Characterization of selenium-enriched wheat by agronomic biofortification

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Abstract Agronomic biofortification of staple 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 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 at an equivalent field rate of 100 g of Se per hectare (ha), using sodium selenate and sodium selenite as Se-supplementation matrices, in actual field conditions throughout. Biotransformation of inorganic Se was evaluated by using 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.

Keywords Agronomy · Biofortification · Nutrition · Selenium · Speciation · Wheat

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—assigned 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). Following 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

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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 Błażej 2013), which can be organic, such as selenoaminoacids, Se-methylated forms and complex selenoproteins, or inorganic, 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; Hidioglou 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-Estépa 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, selenocystathione, selenomethylselenomethionine, selenomethylselenocysteine, 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 every Se species (Seppänen et al. 2010). Another potential 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.

Experimental

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 $118 \pm 6 \mu\text{g kg}^{-1}$ (Galinha et al. 2012b) and $92 \pm 3 \mu\text{g kg}^{-1}$, 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 $10 \mu\text{M}$ 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 $100 \text{ g of Se per ha}$, 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 $1.5 \times 0.5 \text{ m}$) 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.

Instrumentation

Wheat grains were separated from their spikes with the help of a HegeTM 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 TeflonTM 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; Bobadela, Portugal), at a thermal-neutron flux density of $1.7 \times 10^{12} \text{ n cm}^{-2} \text{ s}^{-1}$. 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 ProTM 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 SONOPULSTM 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 $0.22\text{-}\mu\text{m}$, 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 $100\text{-}\mu\text{L}$ injection loop. Selenium species separation was based on a Hamilton[®] PRP-X100 anion-exchange column and an Agilent[®] ZorbaxTM Rx-C8 reversed-phase column. The instrumental (optimal) parameters for HPLC–ICP-MS are listed in Table 1.

Reagents and materials

Reagents for making Se-supplementation solutions (field work) were sodium selenate (Na_2SeO_4 purum p.a. $\geq 98.0 \%$; 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 ($18 \text{ M}\Omega\cdot\text{cm}$) from a Milli-Q[®] water purification system (EMD Millipore Corporation; Billerica MA, USA). Selenomethionine (SeMet), selenomethylselenocysteine (SeMetSeCys) and selenocysteine (SeCys₂) from Sigma-

Table 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® PRP-X100 (250×4.1 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 min
Analytical column	Zorbax™ Rx-C8 (250×4.6 mm; 5 µm)
Mobile phase	0.1 % TFA, 2 % MeOH
Flow rate	1 mL min ⁻¹
Injection volume	100 µL
Elution program	Isocratic
Run time	20 min

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 Na₂SeO₄ and Na₂SeO₃ in 2 % (v/v) nitric acid (HNO₃ 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 (H₂O₂ 35 %; Panreac Química S.L.U.), following the procedure by Sánchez-Martínez et al. (2012).

Acid digestion was carried out using HNO₃ and H₂O₂; 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 (C₆H₈O₇; Sigma-Aldrich®) in 2 % (v/v) methanol (MeOH HPLC grade; Scharlab S.L.), adjusted to pH=5.0 with ammonium hydroxide (NH₄OH; Fluka®), as mobile phase. For reversed-phase, chromatographic separation, a solution of 0.1 % trifluoroacetic acid (TFA; Sigma-Aldrich®) in 2 % MeOH was employed.

Total Selenium determination

Cyclic neutron activation analysis (CNAA)

Wheat-flour samples were heat-sealed in polyethylene vials (1.2 mL), and then placed in mid-sized 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.

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 (3 replicates, whenever possible) were digested with 5 mL of HNO₃ and 1 mL of H₂O₂ in a microwave oven at 130 °C 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 1.

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 0.22 µm 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 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 ⁷⁶Se, ⁷⁷Se, ⁷⁸Se, and ⁸⁰Se isotopes and H₂ 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.

Results and discussion

Selenium accumulation

An overview of the response of wheat cultivars to supplementation is given in Table 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.

Table 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 ₂ SeO ₃	1.00±0.06	—
Na ₂ SeO ₃ +KI	0.85±0.04	0.87±0.01
Na ₂ SeO ₄	1.77±0.06	2.09±0.04
Na ₂ SeO ₄ +KI	1.06±0.04	—
Grain filling		
Na ₂ SeO ₃	1.97±0.07	2.0±0.2
Na ₂ SeO ₃ +KI	2.38±0.19	—
Na ₂ SeO ₄	2.98±0.01	3.0±0.2
Na ₂ SeO ₄ +KI	2.55±0.12	—
Soil addition		
Blank	a	0.07±0.01
Na ₂ SeO ₄	1.064±0.003	—
Na ₂ SeO ₄ +KI	1.51±0.04	—
Bread wheat		
Foliar addition		
Blank	0.06±0.01	—
Booting		
Na ₂ SeO ₃	0.61±0.01	—
Na ₂ SeO ₄	2.7±0.2	—
Grain filling		
Na ₂ SeO ₃	0.92±0.04	—
Na ₂ SeO ₄	0.72±0.09	—
Soil addition		
Blank	a	0.060±0.003
Na ₂ SeO ₄ +KI	0.76±0.01	—

^a Below detection limit

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, ⁷⁸Se⁺ (⁴⁰Ar³⁸Ar⁺) and ⁸⁰Se⁺ (⁴⁰Ar²⁺) (Zhang and Combs 1996). The use of collision/reaction cells is a way to remove polyatomic interferences, which allows ⁷⁸Se and ⁸⁰Se analysis free from interferences (Feldmann et al. 1999). Nevertheless, another interferences as ⁷⁹Br¹H⁺ (*m/z*=80) (Zhang and Combs 1996) could appear when using H₂ as reaction gas, so Se determination was carried out by monitoring ⁷⁸Se isotope in the cell mode to avoid all the 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). Generally speaking, 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, Ylänta (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 though other aspects should be considered for biofortification purposes: field logistics are simpler for soil addition, yet Se supplements could be costlier (higher Se levels are required). A trade-off should thus be considered for an eventual (practical) implementation of any supplementation routine on wheat crops.

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 and 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 min. To optimise Se species separation, both an anion-exchange column and a reverse-phase column were tested. Figure 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 2 illustrates the chromatographic profile of Se species in a standard solution containing $10 \mu\text{g L}^{-1}$ of each, after elution through an anion-exchange column, and Fig. 3a and b 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 (Fig. 3a and b). 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 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 $3448 \pm 146 \text{ mg kg}^{-1}$ and $3215 \pm 122 \text{ mg kg}^{-1}$, 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 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 Se used in the supplementation (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

Fig. 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

Fig. 2 Chromatographic profile of a standard solution containing $10 \mu\text{g L}^{-1}$ 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

stage ($63 \pm 8 \%$) 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 3) was determined in Marialva (durum wheat) flour samples, from crops

Fig. 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 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^{VI}), 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

	<i>n</i>	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 ₂ SeO ₃	3	0.6±0.2	58±20	0.04±0.01	3.6±1.1
Na ₂ SeO ₃ +KI	1	0.995	100	0.020	2
Na ₂ SeO ₄	3	1.0±0.1	60±8	0.064±0.019	3.6±1.1
Na ₂ SeO ₄ +KI	3	0.6±0.04	54±4	0.02±0.013	1.8±1.2
Grain filling					
Na ₂ SeO ₃	2	1.34±0.03	69±2	0.09±0.04	5.1±2.1
Na ₂ SeO ₃ +KI	3	1.50±0.05	63±2	—	—
Na ₂ SeO ₄	2	2.0±0.3	68±11	0.333±0.003	11.1±0.1
Na ₂ SeO 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 ₂ SeO ₄	2	1.0±0.2	95±17	0.006±0.004	0.5±0.4
Na ₂ SeO 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 ₂ SeO ₃	3	0.36±0.06	86±13	—	—
Na ₂ SeO ₄	3	1.5±0.1	61±4	0.018±0.004	3.2±0.7
Grain filling					
Na ₂ SeO ₃	3	0.6±0.1	63±9	—	—
Na ₂ SeO 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 ₂ SeO ₄ +KI	3	0.65±0.03	86±4	0.008±0.002	1.1±0.3

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 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 2) and also have SeMet as the major species (Table 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 Fig. 3a. The presence of KI as an additive to supplementation does not seem to affect either the total accumulation of Se in mature grains or the distribution of selenocompounds.

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. 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 human populations with Se-deprived diets through Se-enriched, wheat-based foodstuff.

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