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Original Research Article

Selenium speciation and bioaccessibility in Se-fertilised crops of dietary importance in Malawi

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ABSTRACT

The purpose of this research was to explore the speciation and bioaccessibility of native soil-derived selenium (Se) versus Se applied via fertiliser in the edible portions of maize, groundnut and cowpea grown in Malawi. Fertiliser-derived Se, applied as isotopically labelled selenate, contributed 88–97% of the total Se in the edible portions. Both soil and fertiliser-derived Se were transformed into similar species, with more than 90% of the extracted Se in an organic form. The main form of fertiliser-derived Se in grain was selenomethionine with an abundance of 92.0 ± 7.6% in maize, 63.7 ± 6.2% in cowpea and 85.2 ± 1.9% in groundnut. In addition, cowpea contained 32.7 ± 6.2% of Se-methyl-selenocysteine. The mean bioaccessibility of fertiliser-derived Se was 73.9 ± 8.5% with no statistically-significant difference across all crops despite some variation in speciation. Understanding the contribution of fertiliser-derived Se to the formation of organic forms of Se in crops is crucial, given that organic Se species are more bioaccessible than inorganic forms.

1. Introduction

Selenium (Se) is an essential micro-nutrient incorporated into at least 25 selenoproteins involved in vital metabolic processes in the human body. Selenoproteins have critical roles in the maintenance of a healthy immune system, protection of tissues against oxidative stress, metabolism of thyroid hormones, DNA synthesis and reproduction (Rayman, 2012). The recommended dietary intake of Se varies from country to country but generally ranges between 30 and 80 μg day^{-1} (Fairweather-Tait et al., 2011). Despite the small amounts required, sub-optimal dietary intake of Se is common in many regions of the world, especially in sub-Saharan Africa (SSA) (Ligowe et al., 2020b). The uptake of Se from soils to crops is the main entry point of Se into the food system. Therefore, strategies to alleviate Se deficiencies have largely focused on agronomic biofortification, mainly through the application of Se-enriched fertilisers to soils and crops (Broadley et al., 2010).

Increased Se concentration in crops after agronomic fertilisation has been observed for wheat (Govasmark et al., 2010; Hart et al., 2011), maize (Chilimba et al., 2014), rice (Gong et al., 2018; Wang et al., 2013), mushrooms (Bhatia et al., 2013) and vegetables (Lavu et al., 2012; Pedrero et al., 2006). For biofortification to be successful, the applied Se taken up by crops must be bioavailable for it to achieve the intended effects in the human body; this depends on the chemical form of Se present and the matrix in which it is embedded (Fairweather-Tait et al., 2010). Selenium exists in various chemical forms, but the most relevant species that have been identified in foods include: (i) selenomethionine (SeMet), which can exist within and outside of proteins in plant sources particularly cereals; (ii) selenocysteine (SeCys), found mainly within proteins in animal sources; (iii) selenoneine, recently found to be the predominant form of Se in fish such as tuna and mackerel; (iv) Se-methyl-selenocysteine (SeMeSeCys) and its γ-glutamyl derivative (γ-glutamyl-SeMeSeCys), found in *Allium* and *Brassica* families; (v) selenate and selenite, found in drinking water and some plant sources (Pedrero and Madrid, 2009; Rayman, 2012; Rayman et al., 2008).

Organic forms of Se are known to be more bioavailable than inorganic forms in humans (Fairweather-Tait et al., 2010). Kirby et al. (2008) observed greater Se bioavailability from biofortified wheat

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biscuits containing SeMet as the dominant Se species compared to process-fortified biscuits with selenomethionine selenoxide (SeOMet) as the dominant species suggesting that variations in bioavailability also exist among organic forms of Se. The wheat flour used to produce the process-fortified biscuits was supplemented with SeMet which was subsequently converted to SeOMet during the production process, whereas biofortified wheat biscuits were manufactured from wheat that was biofortified with Se by the application of sodium selenate to the soil around planting time. In addition to influencing bioavailability, the chemical form of Se also determines the nutritional and health effect. For example, there is variation in the efficacy of different Se compounds against cancer, with SeMeSeCys and γ-glutamyl-SeMeSeCys among the most potent anticarcinogenic Se compounds (Ellis et al., 2004; Rayman et al., 2008).

More than 50% of Se in edible portions of crops, including cereals, legumes and vegetables, is in an organic form after application of different levels of selenite or selenate (Hart et al., 2011; Lavu et al., 2012; Li et al., 2010; Poblaciones et al., 2014). Variation in the proportions of accumulated organic and inorganic Se forms in edible portions of crops appears to depend mainly on the level and chemical form of the applied Se. According to Poblaciones et al. (2014), there was an inverse relationship between the concentration of applied selenite or selenate in fertiliser and the concentration of accumulated inorganic forms of Se in the edible portions of chickpea grains. A higher proportion of inorganic Se (c.50%) was found in control chickpea samples (i.e. non-biofortified samples) compared to Se-enriched samples. The authors concluded that the applied Se was more easily transformed into organic forms compared to native Se from soil which was the only source of Se in the control. It is therefore not clear whether Se from soil and applied Se from fertiliser are bio-transformed into similar compounds in the edible portions of crops.

Isotopic labelling of the Se used in biofortification studies provides a way to trace the fate of applied fertiliser Se and native Se from soil to crops. The use of isotopically enriched Se in biofortification has been instrumental in understanding the potential residual effects of fertiliser-derived Se in soils and the uptake mechanisms of fertiliser Se from soils to crops (Chilimba et al., 2012; Ligowe et al., 2020a; Mathers et al., 2017). Ligowe et al. (2020c) recently showed the feasibility of agronomic Se biofortification in Malawi after the application of Se enriched fertilisers at a rate of 20 g ha^{-1} to cereals and legume crops grown under typical agronomic circumstances. However, the effectiveness of the Se biofortified crops to provide absorbable Se is underpinned by the Se bioaccessibility, which is the proportion of Se potentially available for absorption during digestion. At least one other study has examined the bioaccessibility of selenium in biofortified food and feed crops (Lavu, 2013) but, to our knowledge, there are no studies that have distinguished the source of selenium species in the edible portions of crops as either soil or fertiliser. As an extension to the study by Ligowe et al. (2020c), the aim of this study was therefore to determine the speciation and bioaccessibility of soil and fertiliser-derived Se in maize, cowpea and groundnuts from an isotopically labelled Se biofortification field trial in Malawi. Recently, a nationally representative study of 2761 people in Malawi revealed widespread Se deficiency based on plasma Se concentrations which were below the threshold for optimal glutathione peroxidase 3 activity in more than 50% of men and women of reproductive age (Phiri et al., 2019). This is consistent with Joy et al. (2015) who estimated widespread risk of Se deficiency due to dietary shortfalls. Cereals, in particular maize, and legume crops such as cowpea, are of dietary importance in Malawi, with a per capita consumption of 249 g day^{-1} for unrefined maize flour and 71 g day^{-1} for legumes (Joy et al. (2015). Biofortification of these crops with Se could therefore positively impact the millions of people at risk of Se deficiency in Malawi. The findings from this work will be integrated with findings from Joy et al. (2019) to provide a framework for Se agro-biofortification in Sub-Saharan Africa. Joy et al. (2019) are testing the efficacy of Se-enriched maize to improve the Se status in women of reproductive age (WRA) and school-age children (SAC) in Malawi.

2. Materials and methods

Grain samples (maize, cowpea and groundnut) were obtained from a long-term Conservation Agriculture (CA) field trial at Chitedze Research Centre, Malawi in May 2017 (Ligowe et al., 2017, 2020c). The CA trial consists of ten agronomic management treatments including a control representing conventional cultivation methods, arranged in a randomised block design with four replicates per treatment. The grains were harvested from a sub-plot of the ten management treatments (Table 1) where an isotopically enriched potassium selenate solution (>99% enriched ^{77}Se, purchased from Isoflex, San Francisco, USA) had been applied to soil at a rate of 20 g ha^{-1} 75 days after planting, or at the maize tasselling stage (Ligowe et al., 2020c). Grains were carefully cleaned and dried to constant weight in an oven at 40 °C after which they were milled using a centrifugal mill (Model SM100, Retsch). Sample preparation was mainly undertaken in Malawi while analyses were done at the University of Nottingham, United Kingdom. Three independent replicates for each treatment were analysed.

2.1. Determination of selenium bioaccessibility

Selenium bioaccessibility was determined after *in-vitro* gastro-intestinal digestion using the standardised static INFOGEST method (Brodkorb et al., 2019). Gastro-intestinal digestion was performed on raw flour samples since the sample sizes available were too small to

Table 1
Treatment description for the CA Se biofortification trial at Chitedze Research Centre.

Treatment	Treatment description	Crop
Control plot^{T1}	Traditional farmers practice using the hand hoe, maize sole crop, no residues are returned to the soil	Maize T1
CA Sole crop basin planting^{T2}	Basin (0.15 m - length x0.15 m - width x0.15 m - depth), maize as a sole crop, crop residues retained	Maize T2
CA Sole crop dibble stick planting^{T3}	Direct seeding with dibble stick, maize as a sole crop, crops residues retained	Maize T3
CA Crop rotation^{T4C}	Direct seeding with a dibble stick, rotation sequence is cowpea – maize – cowpea, crop residues are retained, split plot with T4G	Maize T4
CA Crop rotation^{T4G}	Direct seeding with a dibble stick, the rotation sequence is groundnut – maize – groundnut, crop residues are retained, split plot with T4C	Maize T5
CA Intercropping^{T6}	Direct seeding with a dibble stick, maize intercropped with pigeon pea, crop residues are retained	Maize T6
CA Intercropping^{T7M}	Direct seeding with a dibble stick, maize intercropped with cowpea, crop residues are retained	Maize T7
CA Intercropping^{T8}	Direct seeding with a dibble stick, maize intercropped with velvet bean, crop residues are retained	Maize T8
CA Crop rotation^{T5C}	Direct seeding with a dibble stick, rotation sequence is maize – cowpea – maize (in contrast to T4C), crop residues are retained, split plot with T5G	Cowpea T9
CA Intercropping^{T7}	Direct seeding with a dibble stick, maize intercropped with cowpea, crop residues are retained.	Cowpea T10
CA Crop rotation^{T5G}	Direct seeding with a dibble stick, rotation sequence is maize – groundnut – maize (in contrast to T4G), crop residues are retained, split plot with T5C	Groundnut T11

$^{T1-8}$Treatment labels for the conservation agriculture trial design, comprehensively described by (Ligowe et al., 2017, 2020c). Treatment names have been renamed T1–T11 for simplicity.

process into food products. The following digestion reagents were used: pepsin from porcine gastric mucosa (3200−4500 units mg^{-1} protein), α-amylase from *Bacillus* sp. (≥400 units mg^{-1} protein), pancreatin from porcine pancreas (8 x USP) and bovine bile (Sigma Aldrich, Dorset, UK). Digestion was conducted at 37 °C in a water bath shaking at 200 rpm for all the three phases, i.e. the oral, gastric and intestinal phases. First, the oral phase was simulated by mixing 2.5 g of flour slurry (flour mixed with MilliQ water (18.2 MΩ cm) to make a 30% dry matter (dm) slurry) with 2 mL simulated salivary fluid, 250 μL α-amylase (75 U mL^{-1} in final digestion mixture) and 250 μL of 15 mM $CaCl_2$ (0.75 mM in the final digestion mixture). The pH was adjusted to 7.0 and the tubes were incubated for 2 min. The gastric phase of digestion was followed by adding to the oral digested mixture: 4 mL simulated gastric fluid, 800 μL porcine pepsin solution (2000 U mL^{-1} in the final digestion mixture) and 200 μL of 3.75 mM $CaCl_2$ (0.075 mM in the final digestion mixture). The pH was adjusted to 3.0 and the tubes were incubated for 2 h. Lastly, the intestinal stage of digestion was performed by adding 4 mL porcine pancreatin dissolved in simulated intestinal fluid (100 U mL^{-1} trypsin activity in the final digestion mixture), 4 mL bovine bile also dissolved in simulated intestinal fluid (10 mM bile salt concentration in the final digestion mixture), and finally 2 mL of 3 mM $CaCl_2$ (0.3 mM in the final digestion mixture). The pH was adjusted to 7.0 and the tubes were incubated for a further 2 h. After incubation, the samples were placed on ice for 15 min to stop enzyme activity before being centrifuged for 30 min at 4500 × *g*. The supernatant was decanted from the pellet and further filtered through a 5 μm syringe filter. An aliquot of the filtered supernatant (2 mL) was heated with 4 mL concentrated HNO_3 in the microwave following the programme described for the determination of total Se. Selenium concentration was determined by ICP-MS (see below) and bioaccessibility was calculated using Eq. (1):

$$Se_{bio} = \frac{Se_{dig}}{Se_{grain}} \times 100 \quad (1)$$

where Se_{bio} (%) is the percentage of bioaccessible Se, Se_{dig} (μg kg^{-1}) is the Se concentration in the soluble phase of the digesta and Se_{grain} (μg kg^{-1}) is the total Se concentration in the sample.

2.2. Total selenium analysis

An aliquot of the sample (0.2 g) was heated with 6 mL concentrated HNO_3 (PrimarPlus™ grade) in a microwave (Microwave Pro, Anton Paar GmbH, Graz, Austria) for 45 min. The sample was heated for 10 min to reach 140 °C, held for 20 min at 140 °C and then cooled for 15 min to 55 °C. After cooling, the solution was diluted to 20 mL using Milli-Q water followed by a 10× dilution prior to analysis using a triple quadrupole Inductively Coupled Plasma - Mass Spectrometer (ICP-MS) (iCAP TQ, Thermo-Fisher Scientific, Bremen, Germany). The ICP-MS was operated in oxygen mode to minimise interferences from ^{76}Ge hydride and $^{40}Ar_2$: isotopes monitored were ^{77}Se, mass-shifted to *m/z* 93 ($^{77}Se^{16}O$) and ^{80}Se mass-shifted to *m/z* 96 ($^{80}Se^{16}O$). Samples were introduced at a flow rate of 1.2 mL min^{-1} from an autosampler (ESI SC-4 DX FAST Autosampler) incorporating an ASXpress™ rapid uptake module through a perfluoroalkoxy (PFA) Microflow PFA-ST nebuliser (Thermo-Fisher Scientific). An internal standard of rhodium (5 μg L^{-1}), was introduced to the sample stream on a separate line with an equal flow rate via the ASXpress™ rapid uptake module. A standard calibration was created by serial dilution of SPEX certiprep Se standards to give a concentration ranging from 0 to 100 μg L^{-1}. A rice standard reference material (NIST 1568b) with a Se reference concentration of 365 ± 29 μg kg^{-1} was included in the digestion and Se recovery was 94.6 ± 1.7%. The limits of detection (LOD) and quantification (LOQ) are shown in Table S1, supplementary data. Blank and drift corrections were done on raw intensity data (counts per second) at *m/z* 93 ($^{77}Se^{16}O$) and 96 ($^{80}Se^{16}O$) obtained after ICP-MS analysis. Standard calibrations of both ^{80}Se and ^{77}Se were used to convert intensity data into concentrations (μg L^{-1}). The concentration of native soil-derived Se (μg L^{-1}) was calculated from measurement of ^{80}Se while the concentration of ^{77}Se represents the total ^{77}Se which is a contribution of both fertiliser and native Se. Therefore, to obtain the concentration of ^{77}Se from fertiliser, the concentration of native ^{77}Se (soil-derived ^{77}Se expressed in μg L^{-1}) was calculated using Eq. 2:

$$\text{Soil} - \text{derived } ^{77}\text{Se} = \text{Soil} - \text{derived } ^{80}\text{Se} * \left(\frac{^{77}Se_M}{Se_M}\right) * {}^{77}Se_{IA} \quad (2)$$

where 'Soil-derived ^{80}Se' is the total concentration of soil-derived Se expressed in μg L^{-1}, $^{77}Se_M$ is atomic mass of ^{77}Se (76.92), Se_M is average atomic mass of Se (78.96), and $^{77}Se_{IA}$ is the isotopic abundance of ^{77}Se (0.0763). A mass correction was used to account for mass differences of the Se isotopes. Fertiliser-derived Se (μg L^{-1}) was then calculated using Eq. 3:

$$\text{Fertiliser} - \text{derived Se} = {}^{77}Se_{tot} - \text{Soil} - \text{derived } ^{77}\text{Se} \quad (3)$$

where $^{77}Se_{tot}$ is the total concentration of ^{77}Se (μg L^{-1}). The concentration of soil-derived Se and fertiliser-derived Se were then converted to a gravimetric basis (μg kg^{-1}) based on the sample weights and volumes used for the analysis.

2.3. Identification and quantification of selenium species

Selenium speciation analysis used the method of Li et al. (2010) with minor modifications. Protease from *Streptomyces griseus* (≥3.5 units mg^{-1} protein) and lipase from *Candida rugosa* (≥ 700 units mg^{-1} solid), along with the selenite, selenate, seleno-L-cystine ($SeCys_2$) and SeMet standards were obtained from Sigma Aldrich, while SeMeSeCys was obtained from Fisher Scientific (Loughborough, UK). Briefly, 0.2 g of flour sample was extracted with 20 mg protease and 10 mg lipase dissolved in 5 mL MilliQ water. Extraction was carried out at 37 °C, in a water bath shaking at 60 rpm for 24 h. The sample was centrifuged at 4500 × *g* for 30 min, then the supernatant filtered through a 0.22 μm syringe filter. Separation was undertaken using a Hamilton PRP-X100 column (205 mm × 4.6 mm, 5 μm) on an LC ICS-5000 (Dionex, Thermo-Fisher Scientific) coupled to the ICP-MS. The mobile phase consisted of ammonium citrate (A - 20 mM, B - 50 mM), 2% methanol with pH adjusted to 4.3 using citric acid. An optimised gradient step elution programme was used, i.e. (0–5 min − 100% A, 5–5.5 min − up to 100% B, 5.5–12 min -100% B), with a flow rate of 1.0 mL min^{-1} and an injection volume of 100 μL. The same ICP-MS conditions for the determination of total Se concentration were used. Standard solutions of selenate, selenite, SeMet, $SeCys_2$ and SeMeSeCys were injected at varying concentrations (Figs. S1–S4, supplementary data). Each of the standards were run after every 10 samples in order to monitor, and correct for, instrument drift. Identification of Se species was achieved using retention time matching and the species concentrations were calculated based on calibration standards. The enzyme extract was also analysed for total Se using the same preparation method as described for the gastro-intestinal extracts. The ratio of extracted Se to the total Se concentration in the grain was calculated to give the extraction efficiency (%). The column recovery (%) was calculated as the ratio of the sum of identified and quantified species to the extracted Se. The chromatograms for the standards, LOD's and LOQ's for the Se species which were calculated according to the method described by Knoll (1985), are found in the supplementary data.

2.4. Statistical analysis

The means for total Se concentration, Se species concentration and Se bioaccessibility were compared across the different cropping management treatments using one-way ANOVA ($p < 0.05$) and Tukey's Honest Significant Difference where applicable, in R, Version 3.5.2.

3. Results and discussion

3.1. Total selenium concentration

Due to the inherently low concentrations of Se in crops from Malawi, the use of Se enriched fertilisers in the cultivation of three crops of dietary importance was tested to determine potential Se bioaccessibility. The effectiveness of agronomic Se biofortification was assessed in terms of the total Se concentrations achieved, the Se species formed and the resultant Se bioaccessibility. Soil-derived Se and fertiliser-derived Se concentrations ranged between 10.7–30.7 μg kg^{-1} and 123–836 μg kg^{-1}respectively. The contribution of fertiliser-derived Se and soil-derived Se resulted in Se_{grain} concentrations in the range 134–865 μg kg^{-1} (Fig. 1). The addition of Se fertiliser increased grain Se concentration by an order of magnitude such that fertiliser-derived Se contributed 88–97% of Se_{grain}. In general, Se_{grain} was in the order maize < cowpea < groundnut. A higher accumulation of Se in the grains of legumes than in maize has been observed in other Se biofortification field trials e.g. in Malawi (Chilimba et al., 2014) and in Kenya (Ngigi et al., 2019). According to Poblaciones et al. (2014), there is a positive correlation between protein and Se concentrations in the edible portions of grains because Se is mainly accumulated in proteins. Legumes are likely to accumulate more Se than cereals because of their higher protein content which ranges between 20–40% compared to cereals which contain < 15% protein. The potential of legumes such as beans, chickpea, lentils and peas to accumulate high concentration of Se in Se biofortification studies has been highlighted in several studies (Poblaciones et al., 2015, 2014). Nevertheless, a more than 10-fold increase in Se concentrations achieved in maize grain shows that maize may be better suited to supply Se into the Malawian food system as maize consumption is consistent throughout the year and contributes more than 50% of total energy and Se intake (Joy et al., 2015).

3.2. Selenium speciation

The results of the speciation analysis are shown in Figs. 2 and 3. Typical speciation chromatograms of extracted samples of maize, cowpea and groundnut are shown in Fig. 2. About 57.6–93.2% (mean 73.1 ± 10%) of fertiliser-derived Se was extracted and 72.4–95.8% (mean 82.8 ± 6.3%) of the extracted Se was identified and quantified in terms of its chemical form. A higher extraction efficiency was observed for cowpea and groundnut (mean 83.0 ± 8.1%) than for the maize samples (mean 70.2 ± 8.9%). Similar extraction efficiencies and column recoveries have been reported in the extraction of Se in rice and wheat (Hart et al., 2011; Li et al., 2010; Sun et al., 2010). In some cases, especially for maize samples, relatively low extraction efficiencies coupled with relatively low column recoveries led to a significant proportion of Se not being accounted for (c. 50%). The ability of the proteolytic and lipolytic extraction enzymes to access and hydrolyse specific Se compounds will depend on the localization and distribution of Se in the grain structures. For example, Se contained in the bran could be inaccessible for hydrolysis due to the high fibre content of this fraction which is largely indigestible. There are no reports pertaining to the localization of Se in maize, cowpea and groundnut but evidence for rice and wheat shows that whilst Se is distributed more consistently throughout the grain than iron and zinc, a higher concentration of Se is located in the outer layers of the grains (mainly in the aleurone layer which is part of the bran) than in the endosperm (Hart et al., 2011; Moore et al., 2010). According to Shen et al. (2019), the bran fraction in rice, constituting about 7% of grain weight, contains about 14% of the total Se. Selenium associated with the bran fraction could also be higher in maize than in cowpea and groundnut thereby causing a lower extraction efficiency for maize. In addition, large molecules such as starch and phytate could hinder the accessibility of extraction enzymes for the Se compounds. Selenium has been detected in protein regions surrounding starch granules in the starchy endosperm cells and outside of phytate granules in wheat (Moore et al., 2010). Information on the localization and distribution of Se in the grains under study is crucial, particularly for maize which is often dehulled leaving the starch-rich endosperm to make the flour used for cooking the Malawian staple, *nsima*.

The extracted species derived from soil-derived Se that were present in the flour could not be reliably quantified as the Se concentration was very low after extraction. Nevertheless, the chromatographic profiles of both soil-derived Se and fertiliser-derived Se show the same trend, suggesting that both sources of Se were biotransformed into similar Se species (Fig. 2). This means that crops were able to metabolise both soil and fertiliser-derived Se in the same manner, suggesting that the crops biological controls were not overwhelmed by the application of 20 g ha^{-1} Se.

More than 90% of the extracted fertiliser-derived Se was in an organic form in all the grains which indicates that the plants were efficient at converting inorganic Se into organic Se. The presence of a high proportion of organic forms of Se in all crops also indicate that the application of 20 g ha^{-1} selenate did not 'overload' the capacity of the plants to transform assimilated Se into organic forms (Poblaciones et al., 2014). The dominant organic Se species was SeMet with an abundance of 92.0% in maize, 63.7% in cowpea and 85.2% in groundnut (Fig. 3). The actual concentrations of the Se species in the crops are found in Table S2, supplementary data. This finding is consistent with other studies which have shown that SeMet is normally the principal organic species in Se biofortified and non-biofortified staple crops. For example, SeMet contributed 83–84% of total Se in biofortified rice (Sun et al., 2017), >65% in biofortified wheat (Hart et al., 2011), 61–64% in maize

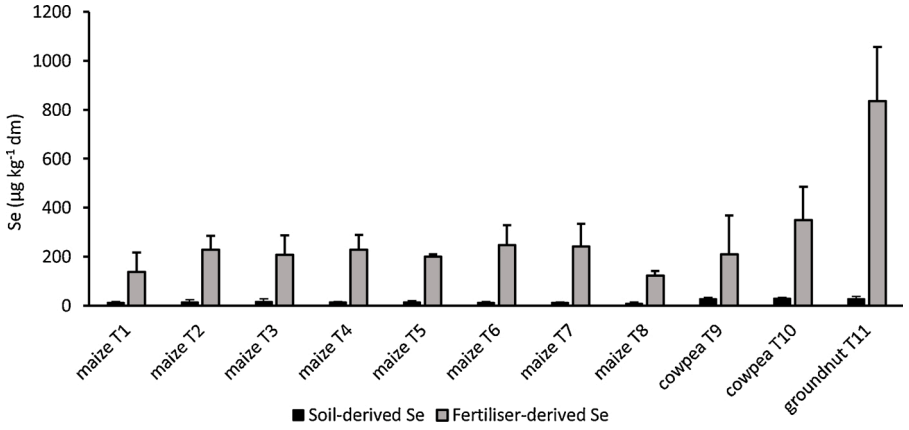

Fig. 1. Concentration of soil-derived Se and fertiliser-derived Se, in maize, cowpea and groundnut grains (μg kg^{-1} dm) grown under different agronomic treatments (T1-T11). Error bars represent standard deviation of three independent replicates. Selenium was applied at a rate of 20 g ha^{-1} as potassium selenate.

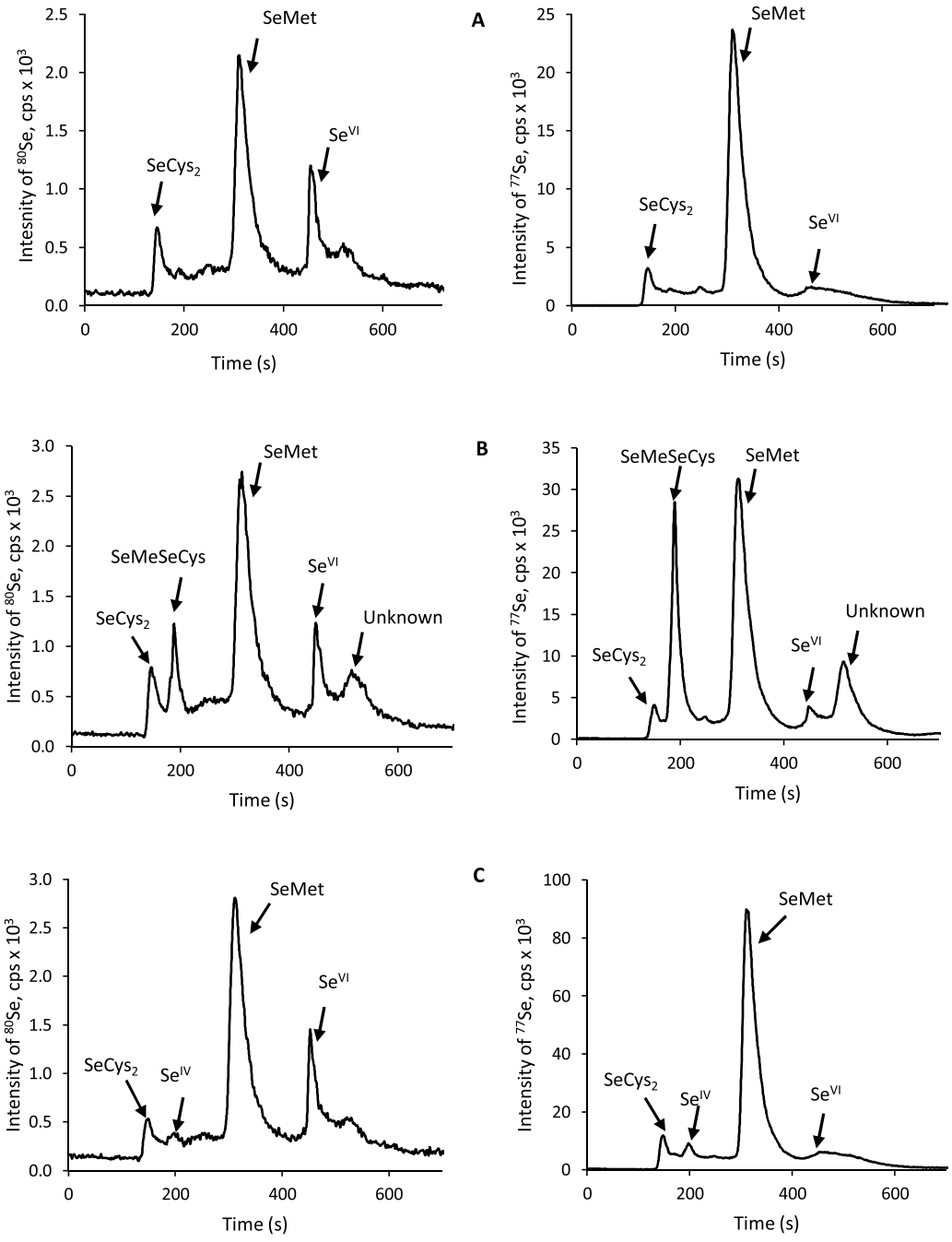

Fig. 2. Representative chromatograms showing the speciation of native soil-derived Se, measured as ^{80}Se (left), and fertiliser-derived Se, ^{77}Se (right) in maize T1 (A), cowpea T10 (B) and groundnut T11 (C). Identification of $SeCys_2$ is tentative.

grown in seleniferous soils (Beilstein et al., 1991) and >70% in bio-fortified chickpea (Poblaciones et al., 2014). According to D'Amato et al. (2019) and Khanam and Platel (2016), the presence of SeMet in Se biofortified crops increases their nutraceutical value because SeMet is more stable to heat processing than other organic forms of Se, such as SeCys and SeMeSeCys. A high proportion of SeMet in Se enriched food products, such as yeast, is also an indicator of their quality and proof of organic character (Bierla et al., 2012).

The metabolic pathway leading to the formation of SeMet in crops involves the uptake of selenate through sulphur transporters in roots, followed by metabolism through the sulphur assimilation pathway. Selenate is incorporated non-specifically into proteins by replacing S in methionine to form SeMet or in cysteine to form SeCys (Ellis et al., 2004). Indeed, SeCys was tentatively identified in all grains, albeit as $SeCys_2$ since it is readily oxidised to this form once exposed to air (Chan et al., 2010). The identification of $SeCys_2$ is tentative at best because its existence should be validated by other separation techniques such as electrospray-TOFMS/MS and LC-ESI-MS/MS. This is because oxidised forms of SeMet and SeMeSeCys also co-elute with $SeCys_2$ at the beginning of the chromatographic separation (Dernovics and Lobinski, 2008; Kubachka et al., 2017).

Many plant species cannot tolerate high Se levels in the environment because the non-specific incorporation of SeCys and SeMet into proteins can result in Se toxicity with SeCys contributing a larger toxic effect than SeMet (Sors et al., 2005). However, Se hyper-accumulating plants have an intrinsic metabolic capacity to circumvent this toxicity effect by producing non-protein Se amino acids which cannot be incorporated into proteins. In these plants, the accumulation of SeMet is limited by the

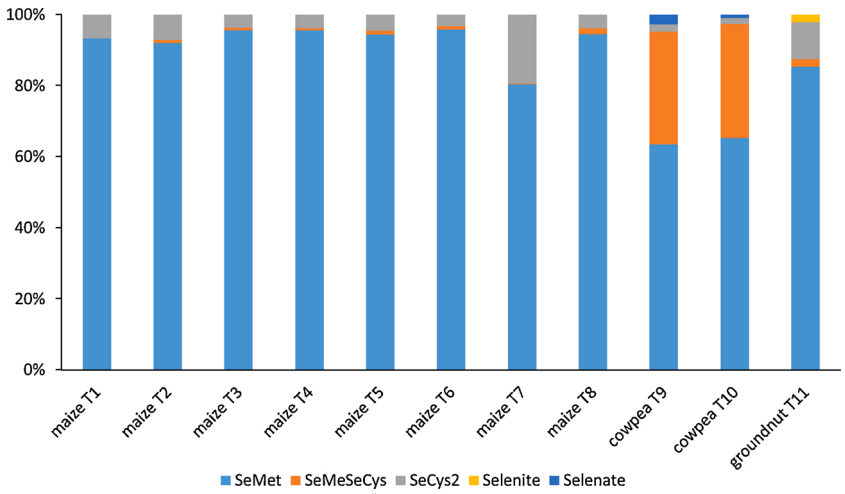

Fig. 3. Speciation of fertiliser-derived Se in maize, cowpea and groundnut grains. Selenium was applied at a rate of 20 g ha^{-1} as potassium selenate. Data presented display the contribution of Se species as a percentage of the total Se extracted from grain samples. Identification of $SeCys_2$ is tentative.

conversion of the precursor SeCys into non-protein Se compounds such as SeMeSeCys, its γ-glutamyl derivative and selenocystathionine (Sors et al., 2005). Among these non-protein amino acids, only SeMeSeCys was identified in the grains with trace levels in maize and groundnut (< 5%) and a surprisingly high abundance of 32.7% in cowpea (Fig. 3). Trace levels of SeMeSeCys are commonly found in Se non-accumulating plants due to the presence of non-specific methyltransferases, but only Se hyperaccumulating plants form this compound at elevated levels (McKenzie et al., 2015). The concentration of SeMeSeCys in the cowpea grains from the two agronomic treatments was approximately 54 μg kg^{-1}. Although the concentration of SeMeSeCys in cowpea was much lower than the concentrations detected in Se hyperaccumulating plants, its proportion in relation to other Se species suggests that a metabolic process in cowpea must be operating, which is distinct from groundnut and maize under the conditions used in this study. The presence of SeMeSeCys in legumes has been reported in a few studies. It was the dominant organic species in soybean fertilised with selenite (Tie et al., 2015) and in different varieties of beans fertilised with selenate where it contributed approximately 20–28% of the total extracted Se (Smrkolj et al., 2007). Clearly, more detailed studies to understand the occurrence of SeMeSeCys in cowpea are needed. SeMeSeCys is known to have chemopreventive properties (Ellis et al., 2004), functions that go beyond meeting the basic nutritional requirement, in this case, the optimal expression of Se enzymes. Crops that accumulate Se compounds with potential health benefits are of increasing interest as they can potentially be classified as nutraceuticals or 'functional foods'.

Trace levels of selenate and selenite were also observed in cowpea and groundnut, respectively (Fig. 3). In addition, an unknown minor Se compound which eluted after selenate, was also observed in cowpea. It is plausible that the unextracted Se in the grains could be inorganic Se species which are located in the outer layers of the grains. According to Carey et al. (2012), during grain filling, organic Se species are able to move to the inner parts of the grain more efficiently than inorganic species, implying a higher concentration of inorganic species in the outer layers of the grain. Therefore, speciation of individual portions of the grains (i.e. bran, endosperm, germ) is critical to provide a complete overview of how Se species are organised throughout the grains. In addition to understanding Se speciation of Se biofortified crops, a deeper insight into their nutraceutical value is needed as an increase of Se in grains has also been associated with an increase in secondary plant metabolites responsible for antioxidant activity such as polyphenols and carotenoids (D'Amato et al., 2019, 2020).

In this study, speciation of gastro-intestinal extracts was not done as no difference in speciation was expected between the two extraction techniques. According to Pedrero et al. (2006), Se species found in gastro-intestinal extracts of radish were not different from species obtained after extraction with protease XIV as used in our study to extract Se species for speciation analysis. In addition, the INFOGEST method used for gastro-intestinal digestion utilises much higher concentrations of enzymes compared to the gastro-intestinal digestion methods used by other studies (Bhatia et al., 2013; do Nascimento da Silva et al., 2017; Pedrero et al., 2006). This means that gastro-intestinal extracts have to be diluted beyond the detection limits for Se speciation (see supplementary data for detection limits). For an understanding of the speciation of the bioaccessible fraction using the INFOGEST gastro-intestinal digestion method, the use of low molecular weight ultra-centrifugal filters to remove enzymes might be useful for future studies.

3.3. *Selenium bioaccessibility*

The mean gastro-intestinal bioaccessibility of fertiliser-derived Se was 73.9% (range 66.6–78.2%) with no significant differences across the different grain types (Table 2). As found for Se speciation, a reliable quantification of bioaccessible soil-derived Se was difficult because of the low concentrations present in the soluble fractions after *in-vitro* gastro-intestinal digestion. Since both soil and fertiliser-derived Se were transformed into similar species, it can be assumed that the bioaccessibility of both sources of Se are the same. Comparison of bioaccessibility results to other published studies can be difficult due to the variation of *in-vitro* gastro-intestinal digestion methods that can differ depending on variables such as pH, enzyme activity units, duration of digestion and the fraction that is considered as bioaccessible. In this

Table 2
Bioaccessibility of fertiliser-derived Se in grains.

Crop	Fertiliser-derived Se bioaccessibility (%)
Maize T1	75.6 ± 10
Maize T2	74.0 ± 6.0
Maize T3	75.7 ± 7.9
Maize T4	76.8 ± 7.1
Maize T5	78.2 ± 13
Maize T6	74.4 ± 9.7
Maize T7	66.6 ± 9.5
Maize T8	75.6 ± 15
Cowpea T9	72.5 ± 2.9
Cowpea T10	66.9 ± 6.2
Groundnut T11	77.1 ± 6.9
Grand mean	73.9 ± 8.5

Values are expressed as mean ± standard deviation, $n = 3$.

study, no dialysis was done to separate Se compounds based on their molecular weights, as such comparison of our results with studies in which bioaccessibility was defined as the dialyzable fraction were done with caution. Selenium bioaccessibility of 67–76% for cooked rice (Sun et al., 2017) was reported in a study that followed a similar approach to ours. In another study, Se bioaccessibility of rice (65%) was higher than that of maize (51%) (Jaiswal et al., 2012).

Despite the differences in Se species, in particular, the presence of substantial amounts of SeMeSeCys in cowpea, there was no evidence of differences in bioaccessibility across grain types. This suggests a high bioaccessibility of all organic Se compounds identified in this study, in line with the assertion that organic forms of Se are highly bioaccessible since these were the dominating Se compounds. The non-bioaccessible Se is likely to be associated with the bran fraction, as whole grains were used in this study. According to Reeves et al. (2007), bioavailability measured in rats was almost 100% for refined wheat flour (containing mainly endosperm), 85% for wheat shorts (containing mainly germ) and 60% for wheat bran. The authors attributed the low bioavailability of Se in the bran to the encapsulation of Se containing proteins by non-digestible fibre contained in this fraction. A similar finding was also observed in an *in-vitro* study by Khanam and Platel (2016) who reported a reduced bioaccessibility for whole legumes compared to their decorticated counterparts. Nonetheless, Se which could not be released after gastro-intestinal digestion could reach the colon where it can be taken up by gut microorganisms for their own metabolism and/or play a role in inhibiting colon carcinogenesis (Lavu et al., 2012; Sun et al., 2017).

According to Ligowe et al. (2020c), biofortified maize consumed as unrefined maize flour would potentially provide 51–58 μg day^{-1} of Se, while cowpea and groundnut would provide 29 and 50 μg day^{-1} respectively (based on an estimated intake of 249 g $capita^{-1}$ day^{-1} for unrefined maize flour and 71 g $capita^{-1}$ day^{-1} for legumes). After accounting for bioaccessibility, it is further estimated that unrefined maize flour will provide up to 47 μg day^{-1} Se, while cowpea and groundnut will provide up to 17 and 46 μg day^{-1} of Se, respectively. Although there is a slight reduction in available Se per day, the bioaccessible Se concentrations are still within a reasonable range to meet the minimum recommended dietary intake levels required to prevent Se deficiency (30–40 μg day^{-1}).

Information on the effect of typical processing of grains used in this study on the Se speciation and bioaccessibility is needed to complement findings from this study. There is no consensus on the effect of processing cereals and legumes on bioaccessibility of Se (Hart et al., 2011; Khanam and Platel, 2016; Lu et al., 2018) and this could be related to variations in the types of processing. Therefore, the processing techniques typically practiced by populations who should benefit from these Se biofortified staple crops should be considered.

4. Conclusion

In this study, it has been shown that Se biofortification through fertilisation with potassium selenate leads to the accumulation of highly bioaccessible Se organic compounds in three crops of dietary importance in Malawi. The dominant organic Se compound, SeMet, serves as an unregulated reserve pool of Se in the body, providing Se when needed, while the presence of SeMeSeCys in cowpea presents an opportunity for its utilization as a nutraceutical. Evidence from this study can be used to guide the formulation of a national strategy for Se fertiliser application in crops and dietary guidelines for nutrition and health.

CRediT authorship contribution statement

Molly Muleya: Methodology, Investigation, Formal analysis, Funding acquisition, Writing - original draft. **Scott D. Young:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition. **Saul Vazquez Reina:** Investigation. **Ivy S. Ligowe:** Resources. **Martin R. Broadley:** Conceptualization, Funding acquisition. **Edward J.M. Joy:** Conceptualization, Funding acquisition. **Prosper Chopera:** Supervision. **Elizabeth H. Bailey:** Conceptualization, Methodology, Writing - review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2021.103841.

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