effectief, maar veldlogistiek en kosteneffectiviteit moeten meegewogen worden in een uiteindelijke biofortificatieroutine.

Hoofdstuk 6 beschouwt de Se speciatie in graanmonsters, nadat deze zijn verrijkt met Se via blad- of bodemaddities. Onafhankelijk van experimentele specificaties bleek selenomethionine (SeMet) de voornaamste species te zijn in alle monsters, wat duidt op een nagenoeg volledige conversie van de gesuppleerde inorganische Se.

Hoofdstuk 7 beschouwt de huidige Se status van een array van 46 verschillende broodtarwes uit een tarwe-archief, met als doel potentiele tarwesoort-kandidaten te vinden voor Se-verbetering via plantenteelt. Onder zowel traditionele- als meer moderne (geavanceerde) cultivars bleken ‘Ideal’, ‘Ribeiro’(b) en ‘Ribeiro’(a) als best mogelijke kandidaten voor de gestelde doelen.

Hoofdstuk 8 geeft een overzicht en een discussie van de verkregen resultaten, en enige suggesties voor verder onderzoek.

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Curriculum vitae

Catarina I. A. L. Galinha was born on the 30th March 1982 in Benavente, Portugal. She obtained her high school degree at Escola Secundária de Benavente in Benavente. Subsequently she studied Biological Engineering at the Technical University of Lisbon and later she obtained her Master degree also in Biological Engineering with a thesis entitled "Different radioanalytical techniques for the determination of selenium in cereal samples". She started a PhD at the Department of Radiation Science & Technology (RST) of the Delft University of Technology the results of which are presented in this thesis.

List of publications

Galinha C, Pacheco AMG, Freitas MC, Costa ARP, Pinheiro NB, Maçãs B, Almeida AS, Wolterbeek HT (2014) Selenium characterization of the Portuguese bread-wheat archival collection. Submitted to *Crop & Pasture Science*

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